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Nesting in reptiles: Natural and anthropogenic threats and evolutionary responses

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Nesting in reptiles: Natural and anthropogenic threats and evolutionary responses

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Editorial: Nesting in reptiles: Natural and anthropogenic threats and evolutionary responses

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Editorial on the Research Topic
Nesting in reptiles: Natural and anthropogenic threats and evolutionary responses

Reptiles, like other animals, are facing the sixth mass extinction event and are challenged with habitat loss, overexploitation, species invasions, pollution and climate change. Although many reptile species have survived past extinction events, others have not. We are at a critical juncture in which we must determine which species require our intervention vs. which species can persist on their own *via* evolutionary rescue. Perhaps the most vulnerable life stage of reptiles is the set of developing embryos in nature, which are rarely attended by the mother. The success of this strategy focuses attention on nest site choice behavior – the last time the mother can influence offspring, and thus, her own evolutionary fitness. Our Research Topic involved a collection of 17 papers on the science of nesting in reptiles within the context of anthropogenic threats and potential evolutionary responses. Although our papers form a somewhat eclectic aggregation, they help funnel us toward a better understanding of reptile populations by providing valuable empirical data and relevant reviews.

Introduction

The vast majority of the world's animals lay eggs and leave them unattended, a strategy that has persisted across two major extinction events spanning hundreds of millions of years. The success of this strategy focuses attention on nest site choice behavior–the last time the mother can influence offspring, and thus, her own evolutionary fitness (e.g., Refsnider and Janzen, 2010). It is likely that nest site choice played some role in getting oviparous animals without parental care through extinction events, large and small.

Reptiles are a prime example of oviparous animals without parental care; more than 80% are oviparous and less than 3% engage in parental care (Shine, 1988; Somma, 1990; Pyron and Burbrink, 2014). Some extinction events, including mass extinctions, involved changing climates, and reptiles and their ancestors may have used nest site choice to offset increasing developmental temperatures (e.g., by shifting the openness of nest sites across generations). Alternatively, but not mutually exclusively, embryonic thermal tolerances may have evolved, or thirdly, reptile ranges may have shifted toward and away from the poles as the climate warmed and cooled, respectively.

We are now on the verge of the sixth mass extinction event (Barnosky et al., 2011) and the climate continues to warm at an unprecedented rate (Allen et al., 2018). Moreover, in addition to climate change, reptiles, like other animals, are under threat from habitat loss and alteration, overexploitation, invasive species and pollution. We are at a critical juncture in which we must determine which species can use evolutionary rescue vs. which species need human intervention. If nesting is the most vulnerable stage for reptiles we need more knowledge about how nest site choice evolves or can evolve, consequences of developmental temperatures for offspring traits and survival, and factors affecting females' nesting decisions in order to predict effects of, and potential responses to, threats in vulnerable species.

The study of behaviors associated with (maternal) nest site choice in reptiles has lagged behind those on birds. For example, there are field guides to the nests and eggs of birds for most continents and many countries but no such field guides exist for reptiles (Doody et al., 2009). Our poor relative knowledge of nesting in reptiles is at least in part due to their secretive lives (Doody et al., 2009), but also to less relative research attention (Doody et al., 2013, 2021). Within reptiles we know more about nesting in turtles and crocodilians than we do for lizards and snakes partly due to nesting turtles of many species leaving tracks in the sand (Doody et al., 2021).

Our Research Topic intends to inch the science behind nesting in reptiles forward within the context of anthropogenic threats and potential evolutionary responses. Although our papers form a somewhat eclectic aggregation, they help funnel us toward a better understanding of reptile populations by providing valuable empirical data and relevant reviews. Herein we summarize these papers by examining common threads in concepts, disciplines, taxa, problems and proposed solutions.

Results

Our Research Topic included 17 papers; 15 of which addressed conservation directly or indirectly with nine considering evolutionary processes. Conceptually, most papers were ecological ($N = 16$), followed by behavioral ($N = 15$), developmental ($N = 14$), physiological ($N = 6$) and morphological ($N = 6$). Taxonomically, most papers focused on turtles ($N = 12$), followed by lizards ($N = 2$), crocodilians ($N = 2$) and snakes ($N = 1$). Geographically, most papers focused on species or populations in U.S. ($N = 7$), followed by Australia ($N = 3$), Africa ($N = 2$) and Mexico ($N = 1$), with four papers explicitly considering reptiles globally. Five of the 18 papers would be considered reviews. Most ($N = 15$) of the papers directly considered nest site choice or nesting biology.

Discussion

Here we summarize how this set of papers inches science forward within the context of our Research Topic. Our Research Topic was dominated by research with turtles. An age-old observation is the apparent link between rainfall and both the propensity to nest and nest survival in turtles. In a study of map turtles by Geller et al., although predation rate was lower when rain fell 24 h after nesting, turtles were more likely to nest on, or after, dry days than wet days. These findings were similar to those from their literature review; of 42 studies on 23 species of freshwater turtles, 29 studies (69%) demonstrated or suggested that rainfall increased the propensity to nest, while 13 (31%) did not. It is expected that future studies will reveal less of an association between rainfall and both propensity to nest and nest survival, because this review will loosen the grip that bias has on turtle researchers in this area. In another review, Geller and Parker used data and a literature review to determine that the main cue used by raccoons to locate turtle nests is soil disturbance. This will come as a surprise to many, as the traditional view is that the cues are the scent of the mother's cloacal fluids and/or the eggs. Recognizing the bias of studies in North America, the authors call for more studies of nest location cues by predators for turtles in other parts of the world where the predators are not raccoons. Keeping with nest survival, Duchak and Burke used natural and laboratory incubation experiments to show that 60% of low hatching success in wood turtles in New Jersey (USA) was linked to maternal identity (maternal effects), compared to 40% attributable to predation, flooding, and other environmental effects. The maternally-linked hatching failure in their population could be due to inbreeding, infertility, senescence, inadequate diet or environmental contamination, and this highlights the fact that the presence of many nests and low nest predation does not ensure sustainable reproductive rates. In contrast to low nest survival, Gravelle and Wyneken found high nest success in loggerhead sea turtle nests under a variety of incubation environments in both warm-temperate and subtropical climates in Florida (USA); nests in both bioclimatic zones differed in location, temperature, moisture levels, and clutch dimensions as well as the subtle genetic differences. There were highly successful hotspots, however, and the authors noted the potential for a simple and effective method for identifying high-priority conservation areas that would facilitate the maintenance of these hotspots for the recovery of imperiled loggerhead sea turtles through the management of essential habitats.

Focusing more on nest site choice within a climate change context, Sullivan et al. showed that mother Florida softshell turtles could offset climate effects on developing eggs by 2–3°C through nesting in more shaded areas. They also showed that canopy openness did not change considerably throughout incubation, giving nesting mothers some scope for predicting incubation temperatures. Finally, they showed that metabolic heating was ecologically negligible for a moderate-sized clutch of small eggs in this hot environment. In an overview paper, Topping and Valenzuela remind us that nest site choice behavior (along with maternal provisioning of the eggs) may be under strong selection because turtles typically lack parental care. To this end they offered three ideas not typically considered in the literature: (1) how water temperature impacts basking behavior, an abiotic factor that influences female physiology, which in turn may alter the timing of nesting and resource allocation to the eggs; (2) how biotic factors such as social facilitation influences nest-site choice; and (3) how water and not just air and soil temperature may affect the conditions experienced by developing embryos in the nest. Finally, they remind us that we lack solid evidence that nest site choice behavior is heritable, a critical trait for forecasting climate change responses. Fukuda et al. showed that while forecasted changes in rainfall and temperatures associated with climate warming could affect the nest success of saltwater crocodiles, sea level rise may be more of an imminent threat: their models predicted a loss of 49% of nesting habitat between 2013 and 2100, compared to mixed effects forecasted for temperature and moisture. The authors, however, underscored the need to determine the expansion of new nesting habitat which could offset those losses. For American crocodiles, Mazzotti et al., after finding a dramatic increase in the number of nests during 1970–2020 and mixed anthropogenic effects on nesting success, noted that the bet-hedging nesting strategy of the species provides a potential evolutionary advantage in climate warming scenarios. However, the authors were careful to suggest that there was likely a limit to the adaptive capacity of the species to face climate change. Anoles have been a model system for studying ectotherm ecology and evolution, including behavioral plasticity, but much less attention has been given to their nest site choice behavior according to Pruett et al., who review nesting behavior and developmental plasticity in the group. In addition to identifying the need for more nesting studies, the authors call for more field-relevant studies of behavioral plasticity using natural nests; most experiments to date poorly reflect natural nest environments but have served as a foundation for our current understanding and future work. Finally, the authors noted that anoles provide a good system for examining the effects of global change due to the marked effects of temperature on their very shallow nests and the wide variety of habitats and microhabitats (including anthropogenic ones) that create temperature- and moisture-related challenges for developing embryos.

A few studies of nest site choice examined issues other than global climate change. For example, Patino-Martinez et al. tackled the complex relationship that can occur among substrate type and color, temperature, moisture, hatchling size, hatchling performance, hatching success and predator abundance in loggerhead sea turtles. In their hatchery experiments, egg incubation in light-colored sand led to higher hatching success and larger and physically fitter hatchlings. However, this was not the case for field data, and mothers chose to nest at similar densities in beaches with light vs. dark sand. They concluded that the population may be exhibiting a bet-hedging strategy in which different clutches might perform better on different substrates. Nesting strategies may also result in different outcomes depending on the life stage considered. Refsnider et al. measured the impacts of nest site characteristics on both hatching success and survival of neonates in eastern box turtles, a declining species. They found that hatching success was highest in nests that were deep and farthest from habitat edges, but survival of neonates was highest from shallow nests under minimal shade cover, which demonstrates that nesting females face a tradeoff between maximizing hatching success vs. neonate survival when constructing a nest cavity. The diamondback terrapin is another turtle species in decline, prompting Butler et al. to locate evidence of terrapins including nesting areas in four counties in Florida. Nests, which were mainly found by finding eggshells from raccoon depredation, were more likely to occur among Christmas Berry bushes but less likely to occur among oaks and wax myrtles. The authors explain the former is usually the first woody vegetation encountered as terrapins proceed inland from the water's edge and provides cover that may provide desirable thermal conditions for terrapin egg development. In contrast, oak and wax myrtle, when present in coastal regions, are typically found further inland; their thicker canopies may lead to lower nest temperatures which could reduce nest survival and lead to the overproduction of male hatchlings. Moreover, this cover may provide cover for mammalian nest predators. Shifting from predators to abiotic factors, Cassill et al., after recording that hatching success of loggerhead sea turtles was 65% in the face of several hurricanes in south Florida, found that hatching success was significantly influenced by distance from the high water line, distance from the vegetation line, and location along the beach axis.

It is typical for some papers in a Research Topic to cover conceptual areas linked to the main subject area rather than the subject area itself. In perhaps the most novel and exciting paper in this issue, Kuchling and Hofmeyr reveal evidence for viviparity – specifically egg retention to the hatching stage by a mother, in an elongated tortoise. This is the first reported case of viviparity in a turtle. The authors proposed that this facultative viviparity may have evolved to buffer embryos from excessively hot temperatures that would have been experienced in a natural nest. This mechanism of thermoregulating the eggs has been offered for the evolution of viviparity in lizards and snakes evolved in cold climates. Brown and Shine tested the possibility that embryogenesis may be affected by shifts in soil microbiota caused by anthropogenic disturbance, translocation of eggs for conservation purposes, or laboratory incubation in sterile media, by incubating the eggs of keelback snakes in untreated vs. autoclaved soil and by injecting lipopolysaccharide (LPS) into the egg to induce an immune response in the embryo. Neither autoclaved soil nor LPS-injected eggs affected hatching success, water uptake, incubation period, or white-blood-cell profiles, but both treatments reduced hatchling size. They concluded that microbiota in the incubation medium can affect viability-relevant phenotypic traits of hatchling reptiles and called for more studies to explore the complex mechanisms and impacts of environmental conditions on reptilian embryogenesis. Unda-Díaz et al. examined side effects of sea turtle egg relocation and hatchery incubation by comparing development and performance between hatchlings of olive ridley sea turtles incubated *ex situ* vs. *in situ*. Turtles from *ex-situ* clutches showed fewer proliferating cells in the dorsal and medial ventricular zones, less mature neurons in the dorsomedial and medial cortices, ovaries with a lesser number of proliferating cells, lower body mass and length at emergence and lower self-righting time. The authors called for future studies to disentangle the differential contribution of egg movement, reburial, nesting environment and parental origin to development. This information, they argue, would likely result in better conservation strategies for sea turtles. Abayarathna and Webb found that incubation temperatures did not influence the thermal preferences of hatchling velvet geckos; however, diet did. They concluded that predicting how future changes in nest temperatures will affect reptiles will require a better understanding of how incubation and post-hatchling environments shape hatchling phenotypes. These three papers, although not directly addressing nest site choice, have real implications for climate change responses in reptile populations.

We anticipate that these 17 papers, taken together, will advance the science of nesting in reptiles within the context of evolutionary and anthropogenic change. Importantly, many of these papers challenge assumptions about nesting ecology of reptiles, and thus have generated many important questions that can be pursued in future endeavors to better understand the likely impacts of anthropogenic impacts on reptiles and their capacity to respond.

Author contributions

JD wrote the first draft. JR contributed to and edited the draft. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Do Incubation Temperatures Affect the Preferred Body Temperatures of Hatchling Velvet Geckos?

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In many lizards, a mother's choice of nest site can influence the thermal and hydric regimes experienced by developing embryos, which in turn can influence key traits putatively linked to fitness, such as body size, learning ability, and locomotor performance. Future increases in nest temperatures predicted under climate warming could potentially influence hatchling traits in many reptiles. In this study, we investigated whether future nest temperatures affected the thermal preferences of hatchling velvet geckos, *Amalosia lesueurii*. We incubated eggs under two fluctuating temperature treatments; the warm treatment mimicked temperatures of currently used communal nests (mean = 24.3°C, range 18.4–31.1°C), while the hot treatment (mean = 28.9°C, range 20.7–38.1°C) mimicked potential temperatures likely to occur during hot summers. We placed hatchlings inside a thermal gradient and measured their preferred body temperatures (T_bs) after they had access to food, and after they had fasted for 5 days. We found that hatchling feeding status significantly affected their preferred T_bs. Hatchlings maintained higher T_bs after feeding (mean = 30.6°C, interquartile range = 29.6–32.0°C) than when they had fasted for 5 d (mean = 25.8°C, interquartile range = 24.7–26.9°C). Surprisingly, we found that incubation temperatures did not influence the thermal preferences of hatchling velvet geckos. Hence, predicting how future changes in nest temperatures will affect reptiles will require a better understanding of how incubation and post-hatchling environments shape hatchling phenotypes.

Keywords: heatwave, nest temperature regulation, reptile, developmental plasticity, climate change

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INTRODUCTION

Developmental plasticity, the changes in the phenotype induced by the environment experienced by the developing embryo, is an important source of variation for many organismal traits that can influence individual fitness (West-Eberhardt, 2003). In most species of oviparous reptiles, females abandon their eggs after laying them in nests (Reynolds et al., 2002). In the absence of parental care, the thermal and hydric conditions inside reptile nests can vary markedly throughout the incubation period. For example, nest temperatures often fluctuate widely on a daily basis (Shine and Harlow, 1996; Andrews and Warner, 2002), and can vary depending on local weather conditions (Shine, 2004). In the last few decades, a large body of experimental research has demonstrated that incubation temperatures can influence a multitude of offspring traits, including sex, morphology, behavior, performance, and cognitive abilities (Deeming and Ferguson, 1991; Deeming, 2004; Noble et al., 2018; While et al., 2018). Some of these developmental effects can be long lasting, and can influence the growth and survival of offspring (Qualls and Andrews, 1999; Andrews et al., 2000; Dayananda et al., 2016) and may influence lifetime reproductive success (Warner and Shine, 2008). Thus, an understanding of thermal developmental plasticity can provide insights into how reptiles may cope with changing environments (Mitchell et al., 2008; Angilletta, 2009; Carlo et al., 2018).

Most research on thermal developmental plasticity has focused on how incubation temperatures affect morphological traits, physiology, sex ratios and incubation duration (While et al., 2018). By contrast, few studies have investigated whether incubation temperatures can also influence the thermal preferences or thermal tolerances of hatchlings (Lang, 1987; Blumberg et al., 2002; Du et al., 2010; Dayananda et al., 2017; Abayarathna et al., 2019; Refsnider et al., 2019). Most lizards maintain their body temperature (hereafter, T_b) within a preferred range by carefully selecting suitable microhabitats, altering their behavior, or by adjusting their posture, shape, or color (Huey, 1982). In turn, selected T_bs influence the physiology, behavior, performance, activity budgets, and growth of individuals, which can influence their survival and reproduction (Huey, 1982; Angilletta, 2009). Thus, incubation-induced plasticity in preferred body temperatures (T_{pref}) may have important fitness consequences for hatchling lizards. More broadly, an understanding of how incubation temperatures influence the T_{pref} of lizards is important for predicting how species may fare under future climates (Huey et al., 2012).

Experimental studies on lizards have found that incubation temperatures may affect the thermoregulatory behavior of hatchlings of some species, but not others (Du et al., 2010; Refsnider et al., 2019). For example, incubation temperatures did not affect the selected T_bs of hatchling veiled chameleons *Chamaeleo calyptratus* (Andrews, 2008), three lined skinks *Bassiana duperreyi* (Du et al., 2010), or Cuban rock iguanas *Cyclura nubila* (Alberts et al., 1997). By contrast, other studies reported the opposite effect. For example, in the Madagascar ground gecko, *Paroedura pictus*, hatchlings from hot incubation temperatures had higher dorsal temperatures prior to crossing between the cold and hot sides of a thermal shuttle apparatus (Blumberg et al., 2002). In *Sceloporus virgatus*, hatchlings from cold temperature incubation (15–25°C) selected higher T_bs, and maintained T_bs more precisely than hatchlings from hot temperature (20–30°C) incubation (Qualls and Andrews, 1999). In a study on Jacky dragons (*Amphibolurus muricatus*) using constant temperature incubation, hatchlings from the 28.1°C treatment had lower T_bs after 2 h in a thermal gradient than hatchlings from 25 or 32°C treatments (Esquerre et al., 2014). Despite evidence that incubation temperatures can affect the T_{pref} of hatchlings, it is unclear whether such incubation induced shifts are ecologically relevant, particularly if the effects are short lived or are masked by interactions with the post-hatching environment (Andrews et al., 2000; Buckley et al., 2007). For example, incubation-induced differences in T_{pref} of hatchlings might have little effect on subsequent growth or survival if lizards shift T_{pref} in response to food availability. In some lizards, individuals may elevate their T_{pref} after feeding, or may select cooler T_{pref} when food is scarce (Brown and Griffin, 2005). Such thermophilic responses to feeding might mask or swamp developmental shifts in thermoregulatory behavior. Hence, to understand the ecological significance of incubation-induced shifts in T_{pref}, we also need to assess whether other sources of variation such as feeding influence the T_{pref} of hatchlings.

In this study, we carried out an experiment to test whether thermal conditions during incubation affected the thermal preferences of hatchling velvet geckos, *Amalosia lesueurii*. Velvet geckos lay eggs communally in nest crevices, and maximum daily nest temperatures are positively correlated with maximum daily air temperatures (Dayananda et al., 2016). Thermal data collected from 21 nests in 2018–2019 revealed that the slope of the relationship between air temperature and nest temperature was greater than one in 24% of nests (Cuartas-Villa and Webb, 2021). Because the frequency and intensity of heatwaves is predicted to increase in future (Cowan et al., 2020; Trancoso et al., 2020), it is likely that some nests will become hotter in future. To determine how such changes might affect phenotypic traits of hatchlings, we incubated eggs under a “cold” (mean = 24.3°C, range 18.4–31.1°C) and “hot” treatment (mean = 28.9°C, range 19.1–38.1°C) to mimic current vs. potential future nest temperatures. We predicted that hatchlings from hot incubation should have higher T_{pref} than hatchlings from cold incubation; i.e., local adaptation hypothesis (Levinton, 1983). Our null hypothesis was that incubation temperature would not influence T_{pref}. However, it is possible that developmental shifts in T_{pref} might not be detectable if T_{pref} is influenced by environmental conditions in the post-hatching environment. To explore these hypotheses, we measured the preferred body temperatures of hatchlings in a cost-free thermal gradient. To assess whether feeding influenced gecko body temperatures, we measured the hatchling’s body temperatures after feeding and fasting.

MATERIALS AND METHODS

Study Species

The velvet gecko, *Amalosia lesueurii,* is a small (up to 65 mm snout to vent length), nocturnal lizard that inhabits sandstone rock outcrops from south eastern New South Wales to south-eastern Queensland (Cogger, 2014). By day the geckos thermoregulate under small, sun-exposed stones (Schlesinger and Shine, 1994; Webb et al., 2008). At dusk, they venture from their rocks or crevices to forage in leaf litter (Cogger, 2014). Female velvet geckos lay eggs in communal nests located in rock crevices in late spring, and the eggs hatch from February to March (Webb et al., 2008). After emergence, hatchlings settle under small stones located near the communal nests, and they spend the first eight months of life sheltering beneath one or two rocks (Webb, 2006). Annual observations of communal nests at three study sites in Morton National Park, NSW, have revealed that gravid geckos have laid eggs inside the same communal nests since 1992 (Webb, unpublished data). Previous studies have shown that maximum daily nest temperatures are positively correlated with maximum daily air temperatures (Dayananda et al., 2016). In some nests, the slope of the relationship between nest and air temperature is greater than one

(Cuartas-Villa and Webb, 2021). Thus, temperatures inside some communal nests may increase in the future if the frequency and duration of summer heatwaves increases.

Egg Incubation Experiment

We carried out an egg incubation experiment to mimic thermal regimes inside currently used nests (hereafter, "cold") and thermal conditions that could occur inside nests during hot summers in 2,050 ("hot"). We programmed two identical incubators (Panasonic MIR 154, 10 step functions) to mimic the cycling temperatures that occur in natural nests at our study sites, in which nest temperatures cycle on a daily basis, but get hotter during summer heatwaves (**Figure 1**). Temperatures inside each incubator were recorded with four miniature data loggers (Thermochron DS1922L-F5#, accuracy of ± 0.5°C) that were placed inside100 ml glass jars filled with egg incubation media (see below), and sealed with cling wrap. These were positioned at the front and rear of the top and bottom shelves of each incubator. Temperatures in the cold treatment (mean = 24.3°C, range 18.4–31.1°C, SD = 3.2°C) were similar to those recorded inside sun-exposed communal nests (Dayananda et al., 2016). Temperatures in the hot treatment (mean = 28.9°C, range 20.7°C–38.1°C, SD = 4.3°C) cycled in exactly the same way as the "cold" treatment (**Figure 1**), except that mean temperatures were 4.6°C higher. Temperatures in the hot treatment were on average, 2°C higher than the temperatures recorded inside four sun-exposed communal nests from Morton National Park over the period 23 November 2018 to 28 January 2019 (mean nest temperature = 26.9°C range 15.8–36.7°C, Cuartas-Villa and Webb, 2021). This treatment simulated the potential future nest temperatures that could occur in 2,050, based on the predicted increases in air temperatures between 2.9 and 4.6°C that are forecast for southeast Australia by climate modelers (Dowdy et al., 2015).

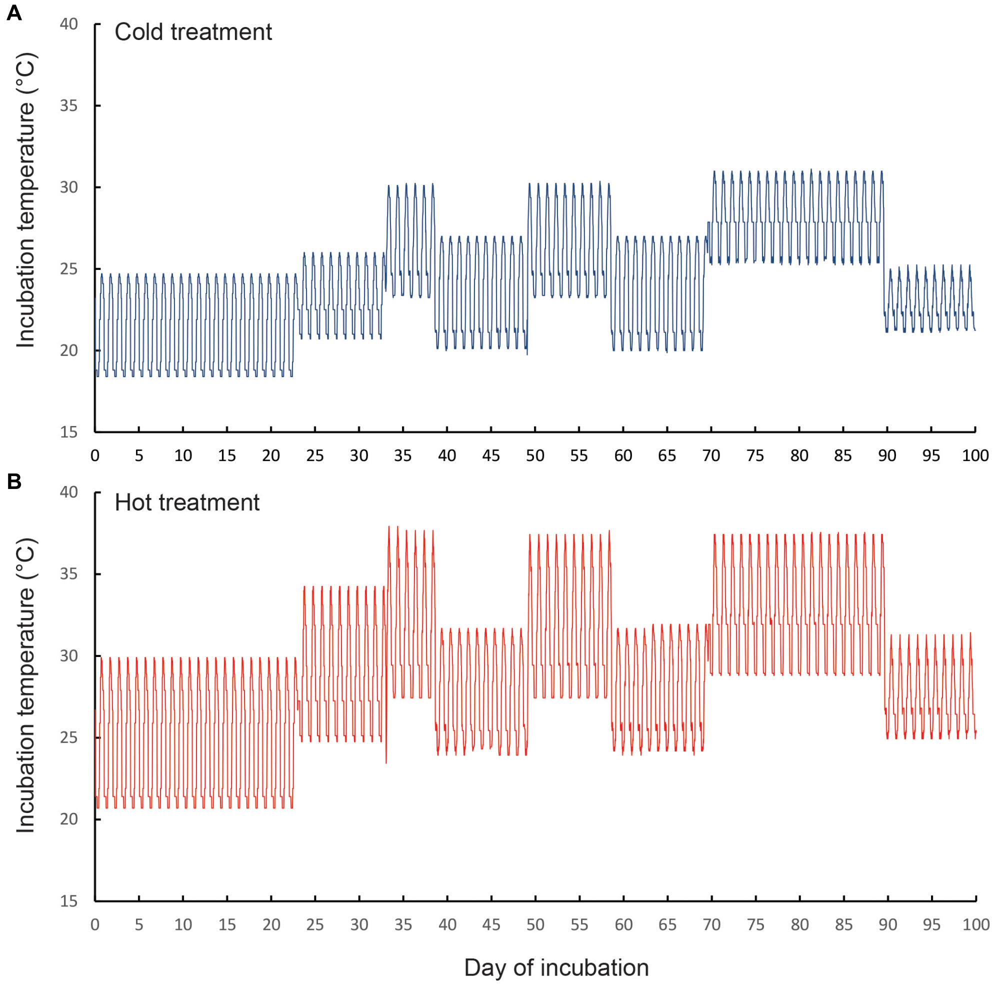

FIGURE 1 | Thermal regimes that we programmed for the **(A)** cold incubation treatment and **(B)** hot incubation treatment. The temperature regimes of the cold treatment mimicked thermal regimes that we have recorded inside the communal nests of our focal species Amalosia lesueurii under the current climate (Cuartas-Villa and Webb, 2021). Temperature regimes of the hot treatment are potential nest temperatures likely to occur under future climates. Note that in both treatments, temperatures fluctuated daily, and increased during the incubation period, to simulate the thermal regimes that occur in natural nests over spring and summer. The three elevated spikes in temperature (at around days 35, 55, and 80 of incubation) correspond to heatwaves of varying durations.

After programming the incubators, we brought gravid females into the lab, and after oviposition, we placed eggs singly inside 100 mL glass jars filled with moist vermiculite (water potential of 200 KPa) and covered each jar with plastic food wrap to prevent the eggs from desiccating. We randomly allocated one egg from each clutch of two eggs produced by each female to each of two programmable incubators. Full details of collection of geckos, husbandry, incubation regimes, and incubation periods and hatching success, are presented elsewhere (Abayarathna et al., 2019).

Measurement of Preferred Body Temperatures

After hatching, we housed hatchlings individually in plastic containers (Sistema NZ 2.0 L, 220 × 150 × 60 mm) with a paper substrate, a plastic half pipe and a water dish. We placed the hatchling cages on timer-controlled heating cables set to 32°C, which created a thermal gradient (23–32°C) inside the cages during the day, while night time temperatures matched the room temperature of 23°C. We fed hatchlings with five pinhead crickets twice weekly, and cleaned their cages at weekly intervals. We recorded the T_bs of 22 four-week old hatchlings (10 hot-incubated and 12 cold-incubated hatchlings, all from Dharawal) inside a thermal gradient. We did not measure the lizards' preferred body temperatures prior to this age as the hatchlings were used in another study in which we measured their learning abilities using a Y maze apparatus (Abayarathna and Webb, 2020). We recognize that testing hatchlings at 4 weeks of age is a limitation of our study; however, if incubation temperatures induce biologically meaningful shifts in hatchling preferred body temperatures, such effects should be detectable in the first eight weeks of life (Buckley et al., 2007).

The thermal gradient consisted of a wooden enclosure (1.5 m long × 0.5 m wide × 0.5 m high) with a mesh lid and a clear glass front at one end (**Figure 2**). We partitioned the enclosure into 8 lanes, each 1.5 m long and 6 cm wide, each of which contained a 1.4 m long white plastic half pipe as a shelter, with a water dish in the middle. To create the thermal gradient, we placed the cage on a wooden base that contained heating cables at one end, and plastic tubes connected to a water bath (Haake F3 K Circulating Water Bath) carrying chilled water (5°C) at the other end (**Figure 2**). Two 250-watt infrared lamps provided additional heating at the hot end. The substrate temperatures within the thermal gradient ranged from 10 to 40°C. To measure the substrate temperature within the gradient, we placed miniature data loggers (Thermochron i-buttons, factory calibrated and accurate to ± 0.1°C) along the floor of each lane. The data loggers recorded the temperature every 60 min.

To measure the preferred T_b of the hatchlings, we placed each hatchling in the middle of each lane of the thermal gradient at 0900 h. We estimated thermal preferences of hatchlings during the day because the geckos thermoregulate under rocks during the day time, as do other geckos (Kearney and Predavec, 2000). After 1 h of acclimation, we observed the location of each hatchling though the front glass wall, and recorded the numbers of the data loggers nearest to the lizard. If we could not see the hatchling, we confirmed its position by gently lifting the half pipe without disturbing the animal. In such cases, we recorded the lizard's T_b with an infrared thermometer (Cool Tech, CT663, spot diameter = 13 mm). We repeated this procedure every hour from 1,000 to 1,700 h. We used substrate temperature

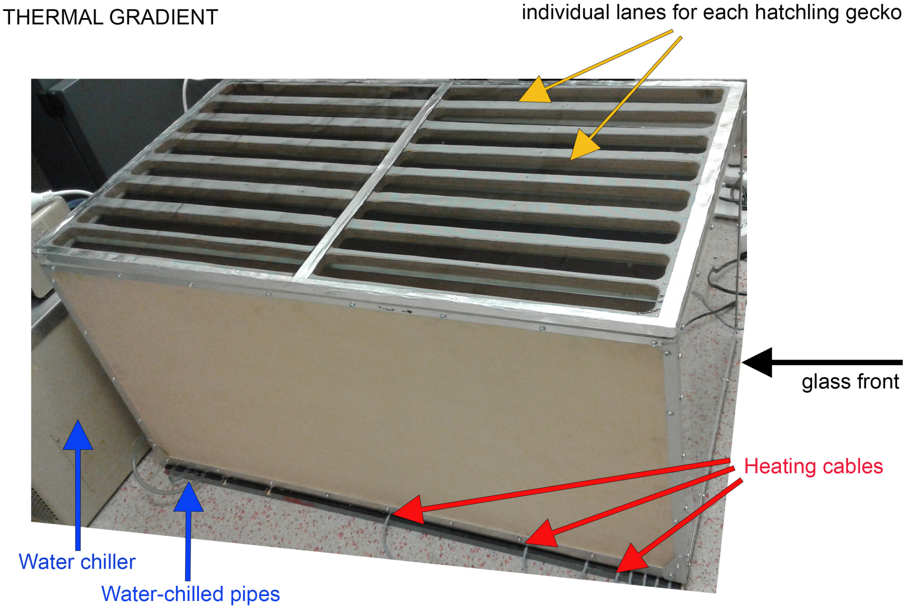

FIGURE 2 | Photograph of the thermal gradient used to measure preferred body temperatures of hatchling velvet geckos. Each gecko was placed in a separate runway, which contained a plastic half pipe for shelter, and a water dish. Two 250-watt infrared lamps provided additional heating at the hot end (not shown in the photo).

as a proxy for lizard T_b (Buckley et al., 2007; Goodman and Walguarnery, 2007) because the hatchlings small body size (SVL < 30 mm, mass < 0.55 g) precluded the use of cloacal probes. In addition, the T_b of small lizards can change rapidly within seconds of handling, so aside from the risk of injuring the lizard, cloacal probes may not provide accurate estimates of hatchling T_bs. In addition, the capture of lizards could affect their subsequent behavior within the thermal gradient, which could affect their T_b. Although our method was crude, substrate temperatures recorded from data loggers near lizards were positively correlated with lizard temperatures that were measured with the IR thermometer ($r^2 = 0.94$, $P < 0.001$). To assess whether feeding influenced the T_b of hatchling geckos, we tested lizards under their normal feeding regime. The order of feeding was counterbalanced across each cohort to avoid any possibility of an order effect influencing results. For the fasted treatment, lizards were not fed for 5 days prior to placement in the thermal gradient, which allowed us to compare our results with other studies on lizards (Brown and Griffin, 2005).

Statistical Analyses

For each individual lizard, we calculated the mean body temperature (T_b) in the thermal gradient, and the 25 and 75% quartiles, after feeding, and prior to feeding (Hertz et al., 1993). These metrics allowed us to compare the preferred body temperatures of hatchlings before and after feeding. To determine whether incubation treatment or feeding status affected hatchling body temperatures, we used repeated-measures ANOVA. In this analysis, hatchling body temperature was the dependent variable, while hour of day, and feeding status were the within subjects effects, and incubation treatment was the between subjects effect. Although we used a split-clutch design, and placed one egg from each clutch of two eggs into each incubator, only two hatchlings had the same mother. For this reason, we did not include maternal ID as a factor in our analyses. Prior to carrying out the analysis, we checked that data met the assumptions of homogeneity of variances (Levene's test, $P = 0.31$) and were normally distributed (Shapiro-Wilks tests). As data transformations did not solve the problem of non-normality, we elected not to transform raw data prior to the analysis, as ANOVA is robust to departures of normality (Schmider et al., 2010). However, because the data did not meet the assumptions of sphericity for hour (Mauchley's $W = 0.038$, $P = 0.001$) or feeding × hour (Mauchley's $W = 0.066$, $P = 0.01$), we used the Greenhouse-Geisser correction for determining the significance of F-tests (Field, 2013). We ran statistical analyses using SPSS version 26.

RESULTS

Lizards from both hot and cold incubation treatments showed very similar patterns of thermoregulation (**Figure 3**), and had similar preferred T_bs before feeding (mean T_bs = 25.9 and 25.7°C, respectively) and after feeding (mean T_bs = 30.7 and 30.4°C, respectively). Both cold-incubated and hot-incubated lizards showed similar patterns of thermoregulation, with hatchlings maintaining higher body temperatures (T_bs) after midday than during the morning (Fig. 3). Lizard body temperatures varied significantly with hour of day [$F_{(3\cdot4,\ 68\cdot6)} = 20.73$, $P < 0.01$], but there was no interaction between hour and incubation treatment [$F_{(3\cdot4,\ 85\cdot4)} = 2.25$, $P = 0.08$] nor between feeding status, incubation treatment and hour [$F_{(3\cdot4,\ 85\cdot4)} = 0.99$, $P = 0.42$]. However, lizard feeding status significantly affected body temperatures, with lizards maintaining higher T_bs after feeding than prior to feeding [$F_{(1,20)} = 206.78$, $P = 0.0001$, **Figure 3**]. There was also a significant interaction between feeding status and hour [$F_{(4\cdot3,\ 85\cdot4)} = 3.97$, $P = 0.004$], reflecting the fact that at 10 a.m., 1 h after being placed in the gradient, body temperatures of fed and unfed lizards were similar (**Figure 3**). Thereafter, body temperatures of recently-fed lizards were higher than those of fasted lizards during each hour of the day (**Figure 3**). Lizards selected higher body temperatures in the thermal gradient after feeding (mean T_b = 30.6°C, IQR = 29.6–32.0°C, range = 23.5–35.5°C) than before feeding (mean T_b = 25.8°C, IQR = 24.7–26.9°C, range = 20.0–32.5°C). Overall, there was no significant effect of incubation treatment on hatchling T_bs [$F_{(1,\ 20)} = 0.13$, $P = 0.73$] and no interaction between feeding status and incubation treatment [$F_{(1,20)} = 0.04$, $P = 0.84$].

DISCUSSION

We predicted that hatchlings from the hot incubation treatment would select higher preferred body temperatures (T_{pref}) than hatchlings from the cold treatment. Contrary to our prediction, we found no evidence that incubation temperatures affected the thermal preferences of 4-week old hatchlings. Indeed, mean selected T_bs of cold- and hot-incubated hatchlings were very similar, as was the precision of thermoregulation (**Figure 3**). Although we measured body temperatures of hatchlings at age four weeks, our findings agree with the results of previous studies, which found no effect of incubation temperatures on selected T_bs of hatchlings during the 2 months of life in veiled chameleons *Chamaeleo calyptratus* (Andrews, 2008), and western fence lizards *Sceloporus occidentalis* (Buckley et al., 2007). Other studies reported no effect of incubation on selected T_bs of 1-week old three lined skinks *Bassiana duperreyi* (Du et al., 2010) or 14–16 month old Cuban rock iguanas *Cyclura nubila* (Alberts et al., 1997). By contrast, other studies have reported that incubation temperatures can influence the thermoregulatory behavior of hatchlings. For example, in western fence lizards hatchlings from a warm-incubation treatment thermoregulated more precisely than lizards from a cool-incubation treatment, and this effect persisted for at least seven weeks post hatching (Buckley et al., 2007). In the Madagascar ground gecko, *Paroedura pictus*, hatchlings from a hot incubation treatment maintained higher dorsal temperatures prior to crossing between the cold and hot sides of a thermal shuttle apparatus at night, and this effect persisted for several weeks after hatching (Blumberg et al., 2002). Studies on other ectotherms suggest that as for reptiles, the effects of developmental temperatures on preferred body temperatures are mixed (Dillon et al., 2009). For example, in some *Drosophila*

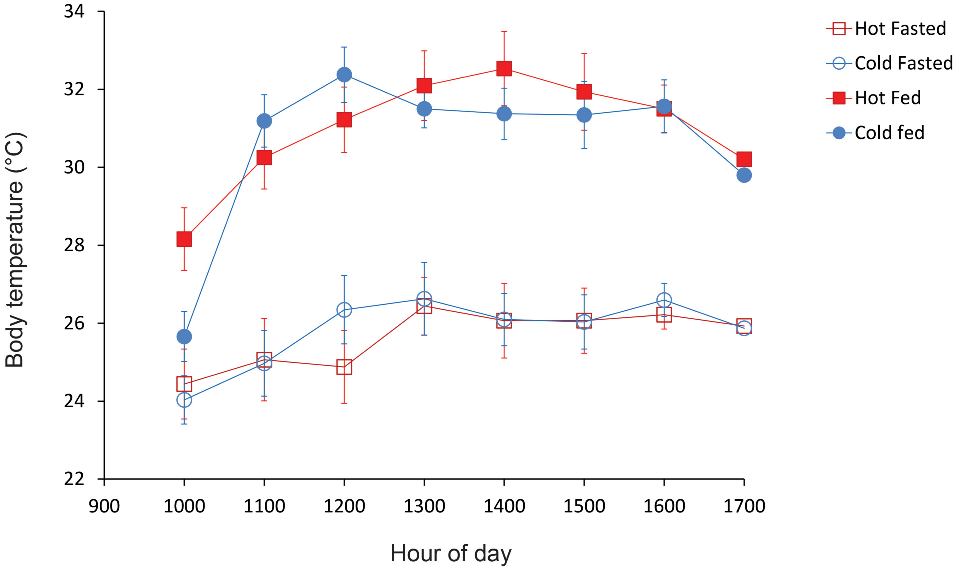

FIGURE 3 | Mean T_bs of cold-incubated and hot-incubated hatchling velvet geckos that were placed inside a thermal gradient between 0900 and 1,500 h. Figure shows the temperature profiles of recently fed lizards and lizards that were fasted for 5 days. Error bars denote standard errors.

species, flies reared at 25°C had higher T_{pref} than those reared at 20°C (Yamamoto and Ohba, 1984). In *Drosophila melanogaster,* adults selected lower temperatures when reared at 28°C than when reared at 19 or 25°C (Krstevska and Hoffmann, 1994). These mixed results suggest that like other traits, reaction norms for thermal preferences may be non-linear (Noble et al., 2018). Hence, we cannot rule out the possibility that intermediate incubation temperatures might affect preferred T_bs of velvet geckos. Future studies, using intermediate temperatures, and larger sample sizes, would help resolve this issue.

Ultimately, the biological relevance of incubation-induced shifts in preferred T_bs will depend on the magnitude and duration of such effects relative to other sources of environmental variation (Booth, 2018). Notably, several studies have shown that incubation-induced shifts in T_b are transitory, so are unlikely to influence traits linked to fitness (Buckley et al., 2007; Goodman and Walguarnery, 2007). In the present study, hatchlings maintained significantly higher T_bs after feeding (fed: mean $T_b = 30.6$°C; fasted mean $T_b = 25.8$°C), demonstrating that food availability has large effects on hatchling T_bs. Thermophilic responses to feeding are widespread in snakes (Blouin-Demers and Weatherhead, 2001) but are less common in lizards (Wall and Shine, 2008; Schuler et al., 2011). Notably, the 4.8°C increase in mean T_b of hatchling geckos after feeding is similar to that reported for snakes in thermal gradients (typically, increases of 2–6°C (Lysenko and Gillis, 1980; Slip and Shine, 1988; Tsai and Tu, 2005), but is higher than the 3.1°C increase reported for adults of our study species (Dayananda and Webb, 2020), or the modest increases (typically, <2°C), reported for lizards such as *Heloderma suspectum* (Gienger et al., 2013) and *Anolis carolinensis* (Brown and Griffin, 2005). Future studies on hatchlings of other lizard species in this respect, particularly geckos, would help to evaluate the generality of our results.

Why do fasted hatchlings select lower T_bs than recently fed individuals? After feeding, selection of higher T_bs likely maximizes digestive efficiency and rates of energy assimilation (Harlow et al., 1976; Beaupre et al., 1993). However, because metabolic rates scale with T_b, maintenance of high T_bs increases energy expenditure (Angilletta, 2009). Therefore, in the absence of food, hatchlings may select lower T_bs to reduce energy expenditure. Conserving energy might be particularly important for hatchlings, as they may lack sufficient energy reserves in their tails to survive long periods in the absence of food (Greer, 1989). Ultimately, shifts in T_b in response to food availability may represent a trade-off between energy conservation vs. maintenance of other fitness related behaviors (Huey, 1982). For example, adults of Yarrow's spiny lizard *Sceloporus jarrovi* that were deprived of food for five days maintained high T_bs, presumably so they could maximize important fitness related behaviors such as territory defense (Schuler et al., 2011). In the wild, hatchling velvet geckos congregate under rocks near communal nest sites, and hatchlings often share rocks with conspecifics during the first few months of life (Webb, 2006), so territory defense may be unimportant during this period.

Irrespective of feeding status, hatchling geckos displayed diel variation in preferred T_bs. Hatchlings selected low T_bs in the morning, and thereafter they raised their T_b and maintained elevated temperatures throughout the afternoon (**Figure 3**). Similar diel patterns of thermoregulation were reported for individuals of two gecko species, *Eublepharis macularius* and *Oedura marmorata*, that were fasted for 3 days before being placed in a thermal gradient (Angilletta et al., 1999). Similarly, individuals of the gecko *Tarentola mauritanica* increased their T_bs during the day (Gill, 1994). The underlying cause for this pattern of thermoregulation in geckos is not known, but we note that T_bs of hatchling *A. lesueurii* follow the same pattern as rock temperatures; i.e., delayed heating, reaching a peak in early afternoon (Webb and Shine, 1998). Potentially, this pattern might represent an entrained circadian rhythm for activity or thermoregulation (Refinetti and Susalka, 1997; Tawa et al., 2014). Because hatchling geckos commence foraging shortly after dusk, maintaining high T_bs around dusk would aid in prey capture and potentially, escape from predators (Christian and Tracy, 1981). As for diurnal lizards, maintenance of high T_bs during daylight hours would facilitate physiological processes such as digestion, growth and sloughing (Huey, 1982; Angilletta et al., 1999).

CONCLUSION

In conclusion, we found no effects of incubation temperature on the thermal preference of hatchling velvet geckos. However, there was a strong effect of feeding status on the hatchlings thermal preference, suggesting that food availability may influence thermoregulation by hatchlings in the wild. To evaluate the role of thermal developmental plasticity on the thermal preferences of hatchling lizards, future studies should not only estimate the duration of such effects, but also, their magnitude relative to plasticity caused by the post-hatching environment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Technology Animal Care and Ethics Committee.

AUTHOR CONTRIBUTIONS

TA and JW contributed to conception, design of the study, contributed to manuscript revision, read, and approved the submitted and revised versions. TA carried out the experiments, wrote the first draft of the manuscript, and organized the database. JW performed the statistical analysis and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.727602/full#supplementary-material

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Turtle Nest-Site Choice, Anthropogenic Challenges, and Evolutionary Potential for Adaptation

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Oviparous animals, such as turtles, lay eggs whose success or demise depends on environmental conditions that influence offspring phenotype (morphology, physiology, and in many reptiles, also sex determination), growth, and survival, while in the nest and post-hatching. Consequently, because turtles display little parental care, maternal provisioning of the eggs and female nesting behavior are under strong selection. But the consequences of when and where nests are laid are affected by anthropogenic habitat disturbances that alter suitable nesting areas, expose eggs to contaminants in the wild, and modify the thermal and hydric environment experienced by developing embryos, thus impacting hatchling survival and the sexual fate of taxa with temperature-dependent sex determination (TSD) and genotypic sex determination (GSD). Indeed, global and local environmental change influences air, water, and soil temperature and moisture, which impact basking behavior, egg development, and conditions within the nest, potentially rendering current nesting strategies maladaptive as offspring mortality increases and TSD sex ratios become drastically skewed. Endocrine disruptors can sex reverse TSD and GSD embryos alike. Adapting to these challenges depends on genetic variation, and little to no heritability has been detected for nest-site behavior. However, modest heritability in threshold temperature (above and below which females or males develop in TSD taxa, respectively) exists in the wild, as well as interpopulation differences in the reaction norm of sex ratio to temperature, and potentially also in the expression of gene regulators of sexual development. If this variation reflects additive genetic components, some adaptation might be expected, provided that the pace of environmental change does not exceed the rate of evolution. Research remains urgently needed to fill current gaps in our understanding of the ecology and evolution of nest-site choice and its adaptive potential, integrating across multiple levels of organization.

Keywords: oviposition-site selection, freshwater and marine reptile vertebrate, temperature-dependent sex determination, natural selection and heritability, genotypic sex determination, maternal effects and egg allocation, hatchling success and female fitness, adaptation to climate change

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INTRODUCTION

Reproduction is an essential component of individual fitness, and it must take place at the appropriate place and time for it to be successful. For oviparous animals such as turtles, nesting is a fundamental part of their reproductive cycle. Diverse strategies have evolved of nesting behavior combined with other maternal effects that can potentially maximize offspring and female fitness.

The main elements that make up a female reproductive strategy are: when, where, and how nesting takes place and the allocation of resources to the eggs laid in those nests. These variables are important given the thermal requirements for egg formation and embryonic development of ectotherms, such as turtles, and how embryogenesis is also restricted by moisture conditions in the nesting substrate in many species (Kamel and Mrosovsky, 2004). Here we first briefly review turtle maternal effects and nesting behavior, then the challenges posed by climate change and other anthropogenic disturbances, mostly in turtles with environmental sex determination, but also in turtles with sex ratios insensitive to temperature whose biology is still vulnerable to environmental change. We concentrate, not exclusively, on North American freshwater turtles, particularly those for which more data are available. **Figure 1** illustrates the various causes and consequences of nest-site choice.

MATERNAL EFFECTS OTHER THAN NEST-SITE SELECTION

Maternal effects in animals contribute significantly to their offspring's phenotype and fitness (Bernardo, 1996; Moore et al., 2019). One potential maternal effect is parental care after oviposition, which researchers often describe as lacking in turtles, but examples of parental care exist in at least a few species. These include nest guarding in natural populations of the mud turtle, *Kinosternon flavescens*, potentially reducing predation and altering the nest moisture conditions in ways that improve offspring fitness (Iverson, 1990), as well as nest guarding in a captive Asian forest tortoise, *Manouria emys* (McKeown et al., 1982). Recently, vocalizations were recorded between hatchlings of the Amazonian giant river turtle (*Podocnemis expansa*) and females who waited nearby nesting beaches and communicated with newborns to guide them to feeding areas immediately after hatching (Ferrara et al., 2013). Thus, while few examples exist of parental care in turtles, growing evidence suggests that it plays a more prominent role in some species than previously thought. Nonetheless, because parental care is less extensive in turtles than in crocodilians and some python snakes (Shine, 1988; Balshine, 2012), nutrient and hormonal allocation to the eggs remain dominant maternal effects observed in this clade (Roosenburg and Dunham, 1997) other than nest-site choice.

Indeed, reproductive female turtles invest heavily into egg production, and different life-history traits have evolved to improve hatchling fitness. For instance, in several turtles, larger females, who possess more energy to allocate to reproduction, tend to produce more and/or larger eggs compared to smaller females, who may compensate by elongating egg shape (Rowe, 1994; Valenzuela, 2001a; Walde et al., 2007; Escalona et al., 2018). Yet, many turtles defy expectations from optimality models, such that egg size never reaches a plateau as female size increases. The positive correlation between female size and egg size can be vital because larger eggs produce larger hatchlings who are better at surviving (e.g., Miller, 1993; Valenzuela, 2001a; Rollinson and Brooks, 2008; Ceballos et al., 2014; reviewed for sea turtle hatchlings in Booth, 2017). Besides nutrients, females allocate hormones such as estrogen, progesterone, and testosterone into their eggs, affecting embryonic sexual development in turtles (Bowden and Paitz, 2021), of which only a brief overview is provided here.

Turtles exhibit two main types of sex-determining mechanisms by which embryos commit to their sexual fate: temperature-dependent sex determination (TSD) of various patterns and genotypic sex determination (GSD) with independently evolved female and male heterogametic sex chromosomes (Valenzuela and Lance, 2004; Tree of Sex Consortium, 2014; Bista and Valenzuela, 2020). No reported mixed system in turtles has withstood empirical scrutiny (Valenzuela et al., 2014; Mu et al., 2015), whereas sex reversals (Valenzuela et al., 2003) are documented in natural populations of other reptiles (reviewed in Whiteley et al., 2021). TSD is more prevalent in turtles than GSD, and its potential adaptive value has been studied extensively and is reviewed elsewhere (see Charnov and Bull, 1977; Valenzuela, 2004, 2021; Schwanz and Georges, 2021). TSD has received much attention in the last few decades, especially as TSD taxa must adapt to overcome environmental challenges such as rising global temperatures to avoid extinction. This is true for TSD turtles that produce males at low temperatures and females at high temperatures (TSD Ia) as well as for turtles that produce males at intermediate temperatures and females above and below (TSD II). Moreover, reptiles like tuatara, some lizards, and crocodilians that produce females at colder temperatures and males at warmer values (TSD Ib) (Valenzuela and Lance, 2004) might be even more vulnerable to global warming as population growth is severely reduced when females are scarce. Temperatures within turtle nests are influenced not only by global climate but by multiple environmental factors at micro-geographic and micro-temporal scales, such that the temperatures experienced by the developing embryos, including during the thermosensitive period for sex determination (Valenzuela, 2008; Mitchell et al., 2013; but see Gómez-Saldarriaga et al., 2016), are affected by the nest-site choices females make.

Maternal effects via the allocation of hormones also matter for sexual development because higher concentrations of yolk estrogen have a feminizing effect within clutches of TSD taxa, as in painted (*Chrysemys picta*) and red-eared slider (*Trachemys scripta*) turtles, particularly at intermediate temperatures around the pivotal value that produces 1:1 population sex ratios (Bowden et al., 2000, 2002; Bowden and Paitz, 2018). These yolk estrogen concentrations increase during the breeding season (Bowden et al., 2000, 2002), yielding more female-biased clutches later in the season (Bowden and Paitz, 2018). Thus, nesting phenology must be taken into account when analyzing sex ratio dynamics in populations. Turtles with GSD have been understudied in this respect, although no association between offspring sex and maternal yolk hormone levels was detected in two GSD softshell turtles, *Apalone mutica* and *A. spinifera* (Radder, 2007). Subtle effects of maternal age on sex ratios have also been documented in TSD turtles and leopard geckos (Roush and Rhen, 2018). Notably, females nesting in habitats polluted with endocrine-disrupting chemicals (EDCs) may pay fitness costs due to the increased mortality or suboptimal offspring phenotypes induced by these

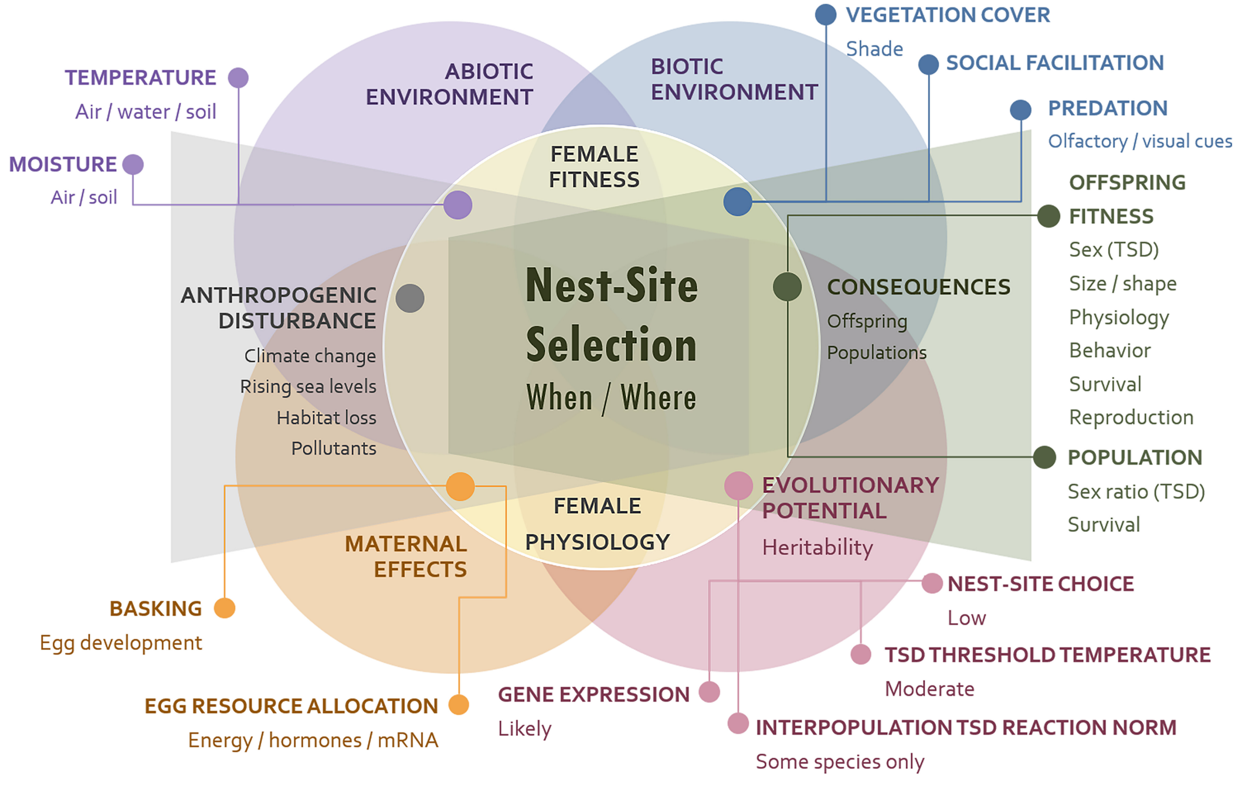

FIGURE 1 | Causes and consequences of nest-site choice. Biotic factors, e.g., predation risk, social facilitation, and vegetation cover, impact nest-site selection directly, whereas abiotic factors may have indirect effects, via their influence of air and water temperature on basking behavior and egg resource allocation. Vegetation cover plus air temperature and humidity affect the thermal and moisture conditions of the soil and those experienced by the developing embryos, thus impacting offspring phenotype and fitness, and population dynamics. Anthropogenic disturbances are detrimental for habitat quality and incubation conditions, and adaptive responses are less likely for nesting behaviors than for TSD reaction norms or for developmental gene networks. In TSD species whose reaction norms vary among localities, habitat loss may preclude recolonization of populations extirpated by environmental change from other populations pre-adapted to altered conditions.

substances, including altered sexual development (Mizoguchi and Valenzuela, 2016). Pollutants may be transferred maternally to the offspring, or eggs could be contaminated directly in the nesting substrate, leading to developmental abnormalities, sex reversal, abnormal sex steroid production, and reduced hatching success (Barraza et al., 2021).

WHEN TO NEST: BASKING AND TIMING OF OVIPOSITION

Egg formation concludes after fertilization but before females are ready to nest, and air temperature and basking behavior are crucial factors in that process as they influence body temperature. Basking elevates the internal body temperature of turtles above that of the water in which they live and toward their preferred temperature (Crawford et al., 1983). Some turtle species bask more than others, either on the shoreline, on floating logs, on debris in the water, and often using aquatic plants to support their weight (Schwarzkopf and Brooks, 1985). Basking in painted turtles differs by age (adults bask more than juveniles) and time of day (concentrating around 10–11 a.m.), but not by sex. In temperate turtles, basking occurs more frequently in the Spring and Fall when the difference between air and water temperature is greatest (Crawford et al., 1983). Still, as the water temperatures warm in the Spring and into the early Summer, basking events are less frequent and shorter in duration, as observed in painted turtles (Lefevre and Brooks, 1995).

Air temperatures differ seasonally in temperate regions, and most temperate turtle species lay eggs only during a restricted breeding season from Spring to Fall (Kennett, 1999), whereas some species and populations living in warmer climates, such as the gopher tortoise (*Gopherus polyphemus*), breed throughout the year (Allman et al., 2019). Environmental temperature affects the gonadal cycles of turtles, which determines the first date of nesting for a population. Not surprisingly, increased basking events raise body temperature and are associated with earlier nesting (these clutches hatch earlier), as shown in snapping turtles (*Chelydra serpentina*) (Bobyn and Brooks, 1994). Such earlier nesting in painted turtles increases the likelihood that a female produces a second clutch in the same season (Iverson and Smith, 1993; Krawchuk and Brooks, 1998). Likewise, warmer Springs elicit earlier onset of nesting in both painted and snapping turtles (Obbard and Brooks, 1987; Rowe et al., 2003; Grayson and Dorcas, 2004; Schwanz and Janzen, 2008). Thus, thermal pollution in aquatic systems from power plants (e.g., Crear et al., 2016) and global warming can potentially alter female physiology and nesting phenology (Hedrick et al., 2021). On the other hand, clutches laid too late in the Summer (typically or during protracted seasons due to global warming) may risk failure if temperatures drop below their thermal minimum before embryonic development is completed. Yet some species, such as the striped mud turtle, *Kinosternon baurii* that lay 1–3 clutches per year, avoid that risk as later-season clutches enter embryonic diapause and resume development in the Spring when temperatures warm (Wilson et al., 1999). However, embryonic diapause is rare among turtles [*K. scorpioides cruentatum* (Iverson, 2010), *Chelodina rugosa* (Kennett et al., 1993), perhaps others (Hernández-Montoya et al., 2017)], and thus, restricted to few multiple-clutch producing species as a potential response to climate change.

WHERE TO NEST: FEMALE NEST-SITE CHOICE

Turtles do not nest at random. Instead, natural selection shapes nesting behavior, and adult females select nesting sites that differ in their characteristics (e.g., vegetation cover, soil moisture, distance from water) from random locations (reviewed in Refsnider and Janzen, 2010). Nest-site choice can influence female survival, offspring phenotype, and offspring survival (during embryonic development or post-hatching) (Refsnider and Janzen, 2010) compared to random locations (Hughes and Brooks, 2006). For instance, female striped mud turtles (*Kinosternon baurii*) choose sites closer to vegetation cover that are 2°C cooler than random sites (Wilson, 1998), while snapping turtles (*Chelydra serpentina*) select nesting areas with shorter vegetation, more sand, and lacking cacti compared to other available locations (Kolbe and Janzen, 2002). The choices by these two TSD species influenced nest temperatures, potentially sex ratios, and in the case of *C. serpentina*, the survival probability of hatchlings traveling from their nest to the wetlands (Kolbe and Janzen, 2002). Similarly, some leatherback turtles (*Dermochelys coriacea*) lay nests close to ocean currents that will guide their offspring to suitable post-hatching habitats (Lohmann et al., 2008). In contrast, in the Australian GSD Murray River Turtle (*Emydura macquarii*), females select sites based on predation levels, closer to the shore in areas with high predation and further from the beach in low predation areas, thus trading off survival gains from optimal temperatures for development against predation risk (Spencer, 2002; Spencer and Thompson, 2003). This factor is essential because clutches are unprotected after oviposition, and predators (e.g., foxes, raccoons) may raid nests to eat the eggs, sometimes causing 90% loss of nests in a given location (Feinberg and Burke, 2003). This risk is highest soon after oviposition, as observed in diamond-backed terrapins (*Malaclemys terrapin*), whose nests suffered 71% predation on the first night (Burke et al., 2005). While predator nest detection was attributed to visual cues earlier, recent research demonstrated that predators likely use olfactory cues from disturbed soil to locate the nests, which may decrease in intensity over time, as observed in several turtle species (e.g., Buzuleciu et al., 2016; Edmunds et al., 2018; and references therein). Perhaps the most remarkable nest-site choice is observed in *Chelodina rugosa* turtles, whose females nest underwater in the inundated billabongs of Australia, where embryos remain in diapause until the substrate dries out and development proceeds (Kennett et al., 1993).

Importantly, females choose oviposition sites that decrease predation risk to themselves during nesting (Rand and Dugan, 1983) while also maximizing the fitness of their clutches as mentioned above. Yet, disturbance experienced by females while nesting can negatively affect their oviposition site choices. For instance, female painted turtles exposed to simulated predation (handled after they started excavating a nest), who were then released and allowed complete nesting, chose new nest sites that suffered higher predation than the first nest site they had selected (Delaney and Janzen, 2020). Although the cause of increased mortality is unknown (distance to water was the same for first and second nest sites) (Delaney and Janzen, 2020), more frequent predator attacks or human disturbances induced by anthropogenic environmental perturbation could lead to maladaptive nesting behavior for offspring survival.

Additionally, the sites a female selects to oviposit may be influenced by the behavior of other females in the population. For instance, social facilitation is an important component in some species, such as the yellow-spotted Amazon river turtle (*Podocnemis unifilis*). Some females nest in large groups, following other females (perhaps more experienced nesters), particularly during full moon (Escalona et al., 2019), and laying their eggs at nearby sites (Escalona et al., 2009). Social facilitation is adaptive in this species because eggs of females that nest in larger groups suffer lower predation (Escalona et al., 2009). A consequence of this behavior is that clutches may experience comparable environmental conditions resulting in more similar sex ratios than otherwise. Communal nesting may also decrease the risk that females get predated (Doody et al., 2009). In painted turtles, females appeared attracted to olfactory cues left by previous nesting females (tested experimentally using female urine), which they follow by ground-nuzzling, such that clutches may cluster in areas of seemingly uniform characteristics (Iverson et al., 2016). Although nesting painted turtles do not use ground-nuzzling to select oviposition sites based on thermal properties (Morjan and Valenzuela, 2001), their chosen sites may be warmer than random sites (Schwarzkopf and Brooks, 1987), perhaps because females cued on slope or vegetation cover.

Importantly, nest-site choice may have fitness effects after the eggs hatch. A remarkable example comes from species in temperate regions whose hatchlings often overwinter in their nest, such as *Chrysemys picta*, *Graptemys geographica*, *Trachemys scripta*, and some *Emys orbicularis* (reviewed in Ultsch, 2006; Costanzo et al., 2008 and references therein), where they may be exposed to temperatures below freezing. For instance, vegetation not only cools painted turtle nests during the breeding season by impeding direct solar radiation to reach the nest surface, but also in the Winter, increasing the risk of mortality for these hatchlings when temperatures fall below −8°C (Weisrock and Janzen, 1999) (the lowest value they can survive via supercooling), and affecting their time of emergence in the Spring (Murphy et al., 2020). Hatchlings of pig-nosed turtles (*Carettochelys insculpta*) delay emergence from their nest via diapause, until the first floods of the wet season indicate improved conditions for survival (Doody et al., 2001). Further, females of several freshwater and sea turtle species return to the same or similar nesting sites yearly when available (Meylan et al., 1990; Lindeman, 1992; Allard et al., 1994; Valenzuela, 2001b; Valenzuela and Janzen, 2001; Morjan, 2003; Freedberg et al., 2005). While such choices may be adaptive and yield high-quality offspring today, they may become detrimental in the future if conditions change over time due to anthropogenic environmental change, and if females do not alter their nesting behavior accordingly.

FACTORS INFLUENCING ENVIRONMENTAL CONDITIONS AT THE CHOSEN NEST-SITE LOCATIONS

The outcomes of female nest-site choices are not only affected by biotic drivers such as predation pressure or vegetation cover, but also by multiple abiotic factors that impose selection due to their effect on nest temperatures and moisture, and thus, on whether developing offspring are exposed to optimal conditions for survival and growth or not. Indeed, the temperature experienced during embryogenesis impacts morphology, physiology, performance, behavior, and survival of turtles (Noble et al., 2018; While et al., 2018), which may be under stronger natural selection than sex ratios of TSD turtles as these effects are profound and long-lasting over the offspring lifetime (Noble et al., 2018). But perhaps embryos are not passive in their phenotypic response to the conditions at the selected nest sites, as documented for several turtles, some crocodilians and snakes, some birds, but not lizards (Li et al., 2014). For instance, embryos of *Mauremys reevesii* turtles are capable of thermoregulating within the egg in the laboratory in ways that impact their survival and sexual development (Ye et al., 2019). However, because this thermotaxis alters sex ratios only around the pivotal temperature, takes place over a week (during which thermal conditions are likely to change in the wild), and occurs only when a gradient is set between the egg poles, the ecological and evolutionary relevance of this intriguing behavior for sex ratio adjustment remains debatable (While and Wapstra, 2019). But it may be an important means to maintain embryonic development within their thermal tolerance (Zhao et al., 2013).

Air Temperature

The ambient temperature eggs experience depends on many external factors (Czaja et al., 2020), including air temperature, which is influenced by clouds, solar radiation, and time of day. Cloud cover reduces the shortwave solar radiation reaching the ground, leading to a lower maximum daily temperature and increasing the minimum daily temperature by enhancing the longwave radiation (Janzen and Morjan, 2001). Also, air temperature is warmer on average during the day and cooler at night (Pyrgou et al., 2019). Ambient air temperature is a crucial driver of nest temperature because it warms or cools the soil, thus affecting survival and sexual development during the thermosensitive period of TSD taxa such as painted turtles (Janzen, 1994b; Bowen et al., 2005; Warner and Shine, 2011), and it may be a prevalent factor for nests placed in unvegetated areas in many species, e.g., *Podocnemis* (Escalona and Fa, 1998; Valenzuela, 2001a). Not surprisingly, global warming poses a risk by altering air temperature.

Water Temperature

Water temperature also influences nest temperature, mainly in turtles that nest near the shore (Ackerman, 1997; Rasmussen et al., 2011). Because the nest position in relation to the water table influences the moisture levels in the nest chamber, higher moisture will insulate the soil against rapid air temperature fluctuations (Seybold et al., 2002). This insulation is due to the high specific heat of water which allows it to store more heat before registering temperature changes compared to other substances. In rivers and streams, water temperature correlates strongly to the ambient air temperature in the long term (months, years). In the short term (days, weeks), river and stream temperatures vary substantially due to solar radiation, ambient air temperature, the movement of water flowing over the streambed, and precipitation events (Isaak et al., 2012). Lake temperatures change throughout the year, with streams warming the fastest during the Spring (Woolway and Merchant, 2019). Lake water circulates, allowing for the mixing of water layers with different temperatures. Ice-covered lakes warm faster than the ambient air temperature in the Spring, yet about 10% of lakes, streams, and rivers cool due to increased glacial runoff in the Spring (O'Reilly et al., 2015). These dynamics (combined with air temperature) would affect the conditions experienced by basking females and developing embryos of freshwater turtles living in these habitats. Additionally, the oceans and large lakes act as a heat sink on Earth, absorbing, redistributing, and storing heat over long timescales. Due to the high specific heat of water, oceans lose heat energy slower than air or soil. Most of the heat absorption occurs in the Southern Hemisphere, at a rate four times faster than the Northern Hemisphere (Wijffels et al., 2016). Thus, basking females and developing embryos of sea turtle populations inhabiting Northern and Southern latitudes are expected to be differentially affected by changes in ocean thermal dynamics due to global warming.

Soil Temperature

Several factors affect soil temperature, including ambient air temperature, moisture, solar radiation, and precipitation (Al-Kayssi et al., 1990; Seybold et al., 2002; Parrott and Logan, 2010). Solar radiation occurs only during the daytime but is also very stochastic (Parrott and Logan, 2010). The radiation reaching the soil is affected by cloud cover, vegetation cover, and other abiotic elements within any given day, such that shaded nests are cooler (Weisrock and Janzen, 1999) and would tend to produce more males in TSD Ia taxa, such as painted turtles (Janzen, 1994b). Soil moisture is influenced by rising and falling water tables and increases with proximity to the shore and precipitation events, affecting the temperatures that eggs experience (Kraemer and Bell, 1980; Janzen, 1994a). In spotted turtles (*Clemmys guttata)*, the higher the soil moisture, the more stable and cooler nests are (Al-Kayssi et al., 1990; Ernst and Zug, 1994) because moist soil has a higher specific heat than dry soil and more heat energy is required to warm the nest. Soil types also influence nest temperatures, with some soil types and granule sizes absorbing more moisture/thermal energy than others (Parrott and Logan, 2010; Mitchell and Janzen, 2019). Soil temperature and moisture can also be influenced by supra-annual natural climatic oscillations, such as El Niño events, which reduce average precipitation and increase mean temperature and irradiance, potentially affecting nest temperatures of South American turtles (Valenzuela, 2021). Nest depth is another important factor altering nest temperatures. Deeper nests are cooler than shallower nests and are less affected by extreme temperature fluctuations detrimental to hatchling viability (Valenzuela, 2001a; Marco et al., 2018). For instance, hatching success in diamondback terrapins (*Malaclemys terrapin*) improved with nest depth (which ranges from 13 to 17 cm) during an unusually hot and dry season, but not during a more favorable season (Czaja et al., 2020). Further, no evidence was detected that females altered their nesting location to improve offspring survival short-term. In contrast, nest depth of shallower nesting species such as painted turtles (7–11 cm) did not affect hatchling sex ratio, survival, or growth (Refsnider et al., 2013). Female limb length and body size constrain the maximum nest depth possibly reached for turtles, as observed in giant Amazonian river turtles, *Podocnemis expansa* (e.g., Valenzuela, 2001a).

ANTHROPOGENIC CHALLENGES TO NESTING BEHAVIOR

Humans have altered and destroyed natural habitats, leading to a drastic decrease in the land available for nesting (Mainwaring et al., 2017) and forcing some turtle species to tolerate human presence in their nesting habitat. Habitat disturbance includes urban development, which increases absorbed solar radiation and thus, raises the temperature of urban landscapes (Yang et al., 2016). Moreover, urban lights have a disorienting effect on nesting females and hatchling sea turtles (McFarlane, 1963; Perry et al., 2008). Human recreational activity may also alter nesting behavior, as in painted turtles nesting around R.V.'s and campsites (Bowen and Janzen, 2008), leading to higher mortality by vehicles, removal as pets, or exposure to pollutants. Additionally, anthropogenic disturbance may attract predators that raid turtle nests, making predator removal programs necessary in some locations (Wirsing et al., 2012). In some cases, removal of predators from turtle habitats can decrease predation rates by > 50%, significantly improving hatching success (Spencer, 2002).

Adding to the local dangers of human-altered ecosystems is global anthropogenic climate change, which poses another significant threat to turtles, particularly TSD turtles, but could also affect turtles in general. According to NASA's Goddard Institute for Space Studies (GISS), the average temperature on Earth has increased by 1.0°C since 1880, with 67% of that warming occurring since 1975 at a pace of 0.20°C per decade, on average. The Earth is predicted to warm between 2.0 and 4.5°C by the end of the century (Rogelj et al., 2012). Land air temperatures will increase on average, but with much variability on different spatial and temporal scales across the globe (Ji et al., 2014). Egg and hatchling mortality are expected to increase with increasing global temperature (Santidrián Tomillo et al., 2015; Hays et al., 2017). Increasing temperatures will lead to rising sea levels at frequently used nesting sites of sea turtles due to rising temperatures melting ice caps in the poles, reducing the nesting area of specific beaches by 86% (Patrício et al., 2019). Rising oceans often flood green sea turtle nests, killing the developing embryos as time underwater increases (Fuentes et al., 2010; Mainwaring et al., 2017). This happens when storms increase the wave runup on the shore, and these events can cover a large portion of a nesting area. Rising oceans are predicted to inundate nesting areas of many sea turtle populations, as well as some species inhabiting brackish water, such as the diamondback terrapin (*Malaclemys terrapin*) (Woodland et al., 2017).

Additionally, fully masculinizing and fully feminizing temperatures in TSD turtles are separated by a few degrees Celsius, called the transitional range of temperatures (TRT), often 1–3°C (Mrosovsky, 1994; Valenzuela and Lance, 2004). For instance, 26°C produce only males and 30°C only females in painted turtles (Valenzuela, 2009). Leatherback sea turtles have a very narrow TRT of about 1°C (Binckley et al., 1998), whereas the TRT of red-eared sliders spans about 2.5°C (Godfrey et al., 2003). Not surprisingly, warming global temperatures raise the thermal profile of nests, often negatively affecting the offspring via sex ratio distortions, developmental abnormalities, and reducing embryonic survival (Janzen, 1994b; Seybold et al., 2002; Telemeco et al., 2013; Hays et al., 2017). Indeed, global warming can potentially cause dangerous feminization of TSD turtle populations (Blechschmidt et al., 2020), as reported in some Australian populations of green sea turtles (Jensen et al., 2018) whose sex ratio reached 99.1% female (99.8% in juveniles and 86.8% in adults). This population has been female-biased for over 20 years and will potentially feminize entirely in the near future. If temperatures continue to warm as predicted, populations of other TSD turtles could eventually become fully feminized, but species with a broader TRT are less vulnerable to such risks because they produce mixed sex ratios over a wider range of temperatures than taxa with a narrow TRT (Hulin et al., 2009). Sex ratio distortion by global warming is not a risk unique to turtles, but one with taxonomically broad impact in other TSD groups and in GSD taxa susceptible to thermal sex reversal (Valenzuela et al., 2003; Edmands, 2021).

And to make things worse, climate change predictions suggest temperature variability within nests will increase in addition to rising averages (Deschênes and Greenstone, 2007; Stouffer and Wetherald, 2007; Neuwald and Valenzuela, 2011; Valenzuela et al., 2019). Temperature variability within nests can accelerate the feminization of TSD turtle populations and cause increased mortality of developing embryos, such as in painted turtles (Valenzuela et al., 2019) and potentially in other shallow nesting taxa, with long-lasting consequences (Noble et al., 2018). In contrast, the observation in *Malaclemys terrapin* that greater nest depth can protect against extreme and fluctuating temperatures (Czaja et al., 2020), even though nests in this turtle are only 13–17 cm below the surface, suggests that deeper-nesting taxa may be somewhat buffered against climate change. But shallower-nesting taxa, such as painted turtles, whose nests are 7–11 cm below the surface, remain vulnerable to accentuated thermal fluctuations. For green sea turtles and painted turtles, warmer nests also decrease hatchling success, further reducing the species survival (Janzen, 1994a; Cavallo et al., 2015; Laloë et al., 2017; Valenzuela et al., 2019).

TSD and GSD turtles in a warming world may bask and nest earlier in the year (Hedrick et al., 2021), as temperature is a primary factor driving the onset of nesting (Rowe et al., 2003; Bowen et al., 2005). Earlier nesting due to climate change is observed in some turtles but not others (Hedrick et al., 2021), and it might appear as an adaptive response that might mitigate some of the detrimental impacts of climate change on offspring survival and sex ratio for TSD species. Yet, the opposite may be true because earlier nesting and a protracted reproductive season due to global warming can induce females to produce an extra clutch too late in the year for eggs to complete development. The failure of these late-season clutches to develop fully represents a large waste of energy that would be better invested in overwintering and producing the first clutch the following season (Schwanz and Janzen, 2008). Warmer air and water can also influence food availability for reproductive females, and consequently, alter their resource allocation to the eggs in both TSD and GSD turtles (Hedrick et al., 2021).

Climate change will also impact the behavior of marine turtles. Many sea turtles nest in the tropical and subtropical regions, and very rarely in more temperate areas. Over the last 30 years, Kemp's Ridley sea turtles have shifted their nesting range from these tropical regions to further North away from the equator (Pike, 2013), resulting in cooler nest temperatures than experienced toward the equator, and perhaps more sea turtles will begin nesting further away from the equator. If global warming continues to alter the environment at the pace of these predictions, turtle biodiversity will significantly decrease over the next century, with over 50% threatened with extinction (Stanford et al., 2020). Much has been elucidated about the factors that influence nest temperature and offspring survival, but more questions remain unanswered, as described below.

CAN FEMALE NESTING BEHAVIOR RESPOND ADAPTIVELY TO ANTHROPOGENIC CHALLENGES?

Our ability to predict the effects of contemporary climate change on sex ratios of TSD turtles depends on how reliable our models are to explain sexual development under natural nesting conditions (pristine conditions or under climate change scenarios). Many incubation studies have shifted from constant temperature experiments, to simple thermal fluctuating regimes, to replicating natural thermal profiles from nests in ecologically relevant studies (Neuwald and Valenzuela, 2011; Valenzuela et al., 2019; Bowden and Paitz, 2021). Simple fluctuations around a mean temperature that produces only males or only females, if constant, can induce sex reversal in painted turtles from that expected by the mean temperature alone (Neuwald and Valenzuela, 2011). But when the amplitude of fluctuations is accentuated experimentally around naturally fluctuating profiles, only the male-to-female sex reversal is observed around an otherwise male-producing profile, whereas no sex reversal and high mortality are induced by wider fluctuations around an otherwise female-producing profile (Valenzuela et al., 2019). These simplistic and natural fluctuation experiments have demonstrated that high temperatures have greater feminizing potency than the masculinizing potency of low temperatures due to their effect on developmental rate (Georges, 1989; Valenzuela, 2001c; Georges et al., 2005; Valenzuela et al., 2019). Similarly, short periods (days) of increased incubation temperatures (heat waves) have a higher likelihood of feminizing red-eared slider nests compared to nests experiencing more constant conditions (Carter et al., 2018). Findings like these helped develop new models to predict sex ratio responses to naturally fluctuating thermal regimes (Fuentes et al., 2017; Carter et al., 2018, 2020; Valenzuela et al., 2019) and temperature profiles altered by anthropogenic environmental disturbance. Future thermal profiles of nests in the wild will depend on the oviposition site choices made by females in disturbed habitats, such that the following are some outstanding questions that warrant further research.

Although it is clear that female nesting behavior impacts the phenotype (including sex in TSD taxa) and survival of her offspring, the genetic basis of these behaviors remains unclear. Existing data from an Illinois population of painted turtles indicate that the heritability of nest-site choice is low or nil (Morjan, 2003; McGaugh et al., 2010; Janzen et al., 2019; Delaney et al., 2020), and without heritability, adaptive behavioral responses to environmental change are precluded. Furthermore, the evolutionary potential of these nesting behaviors is hampered because nest predation, hatching success, and sex ratio are not repeatable at micro-geographic nesting sites or at vegetation cover types in this painted turtle population (Valenzuela and Janzen, 2001), such that female nest-site choices are overwhelmed by interannual environmental variation in biotic and abiotic conditions. Additionally, these potential responses must offset the lethal incubation temperatures predicted to become more frequent in the wild with climate change (Telemeco et al., 2013; Refsnider and Janzen, 2016; Valenzuela et al., 2019), and which will impact TSD and GSD turtles alike.

An alternative route for adaptation by TSD taxa facing global warming is via the evolution of the reaction norm of sex ratios to temperature (Bull et al., 1982; Valenzuela, 2004). Likewise, evolution of the embryonic thermal tolerance could also help both TSD and GSD turtles respond to climate change for traits other than sexual development that are affected by global warming (Du and Shine, 2015). A caveat is that heritability estimates of the threshold temperature (the inflection value between male and female development) in turtle populations are scarce, limited to painted and snapping turtles, inflated when calculated under constant temperature (Bull et al., 1982), and inflated by confounding maternal effects (Bull et al., 1982; Janzen, 1992; Rhen and Lang, 1998; McGaugh and Janzen, 2011), and potentially by embryonic behavioral thermoregulation (Ye et al., 2019), such that they represent broad sense heritability and not additive genetic variance alone. Nonetheless, narrow sense heritability for threshold temperature, estimated for natural nests of painted turtles, ranges from $h^2 = 0$ to $h^2 = 0.35$ (when including clutches with many or with only fewer fathers, respectively) (McGaugh et al., 2011). Further, broad sense heritability (clutch effects) was detected for the expression of gene regulators of gonadal development in painted turtles (Valenzuela et al., 2013). If these clutch effects reflect additive genetic variation, then natural selection could act on it and drive transcriptional evolution underlying sex determination in the face of climate change. This type of variation may also exist among populations and underpin the differences observed in the reaction norm of TSD within turtle species (Ewert et al., 2004). This interpopulation variation may also counter extinction risk because individuals from populations that produce males at warmer temperatures could colonize areas left open by extirpated populations. However, isolated populations (either due to habitat fragmentation or freshwater turtles and tortoises living on islands) likely cannot alter their home range in response to a warming climate. These species must embrace a decrease in island habitat from rising sea levels and warmer nesting areas (Gibbons et al., 2000). Research is needed to test whether extinction risk differs among turtle species across different geographical locations.

CONCLUSION

Turtles play essential roles in the ecosystem, occupying many different and unique niches around the world. As these species head toward extinction, their communities will be negatively impacted (Lovich et al., 2018). Further research is urgently needed to fill the gaps in our understanding of the ecology and evolution of nest-site choice, and its evolutionary potential at multiple levels of organization. First, a taxonomic bias exists because relatively few turtle species are the focus of extensive research while a substantial proportion, including some highly endangered taxa, are poorly studied. A bias also exists in how we address the questions of nest-site choice. For instance, here we show that variables not typically considered in the literature on nesting behavior can have profound effects, such as (1) how water temperature impacts basking behavior, an abiotic factor that influences female physiology, which in turn may alter the timing of nesting and resource allocation to the eggs; or (2) how biotic factors such as social facilitation influences nest-site choice; or (3) how water and not just air and soil temperature may affect the conditions experienced by developing embryos in the nest. Filling these gaps is important to assess what are the rules and what are the exceptions in turtle nesting biology, and to predict and evaluate the impact of environmental change. Indeed, anthropogenic climate change is detrimental to animals that have few options to overcome the effects of rising temperatures. TSD turtles have few options to survive highly biased sex ratios and decreased offspring viability in a warming world. And while the general long lifespan of turtles offers some buffering for these biased sex ratios (Sabath et al., 2016) and some heritability exists for adaptation via the threshold temperature for sexual development of TSD embryos, the rate of warming likely exceeds the evolutionary potential of many turtle taxa (Morjan, 2003). But rising temperatures also imperil the survival of GSD turtles because extreme temperatures induce high embryonic mortality, and endocrine disruptors affect sexual development across species irrespective of their sex-determining mechanism, yet little attention has been paid to GSD taxa in these respects. Likewise, predation risks accentuated by anthropogenic disturbance interact negatively with female nesting behavior and clutch survival, both for TSD and GSD turtles. Breeding programs that control the sex of eggs will probably need to be implemented to delay the demise of the most endangered TSD species, but highly imperiled GSD taxa may also need such measures. Ultimately, humans must curb carbon emissions, sequester carbon excess in the atmosphere, mitigate pollution, and ameliorate other environmental disturbances to protect these species and their native habitats, as evolutionary responses alone may not be enough.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Do Microbiota in the Soil Affect Embryonic Development and Immunocompetence in Hatchling Reptiles?

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Reptile eggs develop in intimate association with microbiota in the soil, raising the possibility that embryogenesis may be affected by shifts in soil microbiota caused by anthropogenic disturbance, translocation of eggs for conservation purposes, or laboratory incubation in sterile media. To test this idea we incubated eggs of keelback snakes (*Tropidonophis mairii*, Colubridae) in untreated *versus* autoclaved soil, and injected lipopolysaccharide (LPS) into the egg to induce an immune response in the embryo. Neither treatment modified hatching success, water uptake, incubation period, or white-blood-cell profiles, but both treatments affected hatchling size. Eggs incubated on autoclaved soil produced smaller hatchlings than did eggs on untreated soil, suggesting that heat and/or pressure treatment decrease the soil's suitability for incubation. Injection of LPS reduced hatchling size, suggesting that the presence of pathogen cues disrupts embryogenesis, possibly by initiating immune reactions unassociated with white-blood-cell profiles. Smaller neonates had higher ratios of heterophils to leucocytes, consistent with higher stress in smaller snakes, or body-size effects on investment into different types of immune cells. Microbiota in the incubation medium thus can affect viability-relevant phenotypic traits of hatchling reptiles. We need further studies to explore the complex mechanisms and impacts of environmental conditions on reptilian embryogenesis.

Keywords: lipopolysaccharide, LPS, leukogram, heterophil, immunocompetence, lymphocyte, Natricidae

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INTRODUCTION

The embryos of most species of oviparous reptiles spend the majority of their total developmental period (typically, the latter two-thirds) outside the mother's body, in external nests (e.g., Shine, 1983). As a result, embryos of many species are exposed to a wide range in both the average values and temporal variance of abiotic factors such as temperature and moisture (e.g., Singh et al., 2020). Experimental studies confirm that even minor variation in such factors can significantly modify fitness-relevant traits such as incubation duration, and the phenotypic traits (morphological, physiological, and behavioral) of hatchlings (e.g., Georges et al., 2005; Amiel and Shine, 2012; Bodensteiner et al., 2015). Importantly, embryos in external nests are also exposed to biotic challenges including predation and infection by pathogens (Moreira and Barata, 2005), potentially inducing adaptive responses (e.g., immediate hatching of eggs induced by physical disturbance: Doody, 2011).

In the current article, we explore a neglected aspect of embryo-environment interactions in reptiles: the possibility that microbiota (bacteria, viruses, fungi, etc.) in the soil within a nest may penetrate the eggshell and affect the embryo. Although we are not aware of any studies that have looked for such penetration, it is feasible on physical grounds because soil microbiota are diverse and abundant (e.g., Wolmarans and Swart, 2014), pores within the squamate eggshell are large enough to permit ingress of micro-organisms (Packard and DeMarco, 1991), and eggs take up substantial water from the incubation substrate during development (thereby providing a straightforward means of entry for microbiota: Warner et al., 2011).

Eggs might also be exposed to potentially harmful microbiota even before oviposition. Pathogens could be present as the eggs pass through the oviduct (e.g., vertical transmission of *Salmonella*) and through the cloaca (fecal contamination) (D'Alba and Shawkey, 2015; Zając et al., 2021). Hydric and thermal conditions inside nests also may expose eggs to high concentrations of saprophytic microbes (e.g., Löwenborg et al., 2011).

The most critical factor thus may not be whether or not microbiota can find their way into developing eggs (surely, they can) but the effects of such exposure on embryos. Studies on later life stages (free-living reptiles) document many effects of infection, including immune responses and stress reactions (e.g., Wellehan and Johnson, 2005; Jacobson, 2007). By analogy, embryos might exhibit such responses as well (as they do in other vertebrates: e.g., Ygberg and Nilsson, 2012). The selective benefit of responding to microbial invasion might include adjusting immune-system development to better match the pathogens likely to be encountered after hatching (Grindstaff et al., 2006). Plausibly, natural selection might fine-tune embryonic responses to microbial exposure either directly, or via facultative manipulation of maternal allocation of resources to the egg (e.g., transfer of immune compounds such as antibodies) in response to the mother's own history of encounters with pathogens.

The potential impacts of soil microbiota on reptilian embryogenesis are relevant to anthropogenic disturbances to the nest environment. For example, human activities such as agriculture and pollution greatly modify microbial communities within the soil (Wolmarans and Swart, 2014), humans affect nest-site selection of reptiles by destroying traditional nest-sites and/or creating new options (Francis et al., 2019; Doherty et al., 2020), and conservation-focused management frequently involves incubation of eggs in places other than where they were laid (e.g., Jarvie et al., 2014; Mitchell et al., 2016) or in substrates that contain very different microbiota than are found in natural nest-sites. In the extreme, captive breeding of oviparous reptiles often includes incubation of eggs in artificial media such as vermiculite, which may be virtually sterile (Köhler, 2005; Tefera and Vidal, 2009). In humans, artificial elimination of contact with the vaginal microbiota in surgically delivered (cesarean) babies can have deleterious long-term effects on infant health (Dominguez-Bello et al., 2016).

As a first step toward addressing this gap in knowledge, we conducted experimental incubation trials with the eggs of an oviparous tropical snake. In the first experiment, we incubated eggs in soil that either contained or did not contain the natural microbiota (i.e., was or was not autoclaved prior to use). Because any null result from such a study may be due to lack of penetration of the shell by local micro-organisms, we then ran another study in which we directly introduced lipopolysaccharide (LPS, a component of bacterial cell walls) into the egg to stimulate an immune response by the embryo. This second experiment allowed us to assess the effects of chemically signaling the presence of pathogenic microbiota without introducing any confounding deleterious affects (such as propagation) that would arise from actually introducing harmful bacteria. To quantify the effects of these manipulations, we measured aspects of developmental rate (incubation period), hatchling morphology, and circulating immune cells (leucocytes). Because the ratio of certain leucocytes (heterophils: lymphocytes; H:L) provides an index of stress (Davis et al., 2008), we looked specifically at levels of those cell types. Specifically, we predicted that the presence of microbiota (or their chemical cues) could impose developmental costs on embryos that would be manifested in altered morphological or immunological measures.

MATERIALS AND METHODS

Study Site

Fogg Dam (12.56°S, 13130°E) is located 50 km southeast of the city of Darwin in Australia's Northern Territory. The 1,500-m long man-made wall of the dam impounds a 250-ha body of water. The area experiences a wet–dry tropical climate, with high temperatures all year (mean daily maximum >30°C every month) but with rainfall primarily restricted to a 6-month (November–April) "wet season" (Brown and Shine, 2006a,b).

Study Species

Keelbacks (*Tropidonophis mairii*, **Figure 1**) are non-venomous natricine colubrid snakes (a group sometimes elevated to a separate family, Natricidae). Keelbacks are found in freshwater habitats across northern Australia (Cogger, 2015). At Fogg Dam, maximum body size (snout-vent length; SVL) of females is 82.2 cm and maximum body size of males is 67.7 cm. Males and females both grow rapidly and mature in <12 months (Brown et al., 2017).

At our study site, keelbacks are active year-round with a diet consisting largely of amphibians (Brown and Shine, 2002). Female keelbacks produce one or two clutches of eggs each year during the dry season (April–October; Brown and Shine, 2002). When females are ready to oviposit, they move from riparian habitats to the higher, well-drained soil of the dam wall to nest (Brown and Shine, 2005b). Eggs typically are laid around 10–20 cm beneath the soil surface, and nests are often communal (Brown and Shine, 2005b, 2009). Female keelbacks exhibit natal philopatry, returning to the site at which their mother oviposited when it is time to lay their own eggs (Brown and Shine, 2007a). Incubation requires approximately 8 weeks, and the phenotypic

FIGURE 1 | Recently emerged hatchling keelback, *Tropidonophis mairii*. Photograph by G. P. Brown.

traits of hatchlings are influenced by the thermal and hydric conditions that they experience during this period (Webb et al., 2001; Brown and Shine, 2004).

In laboratory studies, female keelbacks actively selected moist rather than dry substrates for oviposition; this nest-site choice resulted in larger hatchlings with a higher probability of surviving their first year of life in the field (Brown and Shine, 2004). Moisture levels in the incubation medium also affect immune function of hatchlings. Eggs incubated on moist substrate had higher lymphocyte counts, a trait that was linked to increased probability of survival in the field (Brown and Shine, 2018).

Collection of Eggs

We hand-captured female keelbacks at night on the wall of Fogg Dam during May 2015 and March–April 2017. Snakes were returned to the lab and kept overnight in cloth bags. The following day they were weighed, measured (snout-vent length, SVL), individually marked by scale-clipping, and palpated for the presence of shelled eggs. Gravid females were held in captivity in 12-L plastic cages lined with newspaper and equipped with a nest box containing damp vermiculite and a small water dish. For the present study, we used clutches from five females in 2015 and from four females in 2017. After laying, females were returned to their original capture location and released. At the conclusion of experimental studies (below), hatchlings were released at the mother's site of capture.

Experiment #1: Effect of Incubation Substrate Disinfection on Hatchling Snakes

In 2015 we used a split-clutch design to incubate 68 eggs from five clutches on either natural soil substrate, or soil substrate that had been autoclaved to kill microbes and other potential egg pathogens. We collected soil from the site where the gravid female keelbacks had been captured. In a location typical of keelback nesting habitat (Brown and Shine, 2005b), we collected 2 L of damp soil from 10 to 20 cm below the surface. We placed 40 g of the damp soil into 100-mL plastic cups, and autoclaved half of those cups at 131°C and 0.22 Bar for 4 min.

Within 24 h of oviposition, eggs were measured and weighed. Forty-eight hours after oviposition, eggs within each clutch were paired by mass as closely as possible and one member of each mass-matched pair was assigned to the "Dirty" soil treatment and the other to the "Clean" soil treatment. We covered the cups with lids and placed them into an insulated coolbox at ambient room temperature (25°C). Eggs were re-weighed after 2–3 weeks and again after 5–6 weeks.

Experiment #2: Effect of Lipopolysaccharide Injection on Hatchling Snakes

In 2017, this experiment was conducted on four clutches consisting of 46 eggs. Within 24 h of oviposition, eggs were measured and weighed. Each clutch was then placed in a plastic bag containing 20 g of vermiculite and 5 g water (i.e., 25% water by weight). This level of substrate moisture causes slight water loss in keelback eggs (Brown and Shine, 2006a), generating slightly desiccated eggs that facilitated injection of 0.03 mL of fluid into each one. Bags containing each clutch were placed in an insulated coolbox and incubated at room temperature (25°C).

After 24 days, eggs were reweighed and half the eggs in each clutch were injected with 0.03 mL of a 2 mg/mL solution of lipopolysaccharide (LPS, Sigma L2630) dissolved in sterile phosphate buffered saline (PBS), under sterile conditions. We were unable to find any published reports in which LPS doses were administered to reptile embryos. Thus we chose a dosage rate (approximately 0.02 mg/g) capable of inducing behavioral changes in adult reptiles (e.g., Deen and Hutchison, 2001; do Amaral et al., 2002). This dosage of LPS also elicited significant changes in white blood cell (WBC) differential counts (specifically an increase in H:L ratios) in adult rattlesnakes (*Sistrurus miliarius*) (Lind et al., 2020). Because LPS is a component of bacterial cell walls, its injection into an organism typically elicits an immune response because the animal's immune system recognizes LPS as indicative of bacterial infection (e.g., Bonneaud et al., 2003; de Figueiredo et al., 2021). As controls, the other half of the eggs in each clutch were injected with 30 μL of sterile phosphate buffered saline (PBS), which is not expected to induce any physiological response (e.g., Bonneaud et al., 2003). To inject each egg, the apical tip was swabbed with 70% ethanol and a 50-μL micro-injector used to administer either LPS or PBS into the yolk, taking care to avoid the embryo. Following injections, eggs were individually placed in covered 100-mL cups containing 3 g vermiculite and 9 g water (i.e., 300% water by weight) and placed back into the insulated coolbox to complete incubation. Eggs were reweighed after 5–6 weeks.

Scoring of Phenotypic Traits of Hatchlings (Experiments #1 and #2)

As hatching dates approached, we checked eggs daily for pipping. Within 25 h of emerging, each hatchling was checked for sex (based on tail shape and eversion of hemipenes: Brown and Shine, 2002), measured for SVL, head length and tail length, and weighed. We excised the terminal 1 mm from each snake's tail with a sterile scalpel blade, and smeared the droplet of blood onto a clean glass slide. We fixed blood smears in methanol, stained them with modified Wright's stain and fitted them with cover slips. We examined slides under 1,000× magnification until 100 white blood cells (WBCs)

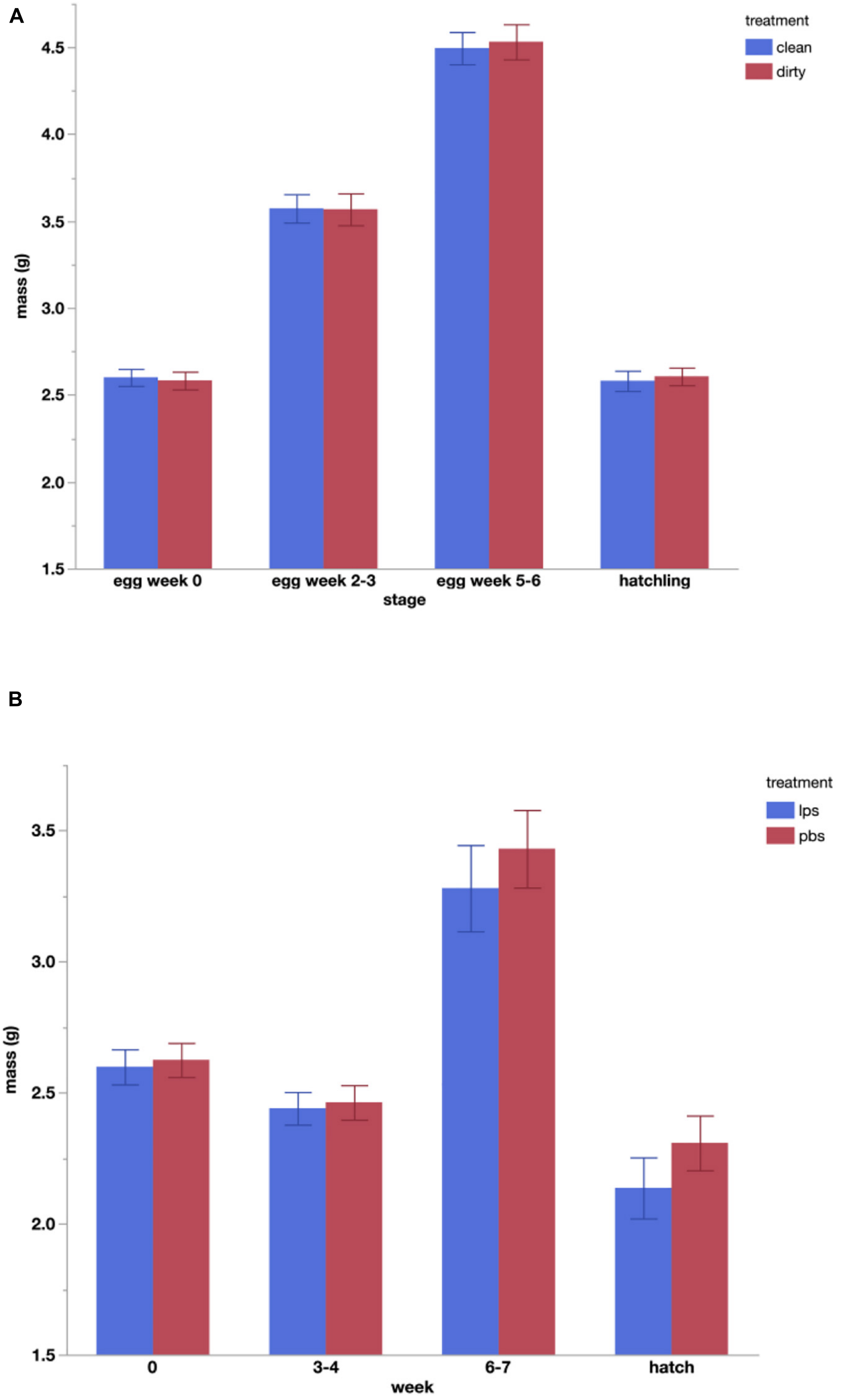

FIGURE 2 | Changes in mean (±standard error) mass of eggs over the course of incubation and resultant hatchling mass of keelback snakes (*Tropidonophis mairii*) for the two experiments. **(A)** Eggs incubated on untreated soil from a natural nest site (Dirty) vs. soil from the same site that had been autoclaved (Clean). **(B)** Eggs injected with 30 μL lipopolysaccharide solution after week 4 (LPS) vs. eggs injected with 30 μL phosphate buffered saline (PBS) after week 4.

were encountered and identified as either basophil, heterophil, monocyte, lymphocyte, or azurophil. Eosinophils are rare or absent in snakes (Sykes and Klaphake, 2008) and were not observed on any slides during the present study.

Analysis of Data

We divided heterophil counts by lymphocyte count for each snake to calculate the H:L ratio. We log-transformed WBC differential counts and H:L ratios data prior to analysis to better meet assumptions of multiple regression. Because sex and size of hatchling keelbacks can affect their WBC profiles (Brown and Shine, 2018), we included these factors as independent variables in models assessing the effects of treatment. We also included Maternal ID as a random effect in these models, to recognize relatedness among clutch-mates. Residuals from all analyses were inspected to verify that all model assumptions were met. Analyses were conducted using JMP 14 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Experiment #1: Effect of Incubation Substrate Disinfection on Hatchling Snakes

Mean mass of eggs did not differ significantly between Clean and Dirty treatments at the beginning of the experiment (2.80 g vs. 2.81 g; $F_{1,66} = 0.01$, $P = 0.91$), nor after 2–3 weeks (3.57 g vs. 3.57 g; $F_{1,66} = 0.001$, $P = 0.97$) or after 5–6 weeks (4.50 g vs. 4.53 g; $F_{1,66} = 0.07$, $P = 0.79$) of incubation (**Figure 2**).

Hatching success was 94% (31 of 33 eggs) for eggs incubated on the Clean substrate and 100% for those on the Dirty substrate (35 of 35 eggs) and did not differ significantly between treatments ($\chi^2 = 2.96$, 1 df, $P = 0.09$). The mean duration of the incubation period also did not differ significantly between treatments (39.8 days vs. 39.8 days; $F_{1,66} = 0.001$, $P = 0.99$). However, hatchling snakes from the Dirty incubation treatment were longer than those from the Clean treatment and had longer heads (**Table 1**).

Autoclaving the incubation substrate had no significant effect on any WBC measures (**Table 2**). However, several measures were affected by hatchling body size. Basophils, heterophils and H:L ratios decreased with SVL (**Table 2** and **Figures 3**, **4**) whereas lymphocytes increased with SVL (**Table 2** and **Figure 3**). Monocyte counts were higher in females than in males.

Experiment #2: Effect of Injecting Lipopolysaccharide on Hatchling Snakes

There was no significant difference in the initial mass of eggs that were assigned to each treatment group (means for LPS 2.60 g vs. Control 2.63 g; $F_{1,44} = 0.08$, $P = 0.78$). After 24 days incubation on 25% vermiculite, eggs lost an average of 0.16 g, but mean mass did not differ significantly between groups (LPS 2.44 g vs. Control 2.46 g; $F_{1,44} = 0.06$, $P = 0.81$). This mass loss allowed us to inoculate the eggs with 30 μL of LPS or PBS. After a further 3 weeks' incubation in individual cups with 300% vermiculite, mean mass was again similar between treatment groups (LPS 3.28 g vs. Control 3.43 g; $F_{1,39} = 0.46$, $P = 0.50$) (**Figure 2**).

Hatching success of eggs was 96% (21 of 22 eggs) in the LPS treatment and 92% (22 of 24 eggs) in the Control treatment and did not differ significantly between the groups ($\chi^2 = 0.27$, 1 df, $P = 0.60$). The mean length of the incubation period also did not differ between LPS and Control treatments (44.6 days vs. 44.7 days; $F_{1,43} = 0.001$, $P = 0.95$). However, hatchlings from the Control treatment had longer bodies, tails and head than did those from the LPS treatment (**Table 3**).

Lipopolysaccharide treatment had no significant effect on any WBC counts, nor did sex or body size (**Table 4**). However, H:L ratio decreased with body size (**Table 4** and **Figure 4**) and was lower in females than in males (**Table 4**).

DISCUSSION

In both experiments, incubation treatments generated significant differences in hatchling body size. Although much of the variation among clutches in hatchling size is driven by heritable (genetic) factors in this species (Brown and Shine, 2007b), considerable variation also is induced by the conditions under which eggs are incubated. For example, incubation on dry substrates substantially reduces offspring size in keelbacks (Shine and Brown, 2002; Brown and Shine, 2005a) as in many other species of squamate reptiles (Bodensteiner et al., 2015). Longer incubation also can allow squamate embryos to develop more fully, affecting body size at the time of hatching (Shine and Olsson, 2003). These complicating factors cannot explain the effect of treatment on offspring size in the present study, however, because neither rates of water uptake nor incubation periods were affected by our experimental treatments (above). Thus, exposure to microbiota (Experiment #1) or to cues associated with bacterial infection (Experiment #2) invoked morphological changes in offspring that were independent of egg size, incubation period or moisture balance. Our data thus add yet another variable to the long list of external factors that are known to affect developmental trajectories in embryonic reptiles [e.g., see reviews by Noble et al. (2018) and While et al. (2018)].

In Experiment #1, autoclaving the soil used for incubation resulted in a small but statistically significant reduction in hatchling head and body length but did not affect body

TABLE 1 | Effect of autoclaving soil (the incubation substrate) on hatchling phenotype of keelback snakes (*Tropidonophis mairii*) in Experiment #1.

Variable	Clean ($N = 31$)	Dirty ($N = 35$)	$F_{1,59}$	P
SVL (cm)	16.56 ± 0.19	16.86 ± 0.13	9.49	**0.0031**
Tail (cm)	4.56 ± 0.06	4.63 ± 0.04	2.22	0.1419
Head (mm)	11.03 ± 0.09	11.17 ± 0.09	4.48	**0.0385**
Mass (g)	2.58 ± 0.06	2.61 ± 0.05	1.80	0.1852

Snakes incubated on non-autoclaved (Dirty) soil had longer bodies (SVL, snout-vent length) and heads than did snakes incubated on autoclaved soil (Clean). Boldface values in table indicate significant differences ($P < 0.05$).

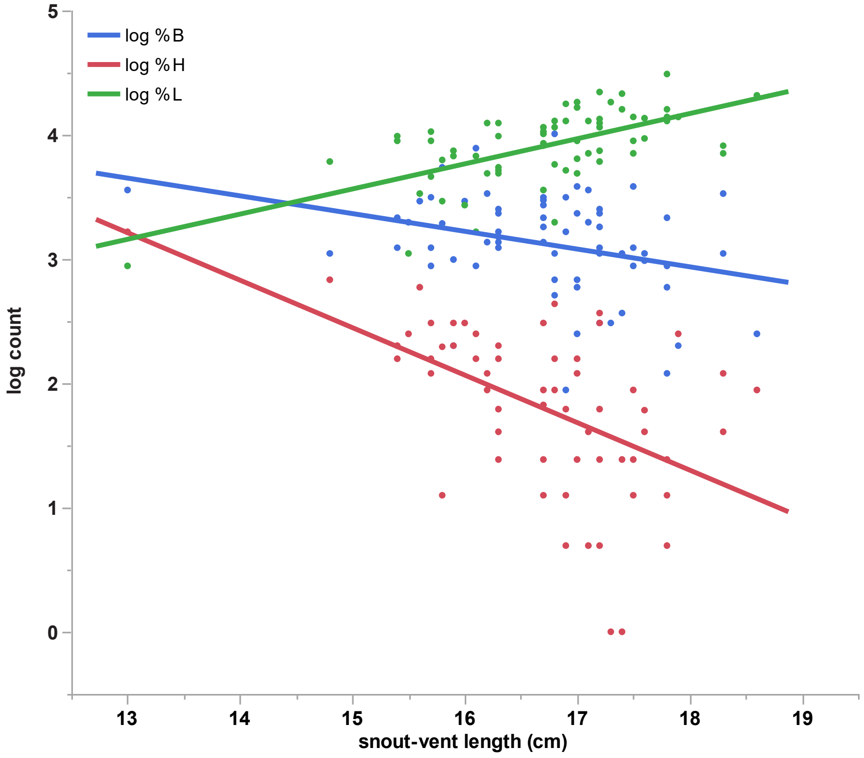

FIGURE 3 | Significant effects of hatchling body size of keelback snakes (*Tropidonophis mairii*) on white-blood-cell measures found in Experiment #1. Basophil and heterophil counts decreased with hatchling body size (SVL, snout-vent length) whereas lymphocyte counts increased with snout-vent length (SVL).

TABLE 2 | Effects of mixed model analyses on the factors affecting white blood cell counts of hatchling keelback snakes (*Tropidonophis mairii*) in Experiment #1.

Cell type	Sex	SVL	Treatment
Log %A	$F_{1,60} = 0.03$ $P = 0.8582$	$F_{1,34} = 0.001$ $P = 0.9700$	$F_{1,59} = 1.20$ $P = 0.2784$
Log %B	$F_{1,62} = 1.93$ $P = 0.1695$	$F_{1,62} = 8.09$ **$P < 0.0060$**	$F_{1,62} = 0.01$ $P = 0.9071$
Log %H	$F_{1,62} = 1.04$ $P = 0.3116$	$F_{1,15} = 7.55$ **$P < 0.0149$**	$F_{1,61} = 1.96$ $P = 0.1661$
Log %L	$F_{1,62} = 1.19$ $P = 0.2805$	$F_{1,9} = 19.10$ **$P < 0.0017$**	$F_{1,61} = 1.01$ $P = 0.3196$
Log %M	$F_{1,61} = 7.48$ **$P < 0.0082$**	$F_{1,25} = 0.04$ $P = 0.8524$	$F_{1,61} = 0.63$ $P = 0.4311$
Log H:L	$F_{1,62} = 1.23$ $P = 0.2717$	$F_{1,11} = 35.55$ **$P < 0.0001$**	$F_{1,61} = 1.07$ $P = 0.3040$

Each model included hatchling sex, body size (SVL, snout-vent length) and treatment (autoclaved vs. untreated incubation soil) as independent variables and Maternal ID as a random effect. A, azurophil; B, basophil; H, heterophil; M, monocyte; L, lymphocyte. Boldface values in table indicate significant differences ($P < 0.05$).

mass (**Table 1**). The mechanism behind this shift in hatchling morphology is unknown. Soil is difficult to sterilize (Nowak and Wronkowska, 1987) so our single bout of autoclaving might only have resulted in a temporary decrease or alteration of the microbiotic community. Further, the high temperature (131°C) and pressure (2.2 Bar) associated with autoclaving might also have altered physical or chemical (e.g., pH) characteristics of the soil. Changes in the substrate characteristics such as these could have caused subtle developmental shifts in embryos.

In contrast, WBC profiles of hatchling snakes were not significantly affected by exposure to the immune challenges that were manipulated in the present study. The lack of a significant impact of microbiota presence on hatchling immune function in Experiment #1 could be attributed to the eggshell blocking signals regarding external bacterial levels from reaching the embryo. If this were the case, then studies on other systems (different focal species, different soil microbiota, etc.) might generate different results. To test that possibility, our second experiment inoculated pathogen signals directly into the egg, bypassing any shielding

TABLE 3 | Effect of inoculating the eggs of keelback snakes (*Tropidonophis mairii*) with lipopolysaccharide (LPS) or phosphate buffered saline (Control) on phenotypes of hatchlings in Experiment #2.

Variable	LPS ($N = 22$)	Control ($N = 24$)	$F_{1,38}$	P
SVL (cm)	14.54 ± 0.34	15.27 ± 0.31	4.35	**0.0437**
Tail (cm)	3.86 ± 0.12	4.06 ± 0.09	4.12	**0.0496**
Head (cm)	10.02 ± 0.14	10.36 ± 0.10	6.54	**0.0146**
Mass (g)	2.14 ± 0.12	2.31 ± 0.10	2.74	0.1062

Control snakes had longer bodies (SVL, snout-vent length), heads and tails. Boldface values in table indicate significant differences ($P < 0.05$).

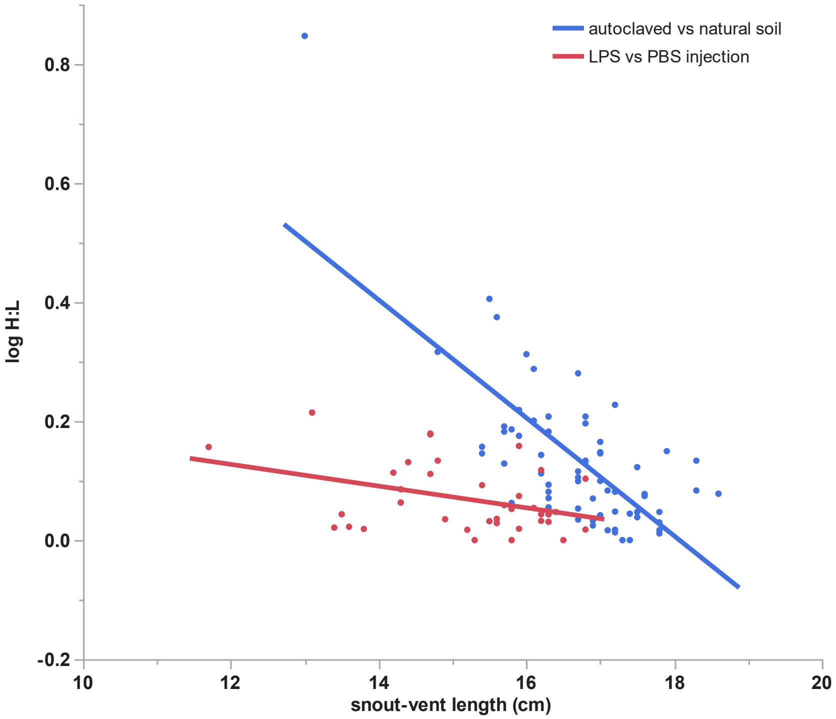

FIGURE 4 | Effects of hatchling body size (SVL, snout-vent length) of keelback snakes (*Tropidonophis mairii*) on heterophil:lymphocyte (H:L) ratios. H:L ratio decreased significantly with snout-vent length (SVL) in both experiments.

provided by the shell. Again, however, we saw no significant immune response in WBC counts of hatchlings.

That lack of response in Experiment #2 is surprising, because administration of LPS into an animal typically initiates a strong immune reaction (Lind et al., 2020; de Figueiredo et al., 2021). By exposing the individual to a component of bacterial cell walls, injection of LPS signals the presence of a pathogen, and thereby triggers a range of immunological and behavioral responses. In particular, WBC profiles often change in response to LPS injection, as specific cell types are recruited to fight the apparent challenge (Keller et al., 2006; Bowen et al., 2009). Why, then, did we find no difference in WBC profiles between treatment and control hatchlings in either of our experiments?

TABLE 4 | Effects of mixed model analyses on the factors affecting white blood cell counts of hatchling keelback snakes (*Tropidonophis mairii*) in Experiment #2.

Cell type	Sex	SVL	Treatment
Log %A	$F_{1,34} = 0.04$ $P = 0.8493$	$F_{1,34} = 0.45$ $P = 0.5073$	$F_{1,34} = 0.001$ $P = 0.9943$
Log %B	$F_{1,33} = 0.05$ $P = 0.8221$	$F_{1,14} = 0.21$ $P = 0.6503$	$F_{1,32} = 0.24$ $P = 0.6300$
Log %H	$F_{1,34} = 1.41$ $P = 0.2427$	$F_{1,34} = 2.67$ $P = 0.1115$	$F_{1,34} = 0.80$ $P = 0.3770$
Log %L	$F_{1,33} = 0.19$ $P = 0.6632$	$F_{1,16} = 0.38$ $P = 0.5456$	$F_{1,32} = 0.18$ $P = 0.6703$
Log %M	$F_{1,34} = 1.31$ $P = 0.2600$	$F_{1,10} = 2.44$ $P = 0.1509$	$F_{1,33} = 2.98$ $P = 0.0937$
Log H:L	$F_{1,34} = 4.46$ **$P < 0.0420$**	$F_{1,34} = 4.91$ **$P < 0.0335$**	$F_{1,34} = 2.67$ $P = 0.1113$

Each model included sex, body size (SVL, snout-vent length) and treatment (injection with lipopolysaccharide vs. phosphate buffered saline) as independent variables and Maternal ID as a random effect. A, azurophil; B, basophil; H, heterophil; M, monocyte; L, lymphocyte. Boldface values in table indicate significant differences ($P < 0.05$).

One possible explanation is that both egg yolk and albumin contain compounds with immune functions. Because reptile eggs are in close contact with soil microbiota, and typically without parental care (i.e., cleaning) of incubating eggs, shells and egg contents may have been under strong selection to develop antimicrobial capability (Gayen et al., 1977; Araki et al., 1998; Zimmerman et al., 2010; van Hoek, 2014). Compounds with antimicrobial abilities (e.g., lysozyme, defensin, ovotransferrin) have been isolated from reptile eggs and others likely remain to be identified (van Hoek, 2014). Furthermore, embryos may be chemically protected by specific maternally provisioned immune compounds (such as antibodies) based on the mother's previous exposure to pathogens or her general health (Hasselquist et al., 2012). Such maternal effects on offspring immunocompetence have been documented in reptiles, but identifying the precise mechanisms through which such effects operate remains a nascent field (Uller et al., 2006; Itonaga et al., 2011; Brown and Shine, 2016). LPS may possibly be destroyed or altered by these compounds before reaching the embryo, thereby preventing any immune response involving WBC populations.

It remains possible that other components of the immune system were activated, without altering WBC profiles. Reptiles in general and snakes in particular are known to possess several extracellular immune mechanisms (Zimmerman et al., 2010). For example, natural antibodies and complement provide rapid non-specific protection against general common pathogens (Madsen et al., 2007; Brusch et al., 2019, 2020). Notably, exposing embryos to LPS produced significantly smaller offspring, consistent with energy allocation trade-offs. The nature of these trade-offs, and the specific components involved, remain to be identified.

Although our experimental treatments did not affect the WBC profiles of hatchling snakes, we did identify strong correlates of WBC variation among individuals. Notably, both the sex and body size of a hatchling snake affected its WBC profile, consistent with our previous research in keelbacks. Two earlier studies reported a negative correlation between H:L ratio and SVL in this species (Brown and Shine, 2016, 2018). This congruence among disparate studies suggest that the association is a consistent feature of keelback biology. The H:L ratio is often described as a stress leukogram because the number of heterophils relative to lymphocytes increases after exposure to known stressors (Davis et al., 2008; Davis and Maney, 2018). However, constitutive differences in H:L ratios among hatchlings might also reflect different allocation to “cheap” vs. “expensive” immune mechanisms (Brown and Shine, 2016).

If H:L ratio does indeed represent a stress response, our results suggest that the treatments we imposed in the present study were not stressful. The lack of stress response following injection of bacterial-derived compound (LPS) directly into the egg suggests that either the embryo’s immune system is not yet sufficiently well-developed to respond to such a challenge; or that as-yet-unknown defense mechanisms protect a growing embryo either by shielding it from these hostile incursions, or by buffering the effects of infective agents by changes other than those revealed by WBC counts.

Lastly, we address the implications of our results for anthropogenic disruptions to microbiota in the soil. As noted in the Introduction to this article, humans may modify the incubation-associated microbiota either inadvertently (e.g., through pollution) or intentionally (e.g., by translocating eggs to new nest sites, or by incubating eggs in sterile media). Our results suggest that (contrary to our *a priori* hypotheses), such manipulations do not affect immune-system function in hatchlings; but do affect hatchling body sizes, which in turn are strongly linked to immune-function parameters. In keelbacks (as is likely the case in many reptile populations), hatchling size is under strong directional selection in the field (Brown and Shine, 2004); and thus, managers need to evaluate the impacts of their manipulations on such traits in order to maximize the viability of hatchlings produced either in the field or the laboratory.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The University of Sydney Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

GB devised the study, gathered data, and conducted statistical analyses. Both authors drafted the manuscript.

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BRIEF RESEARCH REPORT
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Too Hot to Nest? In a Hot Summer the Tortoise *Chersina angulata* Can Switch From Nesting to Facultative Viviparity

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In a captive colony of *Chersina angulata* in Cape Town, South Africa, we observed in 2015/16 retention of the last egg clutch inside the female until the hatching stage was reached, conforming to the generally accepted definition of viviparity. Retrospective climatic analysis indicates egg retention until the hatching stage co-occurred with unusually hot summer weather: the average air temperatures in December 2015 and January and February 2016 were higher than during the preceding five and the following 5 years when facultative viviparity could not be observed. Late December and January appears to be the critical period for females to either deposit their last clutch of the nesting season into a nest, or to retain the last clutch for embryonic development inside the female. Over the 28 December to 24 January period the minimum, average and maximum air temperatures in 2015–16 were about 3°C higher than in the five following years. This association of facultative viviparity with unusual summer heat suggests that hot ambient temperatures at the end of the nesting season may cue females to switch from oviposition to facultative viviparity. Compared to incubation in a nest this phenotypic plasticity of the reproductive mode—to retain during hot summers the season's last clutch inside the female—may buffer the developing embryos from excessive heat exposure: females can thermo-regulate by moving among microhabitats whereas sun exposed shallow nests cannot escape high ground temperatures. This novel reproductive strategy has the potential to enhance the resilience of species to global warming.

Keywords: heat, rainfall, climate change, reptile, Testudines, oviparity, facultative viviparity, reproductive strategy

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INTRODUCTION

Among living reptilian orders, Testudines, Crocodylia, and Rhynchocephalia are considered strictly oviparous whereas Squamata include both oviparous and viviparous species (Blackburn and Sidor, 2014). Turtles typically lay their eggs in underground nests constructed with their hind legs, although in humid environments some may also deposit eggs on the ground beneath leaf litter or beneath the edge of fallen logs (Kuchling, 1999). No matter how long turtle females may retain eggs, the generally accepted wisdom is that intrauterine development of embryos is arrested at the gastrula stage until oviposition (Ewert, 1985; Kuchling, 1999; Rafferty and Reina, 2012).

In the family Testudinidae, *Chersina angulata* (Schweigger, 1812) has an unusual reproductive pattern for a tortoise inhabiting climatic zones ranging from winter rainfall with extreme aridity in the northwest (south-western Namibia) to mediterranean in the southwest (Western Cape) to temperate with all-year rainfall in southern South Africa: females produce single-egg clutches nearly year round (March to December) and females lay up to six clutches per year (Hofmeyr, 2004, 2009; Branch, 2008). The nest consists of an about 10 cm wide shallow depression with a small chamber 4 cm wide and deep at its bottom, constructed in sandy soil, in a well-drained, sunny position. After laying, the soil is tamped down by the female with her shell (Branch, 2008). Similar to other tortoises occurring in the winter rainfall region of South Africa, hatching occurs March to April, just before or at the start of the rainy season in autumn (Hofmeyr, 2009). Egg shells crack 6–7 days before hatchlings emerge. Little is known on the ecology of hatchlings and their habitat choice, but high nest and hatchling predation rates from mongooses and jackals have been observed as well as hatchling predation from baboons, rock monitors, secretary birds, sea gulls, and crows (Branch, 2008).

Egg retention time in the oviducts of *C. angulata* varies substantially: it can last from 23 to 212 days (Hofmeyr, 2004). Observations indicate that *C. angulata* females can retain eggs until embryonic development has progressed to the hatching stage (Hofmeyr and Kuchling, 2017), conforming to the generally accepted definition of viviparity in reptiles (Shine, 1985; Van Dyke et al., 2014): eggs produced in early summer (December) are occasionally not deposited in nests, but retained by females until embryonic development has progressed to the hatching stage, until the time hatching normally occurs in the species (Hofmeyr and Kuchling, 2017). To our knowledge, no respective observations have been reported for any other chelonian species. However, we observed facultative viviparity in the second author's long-term captive *C. angulata* colony only over the summer of 2015/16 (Hofmeyr and Kuchling, 2017) and could not repeat this observation over the five following years.

In the present paper we explore if the switch from oviparity to facultative viviparity in *C. angulata* in 2015/16 may have coincided with unusual climatic conditions. Cape Town recorded unusually high temperatures in 2015, including its highest temperature of the last 100 years at 42°C (downloaded 01 June 2021).[1] A multi-year (2015–2017) drought occurred in the South West of the Western Cape from 2015 to 2017. Total annual rainfall in each of those years was lower than the long-term average, with the strongest anomaly in 2017 (Odoulami et al., 2021). The Western Cape drought continued throughout 2015–2019 and was either the longest or third longest drought on record since 1901 (Kam et al., 2021).

Thus, the climatic condition which differed in 2015/16 from those in the following years was that it was an unusually hot summer period, while severe drought conditions continued for several more years. In the present paper we test the hypothesis that viviparity, resulting from retention of the last egg clutch of the nesting season by female *C. angulata*, is a phenotypically plastic response to unusually high environmental temperatures or drought at the time they would normally nest. Late December/early January is the critical time period to follow one of the alternative strategies to either nest or to retain the last egg clutch of the nesting season inside the female. We analyze monthly ambient air temperatures and monthly average rainfall from December to March and weekly ambient air temperatures from late December to late January to explore if environmental temperatures and precipitation over the summer of 2015/16, when we observed facultative viviparity, differed from those over the five previous and the five following summers.

MATERIALS AND METHODS

The reported observations of facultative viviparity are based on routine observations in the long-term captive research colony of *C. angulata* established by author 2 in South Africa to investigation the species' reproductive biology: in 1999 sixteen wild *C. angulata* females and five males from the West' Coast National Park (WCNP; 33′13′S; 18′09′E) were transferred to Kuilsrivier (33″56′S; 18″41′E), 90 km from the WCNP. The animals were maintained in a 20 × 10 m outdoor enclosure under generally natural climatic conditions, but in addition it was irrigated with ground water 3 days a week for 20 min. The enclosure contained Kikuyu grass (*Pennisetum clandestinum*) that provided food and shelter, sandy areas for nesting, dense bushes and artificial shelters; the animals received supplemental food (fresh vegetables, occasional fruits, and chicken eggshell) and drinking water (Hofmeyr, 2004).

At the time of and in the year prior to the reported incidental observation (April 2016) no research interferences occurred with females, nests or eggs in the enclosure. From 2017 until 2021 all 16 females were annually radiographed in late January/early February to establish if any of them retained eggs over the summer, during the period of the year when no new clutches are ovulated (Hofmeyr, 2004). Following the passing of author 2 in February 2020 the captive colony was disbanded in February 2021, with all originally transferred 16 females and five males still alive.

The climate data analysis is based on air temperatures and rainfall collected by the Matroosfontain Meteorological Station at Cape Town International Airport, 10 km from the captive colony, as provided by https://www.wunderground.com/history/monthly/za/matroosfontein/FACT/date. Monthly temperature averages (December–March) from 2011 to 2021 were converted from Degrees Fahrenheit to Degrees Celsius. The daily minimum, average and maximum temperatures for the period 28 December–24 January of the years 2010/11–2020/21 were converted from Degrees Fahrenheit to Degrees Celsius and averaged for 7-day (weekly temperature) and 28-day (four-weekly temperature) periods. The 1979–2000 averages of monthly rainfall are based on a graphic overview provided by https://www.csag.uct.ac.za/current-seasons-rainfall-in-cape-town/ (downloaded 01 June 2021).

RESULTS

Observation of facultative viviparity:

In 2016, the captive females started nesting by mid-March and in the afternoon of 1 April 2016, author 2 found two unburied

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[1] http://www.csag.uct.ac.za/2019/04/08/is-the-rainy-season-shifting/

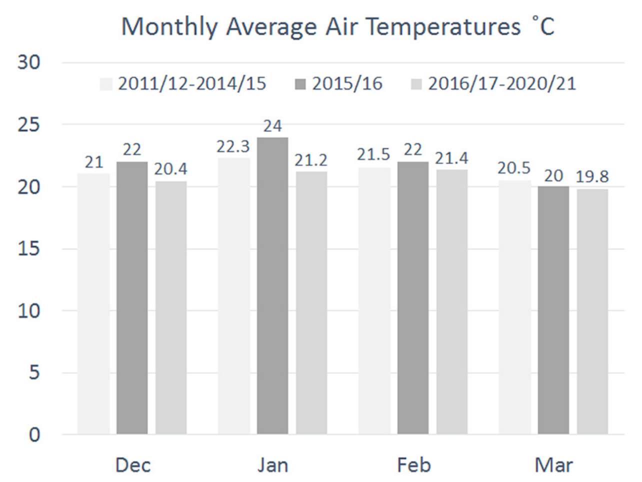

FIGURE 1 | Monthly averages of daily average air temperatures at Matroosfontain Meteorological Station for December to March 2015/16, and the respective averages for the years 2011/12 to 2014/15 and 2016/17 to 2020/21.

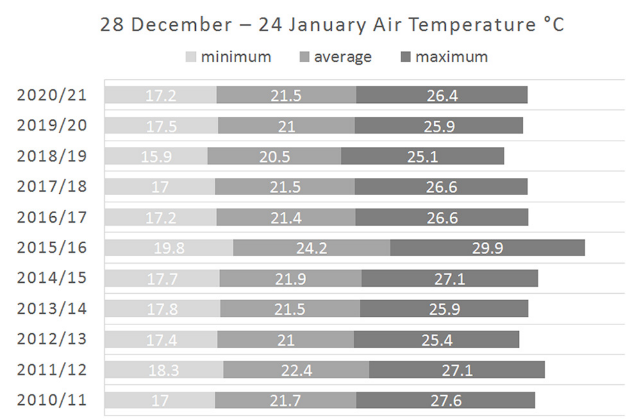

FIGURE 2 | 28 December to 24 January four-weekly averages of daily minimum, average and maximum air temperatures at Matroosfontain Meteorological Station for every year from 2010/11 to 2020/21.

eggs (ca. 2 m apart) in the captives' enclosure with no sign of disturbance of the surrounding ground, indicating that one or two females dropped eggs without digging nests. Although laying one egg at a time is the norm for this species, two-egg clutches occasionally occur and we do not know if one female laid both eggs or if two females laid one egg each. Dropping eggs on the surface is not common but neither unusual for *C. angulata* in the wild or in captivity (author 2, personal observation). The eggs found on 1 April were at most 1-day old, because the tortoises were fed the previous day. Since the eggs have been exposed to full sun, they were left outside to let nature take its course. When the tortoises were fed on the morning of 5 April, each egg had a small opening through which a hatchling and substantial amount of yolk was visible. The eggs were brought indoors, placed in a container, and left so that the hatchlings could internalize the residual yolk. Both hatchlings emerged 4 days later on 9 April 2016. Upon hatching, the hatchlings weighed 19.7 and 16.9 g, respectively, and showed no external abnormalities. Both hatchlings displayed normal activity and growth, and, respectively, weighed 25.9 and 28.5 g 3 months later. Nests from the 2015 nesting season in the enclosure have not been monitored, although some hatchlings emerged in autumn 2016 from nests of the 2015 season we do not know the respective hatching success (percentage of eggs that hatched).

Summer air temperatures:

The monthly average air temperatures for December, January, and February 2015/16 at Matroosfontain Meteorological Station were higher than the monthly averages averaged for the preceding 4 years and the following 5 years. The March 2016 average was only marginally warmer than the average for the five following years, but cooler than the average for the previous 4 years (**Figure 1**).

Regarding the 4-week (28 days) period 28 December to 24 January, the average of daily minimum, average and maximum air temperatures in 2015/16 was, respectively, 2.8, 3.1, and 3.8°C higher than averaged over the five following years and 2.2, 2.5, and 3.3°C higher than averaged over the four previous years. Considering every year from 2016/17 separately, the average of daily minimum temperatures in 2015/16 was still at least 2.3°C higher than in any year from 2016/17 to 2020/21, the average of daily average temperatures in 2015/16 was at least 2.7°C higher than in any year from 2016/17 to 2020/21 and the average of daily maximum temperatures in 2015/16 was at least 3.3°C higher than in any year from 2016/17 to 2020/21 (**Figure 2**).

The weekly averages of the daily minimum, average and maximum air temperatures in the 4-week period 28 December to 24 January were also consistently higher in 2015/16 than the respective temperatures averaged for the years 2016/17 to 2020/21. This was particularly pronounced in the 7-day period 28 December to 02 January, when in 2015/16 the average minimum temperature was 3.7°C, the average of the average temperature 3.4°C and the average maximum temperature 4.3°C higher than the respective averages over the following 5-year period (**Figure 3**).

Monthly rainfall at Matroosfontain Meteorological Station from December 2015 to March 2016 (when facultative viviparity was observed) was higher than the historic monthly rainfall averaged from 1979 to 2000 and December rainfall was higher in 2015 than in 2017 and 2020 (**Figure 4**). December/January rainfall combined was higher in 2015/16 (35.6 mm) than in 2019/20 (34.5 mm) and 2020/21 (18.1 mm).

DISCUSSION

The discovery of facultative viviparity in the captive *C. angulata* colony in 2015/16 was unplanned and involved happenstance. Despite increased monitoring we could not repeat this observation over the five following years. The results of the present investigation demonstrate that, locally for the studied captive population, the summer of 2015/16 was the hottest

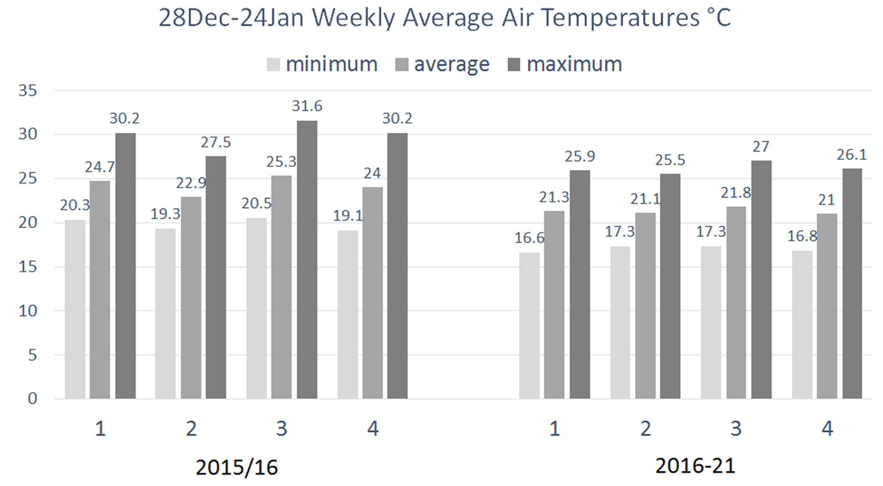

FIGURE 3 | Seven-day averages of daily minimum, average and maximum temperatures (°C) from December 28th to January 24th (1: 28 December–03 January; 2: 04–10 January; 3: 11–17 January; 4: 18–24 January) at Matroosfontain Meteorological Station in the summer of 2015/16 when facultative viviparity was observed and averaged for the five following summers from 2016/17 to 2020/21.

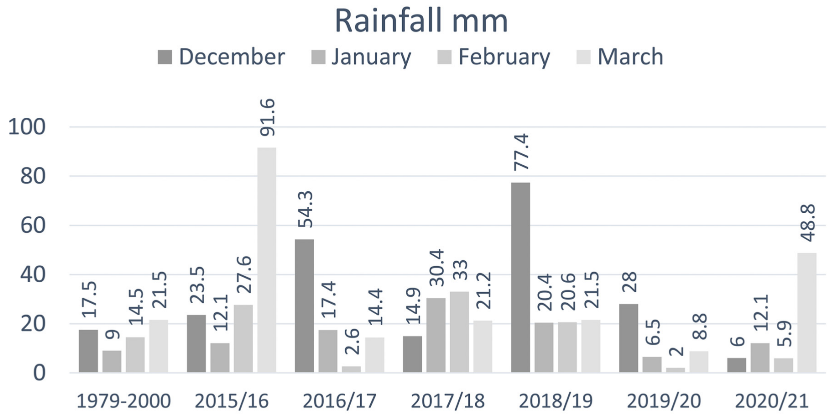

FIGURE 4 | December to March monthly rainfall at Matroosfontain Meteorological Station averaged from 1979 to 2000, monthly rainfall in the summer of 2015/16 when facultative viviparity was observed in the captive colony and in the five following summers when facultative viviparity was not observed.

summer of the last decade. We found no indication for rainfall anomaly in the summer of 2015/16, the 2015–2017 drought in Cape Town was primarily the result of reduced winter rainfall (downloaded 01 June 2021).[2] Opportunistic studies, like the present one, lack information on the situation earlier on: prior to April 2016 observations were insufficient to indicate if facultative viviparity had already occurred in the captive colony in earlier years. However, the results of this study demonstrate that, over the last 6 years when monitoring was increased, facultative viviparity in the *C. angulata* captive colony only co-occurred with a single extreme summer heat event. Extreme climatic events happen rarely, thus there are few opportunities to study their effect on an organism's phenotypically plastic response. However, even observing only one event can tell us what types of responses are possible (Altweg et al., 2017).

Concerning the 2016 case history of the detection of the two fully developed eggs, there was no evidence that the female(s) attempted to dig nests; in fact, the ground surface of the enclosure was completely undisturbed. The two eggs were on open ground close to the enclosure's entrance where they were instantly visible when nearing the enclosure. Because members of the household visit the enclosure and its surroundings on a regular basis, there is no other explanation than that one or two females laid fully developed eggs on the ground surface shortly before the eggs hatched. It could well be that similar cases have gone undetected in past autumns if females laid fully developed eggs among vegetation. Even though our observations do not indicate viviparity in captivity co-occurred with summer droughts, respective triggers might have been mask by the regular irrigation of the enclosure with bore water. Apart from this possibility, Barrows et al. (2016) stressed the general difficulty of separating temperature and

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[2] http://www.csag.uct.ac.za/current-seasons-rainfall-in-cape-town/

rainfall as driving force in semi-arid to hyper-arid winter rainfall areas, in that case for modeling habitat suitability of the desert tortoise (*Gopherus agassizii*): since, in these areas, summer temperature (mean July maximum temperature) and rainfall/humidity are negatively auto correlated they used summer temperature alone to identify habitat refuges under climate change. The natural range of *C. angulata* extends into areas of extreme aridity (south-western Namibia). Low soil humidity may reduce nesting and hatching success and most *C. angulata* eggs are generally laid within 3 days of rainfall (Hofmeyr, 2004). Therefore, in addition to summer heat, we cannot exclude that in natural populations of *C. angulata* drought or low humidity may act as a further potential cue for facultative viviparity.

The only earlier observation suggesting the possibility of facultative viviparity in *C. angulata* was made over two decades ago in a wild population of *C. angulata*, but we were unable to reconstruct the local climatic conditions of that time to analyze them for inclusion into the results of this paper: while doing fieldwork on Dassen Island, an offshore island in the Atlantic Ocean to the south-west of West Coast National Park, author 1 found a *C. angulata* egg (length = 41.0 mm, width = 35.2 mm) in the late afternoon of 20 March 1999. The unburied egg was on open, rocky ground, in full sun, relatively close to a laboratory where we assessed the reproductive condition of *C. angulata* females by ultrasound scanning. No sign of a nest hole could be seen nearby and the eggshell was clean white, without a trace of soil or dirt, as in a freshly laid egg. We did not notice the egg in the morning or in preceding days when field workers frequently passed through this area. Since some females had ovulated by that time, the beginning of a new nesting season, we first assumed that, following the ultrasound examination, a released female had aborted her first shelled egg of the new season and dropped it on the ground without nesting. We assumed it was doomed due to its exposure to the elements, measured the egg and stored it in a glass flask in the laboratory. However, the egg hatched overnight and the following morning we found a fully developed hatchling (mass = 14.5 g, carapace length = 35.6 mm) in the glass flask with all yolk internalized. The hatchling was alert and moved normally, but appeared to have a bilateral microphthalmic condition.

Chersina angulata females occasionally lay eggs on the surface (not into nests underground), but this is not limited to autumn when hatching normally occurs (M. D. Hofmeyr, personal observation). Consequently, egg laying on the surface in itself is not linked to full embryonic development. We detected the fully developed eggs at Dassen Island and in the captives' enclosure because they were out in the open. Although the incident at Dassen Island over two decades ago perplexed us at the time, its viviparity interpretation conflicted with the (still today) generally accepted wisdom that the order Testudines is strictly oviparous. At the time we were hesitant to interpret and report it correctly as viviparity. Our hesitation disappeared with the second incident in the captive colony, which dispelled any other explanation than that *C. angulata* mothers can carry embryos to full term. The reason for the rarity of respective reports for reptiles may well be that such cases can easily be overlooked, or shelved away as odd flukes instead of being reported (as we first did in 1999), or that editors reject their publication in scientific journals (as happened during the late life time of the second author, with exception of a conference abstract: Hofmeyr and Kuchling, 2017). Since then facultative viviparity (capacity to deposit either eggs or developed offspring, depending on circumstances) has been reported for one more reptile, the skink *Saiphos equalis*, with its discovery based on a single female in a captive colony (Laird et al., 2019). Even though this reproductive lability has been rarely described in reptiles, it can be part of a viable reproductive strategy and evolutionary more significant than previously assumed.

The reproductive strategies of oviparity and viviparity both entail advantages and disadvantages, each of which may differ in their applicability to particular species. The universality of internal fertilization in reptiles readily permits the evolution of viviparity which evolved over 100 times in different lineages of the order Squamata, often at subfamilial and subgeneric levels and, in some cases, at the subspecific level (Blackburn, 1999). From the standpoint of life history theory viviparity should evolve only when the benefits of stages that are evolutionarily intermediate outweigh the costs. Selection would only favor uterine retention of eggs if there were a net benefit to female fitness as measured by lifetime reproductive success: the costs of longer egg retention have to be more than counterbalanced by increased egg survivorship through a reduced incubation period (Williams, 1992). Due to the small clutch size of *C. angulata*, the costs of retaining a clutch for longer periods (impaired mobility, higher risk of predation of the egg carrier, reduced potential for feeding impacting subsequent fecundity: Shine, 1980) appear to be small regarding the residual reproductive value of the female.

One explanation for the, until now, presumed absence of steps toward viviparity in the order Testudines is that, in most species, eggs are laid in an environment removed and quite different from the one in which the turtles live, making the intermediate egg retention stages in oviducts ineffective as a means of later placing eggs in the most optimal environment (Tinkle and Gibbons, 1977). However, this reasoning only applies to aquatic and marine turtles, it does not apply to terrestrial tortoises (Family Testudinidae). Another explanation is that viviparity could be disadvantageous in multi-clutching turtles because longer oviducal egg retention times would reduce annual fecundity (Tinkle and Gibbons, 1977). This constraint also appears not to apply to *C. angulata* which usually has single-egg clutches and can ovulate a further clutch while the previous clutch is still in the oviduct (Hofmeyr, 2004). Our data suggest that viviparity in *C. angulata* is facultative and may be restricted to the last clutch of their long nesting season, with gravidity not preventing ovulation of the first clutch of the following nesting season. Facultative viviparity could also occur in other arid-adapted tortoises, particularly in the genus *Chersobius*, *Chersina*'s sister genus which diverged from it during the Late Oligocene warming phase (26 Mya). The ranges of all three species of the genus *Chersobius*, *C. boulangeri*, *C. signatus,* and *C. solus*, are largely embedded in *C. angulata*'s much larger area of distribution and all three species also produce single egg clutches (Hofmeyr et al., 2016).

The co-occurrence of facultative viviparity in *C. angulata* with hot summer temperatures is not in agreement with the "cold-climate hypothesis" for the evolution of viviparity in reptiles, but supports the more general "maternal manipulation hypothesis": that prolonged uterine retention of developing embryos allows a female to provide better incubation conditions for her offspring (Shine, 2014). In the lizard *Zootoca vivipara* females select lower-than-usual temperatures while carrying eggs and this avoidance of high temperatures early in embryogenesis enhances the viability of offspring (Rodríguez-Díaz and Braña, 2011). Under unusually hot summer temperatures facultative viviparity in *C. angulata* may provide comparable temperature advantages for embryonic development: we propose that high risks associated with nesting and egg development during hot and dry summer conditions provide selective pressures for the evolution of facultative viviparity in *C. angulata*. In the captive *C. angulata* colony the environmental cue to initiate facultative viviparity appears to be unusually high temperatures toward the end of their nesting season when, under normal temperature conditions, they would oviposit their last clutch into a nest. Phenotypic plasticity can be a major mechanism of response to environmental variability, which may allow organisms to cope with rapid environmental changes (Bonamour et al., 2019).

Several aspects of facultative viviparity in *C. angulata* need to be studied in more detail. Oviparous reptiles, with the exception of crocodiles, arrest embryonic development prior to oviposition. Squamata most often arrest at stage 30 of a 40-stage chronology (Andrews and Mathies, 2000; Van Dyke et al., 2014), whereas in Rhynchocephalia and, as generalization, in Testudines this pre-ovipositional developmental arrest occurs earlier during development at the gastrula stage (Ewert, 1985; Andrews and Mathies, 2000; Rafferty and Reina, 2012). Under hypoxia, in the oviducts as well as experimentally, turtle embryos arrest development at the gastrula stage, until resumption of their development is again triggered by a normoxic environment (e.g., once deposited into the nest). In the marine turtle *Chelonia mydas* (Cheloniidae) and in the freshwater turtles *Chelodina oblonga*, *Chelodina longicollis,* and *Emydura macquarii* (Chelidae) pre-ovipositional developmental arrest is achieved by a mucus-like substance secreted by the oviducts which retards oxygen diffusion (Rafferty et al., 2013), a mechanism that appears adaptive for these multi-clutching aquatic species. Pre-ovipositional developmental arrest would prevent the transition to viviparity (Andrews and Mathies, 2000), unless it is abolished (as in viviparous squamates) or becomes facultative. Neither details of pre-ovipositional developmental arrest, nor oxygen conditions in the oviducts, nor eggshell morphology and composition have so far been investigated in *C. angulata*. This basic research will be essential to understand the mechanisms behind this species' unique suite of reproductive strategies.

Elucidating mechanisms and strategies available to species plays a significant role in assessing and predicting the susceptibility or resilience of species to future, human-induced environmental change (Franklin and Hoppeler, 2021). Our analysis of air temperatures and rainfall for the summer when we observed facultative viviparity in a captive colony of *C. angulata*, and for the five following summers when we could not observe it, suggests that hot ambient temperatures at the end of this species' long nesting season can function as environmental cue for females to switch from egg deposition into nests to facultative viviparity. The association of this phenotypic plasticity with inter-annual variations in climate has the potential to buffer the developing embryos of the season's last clutch from overheating during heat waves or increasing temperatures due to climate change: females can thermo-regulate by moving among different microhabitats whereas, once the nest site is chosen, nests of non-nest-guarding reptiles are passively exposed to hot ambient temperatures. This study provides insight into a, for the order Testudines, so far unrecognized and novel strategy: to change the reproductive mode during extremely hot summer weather from oviparity to facultative viviparity to facilitate successful embryonic development of the last clutch of the season. This novel reproductive strategy has the potential to enhance the resilience of species to global warming.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Western Cape Research Ethics Committee CWE/23 (SANP), 96/10/15.

AUTHOR CONTRIBUTIONS

MH conceived the idea of facultative viviparity in *C. angulata*, monitored the captive population and wrote an early draft of part of this manuscript with GK providing conceptual input. Following MH's passing in February 2020, GK conceived the idea that climate variability may influence the occurrence of facultative viviparity in *C. angulata*, retrieved and analyzed the temperature and rainfall data, and wrote the final draft. Both authors contributed to the article and GK approved the submitted version.

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REVIEW
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What Are the Primary Cues Used by Mammalian Predators to Locate Freshwater Turtle Nests? A Critical Review of the Evidence

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Turtle biologists have long been interested in the biotic and abiotic factors that influence the detection of freshwater turtle nests by mammalian predators. Increased knowledge of nest predation dynamics may help develop conservation strategies to increase turtle nesting success by altering or reducing the signal strength of predominant nest location cues. However, despite this long-standing interest, the related research has produced inconsistent and sometimes conflicting results across studies. Here we review much of the existing literature on freshwater turtle nest predation by mammalian predators and attempt to synthesize some general, underlying themes. Available data suggest that raccoons (*Procyon lotor*) primarily use olfactory cues associated with nest cavity construction to locate turtle nests. However, some other predators, including red foxes (*Vulpes vulpes*) and other canids, may commonly use a wider array of cues, including scents from nesting turtles and their eggs as well as visual cues, while foraging. The literature also suggests that the length of the period turtle nests remain vulnerable to nest predation is dependent on the predator community, with raccoons exhibiting relatively short timelines relative to some other predators, including canids. This review has revealed a strong North American bias in published work, highlighting the need for additional studies of turtle nest predation dynamics in other areas of the world where chelonians are often imperiled elements of the biota.

Keywords: Reptilia, Testudines, freshwater turtles, *Procyon lotor*, raccoon, sensory cues, nest predation

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INTRODUCTION

For oviparous reptiles, the embryonic stage is one of the most vulnerable phases of the life cycle (Fitch and Fitch, 1967), and high levels of egg predation characterize many chelonian populations (Iverson, 1991). For the majority of turtle species, females provide little or no maternal care, thus successful incubation requires that females select nest sites with appropriate thermal and hydric conditions for incubation (e.g., Schwarzkopf and Brooks, 1987; Wilson, 1998; Morjan, 2003; Pruett et al., 2019) and that nests evade detection by egg predators (Spencer, 2002; Spencer and Thompson, 2003, 2005). Turtle eggs comprise an energy-rich food source (Booth, 2003) and vertebrate egg predators are a predominant source of egg mortality in freshwater turtle species worldwide. Because many turtle species have relatively specific habitat requirements (e.g., Burger and Montevecchi, 1975; Bodie et al., 1996; Wilson, 1998; Paterson et al., 2013) and often exhibit high levels of nest site fidelity (Freedberg et al., 2005), populations are increasingly vulnerable to elevated nest predation rates as habitat loss and degradation (e.g., vegetational succession) reduces the size of nesting habitat and nest predators such as raccoons (*Procyon lotor*) concentrate their foraging efforts in the remaining areas (e.g., Temple, 1987; Jackson and Walker, 1997; Marchand and Litvaitis, 2004).

Turtle nest predation and the factors that influence it have long been of interest to both amateur natural historians and academics alike. Sensory cues used by predators to identify location of turtle nests are of considerable importance to researchers interested in turtle ecology and conservation, as reduction of the signal strength of these cues may aid management efforts to increase hatchling recruitment. Beginning with possibly the first reference to raccoon depredation of turtle nests/eggs (Kennicott, 1858) and the first experimental efforts to understand the underlying nest location dynamics (Moll and Legler, 1971; Wilhoft et al., 1979), numerous studies spanning nearly 70 years have speculated on, or experimentally evaluated, the sensory cues used by mammalian predators to locate turtle nests. In spite of these efforts, little consensus has emerged as to the primary nest location cues used by mammals, and the related literature is inconsistent and sometimes conflicting; especially perhaps, for raccoons, the most commonly studied turtle nest predator in the world.

The purpose of this review is to provide the first comprehensive synthesis of our present understanding of the sensory cues used by mammalian predators to identify and locate nests of freshwater turtles and to suggest productive areas for future research. In our review, we place an emphasis on predation by raccoons, and often compare findings on other predators to them, because of the disproportionately large number of studies where they were documented to be the predominant nest predator in this cue-focused review (**Table 1**). Nonetheless, we have included results from studies of other mammalian species where available.

TABLE 1 | Number of papers in this review reporting predominant mammalian predators of natural or artificial nests (*n* = 46 citations).

Nest predator		Number incorporating natural nests	Number incorporating artificial nests
Family	**Species**		
Procyonidae	*Procyon lotor*	23	12
Canidae	*Canis latrans*	1	0
	Cerdocyon thous	0	1
	Lycalopex gymnocercus	0	1
	Nyctereutes procyonoides	1	0
	Vulpes macrotis	1	0
	Vulpes vulpes	6	3
Mephitidae	*Mephitis mephitis*	5	0
Didelphidae	*Dasypus novemcinctus*	2	1
Mustelidae	*Lontra canadensis*	1	1
	Meles meles	2	0
Suidae	*Sus scrofa*	1	0

METHODS

Literature Review and Analysis

During the course of this review, we surveyed over 90 papers focusing on or incidentally reporting nest predation metrics for freshwater turtles. We found primary literature by on-line searches using Google, Google Scholar, and the academic research databases of the University of Wisconsin Library System (>1100 e-collection content selections from Primo Central Index [PCI] from Ex Libris [ProQuest] including Web of Science and Scopus) using the keywords: "turtle:nest:predation". We also subsequently reviewed the literature-cited sections of all these papers for additional sources relevant to freshwater turtle nest predation. Materials were mostly peer-reviewed, published works, although some unpublished MSc and Ph.D. theses were also included in this review.

For simplicity, we grouped the proposed freshwater turtle nest location cues used by mammalian predators in the reviewed literature into recurring principal types (visual, tactile, olfactory). Within each category we highlight certain studies when, in our view, the presented data are unique or particularly definitive in support of study conclusions or, contrarily, when alternate interpretations appear available for reported results. The provided tables offer an overview of either the claims made or inferences which could be drawn (as in the text, sometimes not stated by the authors) from all reviewed materials. Papers that suggest that certain nest location cues may be operative, but only cite previous publications without providing new data in support, are either not noted or are distinguished as such. Finally, we also made a distinction between nest location cues present soon after nest construction and those present during the hatchling emergence period, and report on these as appropriate. While we recognize that predators likely do not rely exclusively on a single cue, and may use multiple cues to locate turtle nests depending on environmental conditions, our intent is to provide a synthesis of the evidence for the primary sensory cues used by mammalian predators as documented in the literature.

RESULTS

Categories of Proposed Cues

Three broad categories of cues are prominent among those proposed to explain how raccoons and other mammalian predators locate newly created, freshwater turtle nests: (1) olfactory, (2) visual, and (3) tactile. Investigations within each of these categories have attempted to determine the cues of primary importance to nest-foraging predators (**Table 2**), with varying levels of empirical support—from observation-based speculation to supporting data. Consequently, in some cases, there is disagreement among researchers as to which cues

TABLE 2 | Papers proposing cues used by mammalian predators to locate freshwater turtle nests before the emergence period.

Proposed nest location cue	Nest type	Turtle species	Predominant predator(s)	Citation	Comments
Visual					
Nest itself (via visible soil disturbance)	Artificial (cavities, some with turtle or quail eggs)		*Vulpes vulpes*	Spencer, 2002	Artificial nests (all with cavities) with more surface disturbance were excavated at higher rates than those with minimal disturbance.
	Artificial (most with cavities, some with turtle scent, all without eggs)		*Procyon lotor*	Burke et al., 2005	All artificial nests with cavities were smoothed over to resemble natural terrapin nests. Did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Artificial (some with cavities without eggs, some with pond water, etc.)		*Procyon lotor*	Strickland et al., 2010	Surface appearance of artificial nests not described. Did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Natural	*Chelydra serpentina*, *Chrysemys picta*	*Procyon lotor*	Wirsing et al., 2012	Did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Artificial (cavities with chicken eggs)		*Procyon lotor*	Holcomb and Carr, 2013	All artificial nests made the same to resemble natural nests. Did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Artificial (cavities, some with chicken eggs)		*Vulpes vulpes*	Dawson et al., 2014	More visual disturbance (subjectively assessed) associated with higher excavation rates.
	Artificial (some with cavities, some with chicken eggs)		Unspecified	Bernstein et al., 2015	Surface appearance of artificial nests not described. Did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Artificial (cavities without eggs) and natural	*Malaclemys terrapin*	*Procyon lotor*	Czaja et al., 2018	Surface appearance of artificial nests not described. Did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Artificial (cavities, some with quail eggs)		*Lycalopex gymnocercus*, *Cerdocyon thous*	Perazzo et al., 2018	Surface appearance of artificial nests not described. Did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
Tracks of nesting turtles	Natural	*Emydoidea blandingii*	*Procyon lotor, Vulpes vulpes*	Congdon et al., 1983	No supporting data are presented.
	Natural	*Chelydra serpentina*	*Procyon lotor, Vulpes vulpes*	Congdon et al., 1987	No supporting data are presented.
	Artificial		Potentially *Felis silvestris, Meles meles, Sus scrofa, Vulpes vulpes*	Horváth et al., 2021	Camera-based evidence of *Felis silvestris* and *Meles meles* following artificial trails made from turtle-scented water to artificial nests.
Olfactory					
Nesting female turtle (incl. fluids and surrogates)	Natural	*Trachemys scripta*	Unspecified	Cagle, 1950	No supporting data are presented.
	Natural	*Chrysemys picta*	Unknown, but including *Procyon lotor*	Legler, 1954	No supporting data are presented.
	Artificial (most with cavities, some with eggs, turtle urine)		*Ameiva* lizard, *Dasypus novemcinctus*	Moll and Legler, 1971	*Trachemys scripta* turtle urine poured on ground (no cavity) was excavated by lizards and armadillos.
	Artificial (most with cavities, some with turtle scent, all without eggs)		*Procyon lotor*	Burke et al., 2005	Artificial nests with cavities and terrapin-scented sand were excavated at statistically higher rates than those with plain sand in one of two study years.
	Natural	*Kinosternon flavescens*	*Canis latrans, Mephitis mephitis*	Tuma, 2006	*Canis latrans* excavated numerous turtles, including males, from underground burrows in apparent efforts to locate nests. Did not note that soil odor cues may also have been operative.
	Artificial (cavities, some with chicken eggs)		*Vulpes vulpes*	Dawson et al., 2014	Artificial nests (all with cavities) sprayed with pond water were excavated at higher rates than those without pond water.
	Artificial (some with cavities, some with turtle scent or proxies)		*Procyon lotor*	Oddie et al., 2015	Artificial nests with *Chelydra* musk and wetland water (and cavities) were excavated at higher rates than those without applied scents (but no difference when cavity was absent).

(Continued)

TABLE 2 | (Continued)

Proposed nest location cue	Nest type	Turtle species	Predominant predator(s)	Citation	Comments
	Artificial (cavities, several treatments)		Potentially *Felis silvestris, Meles meles, Sus scrofa, Vulpes vulpes*	Horváth et al., 2021	Surmised importance of turtle scent at nests based on camera evidence of scent trail following.
Turtle eggs (and surrogates)	Natural	*Malaclemys terrapin*	*Procyon lotor, Vulpes vulpes*	Burger, 1977	Widespread nest depredation after visible evidence was absent due to wind and rain. Did not resolve the relative importance of olfactory or tactile components of soil disturbance, but suggested olfactory detection of embryonic metabolites or metabolic heat were cues.
	Artificial (cavities, some with turtle or quail eggs)		*Vulpes vulpes*	Spencer, 2002	Artificial nests (all with cavities) with eggs were excavated at higher rates than those without eggs.
	Artificial (cavities, some with chicken eggs)		*Vulpes vulpes*	Dawson et al., 2014	Artificial nests (all with cavities) with eggs were excavated at higher rates than those without eggs.
Soil disturbance (general)	Artificial (cavities, some with chicken, quail, or turtle eggs)		*Procyon lotor*	Wilhoft et al., 1979	Speculated mode relates to soil moisture differences.
	Natural	*Emydoidea blandingii*	*Procyon lotor, Vulpes vulpes*	Congdon et al., 1983	Suggested odor of newly created nest was a major nest location cue. No supporting data are presented.
	Artificial (cavities, some with turtle or quail eggs)		*Vulpes vulpes*	Spencer, 2002	Speculated mode relates to enhancement of turtle and egg scent (possibly also, visual appearance).
	Artificial (most with cavities, some with turtle scent, all without eggs)		*Procyon lotor*	Burke et al., 2005	Found soil disturbance, perhaps detected visually, to be an important nest location cue, but did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Natural	*Chelydra serpentina, Chrysemys picta*	*Procyon lotor*	Wirsing et al., 2012	Did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance. Tested variable was habitat disturbance, not that of nests themselves.
	Artificial (some with cavities, some with chicken eggs)		Unspecified	Bernstein et al., 2015	Found soil disturbance to be a major nest location cue, but did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Artificial (some with cavities, all without eggs)		*Procyon lotor*, *Lontra canadensis*	Rutherford et al., 2016	Concluded that factors associated with soil disturbance, rather than chemical signals from turtles or their eggs, were nest location cues, but did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Artificial (cavities without eggs) and natural	*Malaclemys terrapin*	*Procyon lotor*	Czaja et al., 2018	Suggested that rainfall increased nest success by disrupting soil disturbance cues, but did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
	Artificial (cavities, some with quail eggs)		*Lycalopex gymnocercus*, *Cerdocyon thous*	Perazzo et al., 2018	Concluded that nest location cues were olfactory and related to cavity construction, but did not resolve the relative importance of visual, olfactory, or tactile components of soil disturbance.
Soil disturbance (via moisture differences)	Artificial (cavities, some with chicken, quail, or turtle eggs)		*Procyon lotor*	Wilhoft et al., 1979	Proposed soil moisture signal as speculation based on study data. Soil moisture not tested directly.
Soil disturbance (via geosmin signal)	Artificial (cavities without eggs) and natural	*Graptemys ouachitensis*	*Procyon lotor*	Geller, 2015	Proposed geosmin signal as speculation, not tested directly. Did not resolve the relative importance of tactile components of soil disturbance.
	Artificial (some with cavities, without eggs, some with turtle scent)		*Procyon lotor*	Buzuleciu et al., 2016	Proposed geosmin signal as speculation, not tested directly. Did not resolve the relative importance of tactile components of soil disturbance.
Tactile					
Soil disturbance (via surface hardness differences)	Artificial (some with cavities, some with turtle scent or proxies)		*Procyon lotor*	Oddie et al., 2015	Claim is tenuous. Did not acknowledge that cavity construction also produces olfactory cues.
	Artificial (some with cavities, some with turtle scent, some with geosmin) and natural		*Procyon lotor*	Edmunds et al., 2018	Artificial nests with cavities excavated at higher rates than those without cavities. Proposed the use of a tactile sense, although no supporting data are presented for this mechanism, per se.
	Artificial (cavities without eggs) and natural	*Malaclemys terrapin*	*Procyon lotor*	Czaja et al., 2018	No supporting data are presented.

TABLE 3 | Papers discounting particular cues used by predators to locate freshwater turtle nests before the emergence period.

Proposed nest location cue	Nest type	Turtle species	Predominant predator(s)	Citation	Comments
Visual					
Nest itself (via visible soil disturbance)	Natural	*Malaclemys terrapin*	*Procyon lotor*, *Vulpes vulpes*	Burger, 1977	Widespread nest depredation after visible evidence was absent due to wind and rain.
	Artificial (cavities, all with quail eggs)		Likely mostly *Procyon lotor*	Hamilton et al., 2002	3-m of sand placed over artificial nests did not affect depredation rates.
	Artificial (cavities without eggs) and natural	*Graptemys ouachitensis*	*Procyon lotor*	Geller, 2015	Depredation rates not affected by eliminating visible nest location sign. Facsimiles of nest markings excavated at low rates relative to artificial nests with cavities.
	Artificial (some with cavities, some with turtle scent or proxies)		*Procyon lotor*	Oddie et al., 2015	Inconspicuous artificial nests (with cavities) were depredated at high rates. Facsimiles of nest markings excavated at low rates relative to artificial nests with cavities.
	Natural	*Chrysemys picta*	*Procyon lotor*	Voves et al., 2016	Human-scored degree of nest crypsis was not a significant predictor of survival.
	Artificial (some with cavities, some with turtle scent, some with geosmin) and natural		*Procyon lotor*	Edmunds et al., 2018	Smoothed-over artificial nests (with cavities) were depredated at high rates.
Tracks of nesting turtles	Natural	*Graptemys ouachitensis*	*Procyon lotor*	Geller, 2012a	Camera data showed non-overlap in turtle and depredating raccoon pathways.
	Artificial (cavities without eggs) and natural	*Graptemys ouachitensis*	*Procyon lotor*	Geller, 2015	Eliminating nesting turtle tracks by broom sweeping did not affect depredation rates.
Olfactory					
Nesting female turtle (including fluids and surrogates)	Artificial (cavities, all with quail eggs)		Likely mostly *Procyon lotor*	Hamilton et al., 2002	Tortoise bladder water applied to artificial nests did not affect predation rates.
	Artificial (cavities without eggs, some with pond water)		*Procyon lotor*	Strickland et al., 2010	Slough water applied to artificial nests did not affect predation rates.
	Artificial (cavities with chicken eggs)	*Macrochelys temminckii*	*Procyon lotor*	Holcomb and Carr, 2013	Excavation rates of artificial nests without female turtle scent cues were similar to natural nests
	Artificial (some with cavities, without eggs, some with turtle scent)		*Procyon lotor*	Buzuleciu et al., 2016	Excavation rates of artificial nests with female turtle scent cues were not significantly different from those of treatments with neutral or no scent.
	Artificial (some with cavities, all without eggs)		*Procyon lotor*, *Lontra canadensis*	Rutherford et al., 2016	Turtle-scented water did not affect excavation rates on artificial nests with cavities. No depredation of treatments using turtle water alone.
	Artificial (some with cavities, some with turtle scent, some with geosmin) and natural		*Procyon lotor*	Edmunds et al., 2018	Excavation rates of artificial nests without female turtle scent cues were not significantly different from those of treatments with scent or of natural nests.
Turtle eggs (or surrogates)	Artificial (cavities, some with chicken, quail, or turtle eggs)		*Procyon lotor*	Wilhoft et al., 1979	41% of 83 depredated artificial nests contained only ping-pong balls.
	Artificial (some with cavities, some with chicken eggs)		not specified	Bernstein et al., 2015	Presence of chicken eggs did not significantly affect depredation rates relative to those without eggs.
	Artificial (cavities, some with quail eggs)		*Lycalopex gymnocercus*, *Cerdocyon thous*	Perazzo et al., 2018	Presence of quail eggs did not affect depredation rates relative to those without eggs.
Both turtle scent and eggs (or surrogates)	Artificial (cavities without eggs) and natural	*Graptemys ouachitensis*	*Procyon lotor*	Geller, 2015	Excavation rates on artificial nests without eggs or turtle scent cues were similar to those of natural nests.
	Artificial (cavities without eggs) and natural	*Malaclemys terrapin*	*Procyon lotor*	Czaja et al., 2018	Excavation rates on artificial nests without eggs or turtle scent cues were similar to those of natural nests.
Soil disturbance (via moisture differences)	Artificial (cavities without eggs) and natural	*Graptemys ouachitensis*	*Procyon lotor*	Geller, 2015	Degree of apparent soil moisture did not affect depredation rates on artificial nests.
Soil disturbance (via geosmin signal)	Artificial (some with cavities, some with turtle scent, some with geosmin) and natural		*Procyon lotor*	Edmunds et al., 2018	Although geosmin was found to be a nest location cue, authors dismissed its relative importance.

have most explanatory value in observed nest predation rates (**Tables 2**, **3**).

Visual Cues

Assessment of Nest-Related Visual Cues

Relatively few studies investigating the sensory cues used by mammalian predators to locate freshwater turtle nests have provided definitive evidence of reliance on visual cues, although most studies to-date have focused largely on just raccoons and study designs sometimes limit interpretations (**Table 2**). For example, while experimental studies using artificial nests have suggested that nest-surface markings may be important cues for foraging raccoons, uniformity in surface markings among artificial nest treatments (e.g., all smoothed over or all made to resemble natural nest appearance) reduce the ability to test effects of different visual signal strengths or isolate them from other potentially co-occurring cues inherent in artificially constructed nest cavities: a limitation which is only sometimes implied/acknowledged (Burke et al., 2005; Bernstein et al., 2015; Czaja et al., 2018; Perazzo et al., 2018).

Burger (1977) was apparently the first to suggest that visual cues were not necessary for successful nest location by raccoons and red foxes (*Vulpes vulpes*), based on widespread predation of diamondback terrapin (*Malaclemys terrapin*) nests after visual cues had been eliminated on the sand-substrate nesting site by wind and rain. Results of several subsequent studies align with these results, including the experimental evidence provided by Hamilton et al. (2002) who found that 3-m wide areas of sand smoothed over artificial cavities containing quail eggs did not lower excavation rates by raccoons and other predators relative to unobscured nests (see also Edmunds et al., 2018; **Table 3**). Geller (2015) demonstrated that elimination of surface markings at both natural and artificial nests by broom sweeping did not reduce raccoon depredation rates between experimental and control nests. Additionally, artificial representations of the visual markings made by Ouachita map turtles (*Graptemys ouachitensis*) and snapping turtles (*Chelydra serpentina*) during nesting, but without underlying cavities, were excavated at low rates relative to artificial nests with cavities (≤26 vs. 94%, Geller, 2015; ca. 10 vs. ca. 50%, Oddie et al., 2015, from their Figure 3A; respectively). Finally, Voves et al. (2016) found that human evaluation of the degree of visual conspicuousness of natural painted turtle (*Chrysemys picta*) nests was not a consistent predictor of predation likelihood by raccoons.

In contrast, artificial nest cavities simulating surface disturbance by nesting female Australian Murray River turtles (*Emydura macquarii*) were excavated at greater rates by introduced red foxes than control nests with minimal surface disturbance, independent of whether artificial nests contained turtle eggs (80% depredation on disturbed treatment vs. 60% control), *Coturnix* quail eggs (70% disturbed vs. 57% undisturbed), or were without eggs (Spencer, 2002; his Figure 3). Similarly, Dawson et al. (2014) found that red foxes excavated more artificial cavities simulating oblong turtles *Chelodina colliei* nests initially scored as more obvious than those that were more cryptic.

Collectively, we found no papers that suggest visual cues at nests may be important to foraging raccoons provide unambiguous data in support (see **Table 2**), while several other papers present data to the contrary (**Table 3**). However, foxes may use visual cues associated with surface disturbance, likely in combination with other signals, to locate turtle nests.

Assessment of Visual/Olfactory Cues From Tracks of Nesting Turtles

Congdon et al. (1983) were the first to suggest that predators may use tracks of nesting females (which contain both visual and olfactory elements) to locate turtle nests. However, their statement was likely based on observation or anecdotal reports. The first experimental test of this hypothesis found that eliminating the tracks of nesting females by daily broom sweeping did not reduce depredation rates by raccoons on either natural or artificial nests (Geller, 2015). Camera data has also demonstrated that the foraging paths of nest depredating raccoons do not align with the paths of nesting *Graptemys* (Geller, 2012a, 2015). Moreover, inducing raccoons to follow scent trails in experimental settings has proved difficult (S. Temple, pers. comm.). While these limited data suggest tracks may not be necessary, *per se*, for raccoons to find turtle nests, it is possible that nesting tracks are followed when more evident, such as in moist soils following rainfall or may be otherwise context dependent at the nest site or geographic location level.

For non-raccoons, however, Horváth et al. (2021), in Slovakia, EU, reported camera-based evidence of trail-following by both European wildcats (*Felis silvestris*) and European badgers (*Meles meles*); both of which followed artificially provided scent trails made of turtle-scented water to artificial European pond turtle (*Emys orbicularis*) nests. To our knowledge, this is the first well-documented report of trail following by a predator of freshwater turtle nests to appear in the literature.

Olfactory Cues

Research on which olfactory cues predators use to identify turtle nests appears early in the literature and continues to be an area of great interest for turtle biologists. In one form or another, olfactory cues have generated most research interest to date (referenced in almost all of the studies in **Tables 2**, **3**). Within the context of nest predation, predators can potentially detect nests directly, using scents produced by nesting turtles, turtle eggs, or disturbed soil profiles produced during nest construction, or indirectly by following scent trails (see above) produced by nesting females during travel to and from nesting sites.

Assessment of Olfactory Cues From Nesting Turtles

In some of the earliest studies addressing the question of turtle nest predation, authors suggested that scents directly associated with oviposition could be used by predators to identify nest location. For example, Cagle (1950) suggested, based on anecdotal reports from professional egg collectors, that scent from gravid female turtle urine or oviductal fluids released during oviposition may function as a nest location cue for predators. Similarly, Legler (1954) speculated that "odoriferous fluid voided by the nesting female" may provide an olfactory cue for predators, although he did not provide data in support. In a subsequent study, however, Moll and Legler (1971) found that *Ameiva* lizards and armadillos (*Dasypus novemcinctus*) preferentially excavated artificial nests treated with female turtle urine compared to controls. Although that study was based on a small sample size (n = 1 treatment replicate), to our knowledge, this is the first experimental study to conclude that some vertebrate predators locate nests using scent cues directly produced by gravid female turtles during oviposition.

Subsequent experimental studies have continued to investigate the relative importance of olfactory cues directly associated with gravid female turtles during nesting. In these studies, authors typically applied water from nearby wetlands or from containers housing captive turtles to artificial nests and measured predation rates (**Tables 2**, **3**). Most of this research has not provided evidence that scent from nesting female turtles increases the probability of nest predation by raccoons (**Table 3**). However, Burke et al. (2005) found that raccoons excavated a greater proportion of smoothed-over artificial nests with cavities refilled with *Malaclemys terrapin*-scented sand than when only unscented sand was used (means ca. 93%, n = 56 vs. 50%, n = 56, respectively; estimated from their Figure 1; difference statistically significant in one of two study years). In a follow-up study (Edmunds et al., 2018), 47% (n = 17) of artificial treatments consisting of 100 mL of terrapin-scented sand deposited on the surface were excavated by raccoons, suggesting the use turtle scent as a cue (not emphasized by authors). However, potentially informative controls of same-source sand without turtle scent were absent, and the excavation rate of artificial nests with cavities with terrapin scent (82.7%, n = 75) was similar to that of cavities

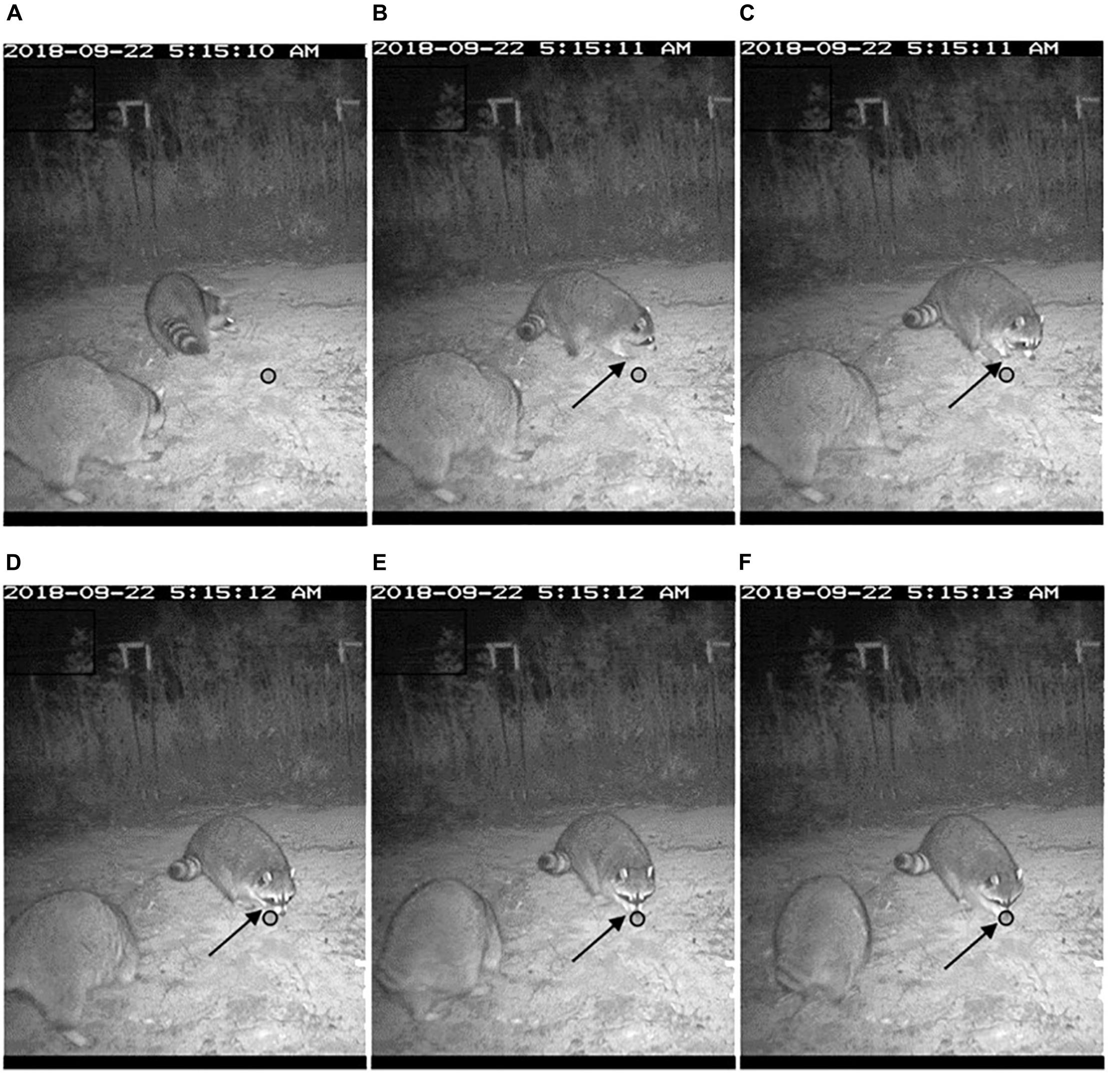

FIGURE 1 | Typical raccoon foraging sequence in finding natural turtle nests and artificial nest cavities: **(A,B)** approach with nose within 2 cm of surface, **(C,D)** nose and head move over detected cavity, **(D–F)** forefoot moves forward into position under nose, **(F)** excavation begins. Forefoot position indicated by arrows; cavity location indicated by dark circle.

without turtle scent (75%, $n = 116$) (2-tailed Fisher's exact test p-value = 0.2831; analysis ours). Similarly, Oddie et al. (2015) found that raccoon excavation rates on their visually inconspicuous artificial nests with cavities supplemented with *Chelydra* musk (ca. 40%) or pond water (ca. 55%) were greater than those without these applied scent cues (ca. 20%; from their Figure 3A). However, locations where these same scents were applied to "artificial nests" that consisted only of visual surface markings were excavated at lower rates (<5% each) than when a cavity was present (overall mean ca. 47%), indicating that some factor associated with nest cavity presence was more influential in nest predation risk than odors directly associated with nesting turtles. Essentially the same conclusion was reached by Oddie et al. (2015), who suggested a synergistic effect of multiple cues in both nest detection and nest predation. However, they considered that cavity-related cue to be tactile, in contrast to some other explanations (such as soil odor, see below).

Related studies on non-raccoon predators are more limited, however Dawson et al. (2014) reported that artificial turtle nests with cavities sprayed with turtle pond water were excavated at higher rates by red foxes than those without pond water, both when they contained chicken eggs (53 vs. 48%) and when chicken eggs were absent (43 vs. 38%). Tuma (2006) noted that coyotes excavated adult yellow mud turtles (*Kinosternon flavescens*), including males, from their underground burrows while searching for nests. He attributed this to coyotes being attracted to the smell of the turtles, rather than to the smell of the eggs, although other cues, such as odors from disturbed soils, may also have been involved. And recently, Horváth et al. (2021) documented scent trail following by two European mesopredators and surmised that odors from nesting *Emys orbicularis* were likely the primary nest location cues.

Overall, although not without exception, most research on raccoons indicates little use of residual turtle scent at nests as nest location cues, while limited evidence suggests that canids and other nest predator taxa may use turtle scent cues to greater extents. More studies on the nest-foraging behaviors of a wider array of mammalian nest predators will be necessary to reveal potential interspecific differences in the reliance on scents associated with gravid female turtles.

Assessment of Olfactory Cues From Turtle Eggs

Artificial nest studies comparing depredation rates of nests with turtle eggs or surrogates to those without eggs provide experimental tests of the importance of egg presence as a factor in nest predation dynamics. In an early study of this type, Wilhoft et al. (1979) found that raccoons actually excavated a greater percentage of the artificial nests depredated in their study ($n = 83$) when they contained only ping-pong balls (41%) than when they contained either turtle eggs (25%) or bird eggs (34%). Similarly, neither the presence nor absence of eggs appeared to influence artificial nest excavation rates in results obtained by Bernstein et al. (2015) in North America (predator species not reported), or by Perazzo et al. (2018) for two native fox species in southern Brazil.

A few raccoon-based studies (Geller, 2015; Oddie et al., 2015; Czaja et al., 2018) have compared concurrent nest depredation rates between natural nests and artificial nests with cavities lacking both turtle scent cues and eggs (i.e., just refilled cavities). With the exception of Oddie et al. (2015), which found predation rates on natural nests (57% within 4 days of oviposition) to be higher than those of artificial nests without applied scent cues, eggs, or visual disturbance (20%; from their Figure 3A), these studies found that raccoon depredation rates of artificial nests lacking these olfactory signals were similar to those of natural nests, where these potential cues were present.

However, these results contrast with those obtained both by Spencer (2002) and Dawson et al. (2014) for red fox depredation of simulated nests in Australia. Spencer (2002) found higher excavation rates of artificial nests with either turtle or quail eggs than those without eggs, both when surface disturbance was reported as more evident (ca. 80% for turtle eggs and 70% for quail eggs vs. 60 and 45%, respectively) and when efforts were made to minimize disturbance (ca. 60% for turtle eggs and 57% for quail eggs vs. 20 and 15%, respectively; from his Figure 3). Dawson et al. (2014) also reported that artificial nests with chicken eggs were excavated by foxes at higher rates than those without eggs both when sprayed with pond water (53 vs. 43%, respectively) and when not sprayed (48 vs. 38%, respectively).

Overall, there is limited definitive evidence to date suggesting that olfactory cues from nesting turtles or turtle eggs are important cues to raccoons foraging for newly constructed freshwater turtle nests. However, as noted regarding visual cues, olfactory cues from eggs or nesting turtles may be used to a greater extent by nest-foraging red foxes and certain other nest predators (**Tables 2**, **3**).

Tactile and Olfactory Cues From Soil Disturbance

Many researchers have suggested that some factor(s) associated with soils disinterred during the course of nest construction may function as location cues to freshwater turtle nest predators, with at least 13 of 26 studies reviewed in **Tables 2**, **3** either suggesting this possibility or providing data demonstrating increased nest predation associated with soil disturbance.

Assessment of Olfactory Cues From Soil Disturbance Versus Tactile Cues

Efforts to resolve which factors associated with soil disturbance function as nest location cues to predators have been complicated by the confounding presence of co-occurring visual, olfactory, and tactile signals inherent in nest cavity construction, resulting in differences in proposed nest detection mechanisms across studies (**Table 2**). For example, in the earliest experimental demonstration of the role of disturbed soil as a scent cue, Wilhoft et al. (1979) speculated that olfactory differences caused by the variation in soil moisture between disturbed and intact soils were responsible for increased raccoon predation of artificial nests. Alternatively, Spencer (2002) suggested that disturbed soils, in addition to increasing the potential visual evidence, may enhance the odors of nesting females or their eggs. Several studies have reported on predator excavation of artificial nests constructed by removing soil and replacing it into the nest cavity without surrogate eggs or the application of additional olfactory cues

(**Tables 2**, **3**). The elimination of experimental additions of olfactory or visual cues from artificial nests presumably limits predators to two options for locating nests: either olfactory cues originating directly from soil disturbance, or tactile cues resulting from differences in soil density between the nest and the surrounding area.

Geller (2015) and Buzuleciu et al. (2016) attempted to decouple the co-occurring sensory cues present at newly constructed turtle nests, each concluding that the odor of disturbed soils is the primary cue used by raccoons when foraging for turtle nests. In Geller (2015), high predation rates on broom-swept, natural *G. ouachitensis* nests and artificial nest cavities without eggs, showed that neither visual evidence of nesting turtles, nor olfactory cues from nesting turtles or their eggs were necessary for raccoons to locate nests. Although surface hardness differences between natural and artificial nests and the surrounding substrates were not tested, camera data consistently documented raccoons foraging for turtle eggs with noses close to the ground as they searched turtle nesting areas, strongly suggesting a reliance on olfactory, rather than tactile nest cues (Geller, 2015; **Figure 1**). Based on these observations, scents derived directly from soils disinterred during nest construction were considered primary nest location cues.

Similarly, Buzuleciu et al. (2016) demonstrated that artificial nests with cavities were associated with three to four times higher raccoon predation than those consisting only of surface-applied scent (gravid female *M. terrapin* scent, neutral scent, and no-scent control). This study also tested the hypothesis that scent of recently disinterred soil was the primary olfactory cue used by raccoons to locate terrapin nests by comparing the predation rate of unprotected, recently constructed artificial nests to that of artificial nests "aged" for 48 h within raccoon exclusion cages. They found that depredation rates of newly created artificial nests (all with 10–12 cm deep, refilled cavities) were about five times higher than that for 48-h-old nests (84 vs. 16%, respectively). Caging artificial nests for 48 h presumably allowed volatile organic compounds to dissipate, resulting in reduced olfactory cues for the raccoons. Given that artificial nests were caged for a relatively brief duration (48 h) and no rainfall occurred during the experimental period, it is unlikely that soil compaction in the caged treatments prevented raccoons from using tactile searching to locate the opening of the artificial nest chamber.

Both Geller (2015) and Buzuleciu et al. (2016) independently proposed that geosmin, an odiferous metabolite from the soil microbe Actinomycetes aerosolized from disturbed soil profiles and recognized by humans as the smell of disturbed soil (Lindbo et al., 2012), may be one of the underlying olfactory cues produced during nest cavity construction. Based on this proposition, the odor of geosmin alone, or in combination with other related microbial hydrocarbons released by soil disturbance during nest cavity construction, serves as a point-source signal identifying locations of turtle nests to raccoons and possibly other nest predators.

Edmunds et al. (2018), in the first direct test of geosmin as a nest location cue, found that locations where small amounts of geosmin were shallowly injected under soil substrates were, indeed, excavated by raccoons. They further noted that greater administered volumes of geosmin at the same concentration (0.5 mg geosmin/1 mL methanol) resulted in higher excavation rates (25% excavation rate at 0.1 mL, $n = 20$ vs. 37% at 0.2 mL, $n = 19$). However, because excavation rates of geosmin treatments were lower than those of both natural nests (67%; $n = 42$) and standard artificial nest cavities without eggs (overall 82.7%, $n = 75$, from Treatment 1 in their Table 1), Edmunds et al. (2018) instead concluded that raccoons primarily use tactile cues associated with differences in soil density between nests and surrounding substrates to identify nest locations, although they did not experimentally test anything related to substrate density. Moreover, they did not explore how additional increases in amounts or concentrations of geosmin in their experimental trials may have influenced nest predation rates or discus how the volatilization timelines of the small amounts of injected geosmin in their study may differ from that within the volumes of soil disturbed during natural nest construction.

The only study purporting to provide data supporting the use of surface hardness as a tactile cue for foraging raccoons is Oddie et al. (2015; **Table 2**). In that study, artificial nests with manufactured cavities (presenting a less-compacted soil surface relative to the surrounding substrate) experienced a higher depredation frequency compared to artificial nests without cavities, independent of whether visual or additional olfactory cues were present. While Oddie et al. (2015) concluded that raccoons probably use more than one sensory cue to locate nests, they proposed that tactile cues resulting from cavity presence were used to make a final determination as to whether to excavate a nest. However, because all treatment combinations used to test a "tactile" (cavity present) predator response (i.e., applied turtle scent, pond water scent, no-scent, or visual treatment) shared hand-excavated and refilled cavities, their results are confounded by scent cues originating from soil disturbance and therefore make the role of tactile cues difficult, if not impossible, to resolve.

How Long Do Nest Location Cues Last?

Assessment of Longevity of Nest Location Cues

With few exceptions, studies indicate that newly constructed turtle nests experience most predation within a few days of construction (**Table 4**). Nonetheless, a few studies (e.g., Wilhoft et al., 1979) have suggested that the risks of predation on freshwater turtle nests can extend much later into reproductive periods (**Table 5**). However, one caveat to interpreting the scent cue longevity observations in Wilhoft et al. (1979) is that their raccoon-depredated artificial nests were made in late July, weeks after natural *Chelydra serpentina* nesting activity had ended. Thus, their results only show that raccoons were still foraging at their study sites and would depredate newly constructed artificial nests during these later dates, not that the aging, natural turtle nests made during the just-completed nesting season were still vulnerable to predators. As a result, some authors have used this paper as support for claims of late-season nest cue retention and vulnerability to depredation, without recognizing this important distinction.

Further examination of papers demonstrating long-term nest predation risk reveals two main commonalities which

TABLE 4 | Papers proposing signal strength of cues used by mammalian predators to locate freshwater turtle nests decreases soon after nest construction.

Nest type	Turtle species	Predominant mammalian predator(s)	Citation	Comments
Natural	*Chrysemys picta*	Unknown, but including *Procyon lotor*	Legler, 1954	Nest predation rate was at least 5/25 (20%). Most nest depredation within 2 days, often within a few hours.
Natural	*Trachemys scripta*	*Dasypus novemcinctus*	Moll and Legler, 1971	Nest predation rate approximated as 78/92 (85%) at one site. Most depredation within 1 or 2 days. Surviving nests relatively free from predation thereafter.
Natural	*Graptemys ernsti*	*Procyon lotor*	Shealy, 1976	Multi-year nest predation rate, including avian, approximated as at least 95% based on field observations. Raccoons found nests up to 4 days after construction (as related in Lahanas, 1982).
Natural	*Chelydra serpentina*	*Procyon lotor*	Petokas and Alexander, 1980	Known nest predation rate was 17/18 (94%). Most of 40 destroyed nests depredated within 24 h.
Natural	*Graptemys ouachitensis, Graptemys pseudogeographica*	*Procyon lotor, Vulpes vulpes* *Lontra canadensis*	Vogt, 1980	Overall nest predation rate not reported. If a nest not depredated within 2 days, it was usually not disturbed thereafter.
Natural	*Chrysemys picta*	Not specified	Tinkle et al., 1981	Multi-year nest predation rate was 9/43 (21%). Most depredation within 24 h (67%), then declining. No depredation after 12 days.
Natural	*Chrysemys picta*	*Procyon lotor*	Christens and Bider, 1987	Predation rate was 7/16 (43.8%). Of these, 85.7% were depredated within 24 h. The remaining nest was destroyed 32 days after nest construction. No predation after 5 August.
Natural	*Emydoidea blandingii*	*Procyon lotor*, *Vulpes vulpes*	Congdon et al., 1983	Multi-year nest predation rate was 46/73 (63%) before the hatchling emergence period. Of these, 47% were depredated within 24 h and 84% within 5 days. An additional 12% of predation occurred from days 6–30. No predation of remaining 27 nests from day 30 until hatchling emergence period.
Natural	*Chelydra serpentina*	*Procyon lotor*, *Vulpes vulpes*	Congdon et al., 1987	Multi-year nest predation rate was 80/114 (70%) before the hatchling emergence period. Of these, 59% were depredated within 24 h and 73% within 6 days. An additional 20% occurred from days 7 to 18, and 6% from days 19 to 31. No predation of 34 available nests after day 32 or during hatchling emergence.
Natural	*Chelydra serpentina*	*Mephitis mephitis*	Robinson and Bider, 1988	Nest predation rate was 113/134 (84%). Of these, 57% were depredated within 3 days. Predation rates declined but ongoing through day 10 with none thereafter.
Natural	*Emydoidea blandingii*	Unknown, but including *Mephitis mephitis*	Ross and Anderson, 1990	All 4 nests found intact were depredated within 24 h.
Natural	*Pseudemys concinna suwanniensis*	*Procyon lotor*	Jackson and Walker, 1997	Multi-year nest predation rate, including avian, was 114/114 (100%). Of these, 100% were depredated within 2 days. Of 30 other nests protected by screening, 29 had digging within 48 h but waned almost completely after 1 week.
Natural	*Malaclemys terrapin*	*Procyon lotor*	Feinberg and Burke, 2003	Nest predation rate was 71/77 (92%). Of 70 depredated nests, 71% were depredated within 24 h and ~89% within 2 days. Longest survival was 7 days, with no depredation thereafter.
Artificial	Mimicking *Malaclemys terrapin*	*Procyon lotor*	Burke et al., 2005	Multi-year artificial nest predation rate was 215/448 (48%). Of these, 71% were depredated within 24 h, 81% within 2 days, 94% within 3 days, and 100% within 4 days.
Natural	*Emys orbicularis*	*Meles meles, Nyctereutes procyonoides*	Najbar and Szuszkiewicz, 2005	Most nests were depredated 0.5–8 days after construction (*n* = 18).
Natural	*Chrysemys picta*	*Procyon lotor*	Rowe et al., 2005	Multi-year nest predation rate was 35/201 (17%). Predation rate on a random subset of nests was 25/122 (20.5%). Of these, 36% were depredated within 24 h, 68% within 2 days. No depredation after 12 days.
Natural	*Terrapene carolina*	Likely *Procyon lotor*	Flitz and Mullin, 2006	Nest predation rate was 21/24 (88%). Of these, 86% were depredated within 24 h and all within 3 days.
Natural	*Emys orbicularis*	*Meles meles, Vulpes vulpes*	Havaŝ and Danko, 2009	Almost all nest depredation occurred within 2 days.

(Continued)

TABLE 4 | (Continued)

Nest type	Turtle species	Predominant mammalian predator(s)	Citation	Comments
Natural	*Malaclemys terrapin*	*Procyon lotor*	Rahman and Burke, 2010	Nest predation rate was 19/28 (68%). Of these, 100% were depredated within 24 h.
Natural	*Graptemys ouachitensis*	*Procyon lotor*	Geller, 2012a	Multi-year nest predation rate was 38/42 (90%). Of 29 nests depredated before the emergence period, 79% were depredated within 24 h, 93% within 2 days. No depredation after 8 days until hatchling emergence period.
Natural	*Chelydra serpentina*, *Chrysemys picta*	*Procyon lotor*	Wirsing et al., 2012	Multi-year nest predation rate was 54/94 (57%) for *Chrysemys picta* and 166/198 (84%) for *Chelydra serpentina*. Most predation within 24 h (74% for *Chrysemys*, 82% for *Chelydra*). In total, 98% depredation occurred within 5 days (both species combined).
Artificial	Mimicking *Macrochelys temminckii*	*Procyon lotor*	Holcomb and Carr, 2013	Multi-year artificial nest predation rate was 90/90 (100%). Of these, 86% were depredated within 24 h, 94% within 2 days. Last depredated nest was ca. 5 days old.
Artificial	Mimicking *Terrapene ornata*	Not specified	Bernstein et al., 2015	Typically, any treatment with disturbed soil was depredated within 2–3 days. Also noted that natural nests were almost never depredated after 2 weeks.
Natural and artificial	*Graptemys ouachitensis*	*Procyon lotor*	Geller, 2015	Multi-year natural nest predation rate was 35/36 (97%). Of these, 35/35 (100%) were depredated within 24 h. In total, 32 of 33 artificial nests excavated within 24 h, 1 within 2 days.
Natural	*Chelydra serpentina*	*Procyon lotor*	Oddie et al., 2015	36 of 63 (57%) nests were depredated within 4 days (not monitored thereafter).
Natural	*Emydoidea blandingii*	not specified	Byer et al., 2018	Modeled and actual nest depredation probabilities were low for nests that survived the first few days after construction.
Natural	*Glyptemys insculpta*	*Procyon lotor, mephitis mephitis*	Bougie et al., 2020	29 of 45 (64%) depredated nests were destroyed within 5 days.

may explain divergence from studies that report shorter nest vulnerability timelines: the inclusion of data from hatchling emergence periods in predation risk assessments or having nesting areas with more diverse predator communities, rather than just raccoons (**Table 5**). Both of these factors have introduced complexity, as well as some confusion, into the literature on turtle nest predation dynamics, and are discussed below.

Evidence for Renewed Nest Location Cues During Hatchling Emergence Periods

Beginning with Burger (1977), many authors have suggested that new nest location cues arise at the onset of, or during, the hatching period as hatchlings fracture eggshells and absorb residual yolk sacs, yet remain in or above the nest chamber prior to emergence. In studies surveying entire reproductive periods, these renewed cues result in a secondary nest predation peak and extend the reported age of depredated nests relative to studies with shorter timelines. Pre-emergence cues of nest location may include olfactory signals from embryonic fluids and disturbed soils created by subterranean hatchling movements, or hatchling vocalizations (Ferrara et al., 2012; Riley and Litzgus, 2014; Geller and Casper, 2019). Upon surface emergence, new nest location cues potentially include odors arising from nest cavities via exit holes (Congdon et al., 1983; Christens and Bider, 1987), and visual cues provided by hatchling tracks (Congdon et al., 1983; S. D. Gillingwater, *pers. comm.*, *in* Riley and Litzgus, 2014). However, the importance of potentially confounding, olfactory cues co-occurring with visible tracks has not been investigated, nor have any published studies attempted to resolve the relative importance of these possible cues on predation frequency during the hatchling emergence period.

Assessment of Differences Among Predator Species in Nest Detection Timelines

A less often recognized source of variance in reported nest survival timelines concerns the composition of the involved predator community. To date, Galois (1996) represents the only experimental work that has attempted to directly assess the differential sensory capabilities of common turtle nest predators, finding that olfactory cues likely play a predominant role in turtle nest detection by both striped skunks (*Mephitis mephitis*) and raccoons, based on food-conditioning and discrimination learning trials. Galois (1996) further determined that striped skunks are likely more narrowly reliant on olfaction than raccoons, in accord with previous research demonstrating the importance of olfaction to foraging skunks (Langley, 1979; Nams, 1991). Raccoons, though also characterized by a well-developed olfactory sense, were found to use both tactile and visual cues to a greater degree than skunks.

Some inferences regarding the role of differential olfactory sense development on nest depredation dynamics in field settings

TABLE 5 | Papers suggesting the signal strength of cues used by mammalian predators to locate freshwater turtle remains long after nest construction.

Nest type	Turtle species	Predominant mammalian predator(s)	Citation	Presented data	Comments
Natural	*Malaclemys terrapin*	*Procyon lotor*, *Vulpes vulpes*	Burger, 1977	Predation rate was 20% of extant nests during first 30 days period, 27% during 30–60 days, and 75% during 60–90 days.	60–90 day period included emergence period (mean emergence = 72 days). Nests were hand-excavated when ≥ 65 days old, possibly affecting nest location signals (however, *see* Burger, 1977, p. 460).
Artificial		*Procyon lotor*	Wilhoft et al., 1979	Artificial nests made in July (various types) were depredated at moderate rates (12–40%) in late July-early August, several weeks after nesting season had ended in early June.	Depredated artificial nests were newly created. Thus, data do not relate to depredation risk of aging natural or artificial nests.
Natural	*Graptemys ouachitensis, Graptemys pseudogeographica*	*Procyon lotor, Vulpes vulpes* *Lontra canadensis*	Vogt, 1980	*Lontra canadensis* depredated nests during June, July, and August.	Hatchling emergence began in August.
Natural	*Chrysemys picta*	*Mephitis mephitis*	Snow, 1982	Most nests depredated on the day of construction (9 of 33), but depredation continued through day 22.	23 of 39 predation events were by skunks, 5 by chipmunks (*Tamias striatus*), and only 4 each by foxes and raccoons. Oldest nest destroyed by skunks was 22 days old, for foxes was 8 days old, for raccoons was probably ≤ 5 days old) (from Figures 2, 3).
Natural	*Glyptemys insculpta*	Unspecified	Brooks et al., 1992	All of 17 depredated nests were destroyed during a single week, around 9 weeks after the last nest was constructed.	Nine-week nest predation date may be at the beginning of the hatching or emergence period.
Natural	*Emydoidea blandingii*	Likely *Procyon lotor*	Standing et al., 1999	Reported evidence of fresh predation during late August and early September.	Noted increases in predator activity in the autumn after a mid-nesting season lull. Hatchling emergence began in early September or October.
Natural	*Gopherus agassizii*	*Vulpes macrotis*	Bjurlin and Bissonette, 2004	In one study year, 1 of 8 nests was depredated within 1 week of oviposition, whereas the rest were depredated during early August, more than one month after nest construction.	Nests were protected from depredation after 70 days. Authors suggested nest visitation rates may have affected predation rates.
Natural	*Malaclemys terrapin*	*Procyon lotor*, *Dasypus novemcinctus*	Butler et al., 2004	Overall nest predation rate was 197/310 (63.5%). Most nest predation within 24 h (74.3% in 1997, *n* = 144 and 53.9%, *n* = 166 in 2000). Older nests were less likely to be depredated, however some (12.1%) nests were depredated 3–53 days, or longer, after construction.	Nests depredated at ages 54–106 days (*n* = 26) were during the emergence period.
Natural	*Graptemys oculifera*	*Dasypus novemcinctus*, *Procyon lotor*	Jones, 2006	86% (*n* = 118) of caged nests were attacked by predators as late as 69 days after nest construction. Over 42% were within the first 24 hrs and 81% within the first 14 days.	Late-season disturbance at caged nests may relate to hatchling emergence activity, as means were 64.4 ± 4.7 days to pipping, 76.3 ± 7.7 days to emergence itself, and 12.0 ± 5.5 days between pipping and emergence.
Natural	*Malaclemys terrapin*	*Procyon lotor*	Rahman and Burke, 2010	In one set of experiments, 9/11 (82%) of nests protected, then unprotected, after 21–25 days were depredated up to 11 days later.	Researcher removal of nest cages may have produced new nest location cues, possibly via stake removal or efforts to visually conceal the location.
Artificial		*Vulpes vulpes*	Dawson et al., 2014	Four types of artificial nests, including some with just refilled cavities, were excavated throughout entire 60-d monitoring periods.	46% of 580 initial artificial nests excavated within 2 months of construction, mostly within 30 d.
Natural	*Chelydra serpentina, Chrysemys picta*	*Vulpes vulpes,* fewer *Procyon lotor*	Riley and Litzgus, 2014	In total, 17% of snapping turtle and 14% of painted turtle nest predation was in first week. Another peak occurred from weeks 10 to 14 and up to 105 days after nest construction for snapping turtles and during weeks 3–4 and 11–12 for painted turtles and up to 109 days after nest construction.	Part of the late period depredation events for both species are just before, and continuing into, hatchling emergence periods (e.g., their Figure 3). Canid predator presence increased later in incubation periods.

can perhaps be gained by comparing depredation timelines in studies of both natural and artificial turtle nests. For example, in Snow (1982), often cited in support of long nest vulnerability timelines, striped skunks were strongly predominant predators, with little on-site presence by raccoons. Although that study did not examine nor discuss the potential implications of differential sensory capabilities among predator species, it is possible to deduce from Figures 2, 3 in that study that the oldest nests destroyed by skunks and foxes were 22 days old and 8 days old, respectively, while the oldest nest depredated by raccoons was likely $\leq$ 5 days old. Similarly, Congdon et al. (1987), proposing a differential, olfactory basis, found that red foxes destroyed older *Chelydra serpentina* nests than did raccoons (mean = 9.4 days, n = 32 vs. mean = 1.9 days, n = 13, respectively). Dawson et al. (2014) also documented long predation timelines by red foxes, with ongoing excavation of artificial nests up to 60 d old occurring throughout monitoring periods. The mid-season nest predation exhibited by skunks and canids likely reflects reliance on turtle egg scent cues themselves, as these odors are presumably the only ones remaining after potential visual, tactile, and other olfactory cues have faded.

In a recent review of nest predation timelines, Riley and Litzgus (2014) presented age-related nest predation data both from their own as well as previously published research. They concluded that nest location cues persist during the several-week period following nest construction and suggested that additional nest predation peaks after week one for the turtle nests in their study were associated with increased numbers of canids at these later dates. They also noted that interspecific differences in cues used by predators, in addition to predator densities and individual behavioral differences, may be partially responsible for reported variation in nest predation timelines. However, while Riley and Litzgus (2014) thus noted the potential for a predator species effect on nest depredation timelines, they did not discuss how differential sensory capabilities among predator species, *per se*, may have impacted their findings or largely explain the results of other research [e.g., the long vs. short predation risk timelines of Snow (1982) and Burke et al. (2005), respectively; see their Table 1]. In fact, red foxes, able to detect and depredate relatively old nests (see above), were strongly predominant in their study and may have been responsible for the somewhat atypical nest predation patterns they observed.

Available evidence thus indicates that the age-related risk of predation is, at least in part, dependent on the species composition of the local predator community due to differential sensory abilities among species.

DISCUSSION

In this review we have attempted to synthesize much of the literature regarding the cues used by mammals in the predation of turtle nests derived from observational and experimental research produced over the past several decades. The bulk of the empirical data from predation studies on both natural and artificial nests suggests that raccoons, the predator most often the subject of such studies, predominantly rely on scent cues of disturbed soils to locate recently constructed freshwater turtle nests. Predation rates of natural nests remain high even after all potential visual cues (i.e., sign of nests and nesting turtle tracks) have been experimentally eliminated, and artificial nests with manufactured cavities are consistently found and excavated by raccoons at higher rates relative to those without cavities, regardless of whether or not they contain turtle scent, egg scent, or visual surface markings. In this regard, suggestions that female turtles make purposeful attempts to visually conceal nest locations by carefully finessing the nesting substrate (sensu Strickland et al., 2010) are, thus, called into question as far as raccoon predation is concerned. However, reducing the visual cues to nest presence is likely important in reducing predation risk by avian predators, whose foraging for turtle nests appears to be primarily visually based (e.g., Burger, 1977; Jackson and Walker, 1997; Butler et al., 2004; see also Voves et al., 2016). In contrast, foxes and other canids, striped skunks, and other predators may not only have better-developed olfactory senses than raccoons, but may also more commonly use a greater number of nest location signals in addition to those from recently disturbed soils, including visual surface disturbances and odors of nesting turtles and their eggs, to locate nests.

Raccoons are particularly effective nest predators because they have the capacity to respond to seasonal changes in food supply (Daglish and Anderson, 1979) and concentrate foraging effort at turtle nesting areas when these new food resources become available (Petokas and Alexander, 1980; Geller, 2012a). Raccoons are naturally inquisitive, and it is likely that they investigate a variety of scents suggestive of food. Typically, when turtle nests are located in soil or sand substrates, both egg and soil odors co-occur in newly constructed nests. Although it is possible that odors from disturbed soils may initially present a stronger olfactory signal than that from buried eggs, raccoons may still be able to resolve the egg-related odors within the olfactory mix, at least when nests are only a few days old. Nonetheless, raccoons appear to use the scent cue produced by disinterred soil as a "default" cue to indicate the presence of turtle eggs or perhaps other food items. Cueing in on olfactory signals of soil disruption would be an efficient foraging strategy because many potential prey items below the substrate may produce these same environmental signs, therefore reducing or eliminating the need for prey-specific scent recognition or detection.

In contrast, reliance on tactile cues to initially locate freshwater turtle nests would seem unlikely from an efficiency perspective, as it is reasonable to assume that raccoons, like other predators, use a foraging strategy that optimizes energetic gain while minimizing expenditure of energy and time (Emlen, 1966). Given the lack of unambiguous data in support of tactile searching by nest-foraging raccoons, and the likely inefficiency of physically detecting point sources of varying surface hardness over large nesting areas compared to "distance" senses of olfaction or vision (Galois, 1996), available evidence suggests that tactile searching is, at best, a complimentary, rather than primary means of locating turtle nests.

Survival of turtle nests is not only a function of nest cue signal strength, but also of predator species, the timing of predator presence after nest construction, and predator proximity to nests. Relatively short nest survival intervals, typically within 2 or 3 days, appear nearly universal where raccoons are the predominant predator (**Table 4**), while at least some nests that initially survive short-term predation are depredated at later dates when the mammalian predator community is more diverse and includes, for example, canids (**Table 5**). Canids, have long been known to possess advanced olfactory senses (Green et al., 2012; Lea and Osthaus, 2018). Although comparative behavioral studies between canids and other carnivores are scarce, at least some canids have large olfactory turbinal surface areas relative to most other carnivores that have been examined (reviewed in Lea and Osthaus, 2018) and red foxes have exceptionally large olfactory turbinal surface areas for their size (Green et al., 2012). We thus suggest that generalized claims as to nest predation risk extending well into nesting seasons be interpreted cautiously, as these timelines appear dependent, at least in part, on the composition of the local predator community. In areas where raccoon predation is dominant, nests may be most at risk shortly after oviposition and then again during hatching and emergence. In contrast, in environments where canids, for example, are dominant, the predation window may extend from oviposition through hatching due to their exceptional olfactory sensitivities.

While this review has allowed us to make generalized statements regarding the primary cues used by predators, the potential for local or context-dependent variation in nest predation dynamics must also be recognized. For example, some individual raccoons or populations may habitually follow the tracks of nesting female turtles to find nests, particularly when tracks are most evident in sparsely vegetated, moist substrates (e.g., see pg. 26 in Buhlmann et al., 2008). Similarly, the occasional excavation of artificially made markings that mimic those of natural nests (Geller, 2015; Oddie et al., 2015), suggests some use of visual cues in locating nests, as does the direct sighting of in-process, nesting turtles themselves (e.g., Jackson and Walker, 1997). Nesting sites also vary in surface soil hardness (e.g., sandy vs. organic soils), influencing the potential for tactile nest detection among sites (Oddie et al., 2015). Thus, while the well-established importance of olfaction to raccoon foraging (Bowman and Harris, 1980; Ruzicka and Conover, 2011) aligns with the primary findings of this review, some variation in cues and sensory modes used to locate nests is to be expected given possible differences in the relative strengths of nest location signals among settings and the wide array of well-developed senses available to these and other turtle nest predators.

Based on the results of this review, we have identified areas of nest predation dynamics in need of further study. We support Edmunds et al. (2018) in calling for more research on the role of geosmin—and possibly other volatile soil hydrocarbons—in turtle nest detection, particularly as it may be a common factor underlying mammalian detection of natural nest cavities globally. More generally, this review has revealed a significant need for expanded research on turtle predation dynamics in areas outside of North America (85.7% of the 49 citations in **Tables 2–5** originate from the United States and Canada). In addition to expanding our knowledge of similarities and differences among mammalian taxa regarding sensory cues used to identify turtle nests, a broader overview of species-specific mammalian nest predation dynamics may help inform local conservation strategies as to which predator species are in greatest need of management and when nest protection measures are most effectively applied. In these efforts, we anticipate that the increasing use of trail cameras will likely lead to larger and better-resolved datasets regarding turtle nest survival timelines and nest predators in both natural and experimental settings. Multi-year studies on both specific turtle populations and across geographies will be particularly useful in understanding how nest predation dynamics may change over space and time (Edmunds et al., 2018) as a result of individual predator variation or culturally influenced learning (e.g., for raccoons, Gehrt, 2004).

Regardless of the potential for targeted management to reduce turtle nest predation by disrupting nest location cues (e.g., by shallow-tilling substrates to obscure the olfactory signals of newly constructed nests), maintaining or optimizing nesting area integrity in terms of vegetative cover, soil condition, and other ecological characteristics over long periods of time remains paramount. In addition to predator exclusion (Buhlmann and Osborn, 2011; Geller, 2012b; Quinn et al., 2015), efforts to increase turtle nesting success where raccoons are the predominant nest predator may benefit from enlarging and maintaining the open habitats often used by nesting turtles, thereby minimizing chance encounters with the point sources of disturbed soils identified as predominant nest location cue for raccoons (see Temple, 1987; Jackson and Walker, 1997; Marchand and Litvaitis, 2004; Vilardell et al., 2008). However, even with such efforts, success may be limited in areas where raccoons have substantially elevated populations due to anthropogenic influence or where foxes are important nest predators, as they, and possibly some other nest predators, may also use scent trails and other cues to individually track and locate nests over greater distances in a more directed fashion.

One of the few ways turtles may be able to reduce the olfactory and visual cues that necessarily arise during nest cavity construction is to nest shortly before significant rainfall. However, to date, there is little evidence that turtles show this propensity (e.g., see Czaja et al., 2018). Total annual precipitation and single event precipitation amounts are expected to increase worldwide under present and projected climate change scenarios, although these effects will not be evenly distributed (Wuebbles et al., 2017). In areas likely to experience increased precipitation frequency and amounts (e.g., northeastern United States) nest success may increase due to greater proportions of nests being constructed before rainfall by chance alone, while the opposite may be true in areas likely to experience historically lower levels of precipitation (e.g., Mediterranean Basin) (see also Czaja et al., 2018). However, as changing rainfall patterns also impact air and substrate temperatures, nest site flooding potentials, and the amount and composition of vegetational cover on nesting areas—all of which have implications for nesting habitat suitability and embryo/hatchling survival—the ultimate impact of anthropogenic precipitation change on worldwide turtle populations is unclear and will likely be context dependent.

AUTHOR CONTRIBUTIONS

GG conducted the literature review and wrote the first draft including 4 of the 5 tables and the figure. SP edited and contributed to the first and subsequent drafts, provided additional citations, and contributed to 1 of 5 tables. Both authors contributed to the article and approved the submitted version.

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Macro- and Microhabitat Predictors of Nest Success and Hatchling Survival in Eastern Box Turtles (*Terrapene carolina carolina*) and Spotted Turtles (*Clemmys guttata*) in Oak Savanna Landscapes

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Differing selection pressures on stationary nest contents compared to mobile offspring mean that the nest-site characteristics resulting in the highest nest success may not be the same characteristics that result in the highest survival of juveniles from those nests. In such cases, maternal nest-site choice may optimize productivity overall by selecting nest sites that balance opposing pressures on nest success and juvenile survival, rather than maximizing survival of either the egg or the juvenile stage. Determining which macro- and microhabitat characteristics best predict overall productivity is critical for ensuring that land management activities increase overall recruitment into a population of interest, rather than benefiting one life stage at the inadvertent expense of another. We characterized nest-site choice at the macro- and microhabitat scale, and then quantified nest success and juvenile survival to overwintering in two declining turtle species, eastern box turtles and spotted turtles, that co-occur in oak savanna landscapes of northwestern Ohio and southern Michigan. Nest success in box turtles was higher in nests farther from macrohabitat edges, constructed later in the year, and at greater total depths. In contrast, survival of juvenile box turtles to overwintering was greater from nests under less shade cover and at shallower total depths. Spotted turtle nest success and juvenile survival were so high that we were unable to detect relationships between nest-site characteristics and the small amount of variation in survival. Our results demonstrate, at least for eastern box turtles, a tradeoff in nest depth between favoring nest success vs. juvenile survival to overwintering. We suggest that heterogeneity in microhabitat structure within nesting areas is important for allowing female turtles to both exercise flexibility in nest-site choice to match nest-site characteristics to prevailing weather conditions, and to place nests in close proximity to habitat that will subsequently be used by hatchlings for overwintering.

Keywords: juvenile, nest depth, nest-site choice, predation, shade cover

INTRODUCTION

In egg-laying animals, a female's choice of nest site must balance a variety of sometimes conflicting selection pressures (Refsnider and Janzen, 2010). The location and microhabitat characteristics of a nest site can impact the survival of multiple life-stages, including the ovipositing or incubating adult female (e.g., Ghalambor and Martin, 2001; Spencer, 2002), the eggs during embryonic development (e.g., Resetarits and Wilbur, 1989; Martin, 1993; Madsen and Shine, 1999), and the juveniles emerging from the nest (e.g., Anders et al., 1998; Kolbe and Janzen, 2001). In addition to directly affecting the survival of different life stages, physical characteristics of nest sites can also impact a variety of phenotypic traits in the offspring produced from those nests, which may influence survival or quality of those offspring later in life, and thereby indirectly impacts the reproductive fitness of the female who chose the nest site in the first place (reviewed in Noble et al., 2018; Refsnider et al., 2019).

Importantly, nest sites that are optimal for one reason, such as minimizing risk to an ovipositing or incubating female, may be sub-optimal for a different reason, such as maximizing likelihood of the eggs hatching (e.g., Madsen and Shine, 1999; Spencer, 2002; Amat and Masero, 2004). In such situations, a female's choice of nest site may have to take into account conflicting selection pressures, which may result in a maternal nest-site choice that optimizes the overall benefits to a female's lifetime reproductive success, while individual components of nest-site choice may appear to be maladaptive if examined in isolation (Martin, 1992; Chalfoun and Schmidt, 2012). For example, in golden-winged warblers, nest success was highest in nests in shrublands farthest from the shrub-forest edge, whereas fledgling survival was highest from nests farthest into the forest from those same edges (Streby et al., 2014a). The opposing selection pressures on nest location for nest success vs. fledgling survival resulted in a population mean nest-site choice of nests located in close proximity to the shrub-forest edge, where neither nest success nor fledgling survival were maximized, but where the number of young raised to independence from adult care was maximized (Streby et al., 2014a).

The potential ramifications to management of nest-site choice having to balance between opposing selection pressures on different life stages are profound. Traditionally, management to impact bird population productivity was, and in most cases still is, based solely on increasing nest success: that is, habitat types that resulted in the highest nest success (defined as the probability of a nest producing at least one fledgling) were assumed to be "best," and management actions were designed to preferentially maintain such habitats over other habitat types with lower nest success (e.g., Hartway and Mills, 2012). The problem with this approach is that it assumes nest success is a complete or representative measure of productivity, and it ignores other life stages that may be impacted differentially by the same habitat type (reviewed in Streby et al., 2014b). In the golden-winged warbler example above, when management decisions are based solely on nest success, management plans assume that shrubland is the "best" habitat for increasing golden-winged warbler productivity, and therefore endeavor to create more shrubland to increase nest success. In reality, the habitat type with the highest overall productivity is actually forest-shrubland edge, which requires a habitat mosaic consisting of forest patches in various stages of succession (Streby et al., 2014b). Thus, management that takes into account only a single life stage could be creating an ecological trap if opposing selection pressures acting on a different life stage are actually driving nest-site choice (e.g., Flaspohler et al., 2001). Therefore, conservation and management decisions made on the basis of nest-site choice need to take into account the consequences of nest-site choice for multiple life stages, as well as the potential indirect effects of nest-site choice on survival or reproduction, to ensure that they are not favoring one life stage at the expense of another and inadvertently lowering overall productivity in a population of interest.

Freshwater turtles are among the world's most imperiled taxa, and threatened turtle species are therefore a common target for conservation and management (Rhodin et al., 2017; Stanford et al., 2020). A major threat to many threatened turtle species, particularly in areas with high anthropogenic disturbance, is nest predation by mammals [in North America, primarily raccoons (*Procyon lotor*) and skunks (*Mephitis* sp.); Kolbe and Janzen, 2002]. Indeed, predation rates sometimes exceed 90% in turtle populations in areas with high human activity (e.g., Strickland et al., 2010; Refsnider et al., 2015). However, turtle nests may also fail to hatch for a variety of other reasons, including infestation by ants, plant roots suffocating eggs, nest substrate that becomes too wet or too dry, or incubation temperatures that are too cool to support embryonic development or become lethally warm (e.g., Schwarzkopf and Brooks, 1987; Packard and Packard, 1988; Buhlmann and Coffman, 2001; Socci et al., 2005). In contrast to the nest stage, generally far less is known about rates of juvenile survival in turtles due to the difficulty in studying this small, cryptic, and mobile life stage (Pike, 2006; Paterson et al., 2012). Upon emerging from the nest, hatchling turtles of many species must travel from the nest site to habitat that is suitable for finding food, shelter, and potentially overwintering (e.g., Salmon et al., 1995; Putman et al., 2010). For many aquatic turtle species, the journey from a nest site in open, sunny habitat to aquatic habitat suitable for the juvenile stage exposes hatchlings to predators, desiccation, and potentially lethal temperature extremes (Janzen, 1993; Wilbur and Morin, 1988; Janzen et al., 2000, 2007). Therefore, as in other species (e.g., Kamel and Mrosovsky, 2004; Streby et al., 2014a), nest-site choice by female turtles likely requires balancing opposing selection pressures on different life stages. For example, nest-site characteristics that produce the highest hatching success may differ from those of nests that experience the highest hatchling survival rates. Determining the characteristics of nest sites that maximize overall recruitment into the population is important, particularly for threatened species, so that management actions such as covering nests with predator-proof cages can be targeted at nests with the greatest likelihood of producing surviving hatchlings that will contribute to the population.

Here, we characterized nest-site choice at the macro- and microhabitat scale in two declining turtle species, eastern box turtles and spotted turtles, that co-occur in oak savanna landscapes in northwestern Ohio and southern Michigan.

We quantified nest success and survival of juveniles to first overwintering in both species to determine which aspects of maternal nest-site choice best predicted overall productivity, so that conservation efforts can prioritize nest sites with the highest chance of contributing offspring to each turtle population.

MATERIALS AND METHODS

Study Sites and Species

Historically, the glacial and lake sand plains of the Great Lakes region in North America contained abundant oak savanna, prairie, and wet prairie habitat (Nuzzo, 1986). More recently, this same landscape and its associated habitats have undergone some of the highest rates of conversion to agricultural land of any native habitats in the United States (Leach and Givnish, 1999). Much of the remaining oak savanna and wet prairie habitat in the Great Lakes region is extensively managed to preserve structure and natural communities. Indeed, these habitat types support a substantial number of rare and declining taxa (Grigore, 2009). In particular, eastern box turtles (*Terrapene carolina carolina*) and spotted turtles (*Clemmys guttata*) co-occur in the oak savanna-wet prairie landscape in the Great Lakes region.

Eastern box turtles and spotted turtles are declining throughout their geographic range due to habitat destruction and, to a lesser extent, over-collection for the pet trade (International Union of Concerned Scientists, 2011a,b). Both species are long-lived, produce relatively small clutches, and often experience high rates of nest predation, meaning that their population sizes are acutely sensitive to losses of even a few adults (Williams and Parker, 1987; Stickel and Bunck, 1989; Hall et al., 1999; Litzgus, 2006; Enneson and Litzgus, 2008; Feng et al., 2019). We studied eastern box turtles in oak savanna and mixed hardwood forest in Lucas County, Ohio, and Calhoun and Kalamazoo Counties, Michigan, United States. We studied spotted turtles in seasonally wet prairie, swamp forest, and fen habitat in Lucas County, Ohio, and Barry County, Michigan, United States. Exact study site locations are being withheld due to the susceptibility of these species to poaching and collection for the pet trade. Management activities at our study sites include prescribed burns, invasive plant species removal, brush-cutting, herbicide application, mowing, and wetland restoration.

Characterizing Nest-Site Choice

We located nests of both turtle species in 2018 and 2019 by intensively radio-tracking gravid females to their nest sites (as in Refsnider and Linck, 2012; **Figure 1**). Turtles of both species were hand-captured during visual encounter surveys of our study sites in April and May, prior to the nesting season, and adult females were fitted with radio-transmitters (R1-2B, Holohil; 14.5 g for box turtles; 9.5 g for spotted turtles). We quantified plastron length of all females as a measure of body size. Radio-marked females were tracked at least once weekly until the nesting season began in approximately mid-May, at which time they were located daily. Females with shelled eggs detected during palpation, traveling toward known nesting areas, or actively moving in late afternoon or early evening were monitored continuously from 1600 and 2200 h until they were either observed nesting, or became inactive for the night. Females actively nesting were monitored from a distance to avoid disturbance. Once a nest had been completed, we recorded GPS coordinates with a handheld GPS unit, covered some nests with a wire cage to prevent mammalian predation (see below), and returned the following day to characterize the microhabitat within 1 m of the nest site.

Within 24 h of nest completion, we briefly excavated each nest to determine clutch size and to measure the total nest depth. We then replaced eggs in the nest cavity and refilled the nest. We used a 180° fisheye lens to take a hemispherical photograph directly over each nest, and we used Gap Light Analysis software (Frazer et al., 1999) to quantify shade cover from the hemispherical photographs over each nest. We classified the macrohabitat (i.e., land cover type within 10 m of the nest site) of each nest as deciduous forest, developed/residential, flooded grassland, grassland, mixed coniferous/deciduous forest, open fen, savanna, shrub swamp, or vernal wetland. We classified the microhabitat of each nest as grass; rocky soil (high organic content, < 1 mm grains interspersed with pebbles > 2 mm); rotten log; sand (well-drained, little organic matter, 1–2 mm grains); sedge; soil (high organic content, < 1 mm grains); or sphagnum. Finally, we re-covered some nests with a wire mesh cage which served both to exclude mammalian predators, and to contain recently emerged hatchlings (as in Refsnider, 2009). In 2019 we left a subset of nests unprotected to estimate mammalian predation rates in each species. For each species, we determined whether a nest would be covered with a predator-proof cage or left unprotected by caging every second nest that was constructed.

Using a combination of aerial photographs and ground-truthing, we created a georeferenced map of land cover types in ArcGIS (ESRI) for study sites in Ohio and Michigan at 0.5 m resolution (1:800). We plotted all nests in this GIS, and used the digitized land-cover map to measure the distance from each nest to the nearest edge of a different macrohabitat type, and the distance from each nest to the nearest road as an indicator of degree of habitat fragmentation in the vicinity of the nest site.

Quantifying Nest Success and Juvenile Survival

From August 1 to late October, we checked nests every 24–72 h for signs of hatchling emergence. If hatchlings did not emerge within 100 days of nest construction, we carefully excavated the nest to determine why the nest had failed (which was most often predation by a burrowing snake or rodent, or desiccation of eggs). For nests where some hatchlings successfully emerged, we excavated the nest cavity, searched the area within the cage to locate remaining hatchlings, and determined the fate of any unhatched eggs or dead hatchlings (e.g., desiccated eggs, eggs entangled by plant roots, egg eaten by ants, dead hatchlings still within the eggshell, or apparently unviable eggs). For each nest, "nest success" was assigned as 1 if any live hatchlings were recovered from the nest. Nest success was 0 if no live hatchlings were recovered and there was evidence of predation or egg mortality as described above.

We weighed hatchlings with a digital scale to the nearest 0.1 g. From each nest, we randomly selected two hatchlings to be radio-tracked, with the caveat that radio-transmitters had to weigh < 10% of a hatchling's mass. We first painted radio-transmitters (Blackburn Transmitters) brown for camouflage, placed a bead of silicone rubber aquarium sealant (Marineland) on the center midline of a hatchling's carapace, and placed the transmitter directly on the sealant bead. We also attached a 5–8 cm piece of blaze orange thread to the sealant bead to aid in locating hatchlings that were not visible on the ground surface. We allowed the glue to dry overnight, and then released radio-marked hatchlings at their nest site the following day. Hatchlings not radio-tracked were released at their nest site after they were measured and weighed.

To quantify juvenile survival to overwintering, we radio-tracked hatchlings 1–3 times per week until the signal was lost, mortality was confirmed, or hatchlings began overwintering. Due to the small size of the transmitters attached to hatchling turtles, batteries lasted for 25–30 days; therefore, we collected active hatchlings after approximately 25 days to replace transmitters, and then continued to radio-track the hatchlings until mortality or onset of overwintering. Each time we tracked a hatchling turtle, we recorded its behavior (e.g., actively moving, burrowed under leaf litter, etc.), microhabitat, and macrohabitat. We also recorded hatchlings' location using a handheld GPS unit with an accuracy of 5 m, and its distance from its previous location using a tape measure. Once a hatchling was observed buried in the same location for > 2 weeks in October or November, we assumed the hatchling had begun overwintering. Juvenile survival to overwintering was assigned as either 0 or 1. Hatchlings that were either found dead or whose transmitters were recovered with damage consistent with a predator attack were assigned a survival value of 0. Hatchlings that were known to have entered hibernation were assigned a survival value of 1. Hatchlings for which we lost the radio signal more than 3 days from predicted transmitter battery expiration were assumed to have been depredated (i.e., the transmitter was either broken or carried out of signal range by a predator) and were assigned a survival value of 0. The final group of hatchlings were those whose radio signals were lost within 3 days of predicted transmitter battery expiration. For these hatchlings, if transmitter expiration occurred after 1 October (the date at which most surviving hatchlings had reached the location at which they subsequently overwintered), we presumed the hatchling had survived to enter hibernation and assigned a survival value of 1. If transmitter expiration occurred before 1 October for hatchlings in this group, we assigned the hatchling an "unknown fate" and excluded it from analysis. All animals were handled in accordance with all required state and local scientific research permits, and with the University of Toledo's Institutional Animal Care and Use Committee (protocol #108797).

Statistical Analysis/Modeling

In all analyses, box turtles and spotted turtles were modeled separately. We first tested for correlations among the variables (i.e., Pearson's correlation coefficient), and subsequently retained all variables because none were strongly correlated (all $r \leq 0.44$). For each species, we modeled the relationship between nest-site macrohabitat variables (distance to nearest road and distance to nearest macrohabitat edge) and nest-site microhabitat variables (day of year, shade cover over the nest, and nest depth) on nest success and juvenile survival to overwintering separately. We used binomial logistic regression to construct models of all possible combinations of macrohabitat and microhabitat variables and their effects on both nest success and juvenile survival. Models for box turtles included year as a random effect because preliminary analysis indicated a difference in nest success between years in this species only. Models for spotted turtles included individual female as a random effect because some female spotted turtles nested twice in the same year (Carter, 2021). We considered the best-supported macrohabitat and microhabitat models predicting nest success and juvenile survival to be those with the lowest AICc. We further evaluated the effect sizes of the parameters in the best-supported models of nest success and juvenile survival. Finally, we used analysis of variance to determine whether either nest success or juvenile survival differed with macrohabitat type or microhabitat type in either species.

RESULTS

We monitored a total of 83 box turtle nests and 36 spotted turtle nests in 2018 and 2019 combined. Mean values for clutch size, hatching success, and nest-site macrohabitat and microhabitat parameters in each species are shown in **Table 1**. The number of nests of each species constructed in each macrohabitat and microhabitat type are shown in **Table 2**. From these nests, we radio-tracked 68 hatchling box turtles and 34 hatchling spotted turtles.

Nest Predation Rates

We caged 42 box turtle nests and 19 spotted turtle nests, and we left 41 box turtle nests and 17 spotted turtle nests unprotected to estimate nest predation rates. Two of the 42 protected box turtle nests were depredated, and 24 of the 41 unprotected nests were depredated, for an estimated natural predation rate of 58.5% in box turtle nests at our study sites. None of the 19 protected spotted turtle nests was depredated, and only 1 of the 17 unprotected nests was depredated, for an estimated natural predation rate of 5.9% in spotted turtle nests (**Table 1**).

Predictors of Nest Success

Only unprotected nests were included in the models predicting nest success. In box turtles, there was no difference in nest success among macrohabitat classes [$F_{(4,36)} = 1.48$, $P = 0.23$]. The best-supported macrohabitat model predicting nest success included only distance to nearest macrohabitat edge (mean = 23 m; range 0–177 m; **Supplementary Table 1**), with approximately a 25% increase in the probability of nest success with every 50-m farther from macrohabitat edge (**Figure 2**). There was also no difference in nest success among microhabitat classes in box turtles [$F_{(3,\ 37)} = 0.46$, $P = 0.71$]. The best-supported microhabitat model predicting nest success in box turtles

TABLE 1 | Summary of clutch, microhabitat, and macrohabitat characteristics of eastern box turtle (*Terrapene carolina carolina*) and spotted turtle (*Clemmys guttata*) nests in northwestern Ohio and southern Michigan in 2018–2019.

	Eastern box turtle ($N = 83$)	**Spotted turtle ($N = 36$)**
Clutch size	6.6 ± 0.2 (2–11)	3.7 ± 0.2 (1–5)
Predation rate (%; estimated from unprotected nests only)	58.5 ($N = 41$)	5.9 ($N = 17$)
Hatchlings produced from successful nests	3.9 ± 0.3 (0–9)	2.6 ± 0.3 (0–5)
Nest distance to nearest road (m)	152.0 ± 13.5 (0–465)	326.2 ± 34.2 (0–644)
Nest distance to nearest habitat edge (m)	22.5 ± 3.8 (0–177)	34.6 ± 4.3 (0–83)
Day of year nest was constructed	166.3 ± 0.7 (151–177)	168.9 ± 1.5 (153–184)
Shade cover over nest (%)	33.7 ± 1.5 (2–71)	38.5 ± 2.4 (3–64)
Total nest depth (mm)	90.4 ± 2.1 (68–137)($N = 57$)	56.9 ± 2.3 (35–82)

Values shown are means ± standard errors (range). For box turtles, nest depth was not measured for 26 nests that were depredated on the night they were constructed.

included nest date (mean = 166; range 151–177) and total nest depth (mean = 90 mm; range 68–137 mm; **Supplementary Table 2**). Probability of nest success increased from ∼15% for the earliest nesting attempts to nearly 100% for those constructed 3 weeks later (**Figure 3A**), and increased from ∼60% in the shallowest nests to nearly 100% for nests 60 mm deeper (**Figure 3B**). That is, nest success in box turtles was greater in deeper nests constructed later in the season. In box turtles, female body size (measured as plastron length) was positively correlated with total nest depth [$F_{(1,\ 52} = 5.47$, $P = 0.023$], although there was substantial among-individual variation in this relationship ($R^2 = 0.08$).

In spotted turtles, only one of the 17 unprotected nests was depredated, and the other 16 unprotected nests successfully produced at least one live hatchling. Due to this lack of variation in spotted turtle nest success, our models of macrohabitat and microhabitat variables predicting nest success failed to converge for this species.

TABLE 2 | Number of nests of eastern box turtle (*Terrapene carolina carolina*) and spotted turtle (*Clemmys guttata*) in northwestern Ohio and southern Michigan in 2018–2019 constructed in each macrohabitat and microhabitat class.

	Eastern box turtle ($N = 83$)	**Spotted turtle ($N = 36$)**
Macrohabitat type		
Deciduous forest	1	0
Mixed coniferous/deciduous forest	7	1
Floodplain forest	0	2
Shrub swamp	0	6
Open fen	0	8
Vernal wetland	1	0
Flooded grassland	0	19
Grassland	52	0
Savanna	20	0
Residential	2	0
Microhabitat type		
Grass	24	9
Sedge	0	1
Sphagnum	1	14
Rotten log	0	3
Sand	17	0
Rocky soil	16	0
Soil	25	9

Predictors of Juvenile Survival to Overwintering

We radio-tracked 68 box turtle hatchlings following nest emergence. Of those, 12 were known mortalities, 27 were observed to have entered or survived overwintering, 16 were assumed to have been depredated when their signals were lost before expected battery expiration, and 13 were assumed to have survived to overwintering because they were observed alive until their transmitter batteries expired after 1 October. Juvenile survival to overwintering did not differ with macrohabitat class in box turtles [$F_{(3,\ 64} = 1.79$, $P = 0.16$], and the null model for predicting juvenile survival to overwintering was the best-supported model for nest-site macrohabitat analysis in this species (**Supplementary Table 1**).

The best-supported nest-site microhabitat model predicting juvenile survival to overwintering in box turtles included shade cover and total nest depth (**Supplementary Table 2**). Overall, probability of juvenile survival to overwintering decreased with both shade cover (mean = 34%; range 2–71%) and nest depth (mean = 90 mm; range 68–137 mm). Probability of hatchling survival to overwintering decreased from ∼80% from a nest with 10% shade cover to ∼40% from a nest with 70% shade cover (**Figure 4A**), and decreased from ∼80% in the shallowest nests to ∼20% in the deepest nests (**Figure 4B**). That is, juvenile survival to overwintering in box turtles was greater for hatchlings from shallower nests with less shade cover.

We radio-tracked 34 spotted turtle hatchlings. One hatchling was found depredated, and 19 were observed to have entered or survived overwintering. Transmitter batteries expired for nine hatchlings after 1 October, and these hatchlings were assumed to have survived to overwintering. The signals for five hatchlings were lost before expected battery expiration, and these hatchlings were assumed to have been depredated.

Juvenile survival to overwintering did not differ with macrohabitat class in spotted turtles [$F_{(4,\ 29)} = 2.24$, $P = 0.09$]. However, juvenile survival to overwintering differed with microhabitat class in spotted turtles [$F_{(4,\ 29} = 4.4$; $P = 0.007$], such that juveniles from nests constructed in soil had lower survival to overwintering than juveniles from nests

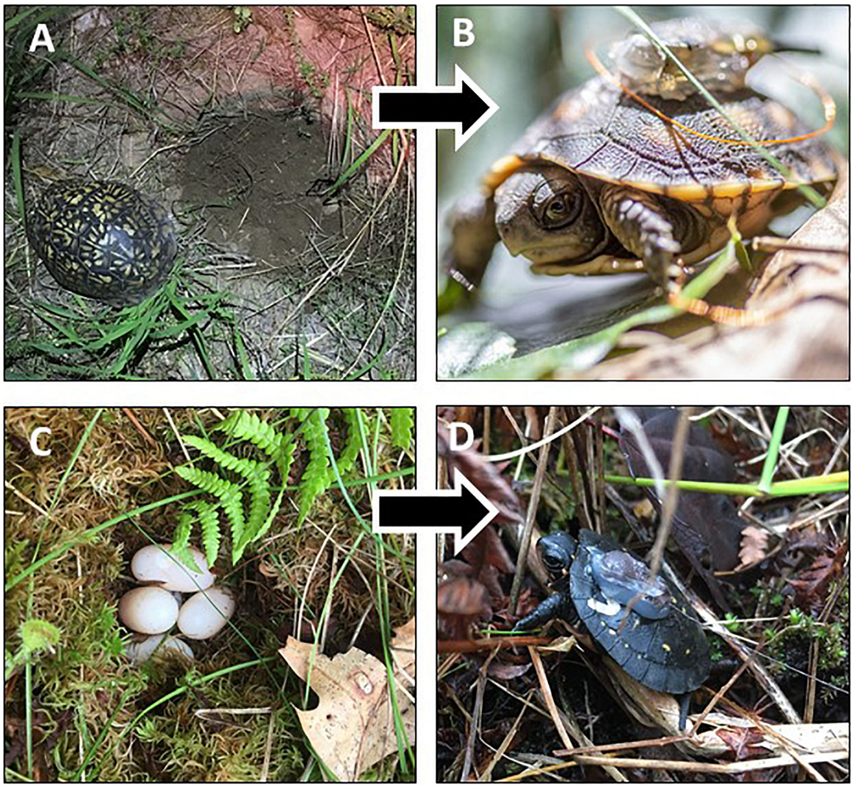

FIGURE 1 | Nest sites were located by radio-tracking gravid female turtles to nest sites in northwestern Ohio and southern Michigan in 2018–2019. We monitored eastern box **(A)** and spotted **(C)** turtle nests until hatchling emergence, at which point miniature radio-transmitters were attached to juvenile eastern box **(B)** and spotted **(D)** turtles. Hatchlings were then monitored via radio-telemetry to determine whether they survived to enter overwintering. Photo credits: A. Hulbert.

in other microhabitat types (**Figure 5**). Finally, the null model for predicting juvenile survival to overwintering was the best-supported model for both nest-site macrohabitat (**Supplementary Table 3**) and microhabitat (**Supplementary Table 4**) in spotted turtles; no other models were competitive.

DISCUSSION

Maternal choice of nest site often entails balancing a variety of potential risks that may differentially affect the nesting female, the successful hatching of eggs, or the survival of juveniles (Refsnider and Janzen, 2010). In balancing these risks, females may select nest sites that minimize risk to one life stage, but at a potential cost to a different life stage. Therefore, if researchers focus only on the effect of maternal nest-site choice on the survival outcome of a single life stage, nest-site choice may appear to be maladaptive, when in fact females are selecting nest sites that optimize overall survival and reproductive output over sites that maximize survival of a single life stage (Mitchell et al., 2013; Streby et al., 2014b).

We modeled effects of nest-site characteristics at the macrohabitat and microhabitat scale on both nest success and juvenile survival to overwintering in two declining turtle species occurring in oak savanna landscapes. We found that spotted turtle nest success overall was very high (∼94%), and did not vary meaningfully with the nest-site variables we measured at either the macrohabitat or microhabitat scale. Survival of juvenile spotted turtles was lower from nests constructed in soil substrate than in other substrates, which predominantly included sphagnum mounds and rotten logs, with a few nests also constructed in sedge or grass mounds. We did not quantify nest hydric conditions in this study, but our results suggest that moisture levels within the nest may play an important role in the survival of emerging spotted turtle hatchlings. It is likely that the spotted turtle nests constructed in soil substrate were drier than nests constructed in sphagnum mounds or rotten logs, and hatchlings of several reptile species are known to have higher survival from nests with greater moisture content than from drier nests (e.g., Miller, 1993; Brown and Shine, 2004; Socci et al., 2005). Future research should investigate the influence of hydric conditions on hatchling survival in spotted turtle nests.

Nest success at our study sites was also relatively high for eastern box turtles, with approximately 40% of unprotected box turtle nests producing at least one live hatchling. Nest predation rates of > 95% have been reported in closely related turtle species (Strickland et al., 2010; Refsnider et al., 2015), so it is encouraging from a conservation standpoint that nearly 40% of nests in our study populations succeeded even without any management intervention such as predator-proof cages. We

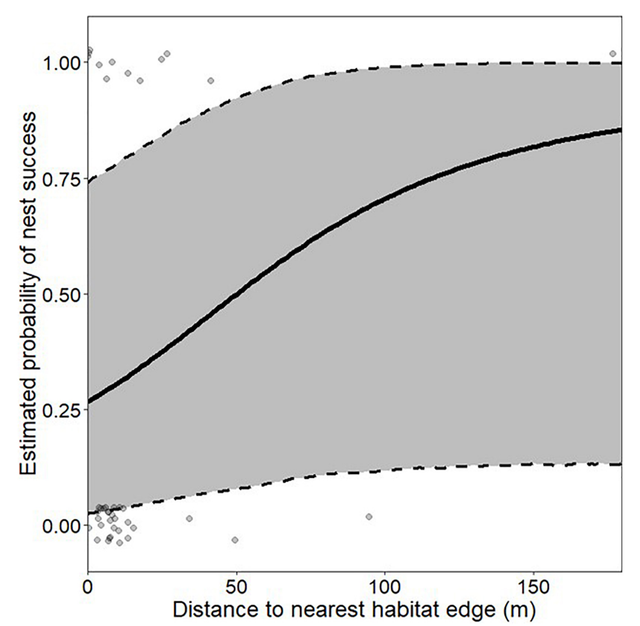

FIGURE 2 | Effect size of nest distance to nearest habitat edge on probability of nest success in eastern box turtles (*Terrapene carolina carolina*) in northwestern Ohio and southern Michigan in 2018–2019. Effect size was estimated from the best-supported macrohabitat model predicting box turtle nest success, which included only distance to nearest macrohabitat edge. All non-depicted variables were held at their mean values.

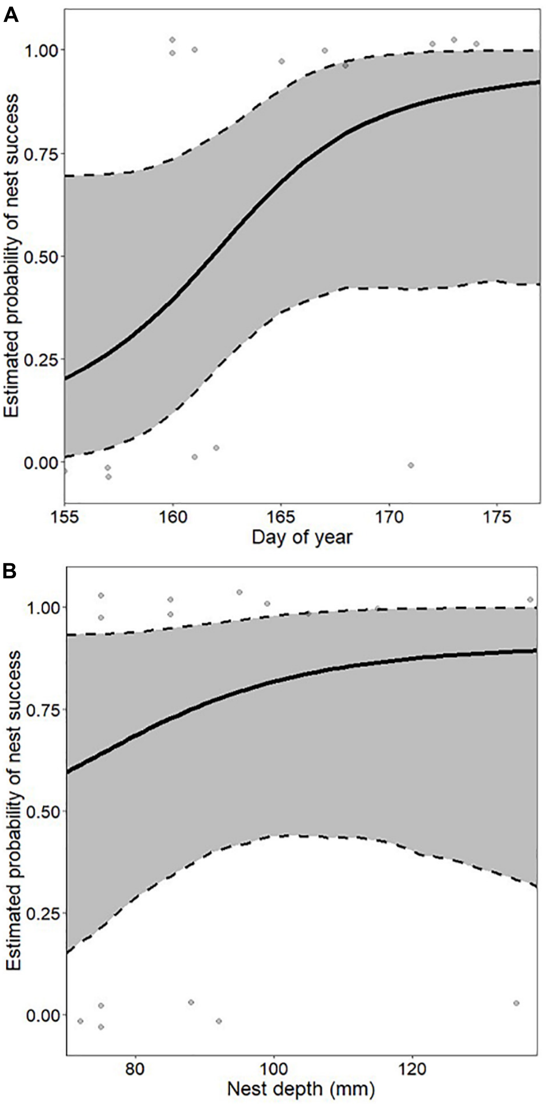

FIGURE 3 | Effect size of nest construction date **(A)** and total nest depth **(B)** on probability of nest success in eastern box turtles (*Terrapene carolina carolina*) in northwestern Ohio and southern Michigan in 2018–2019. Effect sizes were estimated from the best-supported microhabitat model predicting box turtle nest success, which included nest date and total nest depth. In each panel, non-depicted variables were held at their mean values.

found a considerable effect of macrohabitat on nest success, such that nests constructed farther from habitat edges had higher probability of producing hatchlings than nests closer to habitat edges, despite most nests being constructed relatively close to edges. Nesting relatively close to edges, despite lower nest success near edges, is suggestive of a trade-off between selection pressure on a different life stage: perhaps hatchlings' access to other macrohabitat types that may confer higher juvenile survival is an important driver of nesting relatively close to edges. We found no evidence of increased juvenile survival from nests near macrohabitat edges, but nest distance to forest edge was the strongest predictor of a juvenile's eventual overwintering site in a more northern box turtle population (Laarman et al., 2018), and in a separate study on these same populations we found that most hatchling box turtles overwintered in forest or forest edge habitat (Hulbert, 2020). A useful avenue for future research would be to explore other potential life-history tradeoffs that might explain the propensity to nest near edges that confer relatively low nest success or determine if this is simply a negative edge effect, and to identify the mechanism that might underlie such an edge effect. For example, lower nest success closer to habitat edges could be due to increased predator abundance near edges. Alternatively, edges are often warmer and drier compared to core habitat, and both thermal and hydric conditions strongly influence hatchling survival and phenotype in turtles (e.g., Miller et al., 1987; Brooks et al., 1991). It is also possible that nests near edges, while apparently suboptimal for nest success, are safer for females during nest construction. We did not consider risks to nesting females here, but research on a related, aquatic species found that predation risk to nesting females was not related to nest distance from wetland edges (Refsnider et al., 2015).

At the microhabitat scale, box turtle nest success was greater in nests constructed on later dates. The benefits of nesting later are likely highest within a certain date range, with nests constructed after that range resulting in suboptimal phenotypes, lower nest success, or lower hatchling survival (Telemeco et al., 2013). In particular, following completion of embryonic development within the egg, hatchlings of species that do not overwinter within the nest cavity must have sufficient time after nest emergence to reach suitable overwintering habitat before the onset of lethally

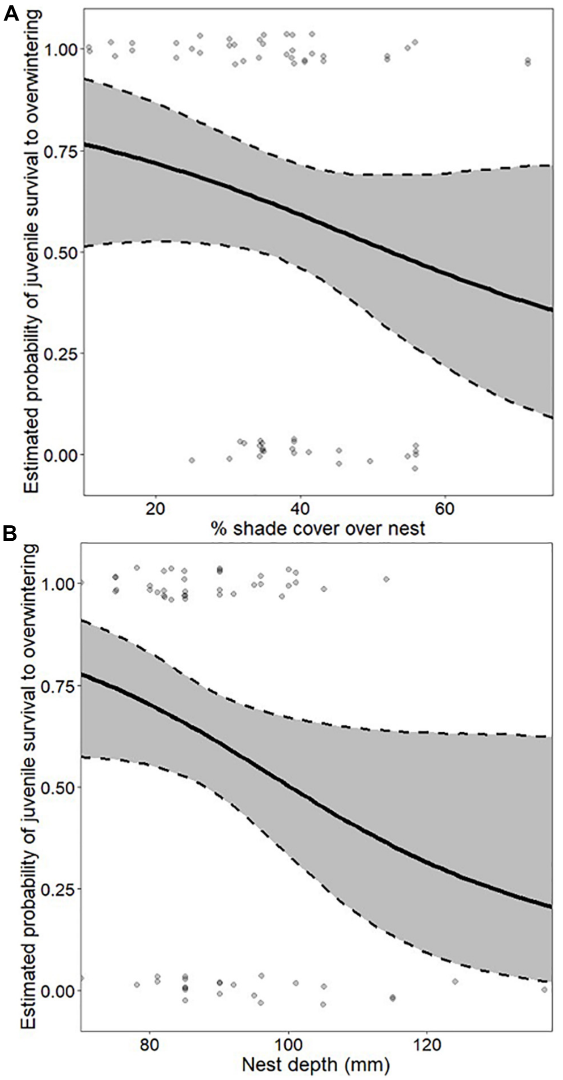

FIGURE 4 | Effect size of shade cover over the nest **(A)** and total nest depth **(B)** on probability of juvenile survival to overwintering in eastern box turtles (*Terrapene carolina carolina*) in northwestern Ohio and southern Michigan in 2018–2019. Effect sizes were estimated from the best-supported microhabitat model predicting juvenile survival to overwintering in box turtles, which included shade cover over the nest and total nest depth. In each panel, non-depicted variables were held at their mean values.

cold weather (e.g., Laarman et al., 2018). Nests constructed too late in the year may hatch with too little time remaining before the onset of winter for hatchlings to successfully reach overwintering habitat.

Box turtle nest success was also higher in nests constructed at greater total depths compared to shallower nests. Although nest temperature decreases with increasing nest depth in large turtle species that construct deep nest cavities (e.g., Roosenburg, 1996), temperature variation in relatively shallow nests such as those constructed by box turtles and closely related painted turtles is minimal and likely insufficient to affect incubation regime or offspring sex (Refsnider et al., 2013a; also see Telemeco et al., 2009). Instead, deeper nests may be more difficult for predators to detect, thereby increasing the probability that deeper nests will successfully produce hatchlings. Importantly, however, turtles construct nests using their rear limbs, meaning that females' maximum nest depths are physically constrained by the length of their rear limbs (Refsnider, 2012). Therefore, older and larger females may be at a reproductive advantage if they can excavate deeper nests with a lower probability of being depredated, compared to younger and smaller females that are constrained to excavating more superficial nests. Indeed, in our study, nest depth increased with female plastron length, suggesting that larger females construct deeper nests than smaller females. Interestingly, there was substantial variation in nest depth even among similarly sized individuals, which could be due to individuals adjusting nest depth rather than constructing the deepest possible nests for their body size. Moreover, painted turtles constructed deeper nests in years where May temperatures were higher (Refsnider et al., 2013a), suggesting that individuals have some capacity to adjust nest depth relative to prevailing environmental conditions. Regardless of the mechanism(s) underlying variation in nest depth, the potential advantages of constructing deeper nests would only hold if increasing nest success confers a reproductive advantage, which would require no opposing selection pressure acting on the same trait (i.e., nest depth) but in a different life stage.

Box turtle hatchlings had a higher probability of surviving to overwintering if they hatched from nests that were shallower and constructed under less shade cover, compared to hatchlings from deeper, more shaded nests. Shade cover is a critical driver of incubation temperature in turtle nests, and is known to influence a variety of hatchling phenotypes, including sex in species with temperature-dependent sex determination (Janzen, 1994a). Furthermore, choice of shade cover over a nest site is a behaviorally plastic trait that females can adjust in order to match nest incubation conditions with prevailing environmental conditions (e.g., Refsnider and Janzen, 2012). Our results suggest that female choice of shade cover can also influence hatchling survival, further emphasizing the importance of this aspect of maternal nest-site choice. Importantly, in order for behavioral plasticity in maternal choice of shade cover over nest sites to be expressed, a range of shade cover options must be available within nesting areas (Refsnider et al., 2013b). For example, in an unusually warm year female turtles may nest at sites with greater shade cover than they would choose in average or cool years, in order to compensate for warmer air temperatures. Indeed, at our study sites mean May air temperatures were 3.4°C (Ohio) and 4.5°C (Michigan) cooler in 2019 than in 2018 (NOAA), and box turtles nested under 40.5% shade cover in 2018, but 30.1% shade cover in 2019 ($t = 3.17$; $P = 0.0027$). Thus, box turtles in our study appear to show similar behavioral plasticity in maternal nest-site choice to the painted turtles in Refsnider and Janzen (2012), wherein female turtles compensate for prevailing climatic conditions on nest incubation conditions by adjusting the amount of shade cover under which they choose to nest. However, if nesting areas lack variability in shade cover (which could include low ground cover as well as tree canopy), females would be unable to express their inherent

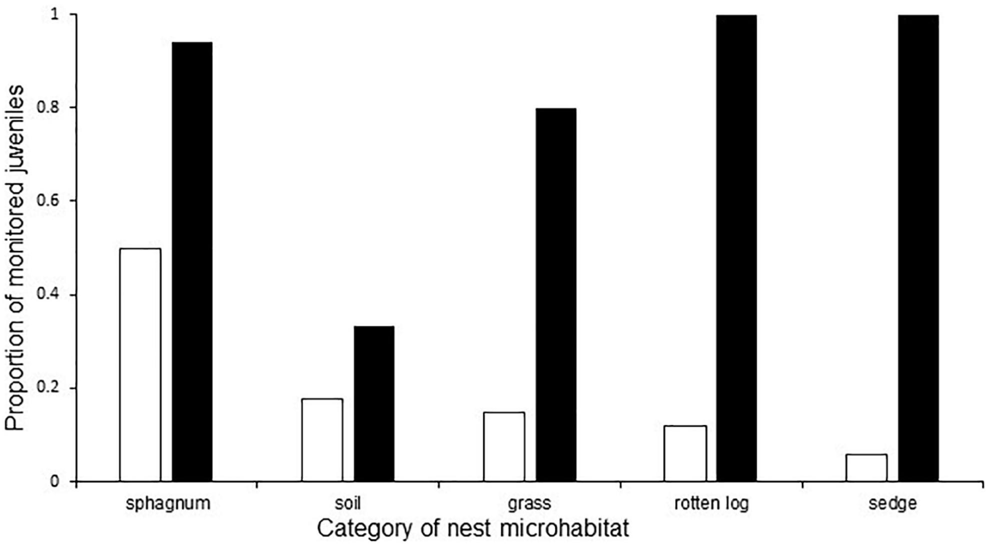

FIGURE 5 | Proportion of spotted turtle (*Clemmys guttata*) juveniles monitored via radio-telemetry from nests constructed in each of five microhabitat types (white bars; sample size indicates total number of nests constructed in each microhabitat type), and rate of survival to overwintering of those juveniles (black bars), in northwestern Ohio and southern Michigan in 2018–2019. Juvenile survival to overwintering differed with nest microhabitat class [$F_{(4,29)} = 4.4$, $P = 0.0067$].

behavioral plasticity and may be forced to nest at sites that could be lethally warm (Refsnider et al., 2013b). We recommend that managers endeavor to maintain heterogeneity in habitat structure at the microhabitat scale (i.e., < 1 m) within known turtle nesting areas such that open, bare patches as well as more densely vegetated patches are interspersed throughout the nesting area.

The effect of nest depth was in opposite directions for nest success compared to juvenile survival to overwintering in box turtles. The probability of nest success was higher from deeper nests, whereas the probability of juvenile survival to overwintering was higher from shallower nests. Predators may have a more difficult time detecting eggs in deeper nests, which likely explains our finding of higher nest success from deeper nests. However, hatchlings likely expend more energy digging their way out of deeper nests compared to shallower nests, which may explain why hatchlings from shallower nests were more likely to survive to overwintering. In painted turtles, deeper nests produced smaller and faster hatchlings than shallower nests, which demonstrates that even if nest depth does not affect incubation temperature or hatchling sex, there may still be effects on other phenotypes in the hatchling stage (Refsnider et al., 2013a), which would further support a potential tradeoff in the benefits of nest depth to the egg stage vs. the hatchling stage. Future research is needed to determine whether energetic costs to emerge from a nest cavity differ with depth, and whether the additional energetic costs of traveling to suitable overwintering sites could be offset in some way, perhaps by providing patches of refuge habitat in areas through which hatchlings are likely to travel from nests to overwintering sites.

Our results identify an important tradeoff between nest success and juvenile survival in box turtles: deeper nests were more likely to successfully hatch, but hatchlings were more likely to survive if they came from shallower nests. This tradeoff means that, when constructing their nests, females must balance opposing risks on two different life stages. Due to physical constraints of body size (i.e., rear limb length) on the maximum depth to which a female is capable of digging, it is likely that small females are unable to adjust their nest depth and therefore may be inadvertently favoring the survival of juveniles over nest success. However, older and larger females may have greater capacity to adjust their total nest depth, in which case they could "choose" to dig a deeper nest that would favor nest success over juvenile survival, perhaps under conditions where nest predators were abundant, or when the nest site is in close proximity to suitable overwintering habitat for juveniles. One way to test for evidence of this ability to adjust nest depth would be to compare nest depth to rear limb length in female box turtles and determine if there is more variation in nest depth in longer-limbed females.

We did not investigate the effects of nest-site characteristics on offspring sex in this study, but this is a critical knowledge gap that needs to be filled in order to better predict the impacts of climate change on these two declining turtle species. Determining the precise incubation temperature ranges that produce each sex in box and spotted turtles, and comparing incubation temperatures in wild nests with those reaction norms, will provide crucial data regarding potential sex ratio skews that could result from a warming climate (Janzen, 1994b). In particular, if specific turtle populations are at risk of producing primarily the warmer sex (females, in the case of box and spotted turtles) as the climate continues to warm, managers may be able to reverse such a trend through strategic placement of shade-providing vegetation within nesting areas such that nesting females could choose shadier nest sites in warmer years, and thereby reduce potential skews in sex ratios (Refsnider and Janzen, 2012).

Maternal nest-site choice is an important mechanism by which females can influence both the survival and the phenotypes of their offspring across multiple life stages. Knowledge of the specific nest-site characteristics chosen by females, as well as the fitness outcomes across multiple life stages resulting from those nest-site characteristics, will inform managers as to the specific habitat characteristics that result in the highest overall productivity. We recommend that nesting areas for eastern box turtles and spotted turtles be maintained with structural variation at the microhabitat scale (i.e., <1 m) to allow nesting females to express plasticity in nest-site choice by matching nest incubation conditions with the prevailing local climate through choice of shade cover over the nest, while continuing to use historical nesting habitat at the macrohabitat scale (i.e., >10 m). Furthermore, managers should avoid fragmenting nesting areas by roads or trails in order to minimize potential edge effects. Finally, a useful avenue for future research would be to investigate whether small refuge microhabitats, such as small patches of dense vegetation or small brush piles, within nesting areas would improve survival rates of box turtle hatchling turtles traveling from nests to overwintering sites in adjacent forest or forest edge habitat, particularly in large nesting areas in which nests are located far from suitable overwintering habitat.

DATA AVAILABILITY STATEMENT

Data are deposited in Mendeley Data, V1, doi: 10.17632/jd64t5prvn.1.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Toledo's Institutional Animal Care and Use Committee (protocol #108797).

AUTHOR CONTRIBUTIONS

JR: conceptualization, formal analysis, investigation, and writing. SC and AH: formal analysis and investigation. AD and PM: investigation. GK: formal analysis. HS: conceptualization, formal analysis, and investigation. All authors contributed to the article and approved the submitted version.

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Population and Nesting Site Evidence for Diamondback Terrapins, *Malaclemys terrapin*, in Northeast Florida

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Diamondback terrapins (*Malaclemys terrapin)* are listed as Vulnerable by the IUCN Red List Index of Threatened Species. Among the challenges terrapins encounter are habitat loss due to coastal development and sea level rise, mortality at all life stages by mammalian and avian predators, road mortality, boat strikes, harvest for the pet trade, and drowning in crab traps. The primary objective of this study was to locate populations and nesting areas of diamondback terrapins in the four northeastern-most counties of Florida (Nassau, Duval, St. Johns, and Flagler). We conducted head counts and performed land surveys of shorelines and high spots for evidence of terrapin presence. During the land surveys we searched for crawls, intact and depredated nests, dead terrapins, and terrapin bones. To evaluate whether woody plant presence affected nest site choices, we recorded the occurrence of 10 common woody plant species during each land survey and compared areas where nesting did and did not occur. We collected 404 records of terrapin activity in 2013 and 2014. Most were from Nassau County (277) and only one was from Flagler County. Most data were in the form of depredated nests (205) and terrapin remains (147). The woody plant data suggest that terrapins were significantly more likely to nest when Christmas berry (*Lycium carolinianum*) was present, and nesting was less likely when either wax myrtle (*Myrica cerifera*) or oak (*Quercus* spp.) were present.

Keywords: ***Malaclemys terrapin*, nesting, nest predation, population, predators, terrapin, turtle**

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INTRODUCTION

Diamondback terrapins (*Malaclemys terrapin)* are listed as Vulnerable by the IUCN Red List Index of Threatened Species (Roosenburg et al., 2019). Among the challenges terrapins encounter are habitat loss due to coastal development and sea level rise, mortality at all life stages by mammalian and avian predators, road mortality (Maerz et al., 2018), boat strikes (Lester et al., 2018), harvest for the pet trade, and drowning in crab traps (Chambers and Maerz, 2018).

Commonly used references concerning reptile natural history (e.g., Ernst and Lovich, 2009; Powell et al., 2016) describe the distribution of diamondback terrapins to be from Cape Cod, Massachusetts to Corpus Christi, Texas. They provide range maps with a continuous line drawn along the Atlantic and Gulf coastlines between those two places. While these maps are useful for general information, terrapin distribution along those lines is not truly continuous due to natural or anthropogenic habitat interruptions. It would be helpful for researchers seeking to study local populations to know where terrapin concentrations exist. Accurate local distribution information can also inform governmental decisions concerning allocation of conservation resources and protection of essential habitats. To this end, one of the major objectives of the Diamondback Terrapin Working Group[1] has been to create a "living" map of historical and current terrapin populations throughout the range (Butler et al., 2006). Historical populations can be identified from existing literature or museum collections, while the distribution and abundance of contemporary populations is determined by surveys.

In Florida, terrapin populations are known from Merritt Island National Wildlife Refuge (Seigel, 1980a,b,c, 1984, 1993), the Keys (Wood, 1992; Baldwin et al., 2005), the western part of Everglades National Park (Hart and McIvor, 2008), Sanibel Island (C. Lechowicz, pers. comm.), Tampa Bay and St. Martins Key (C. Boykin, pers. comm), the Big Bend region (Butler and Heinrich, 2013), and the extreme western Panhandle (R. O'Conner, pers. comm.). In northeast Florida, several discrete terrapin populations in Nassau and Duval counties have been studied (Butler, 2000, 2002; Butler et al., 2004), but no systematic surveys were performed there. In a review of 58 museums only six terrapin records are listed for Florida's four northeastern-most counties (Krysko et al., 2011): one from Nassau, four from Duval, one from St. Johns, and none from Flagler. The primary objective of this study was to identify terrapin populations and nesting sites in these four northeastern Florida counties.

Some oviparous reptiles, including diamondback terrapins, exhibit temperature-dependent sex determination (TSD) where offspring sex is a function of incubation temperature (Bull, 1980; Jeyasuria et al., 1994; Burke and Calichio, 2014; Wibbels et al., 2018). Incubation temperature depends on variables such as nest depth and shading, and in some cases shading by overstory vegetation affects nest depth or nest choice (Kolbe and Janzen, 2002; Czaja et al., 2020). Diamondback terrapins prefer to nest in sandy areas above the high tide line (Palmer and Cordes, 1988; Roosenburg, 1994). These areas often support some woody shrubs and trees that require such soils and do not withstand extensive flooding. Butler and Heinrich (2013) noted that several woody plant species were frequently associated with terrapin nesting areas, and we hypothesized that terrapins may use this vegetation as a distant visual signal that an area is appropriate for nesting. Thus, another objective was to determine if certain woody plant species could be indicators of terrapin nesting sites.

MATERIALS AND METHODS

Study Site

The study area encompassed shorelines, marsh islands, tidal creeks and rivers associated with the Intracoastal Waterway (ICW) of the four northeastern-most coastal Florida counties of Nassau, Duval, St. Johns, and Flagler (**Figure 1**). The northern boundary was the St. Mary's River and we surveyed southward nearly 160 km to the southern border of Flagler County. The area includes inlets at the St. Mary's, Nassau, St. Johns, Fort George rivers, the city of St. Augustine, and the Matanzas Inlet. Most of the habitat adjacent to the ICW in Nassau, Duval and St. Johns counties is described as salt marsh by Montague and Wiegert (1990) with smooth cordgrass (*Spartina alterniflora*) dominating and intermittent stands of needlerush (*Juncus roemerianus*). About midway through St. Johns County, near the Guana-Tolomato-Matanzas National Estuarine Research Reserve (GTM), the northernmost black mangroves (*Avicennia germinans*) occur (Williams et al., 2014), but not until further south in Flagler County do mangroves dominate shorelines.

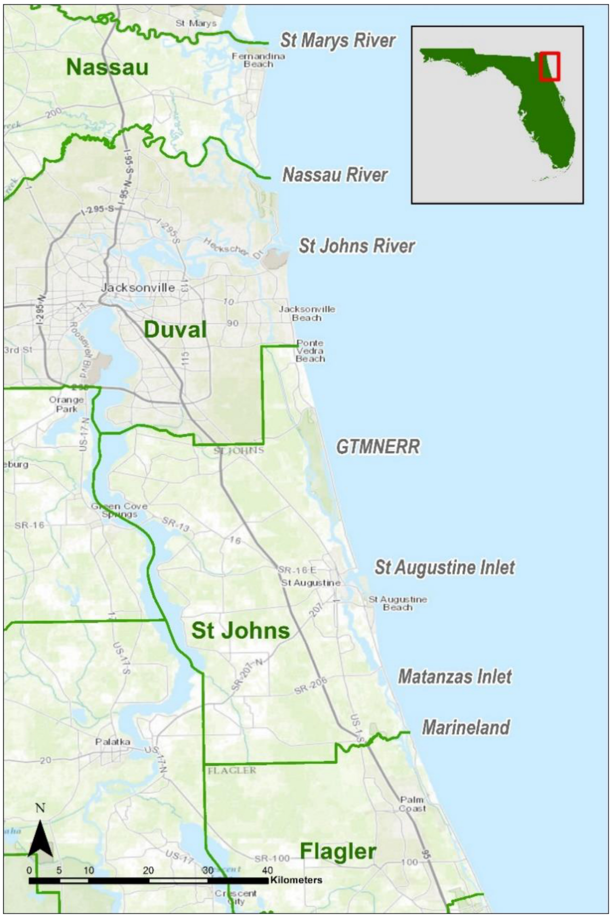

FIGURE 1 | The northeast Florida Diamondback Terrapin study area showing boundaries of the four counties along with major inlets to the ICW.

From May 7 through August 6, 2013, three researchers surveyed 1 day per week for terrapins employing head counts and land surveys (Butler and Heinrich, 2013) in St. Johns County between GTM and Marineland. In 2014, from May 1, through August 1, three researchers surveyed for diamondback terrapins

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[1] www.dtwg.org

3–4 days per week in all four of Florida's northeastern-most counties. Some data collected between 1995 and 2002 from all but Flagler County during earlier studies by JB are included in our maps in the interest of completeness, but they are not counted as new data for this study.

Head Counts

Harden et al. (2009) compared terrapin head counts to population estimates derived from mark-recapture studies. Head counts, while less accurate for quantifying population levels, allow researchers to determine occupancy of an area without needing to engage in time-consuming capture techniques. We counted terrapin heads from our boat as we traveled at idle speed in adjacent tidal creeks, rivers, and occasionally the ICW. For each sighting we recorded GPS locations using a hand-held unit (Garmin GPSMAP 78 SC).

Land Surveys

We conducted walking surveys of all shorelines and dredge spoil or natural marsh islands exhibiting potential terrapin nesting habitat (i.e., above the high tide line with soil composed mostly of sand or sand-shell mix, Palmer and Cordes, 1988; Roosenburg, 1994). At such sites we detected terrapin presence by recording depredated terrapin nests, terrapin remains (carcasses, bones, or scutes), intact nests, or crawls (Butler and Heinrich, 2013; Roosenburg and Burke, 2018). Raccoons (*Procyon lotor*) are major predators of diamondback terrapin nests throughout their range (Burger, 1977; Roosenburg, 1992; Feinberg and Burke, 2003). When raccoons excavate nests, they usually eat the egg contents at the nest site, leaving the eggshells behind. Therefore, we identified depredated nests by finding the eggshells associated with exhumed nests. Further, raccoons often kill female terrapins before they have time to nest (Seigel, 1980a), which was the source of most of the terrapin remnants we found. Occasionally, substrate conditions were such that tracks left in the sand by nesting terrapins could be identified indicating terrapin usage (crawls, Butler, 2002).

Other

Several of our data points resulted from encounters with live terrapins that were not from head counts, not anticipated, and defied placement within our categories. These will be explained below.

Maps

We used Geographic Information System software (ArcGIS 10.3, ESRI, Redlands, CA, United States) to create a geodatabase for all field observations using the GPS locations and field notes. Although vegetation survey locations were included in the geodatabase, maps included here focus on the locations of terrapin evidence and waypoints that indicate the areas surveyed.

Statistical Analysis

We recorded our time spent in the field on most days allowing calculation or the number of data points recorded over time (CPUE as # of records/time).

TABLE 1 | Records of Carolina Diamondback Terrapins in the four northeastern-most Florida counties of Nassau, Duval, St. Johns, and Flagler in 2013 and 2014.

County	Depredated nests	Terrapin remains	Heads	Crawls	Intact nests	Other	Totals
Nassau	137	123	14	0	1	0	275
Duval	39	18	11	11	0	3	82
St. Johns	27	6	6	0	1	6	46
Flagler	1	0	0	0	0	0	1
Totals	204	147	31	11	2	9	404

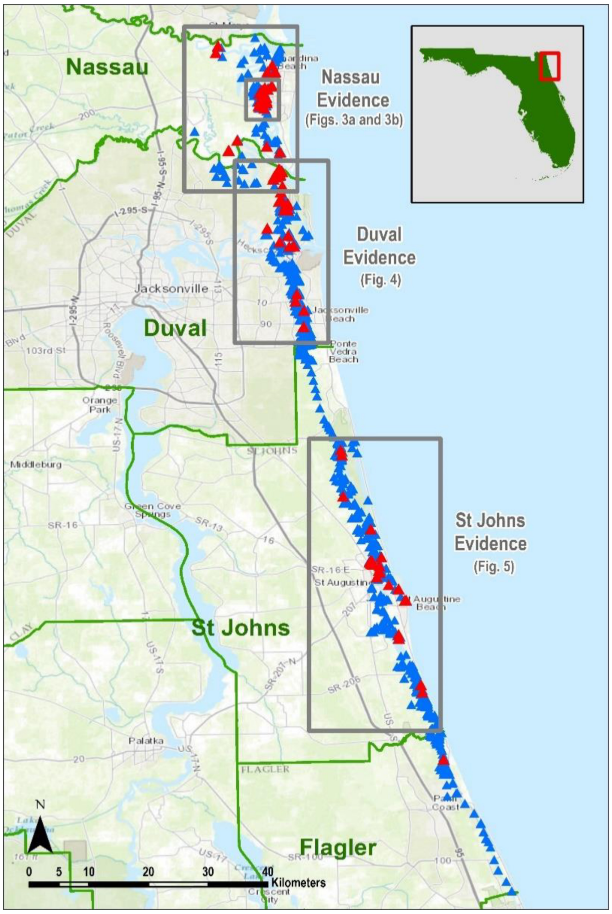

FIGURE 2 | Northeast Florida study area with the three evidence centers outlined and labeled as Nassau, Duval, and St. Johns evidence, with an important Terrapin concentration at Jackson Creek (**Figure 3B**). Blue triangles depict all places where we conducted surveys. Red triangles indicate locations where evidence of Terrapins was found, and each represents between 1 and 102 findings.

To establish whether terrapin evidence data points were distributed randomly, we applied an optimized hot spot analysis (Getis-Ord Gi*) in ArcGIS Pro version 2.9.0 using all terrapin evidence points with the total number of observations as the analysis field.

To determine whether vegetation composition affected nest site choice by terrapins, during the land surveys in 2014, we

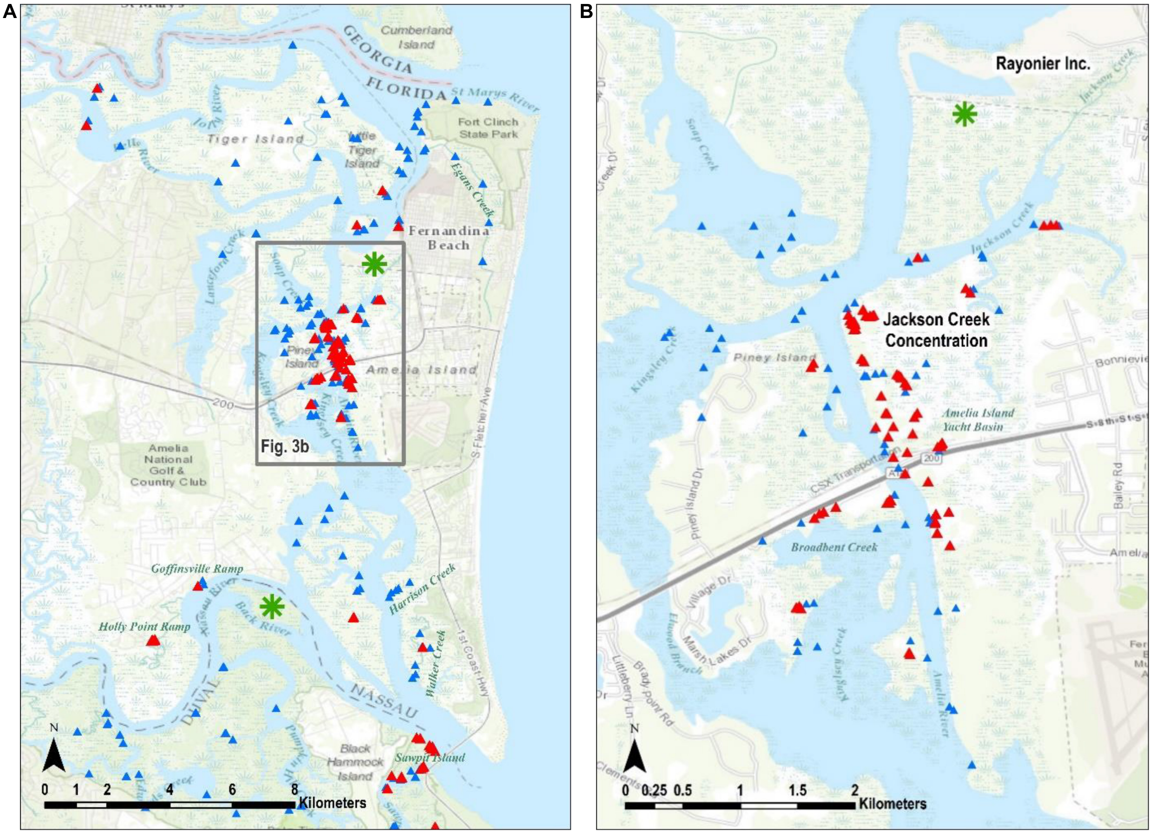

FIGURE 3 | The Nassau Terrapin evidence. All records are indicated in **(A)** along with the Jackson Creek concentration in the box. The Jackson Creek concentration is highlighted in **(B)**. Green asterisks are places where Terrapin evidence was recorded before the current study, and they are discussed in the body of the paper. Triangles as defined in **Figure 2**.

recorded the presence/absence of 10 plant species associated with Florida salt marshes. We limited our records to woody species because most are sensitive to extensive flooding and therefore occur above the high tide line where terrapins normally nest. We did not record the number of each species, only presence/absence. The shrub and tree species we documented are: marsh elder (*Iva frutescens*), saltbush (*Batis halimifolia*), Christmas berry (*Lycium carolinianum*), southern red cedar (*Juniperus silicicola*), wax myrtle (*Myrica cerifera*), cabbage palm (*Sabal palmetto*), saw palmetto (*Serenoa repens*), yaupon (*Ilex vomitoria*), oak (*Quercus* spp.), and pine (*Pinus* spp., *elliotii* or *palustris*).

We compared the presence/absence of the 10 woody species at sites where nesting was detected and locations lacking nests using specialized matched-pairs *t*-testing, to determine if plant species composition differences between nesting and non-nesting sites were statistically significant. We then used decision tree analysis and a generalized ordinal logistic fit regression to model the odds of a nesting or non-nesting event using SAS 9.4 and JMP 15.0.

RESULTS

We documented 404 records of terrapin evidence in northeast Florida (**Table 1** and **Figure 2**). For ease of analysis, we bundled the data into three groups referred to herein as the Nassau evidence (**Figures 3A,B**), the Duval evidence (**Figure 4**), and the St. Johns evidence (**Figure 5**). Sixty-eight percent of our records were from Nassau County, with another 20% from Duval County. St. Johns County records were from several discrete areas, and we had only one record from Flagler County.

Head Counts

Of our 404 observations, 31 (7.7%) were from head counts (**Table 1**). The largest single head count (8) was in Jackson Creek (**Figure 3B**) in Nassau County and most of our head count records occurred in May 2014 (24), which is when we surveyed most of Nassau and Duval counties; we counted only six heads in St. Johns County and none in Flagler.

Land Surveys

Most of our terrapin evidence records were of depredated nests (51%) and again most were in Nassau County (**Table 1**). Among our observations in that county were 88 depredated nests at boat ramps (**Figure 3A**, Holly Point Ramp 58, Goffinsville Ramp 30), 19 on a spoil island in Broadbent Creek, and another 10 along railroad tracks beneath the SR 200 Bridge (**Figure 3B**). In Duval County, we found 12 depredated nests on islands within the marsh between the ICW (called Sisters Creek in this area)

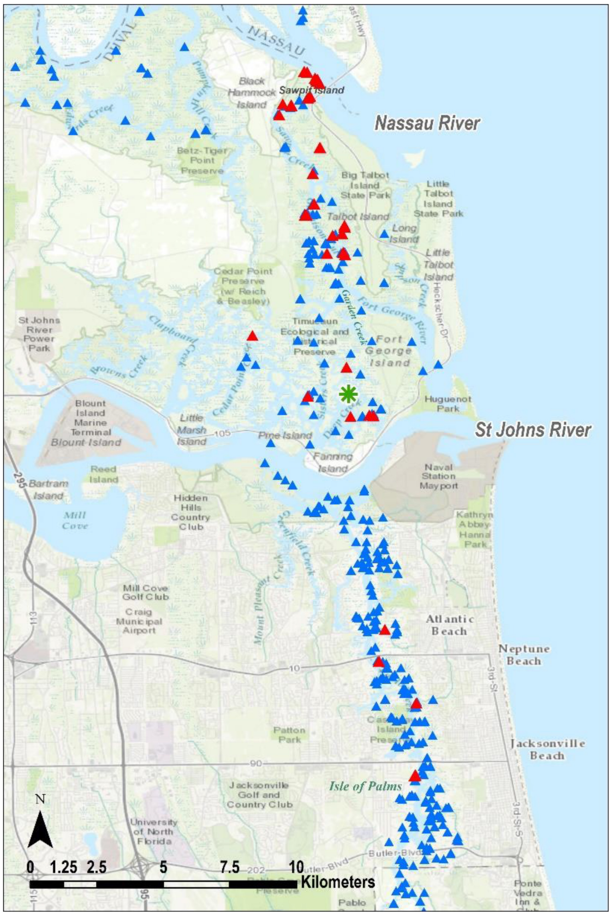

FIGURE 4 | The Duval Terrapin evidence. Triangles and asterisks as in **Figures 2**, **3**, respectively.

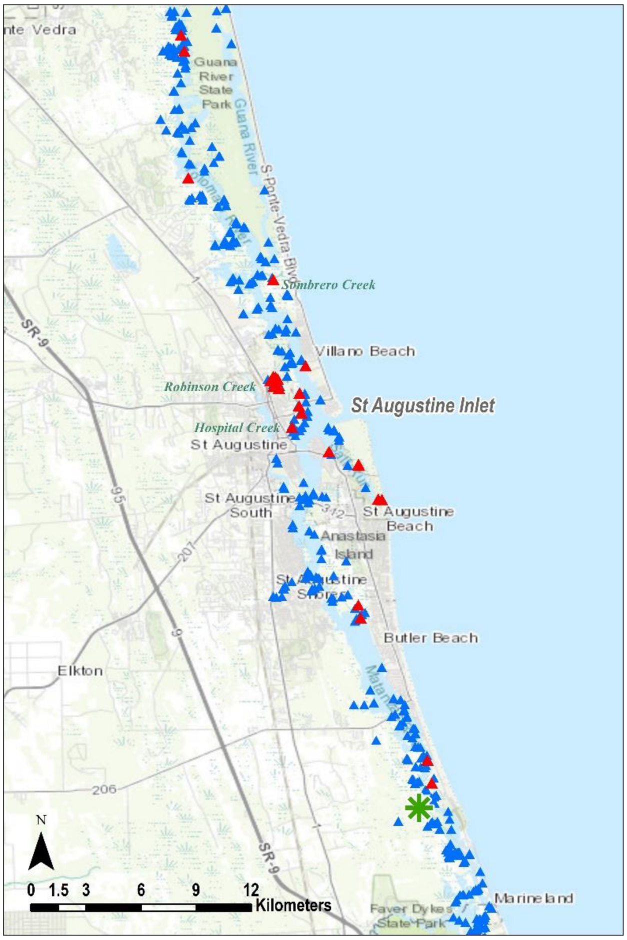

FIGURE 5 | The St. Johns Terrapin evidence. Triangles and asterisks as in **Figures 2**, **3**, respectively.

TABLE 2 | Records of presence of 10 species of woody plants observed during land surveys of Diamondback Terrapin nesting and non-nesting habitats in northeast Florida.

Woody plants genus species	Nesting 153	Non-nesting 758	*Prob* > X^2
Iva frutescens	27	111	0.218
Batis halimifolia	16	81	0.799
Lycium carolinianum	13	23	0.001*
Juniperus silicicola	31	128	0
Myrica cerifera	8	80	0.060*
Sabal palmetto	21	102	0.650
Serenoa repens	5	39	0.386
Ilex vomitoria	22	102	0.401
Quercus spp.	5	65	0.021*
Pinus spp. (*elliotii* or *palustris*)	5	34	0.599

**Denotes a significant statistical value.*

and Fort George Island (**Figure 4**), but most often we found fewer than five at a time. In St. Johns County, we recorded 17 depredated nests at a shoreline upstream in Robinson Creek, three on an island in Sombrero Creek, and other observations were of single raided nests (**Figure 5**). Finally, in Flagler County we located a single depredated nest on a spoil island just south of Marineland. Burke et al. (2009) determined that in Jamaica Bay Wildlife Refuge, New York, raccoons shifted their behavior later in the nesting season such that they devoured eggshells, rather than leaving them near the nest site. If this is the case in northeast Florida, our depredated nest counts would be undercounted.

We discovered most of the terrapin remains in Nassau County (**Table 1**), and it is notable that 87 of these were collected on 1 day from a site near the mouth of Jackson Creek (**Figure 3B**, where we also recorded the most heads). We recorded all 11 crawls on 1 day at Sawpit Island in Duval County (**Figure 4**), a known terrapin nesting site (Butler et al., 2004; Munscher et al., 2012). A terrapin deposited a nest near the Fernandina Harbor Marina (Nassau County, **Figure 3A**) on July 11, 2014 (C. Hoblin, Fernandina resident, pers. comm.). We dug to verify the presence of eggs (not clutch size) and recorded this as one of our intact nests. We discovered a nesting terrapin in St. John's County on 16 July 2013 as it deposited a nest of three eggs on an island adjacent to Hospital Creek in St. Johns County (**Figure 5**). We recorded this as another intact nest (**Table 1**).

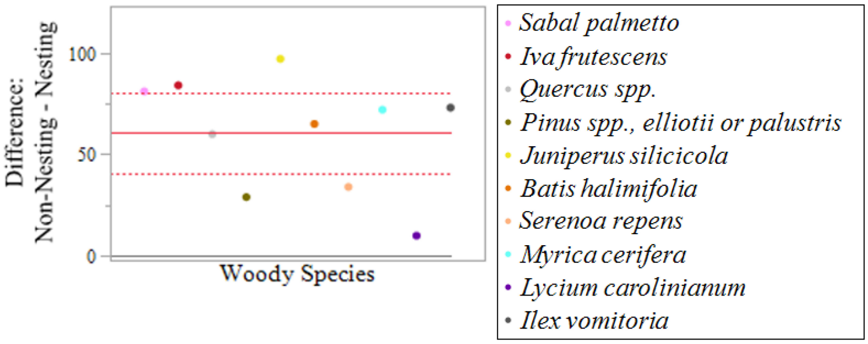

FIGURE 6 | Specialized matched-pairs *t*-testing comparing woody species composition of nesting and non-nesting sites with a |t| ratio of 6.93 with |*t*| < 0.0001. A specialized matched-pairs *t*-test resulted in a mean difference of 52.3 represented by the solid red line and a 95% confidence interval of (40.8, 80.2) represented by the dotted red lines.

In Duval County, we sighted one terrapin abandoning a basking site in the marsh between Sisters Creek and Fort George Island, and we captured two others as they swam at the creek-mouth leading to the Isle of Palms community (**Table 1**, Other; **Figure 4**). Six terrapins were sighted swimming and photographed in Salt Run near a marina and restaurant in St. Johns County (S. Eastman, Florida DEP, and T. Dodson, St. Johns County Govt., pers. comm.). These nine individuals compose the "Other" column in **Table 1**.

Statistical Results

Of the 62 possible field days, we recorded time spent on 56 of them for a total of 263 h and 18 min. During that period, we recorded 276 terrapin evidence data points, so CPUE = 1.05 records/h. We did not include terrapin records obtained from sources other than our own methods in the calculation of CPUE. Further, we did not consider the number of researchers in the calculation, so the hours are not man-hours.

The optimized hot spot analysis calculated optimal neighborhood size as 5003.5 m, so we rounded to 5 k. There is a high level of confidence (95 to 99%) that the only collection of non-random terrapin evidence data points occurs at the "Jackson Creek Concentration" in Nassau County (**Figures 3A,B**).

We documented vegetation data during 184 land surveys: 147 were from non-nesting sites and 37 were from nesting sites which resulted in 911 woody species observations. The most commonly occurring woody species in both nesting and non-nesting areas was southern red cedar (*J. silicicola*), followed closely by marsh elder (*Iva frutescens*), then yaupon (*Ilex vomitoria*) and cabbage palm (*S. palmetto*, **Table 2**).

A specialized matched-pairs *t*-test resulted in a mean difference of 52.3 (i.e., an average difference of 52.3 more non-nesting than nesting sites). The | *t*| ratio provided strong evidence that the difference between non-nesting and nesting was significant (**Figure 6**). In addition, the specialized matched-pairs *t*-test confirmed there are no outliers, indicating that decision tree analysis was an appropriate next step.

Decision tree analysis allowed us to predict terrapin activity based on available choices such as the presence of woody species at potential nesting sites (Stiglic et al., 2012). These results indicate that terrapins prefer to nest when Christmas berry (*L. carolinianum*) is present amongst the other available woody species, with the choices being equally weighted except for saw palmetto and pine, and with oak (*Q.* spp.) and wax myrtle (*M. cerifera*) following closely behind (**Figures 7A,B**). This conclusion is supported as $R^2 = 0.025$ in the least squares regression with four splits using a Classification and Regression Trees (CART) algorithm and a Whole Model Effects test outcome with $|\text{Prob} > \chi^2| = 0.012$ which is statistically significant.

Finally, we used a generalized linear binomial distribution fit model (i.e., GLM with binomial logit and maximum likelihood estimation) to determine whether the results from the decision tree were supported by the overall parameter estimates. Generalized linear binomial distribution fit produced a Whole Model test outcome showing that sites where Christmas berry was present exhibited a significantly higher proportion of terrapin nesting ($|Prob > \chi^2| = 0.001$) than those without (**Table 2**). Conversely, nesting was significantly less likely when either wax myrtle ($|Prob > \chi^2| = 0.060$) or oak ($|Prob > \chi^2| = 0.021$) were present. The presence of the other plant species had no significant statistical effect (**Table 2**). Thus, both tests produced statistically significant results which indicate that terrapins were likely to nest at sites where Christmas berry is present and tended to avoid nesting when oak and/or wax myrtle are present (**Figure 6**).

DISCUSSION

Based upon the evidence, the most active area for terrapins in northeast Florida is in Nassau County. In the northern part of Nassau County, we recorded evidence of terrapins in the Bells River, Little Tiger Island, and in and around the Fernandina Harbor Marina (**Figure 3A**). We found no evidence in the Jolly River, Lanceford Creek, the southern shoreline of the St. Mary's River including that of Ft. Clinch State Park, or in Egan's Creek. We visited most places only once during the survey, therefore not finding evidence is not conclusive; common sense would dictate that terrapins are likely present throughout areas where

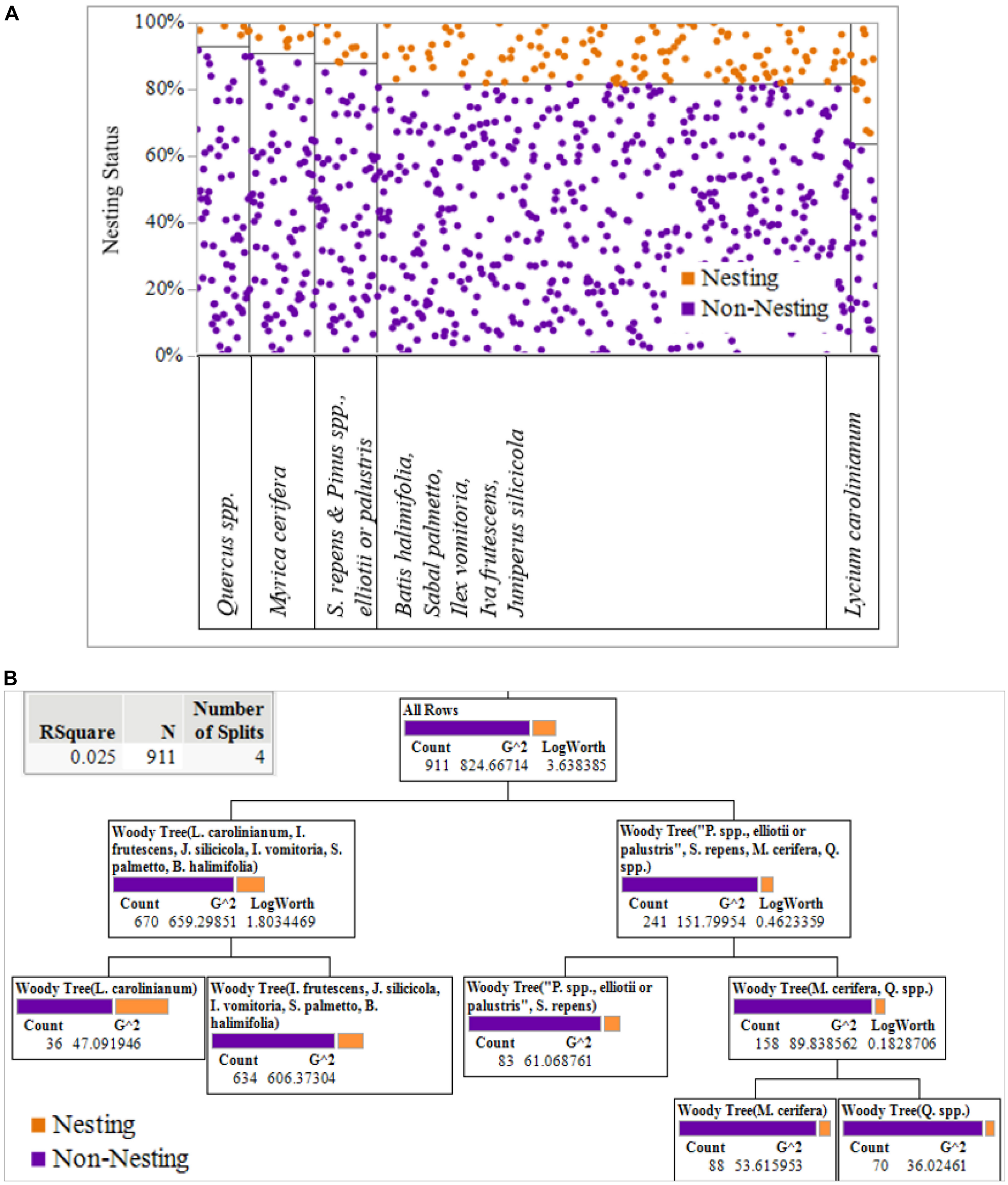

FIGURE 7 | (A) Partition *via* Classification and Regression Trees (CART) analysis supporting the conclusion that woody plant data suggest that significantly more nesting occurred when Christmas Berry (*Lycium carolinianum*) was present, and nesting was less likely when either Wax Myrtle (*Myrica cerifera*) or Oak (*Quercus* spp.) were present. **(B)** Decision tree with R^2 = 0.025 in the least squares regression with four splits using the CART algorithm.

the habitat is appropriate and that are bordered by areas where terrapins have been observed and documented.

In the southern part of Nassau County, the Holly Point and Goffinsville boat ramps had numerous depredated nests, and their shorelines offer the only apparent nesting habitat in the area, even though both are of anthropogenic origin (**Figure 3A**). The unnamed marsh island adjacent to Back River was identified by Butler (2002) as a place where at least some terrapins that nest on Sawpit Island spend the rest of their seasons (**Figure 3A**, green asterisk); we did not visit that marsh during this survey. Further east, we found no terrapin evidence in Harrison Creek and counted only one head in Walker Creek, even though both produced numerous head counts in previous years (Butler, unpubl. data).

The area around Jackson Creek (**Figure 3B**) yielded a variety of terrapin evidence including head counts, depredated nests, and many remains. The marsh area north of Jackson Creek abuts property owned by Rayonier Incorporated, which specializes in pulp, paper, and other cellulose products. Numerous depredated nests were recorded in 1995 on the southern banks of the Rayonier settling pond (Butler, unpubl. data, green asterisk on **Figure 3B**), but we were not granted permission to search the property for the current survey. Jackson Creek and its adjoining tributaries have been intensively trapped for blue crabs (*Callinectes sapidus*) for decades (P. Leary, resident of Jackson Creek, pers. comm.) and many terrapins enter and drown in crab traps in that area (Butler and Heinrich, 2007). Although most crab trappers would likely leave drowned terrapins in their traps as bait, we wonder if the extensive terrapin remains at the mouth of Jackson Creek are related to crab trapping. Conversely, these remains could be the result of extensive raccoon predation. Much of the habitat between Jackson Creek and SR 200 is owned by the Amelia Island Yacht Basin and considerable nesting occurs along the shoreline and some other high spots on the property. We found numerous depredated nests on the shoulders of the railroad tracks just northeast of SR 200.

Some of the Nassau evidence indicates that terrapins are not averse to exploiting habitat opportunities created anthropogenically. Most alterations of habitat by humans (i.e., channel dredging, bulkheading, building roads through salt marshes) are considered threats to terrapin survival (Maerz et al., 2018). However, Seigel (1980c) found terrapins using man-made lagoons and nesting on adjacent dyke roads. Feinberg and Burke (2003) recorded highest depredated terrapin nest densities on man-made trails. Roosenburg et al. (2014) demonstrated that terrapins will sometimes use artificially produced habitats for nesting with some success. The nesting activity associated with the Goffinsville and Holly Point boat ramps would not be possible without the construction associated with the ramps. Further, the construction of the SR 200 bridge and associated railroad tracks created more nesting opportunities at its base.

Within Duval County (**Figure 4**) the most notable area for terrapins is the nesting beach of Sawpit Island, which has been studied and monitored intermittently since 1996 (Butler, 2000, 2002; Butler et al., 2004; Munscher et al., 2012). In recent years, currents at the mouth of the Nassau River have led to extensive beach erosion, and part of the island has been breached to the extent that human passage is difficult or impossible during high tide (M. Simmons, Florida Fish and Wildlife, pers. comm.). Sand loss has decreased the area available for nesting, thus facilitating nest detection by predators. If the shoreline is destroyed, the fate of the terrapins that depend on this beach is unclear, as appropriate nesting habitat is scarce in the area.

Between Sawpit Island and the Ft. George River, terrapin evidence was present on most marsh islands and tributaries of the ICW. The area between the Ft. George and St. Johns rivers was also studied extensively during the late 1990s with head counts recorded in Cedar and Deep creeks nearly daily and radio telemetry studies done in Deep and Garden creeks (Butler, 2000, 2002). The current survey is notable for its paucity of terrapin evidence in all three of those creeks. Butler (2002) reported numerous terrapin heads in Deep Creek nearly every day during the nesting seasons over a three-year period (**Figure 4**, green asterisk). In 2014, despite traveling the entire length of Deep Creek on two separate days in May, we recorded no heads; and we saw no heads in Garden Creek. All creeks in that area were periodic sites of commercial crab trapping in the 1990s, and we fear the terrapin population could have been depleted. From the St. John's River south to the Duval/St. John's County line we found little evidence of terrapins.

In parts of northern St. John's County, we were unable to perform land surveys because both east and west shorelines were privately owned (**Figure 2**); from the boat neither appeared to be appropriate nesting habitat. One locale in that county worthy of further research is the St. Augustine Inlet. On the western shoreline, both Hospital and Robinson creeks produced a variety of terrapin evidence. Further, the numerous adult terrapin sightings in Salt Run, to the southeast, signal what may be a notable population, and it is important to know where they nest so those areas can be protected. We recorded several depredated nests on the adjacent Conch Island, but these were sparse and do not appear to reflect a significant nesting site. Finally, in a May 2002 survey in St. Johns County, 15 swimming terrapins were counted while researchers waded an unnamed creek on the west shoreline of the Matanzas River, directly across from the north end of Rattlesnake Island, which is the home of the Ft. Matanzas National Monument (Butler and Heinrich, unpubl. data; green asterisk, **Figure 4**). We visited this creek during this survey in 2014 and found no evidence of terrapins.

Diamondback terrapins were more likely to nest in areas where Christmas berry was present. We do not suggest that terrapins seek out or even recognize Christmas berry, rather that it is present at most nest sites and can be a useful marker for researchers. This shrub is common in coastal marshes throughout Florida and the southeastern United States and is resistant to high salt concentrations, periodic flooding, and drought (Nelson, 1996). When present, Christmas berry is usually the first woody vegetation encountered as one proceeds inland from the water's edge, and likely affords some cover for nesting terrapins and desirable thermal conditions for egg development. Conversely, terrapins were less likely to nest when oak and/or wax myrtle were present, and both species, when present in coastal regions, are typically found further inland (Johnson and Barbour, 1990). These areas may be unappealing to terrapins because they provide cover to mammalian nest predators (Burger and Montevecchi, 1975). Further, thicker canopies lead to lower nest temperatures (Jeyasuria et al., 1994) which would result in overproduction of male hatchlings, and Roosenburg (1996) suggested that female terrapins choose nesting sites that will produce both sexes. Christmas berry and marsh elder were often associated with terrapin nesting sites in the Big Bend area of Florida and their presence was used by the researchers as a possible nest site indicator (Hackney, 2010; Butler and Heinrich, 2013) found many depredated terrapin nests under wax myrtle in Virginia, and we report that in northeast Florida terrapins are less likely to nest in areas where this species is present. Future research on woody species as terrapin nest site markers could include comparing relative concentrations and canopy cover of each; Mitchell and Walls (2013) looked at this but not at the species level.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee – UNF.

AUTHOR CONTRIBUTIONS

JB contributed as the principal investigator, wrote grants for field study, managed students, and wrote most of the verbiage concerning terrapins. JL oversaw all terrapin data, produced maps for **Figures 1–5**, and provided interpretations when necessary. MD ran all vegetation statistics and provided interpretation. DM did fieldwork with JB and did some lab studies associated with the study. All authors contributed to the article and approved the submitted version.

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Can Reptiles Use Nest Site Choice Behavior to Counter Global Warming Effects on Developing Embryos? Potential Climate Responses in a Turtle

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Climate warming is forecasted to cause extinctions, but populations could theoretically avoid extinction in a rapidly changing environment *via* adaptive evolution (i.e., evolutionary rescue), precluding the need for intervention. Although strong links between a changing climate and the physiology of an organism are expected, climate effects can be buffered by behavior. Nest site choice behavior, for example, can reduce environmental variation that would be experienced by embryos placed randomly with respect to environmental temperatures. We tested four provisions of this prediction by quantifying nest sites and "potential" nest sites in the Florida softshell turtle (*Apalone ferox*). First, turtles chose nest sites with mean canopy openness values (32–47%) that were intermediate between the shadiest (14–17%) and the sunniest potential nest sites (36–57%) available. Second, canopy openness, incident radiation intensity, and nest temperatures were generally, positively related to one another, indicating definitive thermal consequences of nest site choice. Third, our study revealed ample, cooler nest sites available to turtle mothers within close proximity to nest sites utilized; by nesting in the most shaded sites, softshell turtle mothers could depress mean nest temperatures by ∼2°C. Fourth, the growth of vegetative cover throughout incubation had negligible effects on canopy openness, incident radiation intensity, and nest temperatures, supporting the potential for mothers to "predict" developmental temperatures using temperature cues during nest site choice. Finally, our data revealed considerable variation in canopy openness chosen by nesting mothers; such behavior could thus, be subject to natural selection *via* embryonic mortality under future warming. Collectively, our study suggests that Florida softshell turtles, and probably other turtle species nesting in relatively open areas, may be able to counter climate change effects on developing embryos by nesting in more shaded microhabitats, assuming nest site choice behavior is heritable and can evolve at a sufficient rate to keep pace with climate warming.

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The evolutionary and behavioral mechanisms (e.g., assessing substrate temperatures directly vs. indirect choice of canopy cover) in the repertoire of nesting mother turtles for responding to climate warming remain elusive and are required for a more complete understanding of climate responses.

Keywords: climate change, behavior, evolution, nesting, softshell turtle

INTRODUCTION

Current, anthropogenic climate change is projected to continue indefinitely, and is putting pressure on biological systems due to its unprecedented rate (Intergovernmental Panel on Climate Change [IPCC], 2019). Drivers of this change include sea level rises, frequencies of rainfall, wind and storms, ocean acidification, and eutrophication, but the most direct and predictable driver is increased air and surface temperatures (Intergovernmental Panel on Climate Change [IPCC], 2007). As such, understanding potential responses to increasing environmental temperatures has become a major focus of ecological and evolutionary biology (e.g., Parmesan, 2006; Pacifici et al., 2017; Radchuk et al., 2019), especially given the current biodiversity crisis (Barnosky et al., 2011; Urban, 2015). Ideally for conservationists, populations can avoid extinction in a rapidly changing environment *via* adaptive evolution, precluding the need for intervention (i.e., "evolutionary rescue"; Bell, 2017).

Predicting evolutionary responses to climate change is not trivial, and requires understanding links between environmental temperatures and key life processes. Changes in environmental temperatures can alter body temperatures, which in turn affect physiological processes and performance, and thus potentially survival, especially in ectotherms (Huey et al., 2003, 2012; Kearney et al., 2009 and papers cited within). Although an organism's vulnerability to climate warming is complex and could involve other climate change drivers and changes in a plethora of biotic and abiotic interactions, understanding an organism's response to temperature is fundamental to predicting that vulnerability (Kearney et al., 2009; Huey et al., 2012).

Although strong links between a changing climate and the physiology of an organism are expected, climate effects can be buffered by behavior. Using the example that lizards at higher (cooler) elevations basked more, resulting in body temperatures that were more similar among different elevations than would be expected from null models, Huey et al. (2003) coined and described the "Bogert effect," whereby behavioral adjustments can reduce the environmental variation that would be experienced by a non-regulating ectothermic organism. The authors demonstrated that thermoregulatory behaviors likely inhibit selection for evolutionary shifts in thermal physiology across environmental gradients, a notion somewhat counter to the classic "behavioral drive" theory proposing that behavior initiates new evolutionary events (Mayr, 1963).

Doody and Moore (2010) proposed that the Bogert effect could be extended to the egg or embryo stage, after finding (predictable) latitudinal and elevational variation in nest site attributes in a lizard (Doody et al., 2006a; Doody, 2009). In other words, nest site choice behavior can reduce environmental variation that would be experienced by embryos placed randomly with respect to environmental temperatures (Doody and Moore, 2010). It follows that nest site choice could theoretically also buffer developing embryos in nests against *temporal* environmental gradients such as climate change (Doody et al., 2006a; Angilletta et al., 2009).

Turtle nesting behaviors offer desirable systems for studying potential climate change responses in nature; turtles are oviparous ectotherms that lay eggs in ground nests that are typically subject to air and ground temperatures for 2–3 months. Moreover, many turtle species possess temperature-dependent sex determination (Ewert et al., 1994; Valenzuela and Lance, 2004) meaning that choice of nest site can influence offspring sex ratios (Vogt and Bull, 1984; Janzen, 1994). While free-living adults and juveniles can seek cooler microclimates as the climate warms, the eggs cannot. Moreover, like most oviparous ectotherms, turtles rarely exhibit parental care after laying (Shine, 1988), focusing research attention to the mother's nesting decisions as the main avenue for countering climate change effects of temperatures on developing embryos. Indeed, two studies have unequivocally demonstrated mother reptiles used nest site choice behavior—specifically the choice of canopy cover—to offset environmental gradients across latitudes and elevations (Ewert et al., 2005; Doody et al., 2006a). Both snapping turtles (*Chelydra serpentina*) and water dragons (*Intellagama lesueurii*) nested under more open canopies in cooler climates than their counterparts in warmer climates. Such variation strongly suggests that mothers could also use canopy cover to offset climate warming (Doody et al., 2006a; Doody and Moore, 2010) because canopy cover is highly correlated with nest temperatures (Ewert et al., 1994; Janzen, 1994; Janzen and Morjan, 2001).

The Florida softshell turtle, *Apalone ferox*, is a common but geographically restricted species that nests in terrestrial areas around a wide variety of freshwater wetlands in hot climates (Krysko et al., 2019). Although the species is thought to possess genetic sex determination due to the finding of sex chromosomes in a congener (Badenhorst et al., 2013), recent research indicates that offspring sex determination may be thermosensitive in the genus (Bista et al., 2021). Although nests can be difficult to find, nest predation, mainly by raccoons (*Procyon lotor*), is often high in *A. ferox* (G. L. Heinrich, unpubl. data, J. S. Doody, unpubl. data), as in many other turtle populations (e.g., Congdon et al., 1994; Burke et al., 1998; Tomás et al., 2010), providing a solution to finding large numbers of nests. Raccoons preying upon turtle nests leave the eggshells on the surface near the excavated nest. While turtle ecologists aiming to quantify nest site choice generally use intact nests, depredated nests can facilitate quantifying nest site choice attributes such as amount of shading vegetation, distance from and height above water, aspect, and distance to other nests. These attributes reflect some combination of direct and indirect behavioral choices. In particular, canopy or understory openness can be quantified using hemispherical photography, and incident solar radiation intensity striking the nest site can be subsequently estimated using gap light analysis (Frazer et al., 1999; Doody et al., 2006a,b).

Assuming that nest site choice behavior is heritable and can freely evolve at a rate to keep pace with the rate of climate change, we predict that softshell turtle mothers could use nest site choice behavior to offset current and future climate warming by buffering eggs against the effects of increasing temperatures on developing embryos (Doody et al., 2006a), provided that (1) there are potential nest sites with more shading vegetation available to nesting mothers, that (2) exhibit cooler nest temperatures, (3) attributes (e.g., shading vegetation) of nest sites affecting developmental temperatures during nest site choice do not change markedly throughout incubation, and (4) there is sufficient individual variation in canopy openness of nest sites chosen by mothers. We tested these provisions by quantifying nest site choice, potential nest site choice, and consequences of those choices in nest and potential nest temperatures in the Florida softshell turtle in three populations in south Florida. We also quantified seasonal timing of nesting to clarify seasonal changes in nest temperatures. We discuss the implications of our findings for the evolution of nest site choice and climate change responses in turtles, reptiles, and other oviparous ectotherms without parental care.

MATERIALS AND METHODS

Study Species and Study Areas

The Florida softshell turtle is a large, common species inhabiting a wide variety of freshwater habitats in Florida and surrounding states (Krysko et al., 2019). Although it does not range widely across latitudes, it is essentially allopatric with its closest relatives that do span considerable latitudes (*A. mutica* and *A. spinifera*). Florida softshell turtles typically nest in relatively open, sandy areas along watercourses; nests tend to be close to water when suitable sites are available, but have been found > 160 m from water (G. L. Heinrich, unpubl. data). Like most other turtles, *A. ferox* constructs a flask-shaped hole in the ground with the hind feet and backfills the hole after laying. Based on dissections from turtles harvested for meat in south Florida, mothers lay up to 5–6 clutches of 9–38 eggs annually between early March and early August (Iverson and Moler, 1997). Eggs incubate for 56–82 days depending on incubation temperatures (Meylan and Moler, 2006).

We studied softshell nesting at three sites in southwest Florida: Boyd Hill Nature Preserve (BHNP), Myakka River State Park (MRSP), and Sawgrass Lake Park (SLP). BHNP is a 97-ha city park in St. Petersburg, Pinellas County (27.734160°N, -82.65635°W). The BHNP nesting areas bordered Lake Maggiore, a 147-ha permanent lake. MRSP is a 15,054-ha state park in Sarasota and Manatee counties (27.252255°N, -82.295581°W). Nesting areas in MRSP were along canals near the Myakka River. SLP is a 162-ha county park in St. Petersburg, Pinellas County (27.837384°N, -82.665372°W). Nesting areas at SLP were mainly along a manmade pond, but also along a canal feeding Sawgrass Lake. BHNP and SLP are ∼11 km apart, while MRSP is ∼64 km southeast of BHNP and ∼75 km southeast of SLP.

Nest Surveys and Nest Site Choice Data Collection and Analysis

Nest surveys were conducted on foot in riparian areas along lakes, ponds, or canals in 2019 from March to September at BHNP (6 days/week) and MRSP (twice/week), and May to September at SLP (once every 3 weeks). Nests were found by looking for eggshells left by nest predators (**Figure 1**). *Apalone ferox* eggshells are composed of a brittle outer shell that is easily distinguishable from other species. The main predator was the raccoon and depredated nests featured an empty excavated nest chamber with eggshells scattered on the ground within a few meters of the chamber.

Nests were flagged for further processing; data on nest site choice included estimated lay date, aspect (measured with a compass), distance from water (measured with a meter stick), and ground cover (estimated by photographing the square meter surrounding each nest to the nearest 10%). Within 1 week of the estimated lay date, we measured openness of the canopy and understory, and incident radiation intensity using hemispherical photography and gap light analysis (after Doody et al., 2006a,b). Hemispherical photographs were taken by placing a Nikon Coolpix® 900 series digital camera fitted with a fisheye wide-angle lens (Nikon FC-E8®) on each nest and taking a photograph with the camera body facing due north (the fisheye lens pointing upward). Photographs were taken during the first or last hour of daylight to preclude reflection of light off vegetation (i.e., leaves) that can introduce error in the calculation of openness. Openness (%) and incident radiation intensity were calculated by running each photograph through Gap Light Analyzer (GLA) 2.0® (Frazer et al., 1999).

To facilitate comparing our canopy openness and incident radiation data results to those from other species we herein list the settings we used in Gap Light Analyzer 2.0® (Frazer et al., 1999). We pointed the camera due north during hemispherical photographs to allow the program to accurately track the path of the sun across the image. Under configuration/image, we used geographic north rather than magnetic north; although the latter gives a slightly more accurate compass direction due to the dynamic nature of earth's magnetic field, the correction is minor and both attributes provide a relative measure. Under configuration/site, we entered the latitude of the site (allows the program to know where the sun tracks across the photograph), but not elevation or longitude. We used horizontal orientation (no significant slope or aspect) and we turned the topographic mask to "OFF" (there was no need to separate the canopy from mountains on the horizon when determining shading). Under configuration/resolution, we left the solar time step at the default of 2 min., we left the sky regions at the default

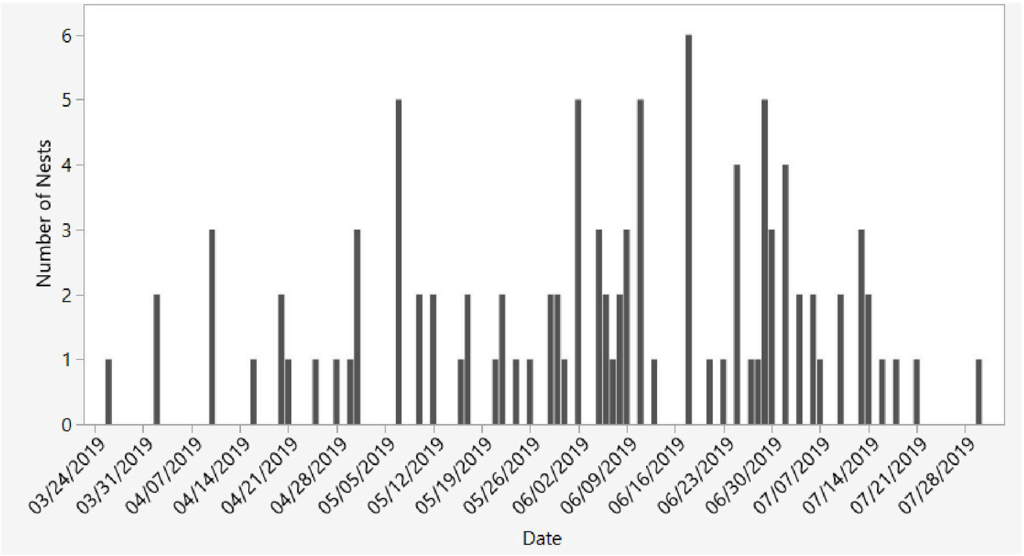

FIGURE 1 | Seasonal timing of nesting of *A. ferox* at BHNP, the study site with the most survey coverage (six visits/week).

(36, 9), and the start date and end date were chosen based on the lay date and hatching date, respectively. The hatching date was estimated to be 60 days after the lay date, based on data from Meylan and Moler (2006). Under configuration/radiation, we used the "modeled data source" (computes above-canopy radiation estimates without entering data from each site); we used Megajoules/m^2/day as the units, the UOC model (assumes that all regions of the sky are equally as bright), and we ignored cloudiness index (this is useful when comparing multiple sites in which there is a consistent difference in cloudiness at one or some sites, but not others). Finally, under calculations, we ticked canopy structure, and we used % openness and "*trans* total radiation" as our outputs for canopy openness and incident radiation intensity, respectively.

To quantify the thermal consequences of nest site choice, specifically openness and incident radiation, we deployed temperature data loggers (Thermochron i-buttons®; DS1921G; accuracy = ± 1.0°C) into 13 randomly-chosen, backfilled, depredated nests at BHNP. Loggers were placed at the average nest chamber depth (16.0 cm), based on the average of an intact nest at BHNP (21.0 cm) and from two nests from previous reports (13.0 cm, 14.0 cm; Hamilton, 1947; Heinrich and Richardson, 1993). Data loggers were deployed within 1 week of the estimated lay date and set to record temperature every 90 min. Analyses using temperature data from data loggers utilized only the first 60 days of data, a typical incubation period for the species (Meylan and Moler, 2006).

To determine if our temperature data from backfilled nests accurately represented real nest temperatures we tested for metabolic heating in one nest (Massey et al., 2019). We employed a data logger into the core of a fresh nest and a second data logger 8.0 cm away in the ground, at the same depth (12.0 cm). The nest was caged with hardware wire to protect the eggs from predators, and data loggers were removed 56 days later for analysis.

To determine if mothers could offset future climate warming by nesting in more shaded areas, we quantified openness and incident radiation from the "most shaded" potential nest site within 20 m of each nesting area (a nesting area here is defined as a cluster of nests within 20 m of one another, within a study site). For comparison, we also did the same for the "sunniest" (most open, canopy-wise) potential nest site for each nesting area (same criteria as for the shaded potential nest sites). As with nest sites, we quantified the thermal consequences of potential nest sites using i-button data loggers. There were 10 such potential nest sites at BHNP and five at MRSP (we did not quantify potential nest sites at SLP). Data loggers recorded temperatures at the same intervals as those in nests (every 90 min) and were buried in artificial nests at the same depth as those in actual nests (16.0 cm).

Because turtle eggs typically incubate in the ground for 2–3 months during the growing season, vegetation such as grasses and understory can grow significantly during that time and potentially change the amount of solar radiation that strikes the nest site. This raises the question of how well mothers can predict nest temperatures throughout incubation by sampling nesting conditions during nest site choice. To address this, we again quantified openness and incident radiation at the nest sites and potential nest sites at 30 and 60 days of incubation for each nest, using the same methods as we did for actual nests (above).

Statistical Analyses

We tested normality and homogeneity of variance using a Shapiro–Wilk test and a Levene's test, respectively. We used *t*-tests to compare openness and incident radiation intensity between nest sites and potential nest sites (sunny and shaded sites), and a Kruskal–Wallis test and Dunn's multiple comparison *ad hoc* test to analyze differences in openness and incident radiation intensity among study sites. To test for seasonal differences in openness, incident radiation intensity, and ground cover we used a Friedman test with a *post hoc* Conover test. To explore the effects of openness, study site, and lay date on incident radiation intensity we used a linear regression model. We fit six regression models with different combinations of lay date, nest site, and percent openness. We used Akaike's Information Criterion with a small sample bias adjustment (AICc) to assess the best model. We used 95% confidence intervals to assess the precision of the parameters of the model. Linear regression analysis was used to test whether canopy openness and incident radiation intensity were related to mean, maximum, and minimum temperatures. All analyses were performed using R 3.6.1 (R Core Team, 2018) with the packages "FSA," "PMCMR," "lmtest," and "MuMIn."

RESULTS

Seasonal Timing of Nesting

We found 156 softshell nests, including 102 from BHNP, 27 from MRSP, and 27 from SLP. Most nesting occurred between late March and mid-July and over 50% of the year's nesting was completed by 1 June (**Figure 1**). All nests were taken by predators; the presence of eggshells on the surface was consistent with predation by raccoons, but it is possible that some were taken by other predators; crows (*Corvus spp.)* are also nest predators at the sites (JSD and GLH, pers. obs.).

Nest Sites vs. "Potential" Nest Sites

Mean canopy openness for all nests was 36.9 ± 13.92% and was significantly different among study sites (**Figure 2**; Kruskal–Wallis: $H = 23$, df = 2, $p < 0.0001$); openness of nest sites at MRSP was significantly greater than openness of nest sites at both BHNP ($p < 0.0001$) and SLP ($P < 0.0002$). Mean canopy openness did not differ between BHNP and SLP ($p = 0.375$). At BHNP, mean canopy openness of nest sites was 35.3 ± 14.55% (range = 8.7–66.7%, $N = 102$), compared to 46.8 ± 10.04% (range = 21.4–70.9%, $N = 27$) at MRSP and 32.7 ± 10.07% (range = 19.3–53.3%, $N = 27$) at SLP.

Canopy openness values of nest sites consistently fell in between those for shaded and sunny potential nest sites. Although openness values for nest sites were more often closer to those of sunny potential nest sites, at some nesting areas they were closer to values of shaded potential nest sites (**Figure 3**). At BHNP, mean canopy openness of nests was significantly greater than shaded potential nest sites (**Figure 3**; $t = 11.26$, df = 110, $p < 0.0001$); although nest sites were generally less open than sunny potential nest sites, the difference was not statistically significant ($t = 0.80$, df = 110, $p = 0.440$). At MRSP, mean canopy openness of nests was significantly greater than shaded potential nest sites (**Figure 3**; $t = 4.89$, df = 30, $p < 0.0001$) and significantly lower than in sunny potential nest sites ($t = 2.60$, df = 30, $p = 0.0145$).

As expected, canopy openness was highly, significantly positively related to incident radiation intensity (**Figure 4**; $r^2 = 0.848$, $F_{1,54} = 836.0$, $p < 0.0001$). The mean incident radiation intensity for all nests combined was 5.6 ± 2.06 MJ/m^2/d, but like canopy openness, was also significantly different among the study sites (**Figure 2**; Kruskal–Wallis: $H = 23.02$, df = 2, $p < 0.0001$). Incident radiation intensity was significantly higher at MRSP than at BHNP ($Z = -4.24$, df = 1, $p < 0.0001$) and at SLP ($Z = 4.41$, df = 1, $p < 0.0001$); incident radiation intensity did not differ between BHNP and SLP ($Z = 1.31$, df = 1, $p = 0.189$).

Mean incident radiation intensity of the sunny potential nest sites at all study sites combined was 7.4 ± 1.92 MJ/m^2/day. As with canopy openness, incident radiation intensity at nest sites generally fell between the values for shaded and sunny potential nest sites (**Figure 3**). Mean incident radiation intensity of sunny potential nest sites was significantly higher than nest sites at MRSP ($t = 2.65$, df = 30, $p = 0.013$), but not at BHNP ($t = 1.82$, df = 110; $p = 0.097$; **Figure 3**). Mean incident radiation intensity of shaded potential nest sites at all study sites was 2.3 ± 1.41 SD MJ/m^2/d (**Figure 3**). Mean incident radiation intensity of shaded potential nest sites was significantly lower than nest sites at both BHNP ($t = 10.74$, df = 110, $p < 0.0001$) and MRSP ($t = 6.13$, df = 30, $p < 0.0001$) (**Figure 3**).

At BHNP, mean incident radiation intensity of nest sites was significantly greater than that of shaded potential nest sites ($t = 10.74$, df = 111, $p < 0.0001$) and significantly lower than that for sunny potential nest sites ($t = 1.82$, df = 110, $p = 0.097$; **Figure 3**). The same relationships were found for MRSP: mean incident radiation intensity of nest sites was significantly greater than that of shaded potential nest sites ($t = 4.28$, df = 30, $p = 0.01$), but significantly lower than that of sunny potential nest sites ($t = 4.60$, df = 30, $p < 0.0001$; **Figure 3**). A linear regression model revealed that incident radiation intensity was explained by canopy openness (**Figure 4**; $r^2 = 0.864$; $t = 26.49$, $p < 0.0001$), lay date ($t = -2.93$, $p = 0.0040$), and study site ($t = 2.20$, $p = 0.0292$).

Seasonal Changes in Nest Site Attributes

Canopy openness and incident radiation intensity decreased significantly with season at BHNP, but not at MRSP (**Figure 5**). At BHNP, openness decreased significantly ($X^2 = 53.33$, df = 2, $p < 0.0001$) between day 0 and day 30 ($p < 0.0001$) and between day 30 and day 60 ($p < 0.0001$) (**Figure 5**). Also at BHNP, incident radiation intensity decreased significantly ($X^2 = 30.62$, df = 2, $p < 0.0001$) between day 30 and day 60 ($p < 0.0001$), but not between day 0 and day 30 ($p = 0.830$; **Figure 5**). In contrast, at MRSP openness did not change significantly over the season ($X^2 = 4.38$, df = 2, $p = 0.112$), nor did incident radiation intensity ($X^2 = 1.46$, df = 2, $p = 0.482$; **Figure 5**).

Ground cover increased significantly with season at both BHNP ($X^2 = 98.22$, df = 2, $p < 0.0001$) and MRSP ($X^2 = 11.28$, df = 2, $p = 0.004$; **Figure 5**). At BHNP, ground cover increased significantly between day 0 and day 30 ($p < 0.0001$) and between day 30 and day 60 ($p < 0.0001$; **Figure 5**). Similarly, ground cover increased significantly between day 0 and day 30 ($p = 0.005$) and between day 30 and day 60 ($p < 0.0001$) at MRSP (**Figure 5**).

Nest Temperatures and Underlying Factors

Nest temperatures tracked air temperatures, warming throughout the nesting season and leveling off in July-August as

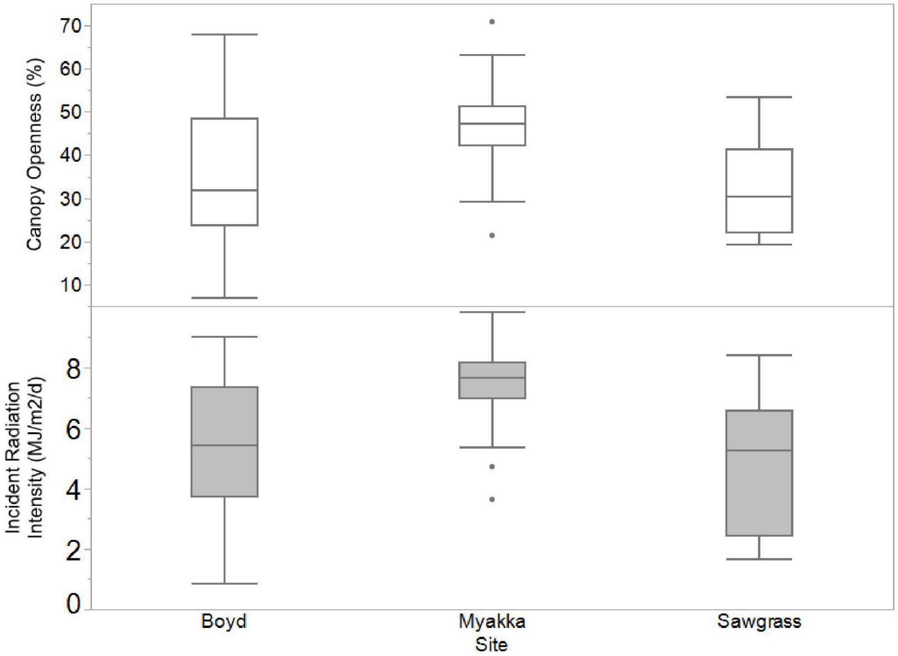

FIGURE 2 | Canopy openness and incident radiation intensity at *A. ferox* nest sites across three study sites. Boyd, BHNP in text; Myakka, MRSP; Sawgrass, SLP.

cloud cover, rainfall, and vegetative cover increased (**Figure 6**). Continuous nest temperatures from the core of a fresh nest were nearly identical to those in the soil 8.0 cm from the nest at the same depth, for most of the incubation period (**Figure 7**). Nest temperatures were ∼0.5 C higher in the last 13 days of incubation (**Figure 7**), indicating a small effect of metabolic heating of eggs in this warm climate.

Grand mean nest site temperatures differed significantly among nest sites and potential nest sites ($F_{2,25}$ = 26.81, $p < 0.0001$) and were, on average, 1.9°C warmer than shaded potential nest site temperatures (Tukey's HSD, $p < 0.0001$), and 0.8°C cooler than sunny potential nest site temperatures ($p = 0.078$). Similarly, grand mean maximum nest site temperatures differed significantly among nest sites and potential nest sites ($F_{2,25} = 15.07$, $p < 0.0001$) and were 2.6°C warmer than those in shaded potential nest sites ($p = 0.009$) and 1.9°C cooler than those in sunny potential nest sites ($p = 0.059$). Conversely, grand mean minimum nest temperatures did not differ significantly among nest sites and potential nest sites ($F_{2,25} = 1.91$, $p = 0.170$).

Nest temperatures generally increased with increasing openness and increasing incident radiation intensity (**Figure 8**), but not all of these relationships were statistically significant (**Table 1**). Grand mean temperatures and mean maximum temperatures were both significantly positively related to canopy openness at shaded and sunny potential nest sites, but not at nest sites (**Figure 8** and **Table 1**). Similarly, grand mean temperatures and grand mean maximum temperatures were both significantly, positively related to incident radiation intensity at nest sites, shaded potential nest sites, and sunny potential nest sites (**Figure 8** and **Table 1**). Grand mean minimum temperatures were not significantly related to openness or incident radiation intensity at nest sites, shaded potential nest sites, or sunny potential nest sites (**Figure 8** and **Table 1**).

Considerable variation existed in both the openness and incident radiation intensity of nest sites at BHNP (**Figure 9**). An order of magnitude separated the lowest and highest values for both openness and incident radiation intensity (**Figure 9**).

DISCUSSION

Oviparous ectothermic animals can theoretically offset climate warming effects on developing embryos by nesting in cooler microhabitats, given certain assumptions such as the heritability of nest site choice behaviors, the rate of climate change relative to the rate of evolutionary response, and the availability of cooler potential nest sites. Surprisingly few studies have quantified the availability of thermally heterogeneous potential nest sites in oviparous animals within the context of climate change (but see Refsnider et al., 2013a; Czaja et al., 2020). Our study produced four lines of evidence that softshell turtle mothers may be able to use nest site choice behavior to offset climate warming effects on incubation temperatures. First, shaded potential nest sites (essentially those in full shade) were available in close proximity to nest sites at all three study sites. Second, we revealed thermal consequences of nesting in shade; variation in openness within and among nest sites and potential nest sites influenced the amount of incident radiation received in nests, and ultimately, nest temperatures (**Figures 3**, **4**, **8**). Thermal traces from data loggers deployed in full shade (e.g., under understory and tree cover) revealed mean temperatures that

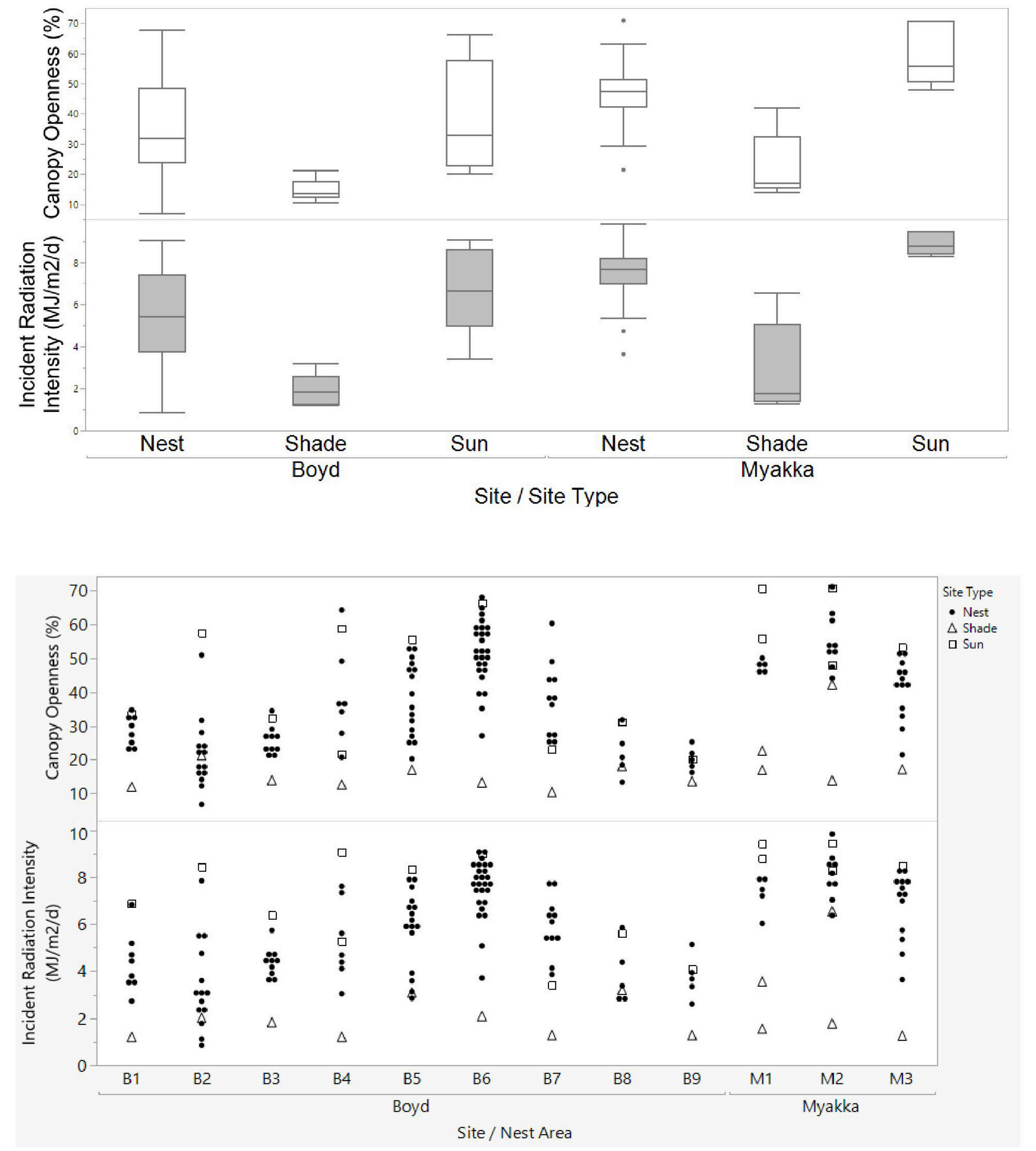

FIGURE 3 | Comparison of canopy openness and incident radiation intensity between *A. ferox* nest sites and potential nest sites across study sites. **(Top graph)** population-level results across the three study sites. **(Bottom graph)** individual-level data within specific nesting areas (e.g., B2) across two study sites. Potential nest sites comprised those in full sun and full shade and were within 20 m of nest sites. Note that values for nest sites generally fell in between sun and shade potential nest sites. Boyd = BHNP in text; Myakka = MRSP.

were ∼2°C lower than temperatures from actual nest sites, while maximum temperatures were ∼2.5°C lower (**Figure 6**). Third, nest temperatures throughout incubation were reasonably predictable from temperatures during nest site choice; that is, vegetative cover had a negligible effect on openness and radiation intensity, and thus, nest temperatures throughout

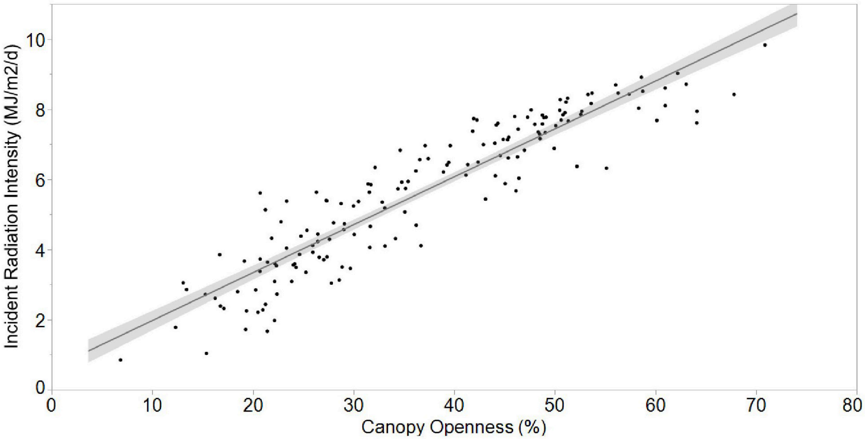

FIGURE 4 | The strongly significant, positive relationship between incident radiation intensity and openness at *A. ferox* nest sites.

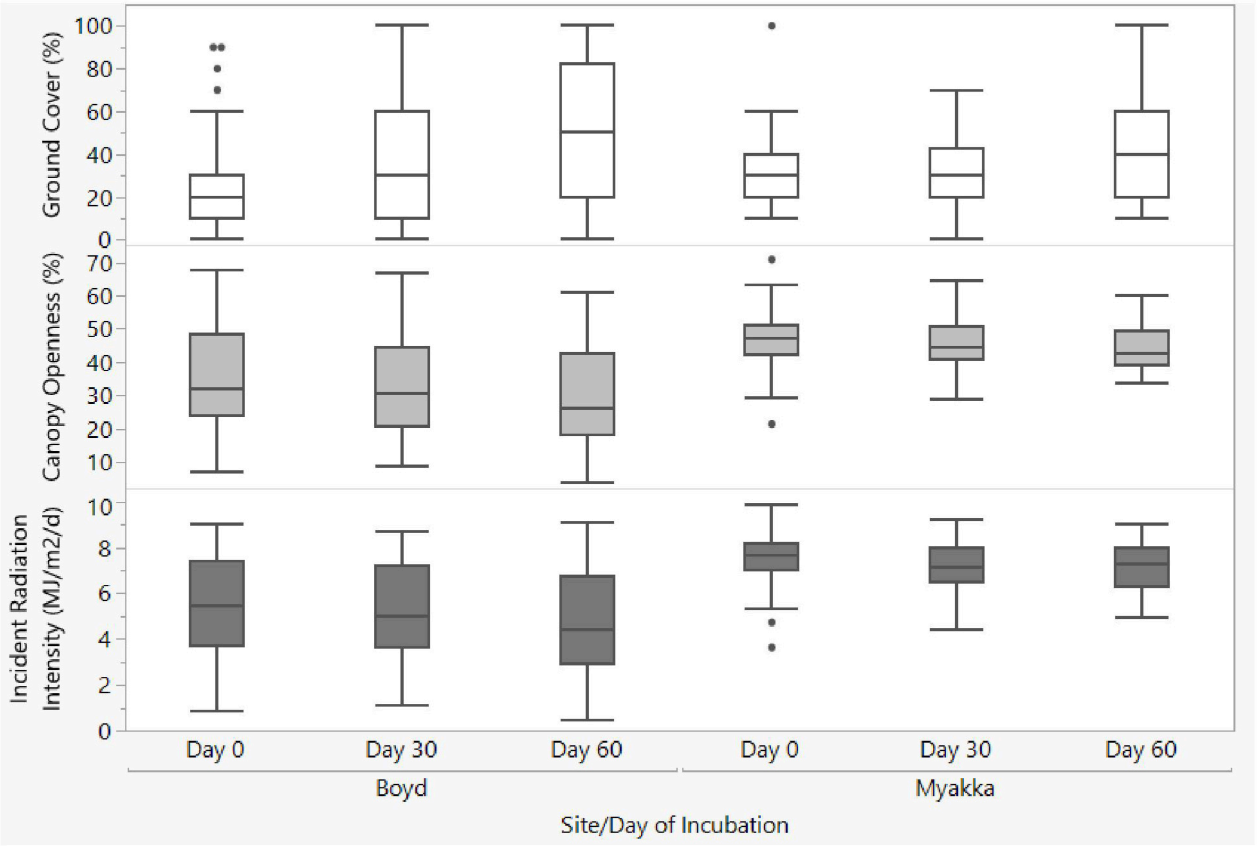

FIGURE 5 | Seasonal changes in ground cover, openness and incident radiation intensity at *A. ferox* nest sites at Boyd (BHNP in text) and Myakka (MRSP).

the incubation period (**Figure 5**). Fourth, our data revealed considerable variation in canopy openness chosen by nesting mothers (**Figure 9**). This variation had predictable consequences in incident radiation striking the nest site (**Figure 9**) and in nest temperatures (**Figure 8**), and presents the opportunity for natural selection to favor the behavioral choice of nesting in more shaded nest sites under current and future climate warming. Our data suggest that this hot-climate, open-nesting species is likely buffered, to some extent, against potential climate-induced declines caused by increasing nest temperatures because cooler microclimates (shaded potential nest sites) are available to nesting mothers.

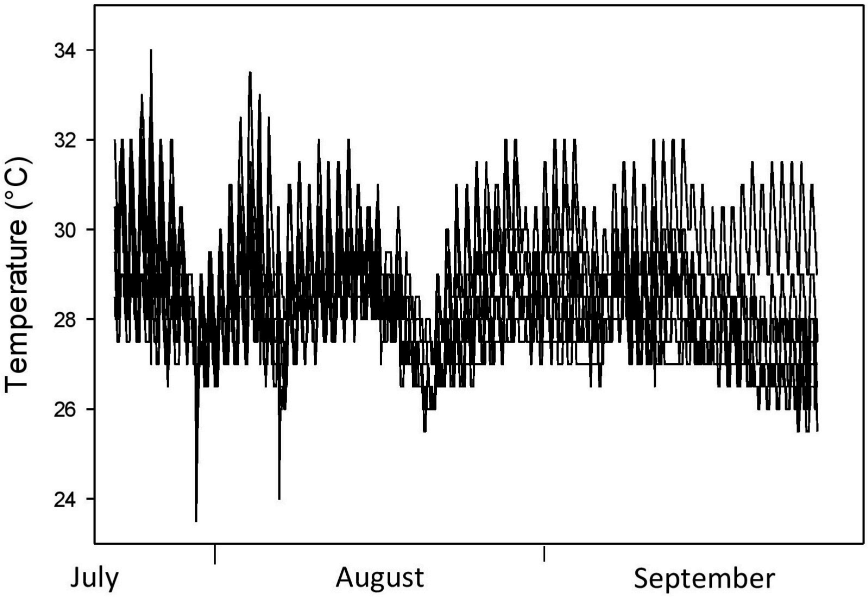

FIGURE 6 | Continuous temperatures from nine *A. ferox* (back-filled) nests from the BHNP site.

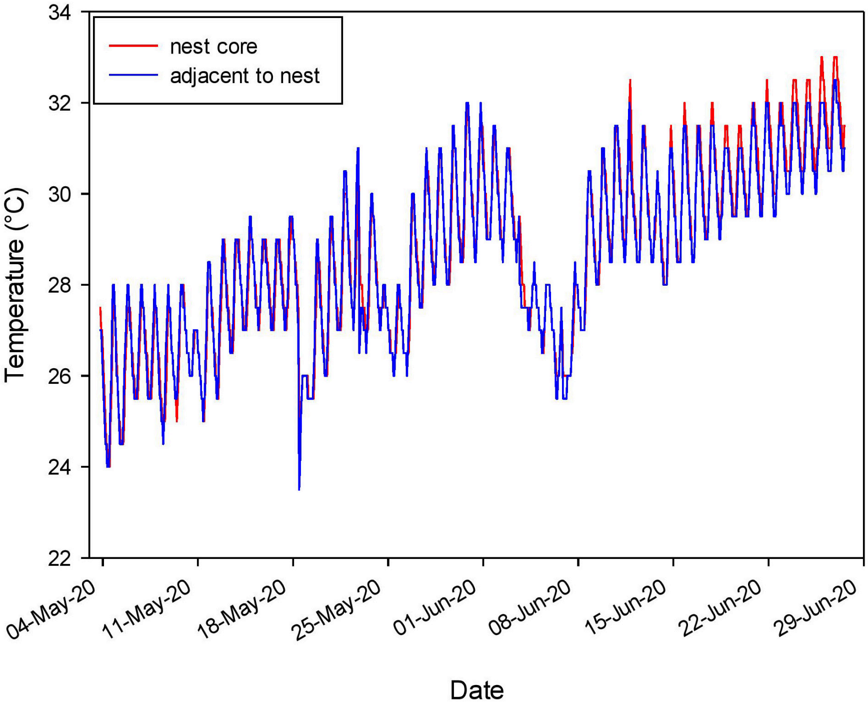

FIGURE 7 | (Minimal) Effect of metabolic heating in a clutch of *A. ferox* eggs incubating *in situ*. Eggs in the core of the clutch were up to ~0.5°C warmer than the soil adjacent to the clutch during the last ~16 days of incubation.

Adjusting nest site choice, either within or among generations, is only possible if there is heterogeneity in ground temperatures. Mothers could then assess microhabitat temperatures directly, or indirectly through the choice of some attribute (e.g., canopy cover) that affects those temperatures, or both. Nesting areas at our three study sites provided an abundance of shaded potential nest sites, generally within just a few meters of nest sites. Mean openness of nest sites at the study site with the most nests (BHNP) was 35%, and only 12 of 102 (12%) nests had canopies with openness values < 20% (**Figure 9**). Those shaded potential

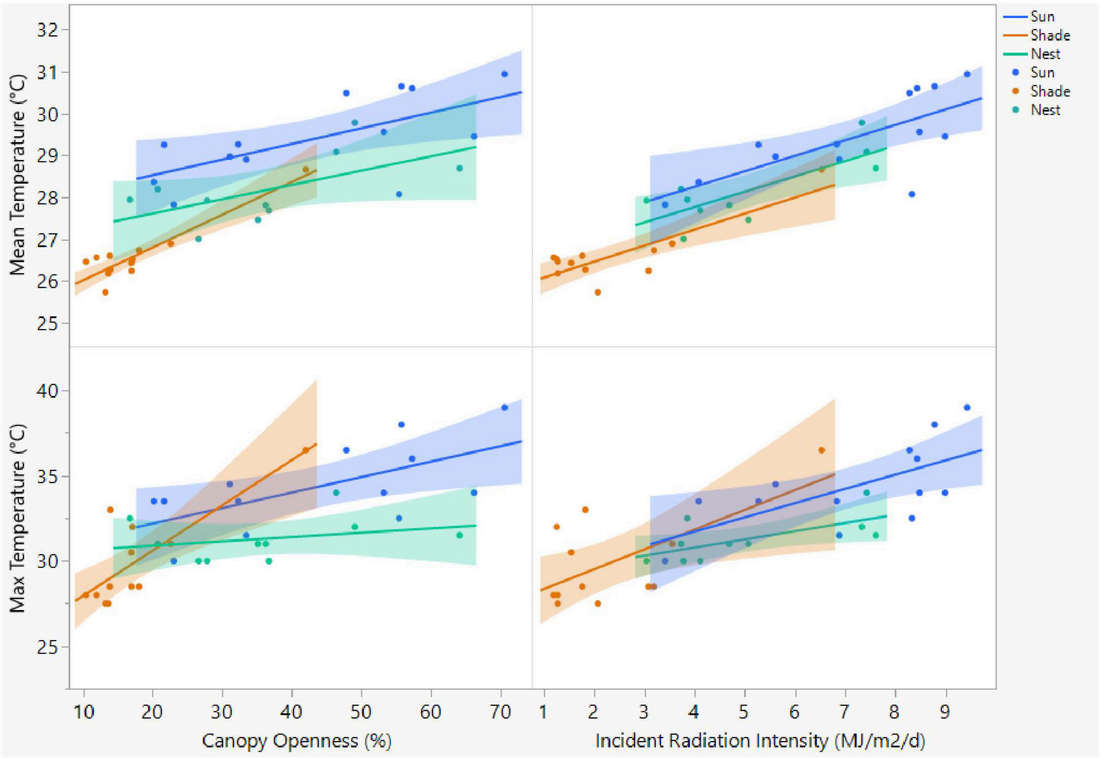

FIGURE 8 | Relationships between mean and maximum (back-filled) nest temperatures and either openness or incident radiation intensity of *A. ferox* nest sites and potential nest sites. Potential nest sites included the shadiest and sunniest sites within 20 m of each nesting area.

nest sites were close to nest sites and easily accessible because nesting softshell turtles are known to move laterally once on land (Fitch and Plummer, 1975; Doody, 1995). Our data revealed that shaded potential nest sites received significantly less solar radiation that, in turn, depressed artificial nest temperatures considerably (mean = ∼2°C, maximum = ∼2.5°C; **Figure 6**). The relationship between canopy cover and nest temperatures has been demonstrated for turtle nests previously (Ewert et al., 1994; Janzen, 1994; Refsnider et al., 2013a). Although shaded areas tended to have more ground cover due to leaf litter, this would be easily swept aside by nesting females and sandy soils were ubiquitous across the study sites. Indeed, we have observed mothers scratch away dead material when constructing a nest.

Although there was some seasonal increase in grasses and shading understory vegetation throughout incubation (the growing season = April–July), the resultant change in openness and incident radiation intensity data was negligible. Openness and incident radiation intensity of nest sites at all three study sites combined decreased by only 3.8 and 6.4%, respectively (**Figure 5**). Similar trends were evident when considering openness and incident radiation intensity for each study site (**Figure 5**). More pronounced seasonal increases in vegetation height and thickness affecting openness and incident radiation intensity may be experienced at other nesting areas for this and other species; seasonal changes in vegetation should thus, be quantified in each study.

Nest sites in the present study exhibited a wide range of openness, and thus incident radiation intensity values (**Figure 9**). For example, openness ranged from 7–70% (**Figure 9**). Although we did not follow individual turtles, the large sample size and thermal consequences of nesting in shade would suggest that (decreased) openness could be the target of natural selection in a climate warming scenario.

Collectively, our data suggest that this open-area nester has reasonable potential to use behavioral means to offset current and future climate warming, at least at a magnitude of ∼2–2.5°C. As the climate continues to warm, softshell mothers have the potential to counter the effects of increasing temperatures in nests by choosing to nest in more shaded areas. Most areas inhabited by softshells contain at least patches of shading vegetation, and among-population variation in chosen and available canopy/understory cover should buffer against declines in the species due to climate effects on developing embryos, provided that certain assumptions are met (heritability of nest site choice, rate of evolutionary response relative to rate of climate change). However, as shaded sites were the coolest microhabitats available, continued warming beyond ∼2–2.5°C could exhaust maternal behavioral avenues for keeping softshell eggs at current suitable temperatures. However, determination of the extent of how much nest site choice could buffer developing embryos from climate warming would require quantifying the thermal limits of developing softshell embryos in the laboratory.

TABLE 1 | Relationships of canopy openness and incident radiation intensity to temperatures (grand mean, maximum, and minimum) from nest sites and potential nest sites (sun, shade).

Nest type	Attribute (X)	Temp	F_{df}	R^2	P
Sun	Incident radiation intensity	Mean	$11.63_{1,11}$	0.47	0.0058^{**}
Sun	Incident radiation intensity	Max	$8.437_{1,11}$	0.38	0.0143^{*}
Sun	Incident radiation intensity	Min	$1.763_{1,11}$	0.13	0.1246
Sun	Canopy openness	Mean	$7.707_{1,11}$	0.36	0.018^{*}
Sun	Canopy openness	Max	$7.408_{1,11}$	0.35	0.0199^{*}
Sun	Canopy openness	Min	$4.128_{1,11}$	0.21	0.067^{a}
Shade	Incident radiation intensity	Mean	$22.61_{1,10}$	0.66	0.0008^{**}
Shade	Incident radiation intensity	Max	$7.351_{1,10}$	0.37	0.0219^{*}
Shade	Incident radiation intensity	Min	$0.028_{1,10}$	0.1	0.870
Shade	Canopy openness	Mean	$54.08_{1,10}$	0.83	$< 0.0001^{***}$
Shade	Canopy openness	Max	$18.24_{1,10}$	0.61	0.0016^{**}
Shade	Canopy openness	Min	$0.043_{1,10}$	0.1	0.8393
Nest	Incident radiation intensity	Mean	$12.23_{1,8}$	0.56	0.0081^{**}
Nest	Incident radiation intensity	Max	$6.027_{1,8}$	0.36	0.0396^{*}
Nest	Incident radiation intensity	Min	$0.134_{1,8}$	0.11	0.7237
Nest	Canopy openness	Mean	$4.349_{1,8}$	0.27	0.0705^{a}
Nest	Canopy openness	Max	$0.700_{1,8}$	0.03	0.4275
Nest	Canopy openness	Min	$0.181_{1,8}$	0.10	0.6816
All	Incident radiation intensity	Mean	$154.1_{1,33}$	0.82	$< 0.0001^{***}$
All	Incident radiation intensity	Max	$53.12_{1,33}$	0.61	$< 0.0001^{***}$
All	Incident radiation intensity	Min	$0.041_{1,33}$	0.03	0.8403
All	Canopy openness	Mean	$64.88_{1,33}$	0.65	$< 0.0001^{***}$
All	Canopy openness	Max	$33.03_{1,33}$	0.49	$< 0.0001^{***}$
All	Canopy openness	Min	$0.131_{1,33}$	0.03	0.7199

Mean, grand mean temperature; max, mean maximum temperature; min, mean minimum temperature.

^{a}*Approaching significance.*

$^{*}p < 0.01$, $^{**}p < 0.001$, *and* $^{***}p < 0.0001$.

Without behavioral compensation, softshells would be forced to either shift their distribution toward the poles, or shift their embryological tolerance to rising nest temperatures. Air temperatures have been predicted to warm by 1.4–4.8°C by 2100 without considerable (new) efforts to reduce greenhouse gas emissions (Intergovernmental Panel on Climate Change [IPCC], 2014). If softshell turtle mothers in our populations do not shift nesting into more shaded areas accordingly, then embryos would spend more time at potentially lethal temperatures; moreover, extreme temperatures could cause severe sub-lethal effects in turtles that do hatch. Although mean temperatures of 35 monitored (back-filled) nests did not exceed 32°C, maximum temperatures in 5 of 35 (7%) nests exceeded 36°C, and one nest recorded 39°C. While there are no data on the thermal limits of developing embryos in *A. ferox*, in the congener *A. spinifera* constant incubation temperatures of 34°C decreased embryonic survival and reduced performance and endurance in neonates that did hatch (Doody, 1999).

Another possible, climate response option is for softshell mothers to excavate deeper nests. Although recently authors have posited that nest depth is constrained by hindleg length in turtles (Refsnider et al., 2013b), there is evidence that softshell mothers can excavate forms or "troughs" prior to nesting (Doody et al., 2020) that can result in deeper nests (for a discussion on congeners see Plummer and Doody, 2010). By digging a form mothers could uncouple hindleg length from nest depth. We were not able to quantify nest depth in the present study because our nests were depredated; chamber depth would have been compromised during excavation by nest predators. A final way in which turtle mothers could behaviorally offset climate change effects is by adjusting seasonal timing of nesting (Doody et al., 2006a; Doody and Moore, 2010; Nelson et al., 2018). In our study, softshells nested from late March to late July (**Figure 1**). It is conceivable that mothers could advance or retreat the nesting season into cooler months to achieve cooler nest temperatures as the climate warms. However, shifting seasonal timing of nesting could be difficult due to tradeoffs and/or constraints on other aspects of the species' biology. For example, seasonal timing of mating or hatching may be adaptive in their own right; shifting those to accommodate shifting timing of nesting could incur survival costs. In another example, the onset of nesting in animals, including turtles, is often underpinned by energy acquisition, which in turn is driven by seasonal climatic conditions (e.g., Rowe, 1994); advancing timing of nesting might thus not be possible due to insufficient energy for vitellogenesis or the production of sex hormones (Kuchling, 1999).

There are two critical missing pieces needed for a fuller understanding of how nesting turtle mothers might respond to climate change to offset increasing temperatures on developing embryos. The first concerns among-generation change vs. phenotypic plasticity. Our conceptual model focuses on among-generation change (adaptation) in nest site choice behavior (see also Janzen and Morjan, 2001), while others have implicated phenotypic plasticity in nesting behavior as a potential response (e.g., Refsnider et al., 2013a). In turn, these mechanisms can only be clarified within the context of the other missing piece, the behavioral mechanism. Do mothers assess ground temperatures directly as suggested by Belzer et al. (2007), or indirectly through canopy cover as posited by others (Janzen, 1994; Morjan and Valenzuela, 2001)? For example, if a mother uses a canopy gap as a surrogate for temperature, that same canopy gap in a future warmer climate will result in hotter developmental (nest) temperatures; continued warming thus, would render canopy cover as a poor predictor of suitable developmental (nest) temperatures. If on the other hand mothers can assess temperature directly, then mothers might use canopy cover as a rough guide to find reasonably thermally-suitable nesting areas, but then fine-tune their choice of nest site by directly assessing substrate temperatures. In this latter case, mothers would not be reliant on a particular canopy cover value or range of values to predict developmental temperatures. More research including experiments is needed to disentangle direct vs. indirect cues for assessing temperature, and to evaluate among-generation change vs. phenotypic plasticity in the potential climate warming responses of turtle mothers.

A potential limitation of our study was that all of our 156 nests were taken by predators (although we did not confirm each event, most of these were by raccoons). We thus assumed that our nests reflected a representative subset of all nests in terms of nest site choice attributes. Although our large sample size and coverage

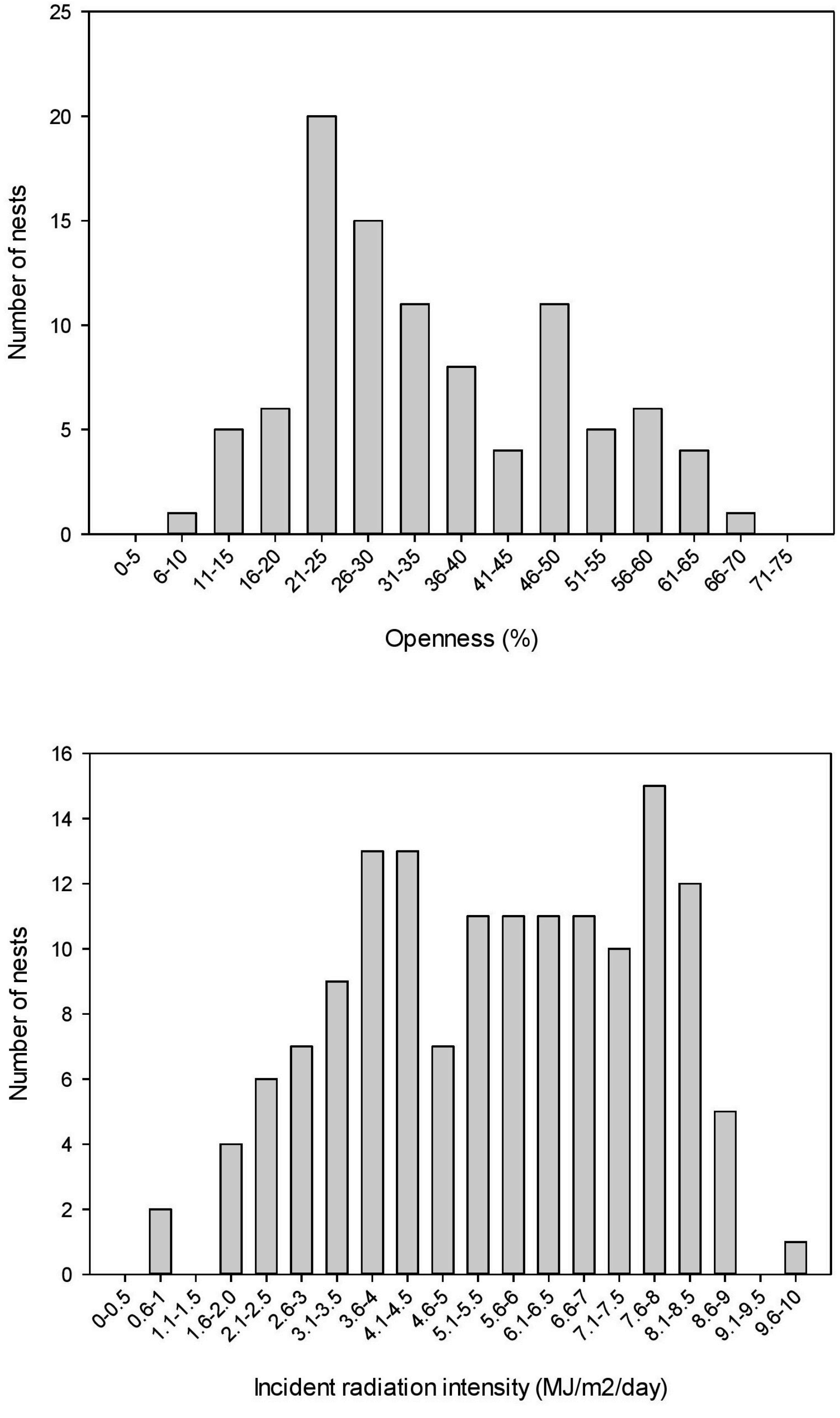

FIGURE 9 | Frequency distribution of openness and incident radiation intensity of *A. ferox* nest sites at BHNP, showing marked variation, most of which reflected the efforts of different mothers.

of multiple nesting areas within three study areas might seem to be robust to bias, there could be differences in nest site location, and thus canopy cover, between intact and depredated nests. For example, predation rates of simulated (chicken egg) nests by raccoons were higher in hedge (84%) than in open habitats (45%) (Grosse et al., 2014). However, raccoons, the main softshell nest predator at our study sites, are olfactory-driven predators that can successfully locate nests regardless of microhabitat. There is no published direct evidence that canopy cover influences the probability of predation by raccoons in turtle nests.

How might our results apply to other turtles, reptiles, and other oviparous ectotherms under future warming? Our study species and its two North American relatives *A. mutica* and *A. spinifera* nest in open areas (Plummer, 1976; Doody, 1995), providing the opportunity for shifting their nesting into shade in a climate warming scenario. Although rarely quantified, other turtle species nest in semi-shaded areas (e.g., *Pseudemys concinna*, Jackson and Walker, 1997; *P. floridana*, J. S. Doody, unpubl. data), a few species nest in full shade (e.g., *Kinosternon baurii*, Wilson, 1998; *K. steindachneri*, J. S. Doody, unpubl. data), and at least one wide-ranging species nests in open areas in cooler climates and more shaded areas in warmer climates (e.g., *Chelydra serpentina*, Ewert et al., 2005; see also Doody et al. (2006a) for an example in lizards). Shade nesters would theoretically be at a disadvantage in a climate warming scenario because they may already be nesting in the shadiest sites (coolest microclimates), especially at the hot end of their range. This behavioral “dead-end” would force a shift in geographic distribution, in the physiological tolerance of developing embryos, or in nest depth, or the population/species could face extirpation or even extinction. Species restricted to hot climates (e.g., the tropics) or with populations at hot climate range margins could also be disadvantaged because the rate of range contraction (extirpations) at the hotter range edge could outpace the rate of range expansion (dispersal) at the cooler range edge. The same should apply to other reptiles and ectotherms. This predicted pattern in the egg life history stage is parallel to the notion that tropical ectotherms (i.e., adults and juveniles) are more vulnerable to climate warming than temperate species because the former need to thermoregulate to keep cool (rather than warm) by using shade, and continued warming could result in heat stress even in deep shade (Deutsch et al., 2008), allowing little or no opportunity for compensation (Kearney et al., 2009).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC.

AUTHOR CONTRIBUTIONS

JSD designed the project with help from GLH. SS collected the data with help from JSD and GLH. DC, SS, and JSD performed the data analysis. JSD and NM accomplished the formatting. JSD wrote the manuscript with help from SS, GLH, NM, and DC. All authors contributed to the article and approved the submitted version.

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Light Sandy Beaches Favour Hatching Success and Best Hatchling Phenotype of Loggerhead Turtles

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We conducted a 5-year field (2017–2021) and laboratory study of the relationship between type of substrate and hatching success, embryonic development, and the quality of hatchlings in loggerhead turtle nests. Our study site, the island of Maio in the archipelago of Cabo Verde, one of the world's largest loggerhead turtle nesting colonies, displays marked heterogeneity of sand colouration, with dark, mixed, and light sandy beaches. We experimentally incubated eggs, comparing different nesting substrates under standard temperature and humidity conditions. Females nest in all sand types without preference. However, both the field and experimental study revealed a significant difference in hatching success depending on the type of substrate. Substrate of volcanic origin, dark in colour, with a lower amount of calcium carbonate, had a lower hatching success (HS; 30.3 ± 20.2%) compared to substrates of mixed (HS = 46.1 ± 26.5%) or light (HS = 78.1 ± 18.2%) colour. Eggs experimentally incubated in substrate that was light-coloured, with a larger grain size and higher calcium carbonate concentration, produced significantly more and larger offspring. Incubation temperatures were significantly higher in dark substrate, which partially explains the lower hatching success in this type of sand. However, experimental incubation with controlled temperatures consistently showed lower hatching success in dark sand. Thus, we found that not only the temperature, but also the specific characteristics of each substrate determine hatching success. The main predator of eggs and hatchlings (the ghost crab *Ocypode cursor*) showed no significant differences in abundance or size between different substrate types. Our results indicate that nest site selection between beaches or even within the same beach with different substrate conditions affects hatching success, hatchling physical condition, and subsequently the reproductive success of each female. The results of this study can inform conservation programmes with nest management and controlled incubation in the field and optimise adaptive nest management under future scenarios of rising global temperatures.

Keywords: egg incubation, Maio Island, sea turtles, incubation substrate, (*Caretta caretta*), conservation, global warming, climate change

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INTRODUCTION

The absence of parental care in some oviparous species, such as sea turtles, makes nest site selection and incubation substrate vital for the success of their nests (Doody, 2011). Sea turtles are long-lived, late-maturing, ectothermic organisms and depend on sandy beaches to lay their eggs, which incubate at the mercy of environmental conditions. Sea turtle females exhibit nesting philopatry but use different beaches in the same area, which may vary in morphology, type of substrate or vegetation (Conrad et al., 2011; Ditmer and Stapleton, 2012). For eggs laid in each type of substrate, abiotic factors such as temperature, humidity and gas exchange, and biotic factors such as presence of roots or predators, determine their final embryonic success (Ackerman, 1997; Wallace et al., 2004; Chen et al., 2010). Dark-coloured sand, for example, absorbs more solar radiation and therefore eggs must withstand higher temperatures than in light-coloured sand (Hays et al., 2001). The temperature range at which turtle eggs develop is relatively narrow and even small changes in incubation temperatures can have dramatic effects on embryo mortality (Laloë et al., 2017). The different compaction, composition, or granulometry of the substrate may also be determining factors in the success of embryonic development in these species, but this is an area that needs further study (Mortimer, 1990; Chen et al., 2007; Yalçın-Özdilek et al., 2007; Salleh et al., 2018; Saito et al., 2019; Stewart et al., 2019). The different environmental incubation conditions in nests causes variation in the incubation time, hatchling phenotype, their sex, and their vitality (Patino-Martinez et al., 2014; Kobayashi et al., 2017; Jensen et al., 2018; Marco et al., 2018). As clutch survival may vary according to substrate type, e.g., dark versus light sandy beaches (Martins et al., 2020), nest site selection has the potential to enhance or reduce this parameter (Hawkes et al., 2010; Patrício et al., 2018). Understanding this behaviour and the consequence for clutch survival is therefore critical, especially for populations of conservation concern.

This study was conducted in Cabo Verde, an archipelago of volcanic origin located on the west coast of Africa, 500 km from Senegal. Recent findings suggest there are more loggerhead turtles nesting in Cabo Verde than previously estimated, and that this might be the largest nesting subpopulation of this species globally (Patino-Martinez et al., 2021).

The loggerhead turtle (*Caretta caretta*) is listed as globally "Vulnerable" by the International Union for the Conservation of Nature (IUCN) and its persistence is conservation dependent (Casale and Tucker, 2017). The subpopulation of Cabo Verde has been identified as a separate genetic stock (Monzon-Arguello et al., 2010; Wallace et al., 2010) with multiple nesting groups inside the rookery (Stiebens et al., 2013; Baltazar-Soares et al., 2020). It is listed as "Endangered" under the IUCN Red List criteria B2, due to the continuing decline in area, extent, and/or quality of its habitat (Casale and Marco, 2015).

Within Cabo Verde, the island of Maio hosts an important proportion of this subpopulation, with 4,063–14,364 nests per year between 2016 and 2019 (Patino-Martinez et al., 2021). The nesting beaches of Maio present a high heterogeneity in the colour and type of sand, varying in a spectrum from dark sands of volcanic origin to light-coloured sands of biogenic origin, and including beaches which are a mixture of the two. Such diversity of sandy beaches provides an excellent natural laboratory for studying the influence of substrate on the incubation of eggs and the reproductive success of sea turtles.

To understand the potential effect of incubation substrates on the reproductive success of the loggerhead turtle, we have quantified the inter-beach variability of sand type and evaluated its effect on (i) nesting success, (ii) nest density, (iii) abundance and size of predators, (iv) hatching success, and (v) physical condition of hatchlings, both *in situ* and in laboratory conditions.

MATERIALS AND METHODS

Study Site

The study was conducted on the island of Maio (269 km^2, 110 km of coastline and 38 km of sandy beaches), one of the ten islands of the archipelago of Cabo Verde (15°13′50′′N 23°09′22′′0; **Figure 1**).

The high-energy beaches of the island of Maio are currently pristine and undeveloped. The colour of the sand and the size of the grain varies over the whole island. Beaches were classified by colour using the Munsell colour chart system (Fan et al., 2017), and all beaches were measured and georeferenced.

Nesting Success and Nest Density

We conducted 615 sampling nights (from 20:00 to 6:00; 44 fieldwork teams per night) during five consecutive nesting seasons (July–October) between 2017 and 2021, along 100% of the island's sandy beaches (38 km). Each time a female turtle emerges from the sea to attempt to nest (nesting activity) she creates a distinctive set of tracks in the sand: with one track ascending to any aborted nesting attempt or a successful (camouflaged) nest, and another track descending to the sea. Following this pattern of tracks, all nesting activities (sometimes with direct observation of the females) were recorded and geolocated (Garmin e-trex Summit) and all turtle tracks were erased using wooden rakes or the ranger's feet. To identify any nesting activity not recorded during the night, a daily early morning track count was carried out. Nesting success was estimated as the proportion of nesting activities resulting in effective nest placement. Nest density was calculated as the total number of nests per beach area (length × average width of three measurements along the beach). Nesting success and nest density were compared between beaches of different substrate (dark, mixed, and light) in the eastern part of the island (with similar wave conditions and accessibility from the sea), to reduce the spatial effect in the analysis.

Sand Type and Ghost Crab Abundance

In 2017, an observational study was undertaken to evaluate the influence of substrate type on the abundance and size of the ghost crab, the main predator of eggs, and offspring (Marco et al., 2015). Two infrared cameras (Browning BTC-5HD)

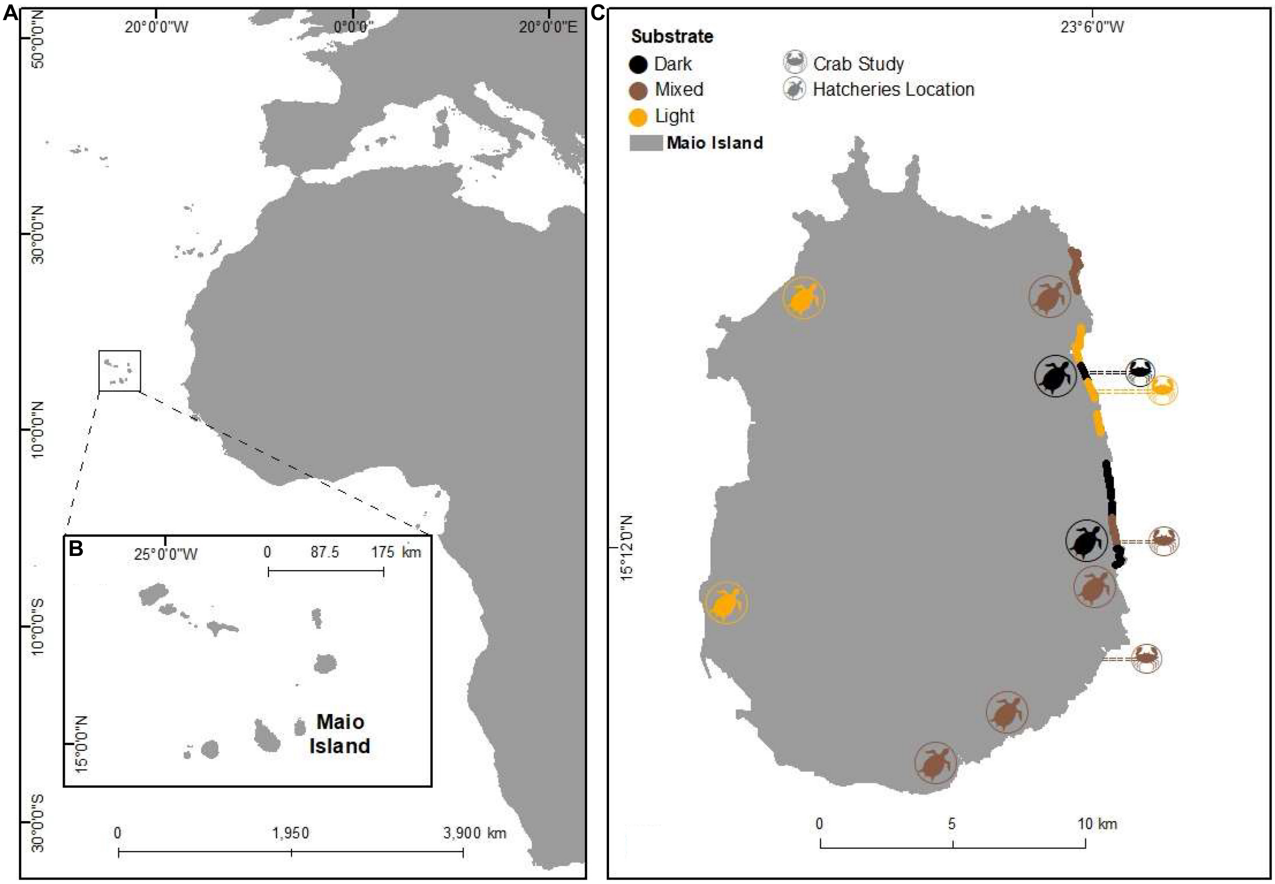

FIGURE 1 | Overview of the study sites. **(A)** Image of the Cabo Verde archipelago, West Africa. **(B)** Detailed map of the Cape Verde archipelago (inset). **(C)** Island of Maio (**C**; 15°12′N—23°6′W) showing the study areas by sand types (by colours) where loggerhead turtles nest. The crab symbol represents the location of the predator survey. Coloured lines (east coast) illustrate nest density and nesting success study areas.

were installed on three dark-coloured beaches (for six nights between July 5, 2017 and October 10, 2017), two mixed-sand beaches (for 10 nights between July 6, 2017 and September 16, 2017), and two light-sand beaches (for 13 nights between July 12, 2017 and October 8, 2017). Bait (unsuccessful eggs from excavated nests, and fresh fish heads) was placed in front of the cameras, and the number of crabs present in an area of 9 m^2 (3 m × 3 m of sand) was counted with one photograph every hour (**Supplementary Figure 1**). The seven beaches were chosen in the eastern part of the island of Maio and are interspersed with each other (**Figure 1**). For the biometric study of the crabs, they were captured using drop traps (in 20-L plastic buckets with a high vertical wall) buried in the sand without a lid. The bait (fresh fish) was tied to a wire across the diameter of the top of the bucket. Three adjacent beaches (**Figure 1**) were chosen (one for each type of sand) and one trap was set per night from 22 to 6 h (dark sand – 10 nights between August 15, 2017 and October 13, 2017; mixed sand – eight nights between August 21, 2017 and September 30, 2017 and light sand – 14 nights between August 16, 2017 and October 11, 2017). Caught crabs were counted and the maximum carapace length and width of each crab were recorded (using Cen-Tech Digital callipers; Harbor Freight Tools, Pittsburgh, PA, United States; accuracy ± 0.01 mm).

Measuring the Effect of Sand Type on Hatchery Nests

To evaluate the influence of substrate type on nest incubation under standardised conditions, we conducted a field experiment. We moved 106 nests in 2017 to eight open beach hatcheries (two dark sand, four mixed substrate, and two light sand; **Figure 1**). In order to avoid any seasonal effect on hatching success the relocation was simultaneous in all three hatchery types (from 04 July to 17 August). The eggs were moved by walking with them in new plastic bags; it took a maximum of 3 h between oviposition and reburial. The relocated nests (28 in dark; 56 in mixed; 22 in light) were buried at a depth of 45 cm, in root-free substrate, and monitored daily during incubation. The hatcheries (enclosures surrounded by a 1 m high plastic mesh and wooden fence; 7 × 7 m; on a 0° slope) were in areas of the beach where tidal flooding does not occur and all of them prevented the entry of predators. Nests were in plots of 1 m^2 for each nest. The number of eggs at the time of relocation was counted for each of the nests studied. The hatching success of each nest was determined as the proportion of eggs culminating in hatching. Incubation temperatures were recorded in three of the hatcheries simultaneously between 18 August and 18 November by one datalogger (Hobo Stow Away TidbiT v2 Onset,[1] ±0.2°C accuracy, measuring 3.0 cm × 1.7 cm; programmed to record temperature at 30 min intervals) for each sand treatment at a depth of 0.37 m in the sand, resulting in 4,417 temperature data points from each sand type.

Temperature-Controlled Experiment

In 2018, we conducted a field experiment to assess the influence of sand type (dark, mixed, and light) on embryonic development, hatching success, and hatchling phenotype into a common temperature incubation environment. The experimental design corrected for spatial effects associated with the hatchery study and standardised the thermal regime as previously described in the study (Patino-Martinez et al., 2014). A total of 225 eggs were collected from five different females, laid during the same night (45 eggs per female). The eggs were collected directly from the female's cloaca, ensuring that they did not touch the sand before the beginning of the experiment. Because females distribute their different nests among different beaches (Patino-Martinez et al., in press), nests were chosen randomly among beaches. Twenty-five eggs (5 per female) were placed in each one of nine plastic containers (0.37 m × 0.27 m × 0.23 m, sterile and dry). All eggs were weighed (using a microbalance Criacr Digital Pocket Scales, 500 g/accuracy ± 0.01 g. Stainless Steel. Energy Class A+), randomly distributed horizontally and completely covered with one of the types of sand. Each container was closed with a lid, with an air space (0.1 m) between the lid and the sand covering the eggs. The closed containers were buried in an open area of a mixed sand beach in three blocks of three containers, each block representing the three sand type treatments randomly distributed. The sand used in the experiment was collected at a depth of 0.45 m in the centre of natural nesting beaches of the different sand types and manually stirred for 3 min, before starting the experiment. All eggs were incubated at a depth of 45 cm (measured from the egg layer) without touching any other sand until hatching. From incubation day 45 onward, the experimental containers were inspected daily to verify hatching. To study temperature variability within the study treatments, data loggers [Hobo Stow Away TidbiT v2 Onset (see footnote 1), ±0.2°C accuracy, measuring 3.0 cm × 1.7 cm; programmed to record temperature at 30 min intervals] were placed in three containers (one per treatment) at the same depth as the eggs. The data loggers were placed at the start of the study, in the middle of each container and left untouched throughout incubation. To assess the effect of controlled incubation on temperatures, a temperature control was placed in the sand (one data every 30 min simultaneously with the experiment) (**Supplementary Figure 2**) on the beach, 1 m outside the experiment and at the same depth (45 cm).

To measure moisture, sand samples (approximately 300 g) were taken from each of the treatment at the beginning of the experiment. The fresh samples were weighed (using a microbalance Criacr Digital Pocket Scales, 500 g/accuracy ± 0.01 g. Stainless Steel. Energy Class A+) and then left to dry in high containers in a sunny location. The samples were reweighed periodically until a constant mass was reached (i.e., all water had been lost). The water content of the sand was calculated as the water loss divided by the total fresh (wet) weight, multiplied by 100. This experimental technique, using closed plastic containers, avoids flooding, and maintains initial moisture levels throughout the study (Patino-Martinez et al., 2014).

Temperature *in situ*

An observational study was conducted in 2019 and 2021 to compare *in situ* sand temperatures (dark, mixed, and light) at different depths (25, 35, 45, and 55 cm), to obtain baseline data on the normal ranges of incubation temperatures at these positions. The period studied was from 5/8 to 17/11 in both years. One temperature logger [Hobo Stow Away TidbiT v2 Onset (see footnote 1), ±0.2°C accuracy, measuring 3.0 cm × 1.7 cm] per depth and substrate type was placed in 2019 and two loggers per substrate type at 25 and 35 cm depth and three loggers per substrate type at 45 and 55 cm depth in 2021. Each year the dataloggers recorded data simultaneously at 30-min intervals. These data were used to calculate average temperatures per substrate and depth.

Measuring Hatchling Size and Performance

Morphology and locomotion of hatchlings affect their survival in the first hours after hatching (Ischer et al., 2009). Therefore, a random sample of eight hatchlings per nest was taken immediately after emergence and 100% of the hatchlings from the temperature-controlled experiment were taken for biometric analysis. For each hatchling, the length and straight width of the shell were recorded (using Cen-Tech Digital callipers; Harbor Freight Tools, Pittsburgh, PA, United States; accuracy ± 0.01 mm). All hatchlings were weighed (using a microbalance Criacr Digital Pocket Scales, 500 g/accuracy ± 0.01 g. Stainless Steel. Energy Class A+). The performance of the offspring was measured as the time taken to turn from the "face up" position to the normal "face down" run position (self-righting ability). These tests were done outdoors on moist, compact sand, repeated three times per individual and then averaged. Self-direction tests were mostly carried out before midnight (66.3%), therefore, in the present study, the possible effects of circadian rhythm on hatchling activity were not standardised. After the physical tests, the hatchlings were immediately released into the sea.

All unhatched eggs were dissected 48 h after the last nest emergence and 60 days later in the eggs from the temperature-controlled experiment. The embryos were assigned to a developmental status category, adapting the criteria proposed by Bilinski et al. (2001). We assigned a value per category, as follows: 0 = undeveloped, 1 = early embryonic death, 2 = middle embryonic death, and 3 = late embryonic death. The success of

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TABLE 1 | Nesting success, nest density, and crab biometric measurements among the three types of substrates on 14 beaches close to each other (5 in dark sand, 5 in mixed sand, and 4 in light sand) on the east coast of the island of Maio (**Figure 1**).

Parameters	Substrate type			Statistical test	*P*-value
	Dark	Mixed	Light		
Nesting success (%)	56.45 ± 17.93	55.78 ± 20.93	56.76 ± 18.51	F(2) = 0.03	=0.973
Nest density (m^2)	0.1 ± 0.09	0.1 ± 0.09	0.09 ± 0.07	H(2) = 310	=0.213
Number of crabs per trap	1.5 ± 2.5	2.1 ± 1.9	4.6 ± 3.9	H(2) = 5.55	=0.062
Crab length (mm)	37.7 ± 5.9	33.1 ± 9.9	35.7 ± 7.9	H(2) = 1.57	=0.456
Crab width (mm)	28.6 ± 5.1	24.9 ± 8.3	26.0 ± 6.3	H(2) = 2.24	=0.326

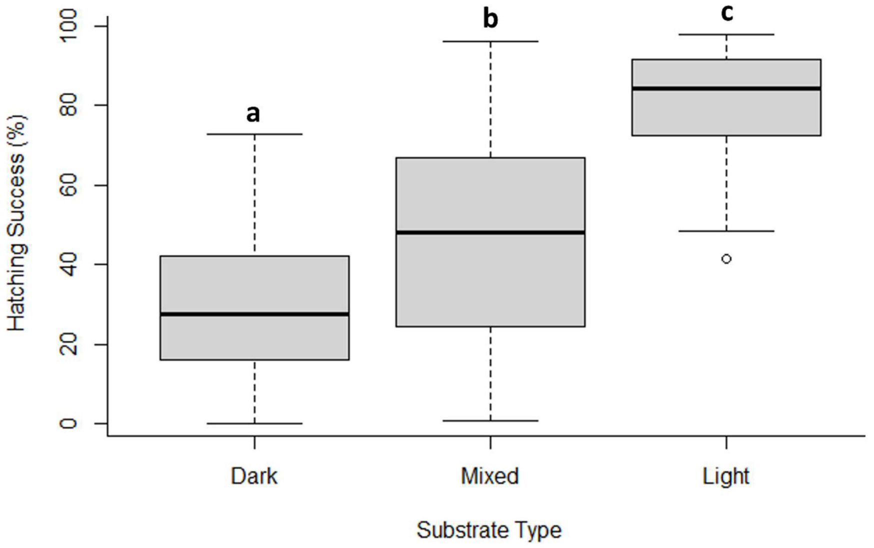

FIGURE 2 | Hatching success in different substrates (dark, mixed, and light) under controlled incubation conditions in the hatchery. Different lowercase letters indicate statistically significant differences among treatments. The median is the bold line as a measure of central tendency, quartiles one (Q1) and three (Q3) marked by upper and lower box limits and maximum (max) and minimum (min) values marked with whiskers above and below.

embryonic development was calculated as the proportion of eggs reaching stages 3 and successful hatching.

Laboratory Analysis

Samples (500 g) of each type of sand were collected at the beginning of the temperature-controlled experiment and were sent for laboratory analysis.

Granulometry, composition and origin were analysed in the laboratory for each type of sand. Salts and impurities were initially removed to avoid errors in the analysis, by washing each sample with 2 L of distilled water. They were then dried in a stove at a moderate temperature (65°C). To obtain the representative fraction for each of the analyses to be carried out, each sample was split as many times as necessary (Folk and Ward, 1957).

The particle size of the sediment was determined by dry sieving of the sample, using a sieving tower composed of 17 sieves, covering the range from gravel to silt and clay (Wentworth, 1922).

The carbonate content was determined by Bernard's Calcimeter (Lamas et al., 2005). This method is based on the reaction of hydrochloric acid (HCl) with calcium carbonate ($CaCO_3$) causing release of carbon dioxide (CO_2). The gas displaces the volume of a liquid contained in a burette that was quantified, indirectly obtaining the carbonate content.

The origin of the grains and their proportions was analysed by a provenance analysis. Each sand sample (dark, mixed, and light) was fixed in sheets with epoxy resin for 24 h. By cutting the sheets and using carborundum abrasive, three thin sections of between 20 and 30 microns were obtained. The characteristics of the grains were determined by observing the sheets under a petrographic microscope (Ortoplan-Leitz) with plane and polarised lights. Each sand grain was identified into subcategories: bioclasts (red algae meshes, molluscs, foraminifera, bryozoans, and echinoderms), lithoclasts (mafic volcanic rocks, intraclasts, olivine, clinopyroxene, opaques -Fe and Ti oxides, and volcanic glass), and carbonate aggregates (bioclastic and lithoclastic grains containing sparite and micrite carbonate cements).

Statistical Analyses

We used R Studio software, version 1.1.463 (Campbell, 2019) to perform non-parametric and parametric tests. When normality and heteroscedasticity were assumed, an ANOVA test was made.

A Kruskal-Wallis test was performed once the assumptions were not assumed, while a Tukey's Test was performed for multiple comparisons. To investigate if nesting success and nest density were significantly different between substrates, we fitted generalised linear models (GLM), with substrate type as predictor (fixed factor) with nesting success and nest density as response (dependent) variables, and year as a random factor. A Chi-squared test for given probabilities was also performed. We arcsine-transformed the percentages of hatching success and development success data. Alpha was set at 0.05.

The granulometric analysis was done through the Gradistat programme (Blott and Pye, 2001).

RESULTS

Field Study

Over the 5 years of the study (2017–2021), on all sand types, we counted 12,806 nesting activities in 2017, 30,075 in 2018, 19,316 in 2019, 38,993 in 2020 and 68,789 in 2021, corresponding to 5,429, 14,364, 7,937, 23,185 and 46,161 clutches respectively (mean number of nests per year = 19415; SD = 16450). According to the Munsell colour charts, 9.1% of the beaches were dark (Munsell = 5Y 3/1), 65.4% mixed (Munsell = 5Y 8/3), and 25.5% light (Munsell = 5Y 8/4). Nesting success was not significantly different between substrates (GLM df = 2; $F = 0.03$; $P = 0.97$; $n = 70$; **Table 1**) and nest density did not differ statistically among the distinct types of sand (K-W df = 2; $H = 310$, $P = 0.21$, $n = 70$; **Table 1**).

Crab abundance (No. of crabs per trap per night) did not differ significantly between beaches on different substrates. Light-coloured sand had an average of 4.6 crabs, which is higher than the averages of 2.1 and 1.5 for mixed and dark sand, respectively (**Table 1**). Crab biometrics were similar in all three sand types (length 37.7 mm in dark, 33.1 mm in mixed, and 35.7 mm in light; $H(2) = 1.57$, $P = 0.456$; **Table 1**).

The Effect of Sand Type on Hatchery Nests

Hatching success differed between incubation substrates, with a range between 30.3 and 78.1% and was significantly lower in dark sand (**Figure 2**). Nests incubated in light sand had the highest hatching success (Dunn test light vs mixed $P < 0.0001$ and light vs dark $P < 0.0001$). Hatchling mass varied as a function of incubation sand (Weight: light 16.8 g ± 2.0 vs mixed 15.1 g ± 1.3 and dark 15.2 g ± 1.1; $H_2 = 10.9$, $p < 0.01$; **Figure 3A**). Both SCL and SCW were significantly higher for hatchlings from light-coloured substrate (SCL: light 41.5 mm ± 3.6 vs mixed 40.5 mm ± 1.2 and dark 40.4 mm ± 1.5, $H_2 = 9.1$, $p = 0.01$, **Figure 3B**; SCW: light 31.8 mm ± 2.5 vs mixed 31.1 mm ± 1.1 and dark 30.3 mm ± 1.0, $H_2 = 14.2$, $p = 0.001$, **Figure 3C**), indicating that embryo growth occurred differently between substrates and produced different phenotypes. Self-righting time showed that the hatchlings from the light substrate were faster (Self-righting time: light 13.2 s ± 7.4 vs mixed 24.2 s ± 11.4 and dark 26.8 s ± 10.6; $F_{2-69} = 9.3$, $p < 0.001$; **Figure 4**). There was a statistically significant difference in incubation temperatures between the different substrates, where the temperature decreased from dark to light sand (**Table 2**).

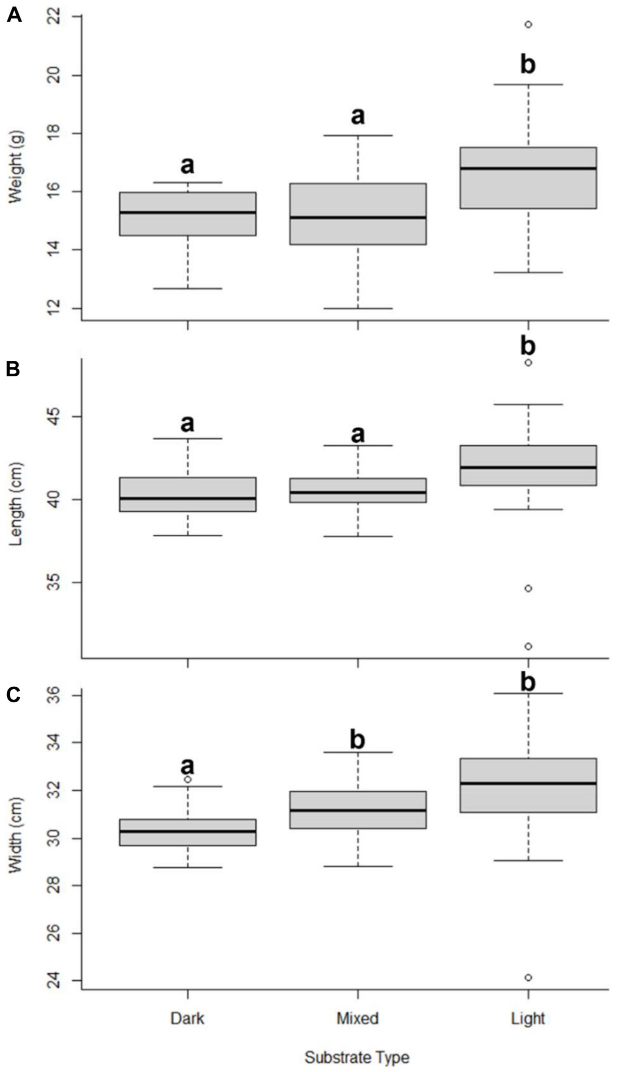

FIGURE 3 | Hatchling phenotype **(A)** weight, **(B)** straight carapace length, and **(C)** straight carapace width in the different incubation substrates (dark, mixed, and light) under controlled conditions in the hatchery. Different lowercase letters indicate statistically significant differences among treatments. The median is the bold line as a measure of central tendency, quartiles one (Q1) and three (Q3) marked by upper and lower box limits and maximum (max) and minimum (min) values marked with whiskers above and below.

Temperature-Controlled Experiment

Hatching success differed among substrates (ANOVA: $F_{2,6} = 30.6$, $P < 0.001$), ranging from 16 to 81% and was significantly lower in the dark sand (16.0% ± 4.0 s.d., 95% CI = 0.2–32) compared to mixed (81.3% ± 15.1 s.d., 95% CI = 65–97) or light (77.3% ± 12.2 s.d, 95% CI = 61–93). Embryonic mortality in the dark substrate occurred earlier than in mixed and light (ANOVA: $F_{2,222} = 16.1$, $P < 0.0001$). While 24% of the eggs experienced

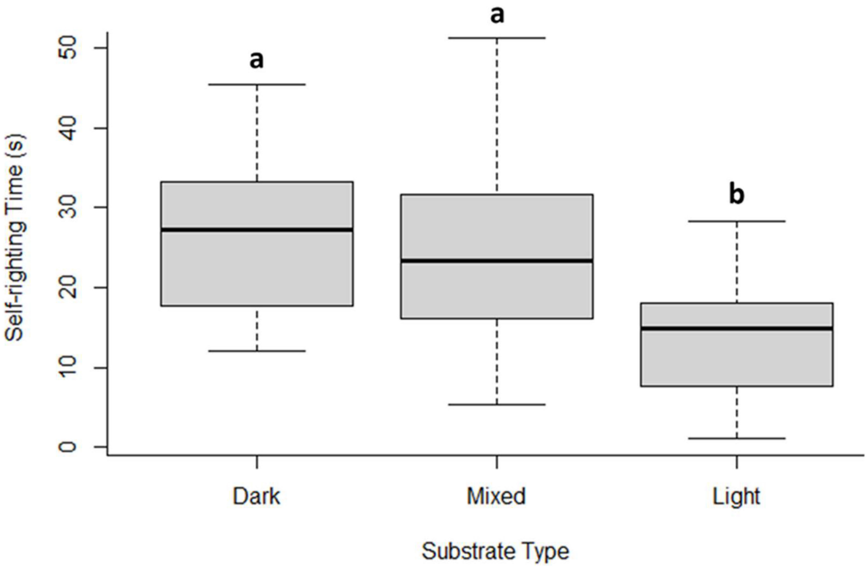

FIGURE 4 | Hatchling self-righting time (time needed to recover the face-down position) in different substrates (dark, mixed, and light) under controlled incubation conditions in the hatchery. Different lowercase letters indicate statistically significant differences among treatments. The median is the bold line as a measure of central tendency, quartiles one (Q1) and three (Q3) marked by upper and lower box limits and maximum (max) and minimum (min) values marked with whiskers above and below.

TABLE 2 | Temperatures recorded in three types of substrates in two study conditions (hatchery and experimental).

		Substrate type			
Study conditions		**Control**	**Dark**	**Mixed**	**Light**
Hatchery conditions (2017)	*N*	ND	4,417	4,417	4,417
	Mean		32.85	31.97	30.49
	σ		1.03	0.72	0.72
	Minimum		30.44	30.07	28.59
	Median		30.60	32.18	33.05
	Maximum		31.71	33.16	35.00
Experimental conditions (2018)	*N*	2,386	2,386	2,386	2,386
	Mean	30.04	29.97	29.94	30.10
	σ	0.79	1.07	1.21	1.22
	Minimum	27.95	27.90	27.70	27.95
	Median	29.99	29.76	29.64	29.79
	Maximum	31.41	31.79	32.10	32.23

early embryonic mortality in dark substrate, only 6.7% of the embryos died in initial stages in light substrate.

The dark substrate produced significantly smaller offspring in shell length (SCL: WS = 40.6 mm ± 1.9 s.d, MS = 40.9 ± 2.1 s.d, BS = 38.3 ± 3.7 s.d ANOVA: $F_{2,119} = 4.9$, $P < 0.01$). Shell width of experimental hatchlings were similar among treatments (SCW: $P = 0.13$). The hatchlings from dark substrate were significantly slower in the self-righting ability test, at 84.8 s ± 42.1s.d (mixed = 28.6 s ± 38.4s.d, light 58.6 s ± 42.4s.d; ANOVA: $F_{2,119} = 11.78$, $P < 0.0001$). The apparent shell malformations of offspring were extremely rare and there was no relationship with the incubation substrate.

The mean incubation temperatures in the experimental treatments were highly and significantly correlated with the control temperatures (Pearson correlation: dark = 0.96, mixed = 0.94, light = 0.95, $P < 0.0001$, **Table 2** and **Supplementary Figure 2**) and the mean difference between treatments was 0.03°C. The eggs were incubated at the natural humidity of the substrate and had a maximum difference of less than 1% between substrate types, and therefore were considered to be within a similar range (Patino-Martinez et al., 2014). The natural humidity of the sand was 2.4% in dark substrate, 1.5% in mixed, and 2.1% in light substrate.

Temperatures *in situ*

Mean incubation temperatures differed significantly between sand types (dark, 32.5°C ± 1.08, mixed 32.38°C ± 1.10, and light 30.40°C ± 1.13; KW $H_2 = 71{,}999$, $p = 0.000$). Temperatures in

TABLE 3 | Temperatures recorded at four depths (25, 35, 45, and 55 cm) between August 05 and November 17 during two nesting seasons (2019 and 2021), under three different substrate types.

Year	Depth (cm)	Substrate type		
		Dark	Mixed	Light
2019	25 (*N* = 5,040)	32.89 ± 1.29	ND	30.89 ± 0.86
	35 (*N* = 5,040)	32.63 ± 0.98	31.91 ± 1.02	30.74 ± 0.72
	45 (*N* = 5,040)	32.50 ± 0.88	31.78 ± 0.96	30.39 ± 0.67
	55 (*N* = 5,040)	32.30 ± 0.78	31.64 ± 0.92	30.31 ± 0.64
2021	27 (*N* = 10,080)	32.79 ± 1.40	33.17 ± 1.07	30.87 ± 1.46
	35 (*N* = 10,080)	32.56 ± 1.12	32.96 ± 0.98	30.72 ± 1.21
	45 (*N* = 15,120)	32.80 ± 1.05	32.83 ± 0.87	30.13 ± 1.11
	55 (*N* = 15,120)	32.28 ± 0.86	32.40 ± 0.80	29.93 ± 1.03

TABLE 4 | Grain characterisation and composition within the three different substrate types.

Grain size analyses		Substrate type		
		Dark	Mixed	Light
Folk and ward Method	Mean (mm)	0.32	0.32	0.41
	Sorting (σ_1)	0.45	0.62	0.57
Description	Mean (mm)	Medium sand	Medium sand	Medium sand
	Sorting (σ_1)	Well sorted	Moderately well sorted	Moderately well sorted
Composition (%)	Gravel	0.03	0.94	0.33
	Sand	99.95	98.88	99.61
	Mud	0.01	0.18	0.06
Grain size distribution (%)	Cobble	0.00	0.00	0.00
	Pebble	0.00	0.50	0.18
	Granule	0.03	0.43	0.15
	V. coarse sand	0.12	1.01	0.29
	Coarse sand	3.71	7.83	22.31
	Medium sand	72.54	60.85	63.09
	Fine sand	23.18	27.37	13.66
	V. Fine sand	0.40	1.82	0.27
	Silt and clay	0.01	0.18	0.06

light sand were on average 2.2°C cooler than black sand (**Table 3**). There was a significant sand type × depth interaction for temperatures (GLM, random factor year: substrate type × depth, $F = 67{,}881$, $p = 0.000$). Temperature decreased with increasing depth in all sand types, although not to the same extent (mean decrease every 10 cm: dark = 0.18°C, range = 0.17–0.19; mixed = 0.19, range = 0.13–0.25; light = 0.25, range = 0.19–0.31).

Analysis of Substrates in the Laboratory

The substrates analysed were composed mostly of medium sand (between 98.9 and 99.9%), with little size variability (sorting; **Table 4**), and with small proportions of mud or gravel (between 0.01 and 0.3%) (**Table 4**).

The proportion of coarse:fine sand was markedly different, with a of 3.7:23.2% in dark, 7.8:27.4% in mixed, and 22.3:13.7% in light substrate.

The proportion of CO3 was markedly different with a percentage of 17.8 ± 0.4 in dark, 44.8 ± 1.85 in mixed, and 78.5 ± 0.5 in light sand. This indicates that the composition of light-coloured sand was comparatively much higher in grains of biogenic origin (red algal and mollusc meshes predominated; foraminifera, bryozoans, and echinoderms were scarce) (**Supplementary Figure 3**). Volcanic lithoclasts (clinopyroxenes, Fe and Ti oxides, and the fragments of mafic volcanic rocks and glasses) were major components of the dark-coloured sand (**Figure 5** and **Supplementary Figure 3**).

DISCUSSION

Field and Experimental Study

Through a comprehensive 5-year field (2017–2021) and laboratory study we have demonstrated the effect of the different types of sand selected by loggerhead sea turtle nesting females on their embryonic development, hatching success, and hatchling phenotype. This information is especially relevant for species that depend on the conservation and protection of their nests, such as sea turtles (Casale and Tucker, 2017). Incubation of eggs in dark-coloured, volcanic, and fine-grained sands may have negative effects on loggerhead reproduction. Dark sand

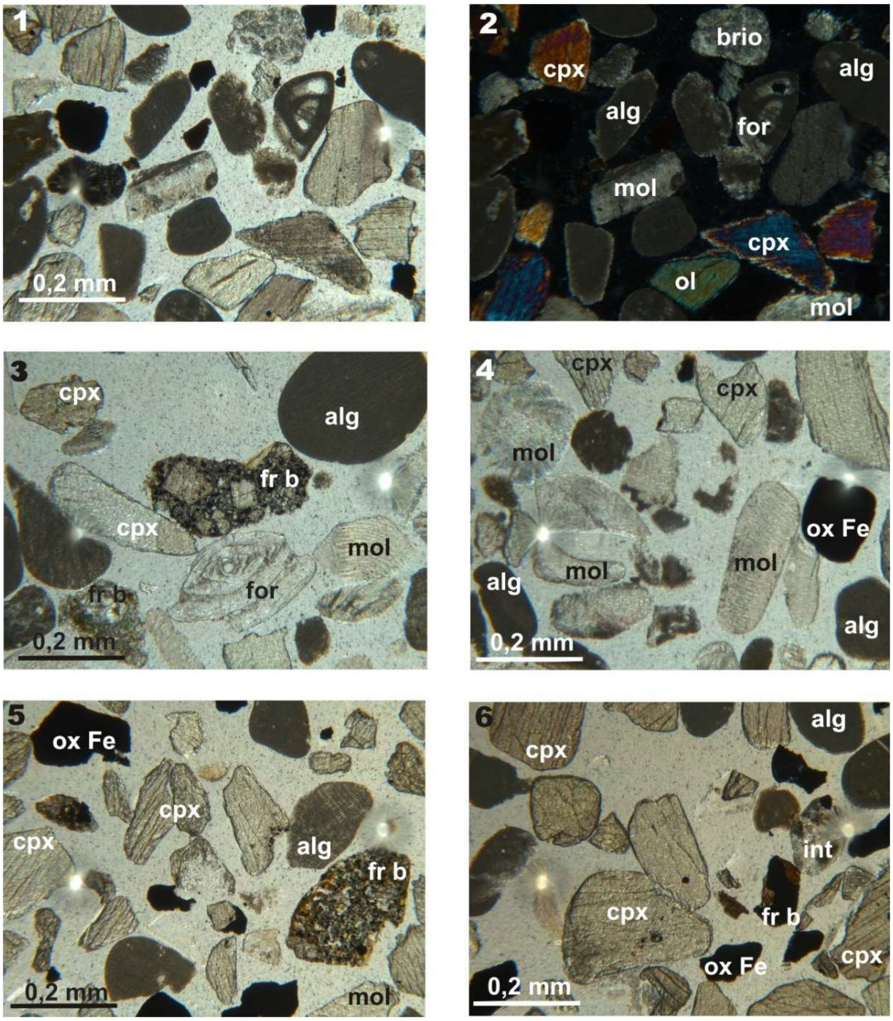

FIGURE 5 | Petrographic microscope photographs at 400× magnification of the samples from the light (**1** and **2**), mixed (**3** and **4**), and dark (**5** and **6**) sands. The difference between polarised light (parallel nicols, Photo 1) and plane light (crossed nicols, Photo 2) is illustrated. Alg, meshes of red algae; mol, molluscs; brio, bryozoans; for, foraminifera; cpx, clinopyroxene; ol, olivine; Fe ox, Fe and Ti oxides; fr g, basalt rock fragment; int, intraclasts.

caused significantly lower hatching success and thus may lead to reproductive loss of some populations. Embryo death tended to occur at an earlier stage of development in the dark-coloured substrate, whereas in the light-coloured sand embryo death occurred mostly at the end of development. The reason for this difference could be desiccation and late death by dehydration, which has been described in coarser sand substrates (Mortimer, 1990; Warner et al., 2011). However, our experimental results, with controlled temperature and humidity, do not support this theory. Dark-coloured substrates have been associated with negative impacts for embryo survival by direct association with high incubation temperatures (Weber et al., 2012; Martins et al., 2020). Interestingly, divergence in the thermal tolerance of female green turtles with philopatry to both dark and light-coloured beaches has also been demonstrated (Weber et al., 2012). Here we have demonstrated experimentally at controlled temperatures that an inherent effect of the substrate determines differences in embryonic development, hatching success, and hatchling performance regardless of temperature. The fundamental differences between the substrates analysed, other than their colour, were the grain size and the composition of calcium carbonate (different origins; dark substrate from volcanic material and light substrate from biogenic material). A higher fraction of fine sand, as in our dark coloured substrates, could hinder gas diffusion and generate lethal hypoxia during embryonic development. On the other hand, the lower percentage of calcium carbonate in this type of sand generates questions about the optimal composition for sea turtle nest incubation substrates. It is possible that the volcanic substrate composition itself may cause dehydration, leading to the death of the embryos (Marco et al., 2017). The mechanism responsible for this lower hatching success and the association with biotic factors linked to each substrate, e.g., microflora, is still under study for sea turtles (Elshafie et al., 2007).

Adaptive Substrate Selection

The incubation substrate on the island of Maio is dynamic, varies temporally and spatially, and consequently it would be difficult for females to choose a specific substrate before their arrival to the beach. The results of this study suggest that females select all sand types for nesting without preference, as no significant difference was found in either nesting success or nest density between beaches with different substrates. Nesting success indicates the number of nesting attempts on the beach that culminate in the effective laying of a nest (there is a proportion of unsuccessful attempts). Nesting success was surprisingly very similar for the different substrates (56% on dark sand, 56% on mixed sand, and 57% on nearby beaches light sand). This suggests that the abandonment of the laying process is not due to the type of substrate present on the beach chosen by the female. Other factors, not sand type, appear to be more important determinants for the choice of the laying site (Wood and Bjorndal, 2000). Therefore, extreme philopatric adaptation to one type of substrate or beach seems unlikely for loggerhead turtles in Maio. The most plausible adaptive mechanism to ensure the survival of some of their embryos seems to be the selection of different nesting beaches for different clutches, with their inherent differences in substrate type, to spread the risk of nest failure (Patino-Martinez et al., in Press).

Hatchling Phenotype and Performance

We found inter-substrate differences in hatchling biometrics and performance. Hatchlings from dark substrates had smaller carapace dimensions than hatchlings from light sand, suggesting that more yolk was converted to hatchling tissue (Ischer et al., 2009) during embryonic development in light sand nests. The relationship between substrate type and hatchling survival may be complex. Previous work has suggested that larger, more vigorous offspring may be better prepared for survival than smaller, weaker offspring (Miller et al., 1987; McGehee, 1990; Packard, 1999; Reece et al., 2002).

Hatchlings from light sand tended to be faster self-righters than hatchlings from dark sand, which could have a further effect on their survival. The amount of time required for a hatchling to self-right is negatively correlated with its physiological condition (Kobayashi et al., 2017). Globally, sea turtle hatchlings are vulnerable to predation by ghost crabs, mammalian predators, and shorebirds (Antworth et al., 2006; Ischer et al., 2009; Marco et al., 2015), and the time of exposure to these predators is inversely related to the ability to self-right when necessary. Assuming a fixed amount of energy to share between embryonic development, nest emergence, beach crawling and swimming offshore (Rusli et al., 2016), those hatchlings that take longer and expend more energy to reach the sea will reduce their subsequent swimming performance (van de Merwe et al., 2013). Interestingly, recent studies found significant correlations between self-righting propensity, crawling speed, and swimming performance in sea turtle hatchlings (Saito et al., 2019; Stewart et al., 2019). It is crucial to have a strong swimming performance once they enter the ocean because of their vulnerability to predation (Pereira et al., 2011; van de Merwe et al., 2013). Therefore, substrates that optimise the performance attributes of sea turtle hatchlings, as light sand did in our study, increase the likelihood of survival in this critical phase of high mortality.

Sand Type and Ghost Crab Abundance

In Maio, about 42% of loggerhead turtle nests suffer from ghost crab predation (Patino-Martinez et al., 2021), making it one of the most important threats to reproductive success on the island, and a strong selection pressure (Skelhorn et al., 2011). In this study, crabs that came to our bait (non-viable eggs from hatched nests, and fish) were of equivalent size and abundance between substrate types. Therefore, if we assume that these two factors determine predation rate, we could conclude that predation risk is similar between substrates. However, here there may be a benefit linked to the dark colour of the hatchlings on dark-coloured substrates where they may be more difficult to detect. One of the anti-predatory traits of some species is to resemble, sometimes almost imperceptibly, the local environment (Skelhorn et al., 2011). On the other hand, the benefit of resembling the environment has been shown to diminish as local prey density increases relative to their camouflage environments (Skelhorn et al., 2011). In this case the decrease in offspring density, resulting from lower hatching success, could decrease predator motivation and provide a density-dependent benefit. Further experimental studies are needed to evaluate predator density and their activity in microhabitats with thermal variability (due to sand type) and assess the effect on nest and hatchling predation.

Incubation Substrates and Climate Change

The Republic of Cabo Verde hosts one of the two largest loggerhead nesting populations in the world (Patino-Martinez et al., 2021), hosting up to 100,000 nests per season during the period of this study. All of the islands in Cabo Verde have some proportion of nesting beaches with dark-coloured sand (Tanner et al., 2019). Assuming an equal distribution, we estimate that 10% of nests currently show a strong decrease in hatching success due to dark incubation substrates. Under probable emission scenarios, lethal temperatures in dark incubation substrates on the islands will be reached before 2100 (Tanner et al., 2019). Furthermore, dark-coloured substrates, and therefore higher incubation temperatures, will only support the globally female-biassed hatchling sex ratios due to anthropogenic global warming (Hays et al., 2014).

Eggs incubated in light-coloured, biogenic substrates with high carbonate levels, larger grain size and lower temperatures are more successful than those on other substrates. They also experience better embryonic development and produce hatchlings in better physical condition. Therefore, in spatially heterogeneous environments, such as the island of Maio, sea turtles may be exposed to contrasting selective regimes that may cause divergence in traits affecting survival and reproduction (Mickelson and Downie, 2010). For example, given predictions of future climate change, adaptive variation in enhanced heat tolerance may have important adaptive consequences in an increasingly warmer world (Weber et al., 2012). Many conservation programmes consider thetranslocation of turtle nests on or between beaches, or to hatcheries, as a useful technique. In the face of foreseeable climate warming scenarios, we propose adaptive management of nests using light-coloured substrates, and at depths slightly deeper than the natural average (for a further decrease in incubation temperature). The aim is to favour a higher primary production of males, which can ideally become reproductive adults and to favour a future colonisation of new, thermally appropriate nesting areas, as an adaptive mechanism.

CONCLUSION

This study investigates (i) the selection of different incubation substrates by loggerhead turtle nesting females and (ii) the reproductive effects associated with incubation. We provide convincing evidence that the benefit of incubation on light-coloured, biogenic-derived sands is greater than on dark-coloured, and volcanic-derived sands. Our results strongly suggest that greater embryonic development and hatching success (a direct measure of fitness), with larger and more physically fit hatchlings (a possible determinant of early survival), implies a reproductive advantage. We recommend replicating this study with other sea turtle species and in different ecosystems. The effect of substrate on predator activity and the intrinsic components of substrate that affect embryonic development are aspects that we suggest investigating in the future. We propose an adaptive management technique of nest incubation on light-coloured substrates (beach or hatchery) and at a greater depth, to favour hatching success, hatchling quality, and to slightly lower incubation temperatures to help mitigate the effects of feminisation linked to global warming.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this research submitted to Frontiers in Ecology and Evolution comply with the journal's Code of Conduct for authors contributing articles. This study does not involve human subjects, experimentation with animals, and/or collection of specimens. All necessary DNA (Direcçao Nacional do Ambiente) authorisations were received. The animal experimentation standards used to protect the species were followed.

AUTHOR CONTRIBUTIONS

JP-M: conceptualization, writing-original draft, and supervision. JP-M, JV, IA, KY, JM-V, and GC: methodology and writing-review and editing. JP-M, JV, JM-V, and IA: formal analysis. JP-M, JV, IA, KY, and GC: investigation. JM-V: lab analysis. JP-M, JV, and GC: project administration. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.823118/full#supplementary-material

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Resilient Eggs: Highly Successful Loggerhead Sea Turtle Nesting Sites Vary in Their Characteristics

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Sea turtle nest success, defined as the number of eggs in a nest that successfully hatch and emerge, is closely linked to environmental conditions. Interacting biotic and abiotic factors influence hatching and hatchling emergence success. To date, combinations of multiple factors interacting together, which result in highly successful sea turtle nests are not well understood. Using 25 years of historic nest data and local expert experience, we identified five historically successful loggerhead (*Caretta caretta*) nesting beaches (hotspots) along the Florida (United States) Atlantic coast and measured nest environments along with the nest success. Principal component analysis was used to reduce 12 environmental variables so that the relative contributions of sand characteristics, nest temperatures, sand moisture, and nest location were considered. The nest environments differed among nesting beaches and were broadly segregated into two distinct climates: subtropical (hot and humid) and warm-temperate (warm and dry). We found that nests at subtropical sites, compared with warm-temperate sites, were characterized by environmental gradients in contrasting ways. Nest locations were predominantly mid-beach in subtropical sites but clustered at higher elevations and closer to the base of the dune at warm-temperate climate sites. Collectively, highly successful nest hotspots represent a mosaic of abiotic factors providing conditions that promote successful hatching and emergence. This new perspective on consistently successful loggerhead nesting beach traits demonstrate that the key traits of sea turtle nesting habitat vary with prevailing climate type and should be managed accordingly.

Keywords: sea turtle, nesting beach, loggerhead, incubation, nest success and survival, turtle eggs, nest, clutch temperature

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INTRODUCTION

Much of our understanding of sea turtle production is based on nest surveys and experiments done on nesting beaches. Focus on nesting beaches is largely due to the ease of access to nesting females, incubating eggs, and the nests; these studies contribute to sea turtle population growth and stability (Fuentes et al., 2013; Butt et al., 2016). While various sensitivity analyses highlight the importance of survival of later life stages found at sea (Crouse et al., 1987; Crowder et al., 1994), life history stages are necessarily linked (Congdon et al., 1993; Heppell, 1997). Therefore, the deterioration of nesting habitat due to coastal development, erosion, and climate change poses a prominent threat to sea turtle populations (Mazaris et al., 2009; Rizkalla and Savage, 2011; Witherington et al., 2011; Lyons et al., 2020). Assessments of nesting beaches frequently focus on the factors that lead to nest failure

(National Marine Fisheries Service and U.S. Fish and Wildlife Service, 2008) while not considering the multiple factors that together define successful nests.

The distributions of successful sea turtle nests are affected by several abiotic and biotic factors acting on different spatial scales. Abiotic factors include large-scale (regional) climates (i.e., subtropical, warm-temperate) to local-scale variables such as beach morphology and sand characteristics. At regional-scales, temperature and precipitation are the main determinants driving the distribution and temporal window of suitable nesting habitats (Pike, 2007, 2013). For example, sea turtle embryonic development is thermally constrained to 24–35°C (Miller, 1985).

Whereas regional-scale factors are important drivers of nest success, their effects may be mediated locally. On local scales, the nest microclimate varies with clutch sizes, nest location, sand characteristics, temperature, and available moisture. Precipitation can mitigate the effects of extreme warm temperatures by evaporative cooling of the nest and eggs (Tezak et al., 2018; Lolavar and Wyneken, 2021). These factors may be critical because they affect the likelihood that the nest is inundated by seawater or exposed to extreme temperatures (Horrocks and Scott, 1991; Zare et al., 2012; McElroy et al., 2015), as well as gas exchange and metabolic processes of developing embryos (Ackerman, 1997). For example, the metabolic heat produced by developing embryos increases during the final third of incubation and contributes to overall clutch temperature, typically above adjacent sand temperatures at an equivalent depth (Godfrey et al., 1997; Broderick et al., 2001). Nests with larger clutches establish greater thermal flux between sand and the egg chamber as metabolic heat increases (Ackerman, 1980). Sand characteristics, particularly bulk density, reflect the percentage of air in a known volume of sand, which, in turn, affects the gas exchange processes of the nest (Ackerman, 1997). Generally, eggs laid in sands with higher bulk density may suffocate the developing embryos or, if the embryos complete development, impede the hatchlings' emergence (McGehee, 1990; Mortimer, 1990; Ackerman, 1997). Nest location along the landward-seaward axis ultimately may determine whether eggs incubate in a suitable environment. A nest's elevation must be high enough to prevent it from being inundated by high tides, ground water, wave runup, or eroded (Katselidis et al., 2013). However, high-elevation nests may also experience higher temperatures (Ackerman, 1997; Wood and Bjorndal, 2000; Matsuzawa et al., 2002; Zárate et al., 2013). Thus, nest success is not dependent upon a single environmental factor but rather a combination of interacting regional- and local-scale factors.

Florida's sea turtle nesting beaches extend along much of the peninsula's Atlantic coast and to a smaller extent along the southwest coast and panhandle. Most loggerhead turtle (*Caretta caretta*) nesting activity in the United States occurs in the southeastern coastal region with ∼90% of the Northwest Atlantic subpopulation nesting in Florida (Dodd, 1988; National Marine Fisheries Service and U.S. Fish and Wildlife Service, 2008; Casale and Tucker, 2017). Florida beaches provide critically important nesting habitat for loggerheads, yet that habitat is being compromised by "coastal squeeze" associated with urban development, alongside the encroachment of rising seas which further reduces available nesting habitat (Mazaris et al., 2009). Beach modifications to protect beachfront property via coastal armoring (e.g., seawalls and revetments) may impede beach access for nesting females. The installation of seawalls may also change beach morphology making the nesting areas less suitable with lower profiles and more frequent inundation (Basco, 2006; Ells and Murray, 2012). Within this context, protecting and maintaining high quality nesting habitat is imperative (National Marine Fisheries Service and U.S. Fish and Wildlife Service, 2008). To address this goal, essential first steps must include both identifying areas of high nest success and the regional- and local-scale environmental factors associated with that success.

Multiple studies assessed the role of the nest environment on sea turtle embryo mortality (e.g., Foley et al., 2006; Awong-Taylor et al., 2008; Camargo et al., 2020). However, information regarding the environment of highly successful nests is sparse, and typically restricted to just a few factors. Here we identify factors that define highly successful loggerhead nesting habitats and address how loggerhead nest success is distributed across nesting beaches along Florida's Atlantic Coast. We noted diversity in regional- and local-scale variables most strongly correlated with successful nests and compared successful nest environments among nesting beaches. The study described here draws approaches from ecological developmental biology and beach geomorphology. To enhance clarity, we provide a glossary to the terms that may be specific to one field or the other (**Supplementary Table 1**). We discuss multiple biotic and abiotic factors acting simultaneously with regional- and local-scale and how they contribute to the success of loggerhead clutches.

MATERIALS AND METHODS

Historical Nest Success

Five highly productive loggerhead nesting beaches were selected based on their consistent long-term sea turtle nest success and beach data (**Figure 1**). Red Reef Park, Boca Raton (RR), Juno Beach (JB), Hutchinson Island (HI), Archie Carr National Wildlife Refuge (AC), and Canaveral National Seashore (CC) span more than 2 degrees in latitude (**Figure 1**). Together, these nesting beaches span approximately 100 km of contiguous Florida loggerhead nesting habitat. The latitudinal breadth crosses the bioclimatic transition zone between subtropical and warm temperate climates (28°00′N). We designate the southeast sites (RR, JB, HI) as in the "subtropical" region and the central east coast sites (AC, CC) as in the "warm-temperate" region, based on relative geographic positions and climatic regions.

Previous studies indicated that individual loggerhead females are capable of nesting for a minimum of 25 years (National Marine Fisheries Service and U.S. Fish and Wildlife Service, 2008), therefore we analyzed 25 years of nest locations and post-emergence nest inventory data spanning 1994–2019. We obtained the study sites' systematic historical data from data owners that follow the Florida's Index Nesting Beach Survey and Statewide Nesting Beach Survey programs

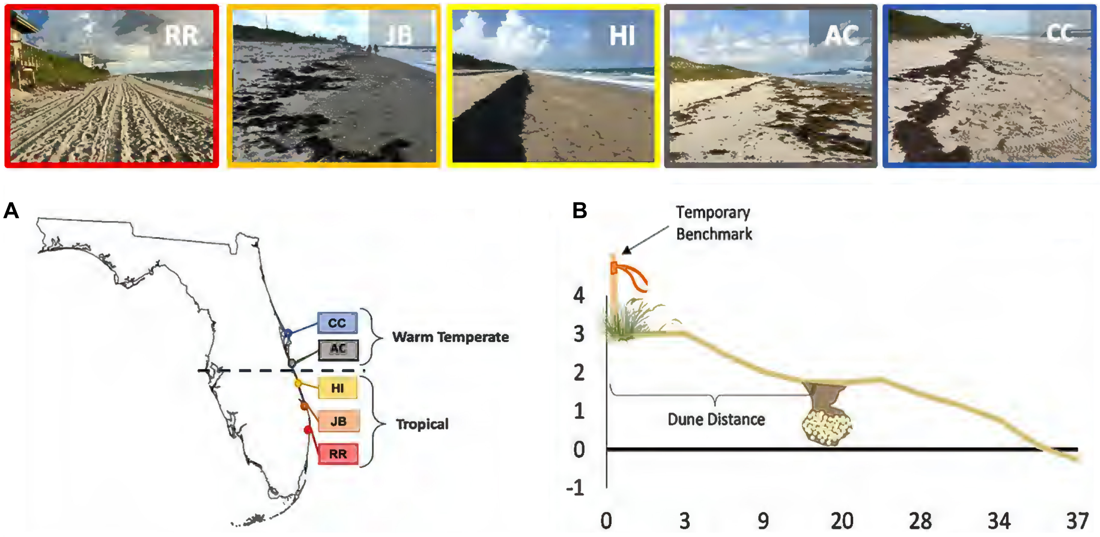

FIGURE 1 | Site map with nesting habitat illustrated for each beach. **(A)** Study beaches by location relative to the bioclimatic break shown by dashed line. **(B)** Diagram profiling a nesting beach with a clutch located at the middle beach. The temporary benchmark was used to establish beach morphology transects. Red Reef Park, Boca Raton (RR), Juno Beach (JB), Hutchinson Island (HI), Archie Carr National Wildlife Refuge (AC), and Canaveral National Seashore (CC).

(Florida Fish and Wildlife Conservation Commission, 2016) for all but one site. The most productive nesting beach areas in AC were identified by the long-term sea turtle survey staff (C. Long, personal communication).

Surveyors verify and mark each clutch location, then record its location with either GPS coordinates or zone and physical landmarks. For nests without latitude and longitude data, we approximated locations by randomly generating a point within the spatial boundary of the zone noted by the surveyor. All historical data were evaluated for potential inconsistencies prior to analyses. We excluded data that were incomplete or manipulated in at least one of the following ways: (1) the nest was not located at the time of inventory; (2) surveyors gave the nest special experimental treatment; (3) a storm event destroyed the nest; (4) the clutch was relocated; (5) nest inventory data had gaps for certain years (i.e., loggerhead nests were not evaluated, only nest counts were recorded). The "imputeTS" package in R estimated missing data using a weighted moving average (Moritz and Bartz-Beielstein, 2017).

To reconstruct hatching and emergence success time series and to define the threshold values of "highly successful" hatching and emergence rates, we used a locally weighted scatterplot smoother (Cleveland et al., 1990). Mean monthly hatching and emergence success was modeled as a function of year. We determined the threshold values of highly successful nests as being greater than or equal to the moving average produced by the model (**Table 1**). To identify hotspots for highly successful nests across years, we analyzed historical data with the heatmap plugin for QGIS version 3.10 (QGIS Development Team, 2019). "Heatmap" generates a density raster through kernel density estimation from sea turtle nest positions. All 25 years of nesting data were represented as a single input layer to generate the overall trend. Nests with success rates lower than the moving average of its respective site were excluded, ensuring that only highly successful nests were plotted on the heatmap.

Nest Parameter Surveys

To characterize the nest environment at these historically successful sites, we measured several biotic and abiotic parameters in 10 nests at each of the five study sites during the 2020 nesting season ($N = 50$ nests). Sampling spanned June–August 2020 when most loggerhead turtle nests were incubating. Clutch temperatures were recorded using HOBO-U22 temperature dataloggers (accuracy ± 0.2°C, resolution ± 0.02°C per manufacturer's specification and verified empirically; Onset Computer Corp., Bourne, Massachusetts, United States). The dataloggers were placed in the middle of the clutch with minimal disturbance by gently excavating the sand from the top of the clutch, removing approximately 40–50 eggs within a few hours of deposition, protecting the eggs from heat and desiccation while inserting the datalogger, then replacing the eggs in the order they were removed, followed by replacing the sand (after Lolavar and Wyneken, 2015). All dataloggers were placed within 12 h of oviposition to maintain embryonic viability while handling the eggs (Limpus et al., 1985; Rafferty et al., 2013). Nest temperatures were recorded every 15 min and the dataloggers were retrieved at nest inventory. To account for the metabolic heat produced by developing embryos, a control datalogger was buried at each site at a depth (~45 cm) representing the approximate middle of a loggerhead egg chamber (Maloney et al., 1990; Matsuzawa et al., 2002; Lolavar and Wyneken, 2015). The control datalogger for each site was buried within 2–3 m of a study nest at the same approximate beach elevation. The sand site was selected randomly among marked nests early in the sampling period without a nearby nest. Daily mean sand or clutch temperatures and maximum temperatures were calculated for the incubation period for all nests and controls.

Depending on site-specific and nest-specific protocols, nests were excavated and inventoried either the day of first major hatchling emergence or 3 days after first emergence. If there was no sign of emergence, nests were excavated 70 days after oviposition per Florida Fish and Wildlife Conservation Commission (2016). Clutch inventory included the number of hatched eggshells, dead and live hatchlings in nest, and the number of unhatched eggs.

Hatching (HS) and emergence success (ES) were determined using the following formulas:

$$\mathrm{HS} = \frac{\mathrm{S}}{\mathrm{C_i}} \times 100 \tag{1}$$

$$\mathrm{ES} = \frac{(\mathrm{S} - \mathrm{R})}{\mathrm{C_i}} \times 100 \tag{2}$$

S is the number of empty eggshells that are $> 50\%$ intact, C_i is the total number of eggs in the nest, and R is the number of hatchlings remaining in the nest at inventory (Miller, 1999). We also recorded the depth of the top egg and bottom of the egg chamber for chamber dimensions, and landward distance from the nest to the dune vegetation line or seawall.

Sand Characteristics

To analyze the sediment surrounding the egg chamber, ~350–500 g of subsurface sand were collected by hand from a hand-dug 45 cm hole along the periphery of each nest but not breaching the egg chamber. Replicate samples (3 replicates/nest × 10 nests × 5 sites $N = 150$ total samples) were collected at the beginning, middle, and end of incubation.

The field water content (θ) was determined gravimetrically by weighing a sample before and after drying in a 52°C oven for a minimum of 24 h (after Gardner, 1986).

$$\theta = \left[\left(\frac{\text{Wet Sand Mass (g)}}{\text{Dry Sand Mass (g)}}\right) - 1\right] \tag{3}$$

Particle density, bulk density, and sand porosity are factors that indicate the density of solid constituents, mass of material contained within a given volume, and amount of pore space contained in a sample, respectively (Mota, 2009). To calculate particle density, 40 g of dried sand was placed in a 100 mL graduated cylinder with 50 mL of deionized water ($\mathrm{diH_2O}$). After stirring to displace air, the water meniscus volume was recorded. This volume, minus the 50 mL initial water volume, equals the volume of sand solids. Particle density ($\mathrm{g \,.\, cm^{-3}}$) was calculated by dividing the weight of dry solid particles by the volume of sand solid particles.

$$\text{Particle Density}\left(\frac{\mathrm{g}}{\mathrm{cm^3}}\right) = \frac{\text{Dry Sand Mass (g)}}{\text{Volume of Water Displaced (cm}^3)} \tag{4}$$

Bulk density is the mass of soil per unit volume (= bulk soil volume; Tan, 1995) and was measured as the mass of sand required to fill a graduated cylinder up to 100 mL (= $\mathrm{cm^3}$).

$$\text{Bulk Density}\left(\frac{\mathrm{g}}{\mathrm{cm^3}}\right) = \frac{\text{Dry Mass of 100cm}^3\text{ of sand (g)}}{100\ (\mathrm{cm^3})} \tag{5}$$

Sand porosity is defined as percentage of sand volume occupied by pore spaces and was calculated as bulk density/particle density (Tan, 1995) converted to a percentage using the following formula.

$$\%\ \text{Pore Space} = 100^{*}\,\frac{(\text{Particle Density} - \text{Bulk Density})}{\text{Particle Density}} \tag{6}$$

The sand salinity was measured using a handheld conductivity meter (model HI98192, accuracy: ± 0.01 mS/cm, resolution: 0.001 mS/cm, Hanna Instruments, Woonsocket, Rhode Island, United States). To measure sand salinity, 40 g of dried sand was mixed with 200 mL of $\mathrm{diH_2O}$. The electrical conductivity of the sand solution, in microsiemens was converted to ppt (after Bennett et al., 1995; Foley et al., 2006).

Sand grain size distribution (i.e., size and sorting) of each sample was measured using a mechanical sediment shaker (RX-29 Ro-Tap, accuracy: ± 0.5-micron, resolution: ± 0.01 micron, W.S. Tyler, Mentor, Ohio) at half-phi sieve intervals between −4 and 2 φ and quarter-phi sieve intervals between 2 and 4 φ .

Percent calcium carbonate ($\%\mathrm{CaCO_3}$) was determined as the change in mass of a sample measured before and after mixing

TABLE 1 | Descriptive statistics for nest success that were made available for 1994–2019.

		RR (***n* = 5,018**)	**JB** (***n* = 4,510**)	**HI** (***n* = 1,018**)	**CC** (***n* = 2,861**)
Hatching success	Mean	71.45	74.12	66.86	72.3
	(± 95% CI)	(± 2.20)	(± 4.7)	(± 3.74)	(± 3.74)
	Max	82.3	82.0	79.1	70.9
	Min	44.1	49.4	35.8	56.1
Emergence success	Mean	67.84	70.97	64.65	70.43
	(± 95% CI)	(± 2.28)	(± 4.69)	(± 3.74)	(± 3.74)
	Max	78.8	79.3	76.4	70.8
	Min	39.9	45.5	33.1	56.0

Moving averages for 1994–2019 with measures of dispersion. No nest success data were made available directly for the Archie Carr National Wildlife Refuge site (AC). n, numbers of nests over the 25 years; CI, the confidence interval.

with 4N hydrochloric acid (HCl) and air-drying for a minimum of 24 h.

$$\%\mathrm{CaCO_3} = \left(\frac{\text{Mass After HCl (g)}}{\text{Mass Before HCl (g)}}\right) \times 100 \qquad (7)$$

Sand color was determined using a Munsell soil color book. Color charts were placed over the sand sample under a fluorescent lamp at a fixed distance and the corresponding chroma and hue were recorded.

Beach Morphology

The spatiotemporal variations in beach morphology were characterized by time series beach profiles in zones with historically high hatching and emergence success hotspots. Temporary benchmarks (wooden stakes in the dune field for the duration of the study, **Figure 1B**) were established for each site using a Real-Time Kinematic Global Positioning System (RTK-GPS, accuracy ± 1.0 cm, resolution ± 0.01 cm; Precision Agriculture, Boston, Massachusetts, United States). Each benchmark allowed for subsequent beach profile surveys to locate the same transect line for temporal comparisons provided an elevation control for the entire profile transect. Beach profiles were measured in April, June, and September 2020 and plotted as cross-sectional profiles to identify morphologic features including dune toe, shoreline position (0 m elevation), dry beach (from dune toe to shoreline), and foreshore (from shoreline to the waterline). To account for cross-shore variability, slope estimates were calculated: (i) between the dune toe and shoreline at the three sampling times. Beach width was calculated as the distance between the dune toe and waterline.

Data Analyses

All statistical analyses were performed in R Studio ver. 3.5.1 (R Core Team, 2018) and MS Excel version 16.46. The Shapiro-Wilkes (for normality) and Levene's tests (for equality of variances) were used to check sand characteristics, temperature, and beach morphology, hatching and emergence success, and clutch sizes data. A nested ANOVA tested for differences in hatching and emergence success among study locations. Region (subtropical, warm-temperate) was treated as a fixed effect, site nested within region was classified as a random effect. Due to the large number of environmental factors and the potential for high spatial autocorrelation (Griffith, 2011), a principal components analysis (PCA) was used for variables related to: (i) nest location and egg chamber dimensions, and (ii) nest microclimate (sand grain size, sorting, porosity, temperature, sand moisture, calcium carbonate content). The PCA reduced the dimensionality and collinearity of the standardized predictor variables collected in the field. The scores of the first two principal components were used as predictor variables in generalized linear mixed models (GLMM) of hatching and emergence success. Multiple models were fit using all possible combinations of PC scores and their interactions. Candidate models were compared using delta AIC scores, with a delta AIC threshold of 4 for model selection (Burnham and Anderson, 2002).

Bioethics

This study did not involve live vertebrates; no IACUC authorizations was needed. The study adhered to guidelines in the FWC Sea Turtle Conservation Handbook (Florida Fish and Wildlife Conservation Commission, 2016).

RESULTS

Historical Nest Success

Hatching and emergence success of 13,407 loggerhead nests from 1994 to 2019 were analyzed for RR, JB, HI, and CC nesting beaches (**Table 1**). Using the kernel density estimates of highly successful nests, a minimum of two hotspots were identified within each study site (**Figures 2A–E**). The surface area and number of the hotspots varied among the study beaches. Although overall hatching and emergence success values fluctuated from year to year, hotspot locations were relatively consistent. Data for AC were not shared for site selection; instead, staff with no less than a decade of experience conducting nest inventory at the site and working knowledge of the multidecadal nest inventory data provided landmarks for highly successful nest areas. Two hotspots within the historically productive areas of each nesting beach were selected for 2020 field measurements of nest sites and clutches.

2020 Nesting Season's Sand and Clutch Characteristics

Of the 50 clutches instrumented, measured, and inventoried, all were instrumented between 3 June and 15 August 2020 and inventoried between 28 July and 14 September 2020. All study nests incubated during the time period when most loggerhead turtle nests in the study region were incubating and hatching. Two major storm events occurred during the study: Hurricane Isaias (30 July to 5 August 2020) and Hurricane Laura (16–27 August 2020). During Hurricane Isaias, two nests at JB, one at AC, and one at CC experienced wave washover and inundation. At HI, two nests were severely depredated. These 6 nests were omitted from analyses. Hatching and emergence success summary statistics for the 5 sites is in **Table 2**. Neither hatching success nor emergence success differed among sites (**Supplementary Table 2**).

Details the local nest environments (clutch temperatures, sand temperatures, sand water content), beach morphology (beach slope, beach width), clutch characteristics (clutch size, top depth, bottom depth), nest location (dune distance), and sand characteristics (size, sorting, bulk density, particle density, porosity, $\mathrm{CaCO_3}$ content, salinity, color) are aggregated in **Supplementary Table 3**. Sand color varied relatively little among sites yet subtropical sites were warmer and had slightly darker sand than the warm-temperate sites.

Climate

Climatic variation spanned the study sites; those south of the climatic break (subtropical; BR, JB, HI) are characterized by hot, humid conditions while those north (AC, CC) have a

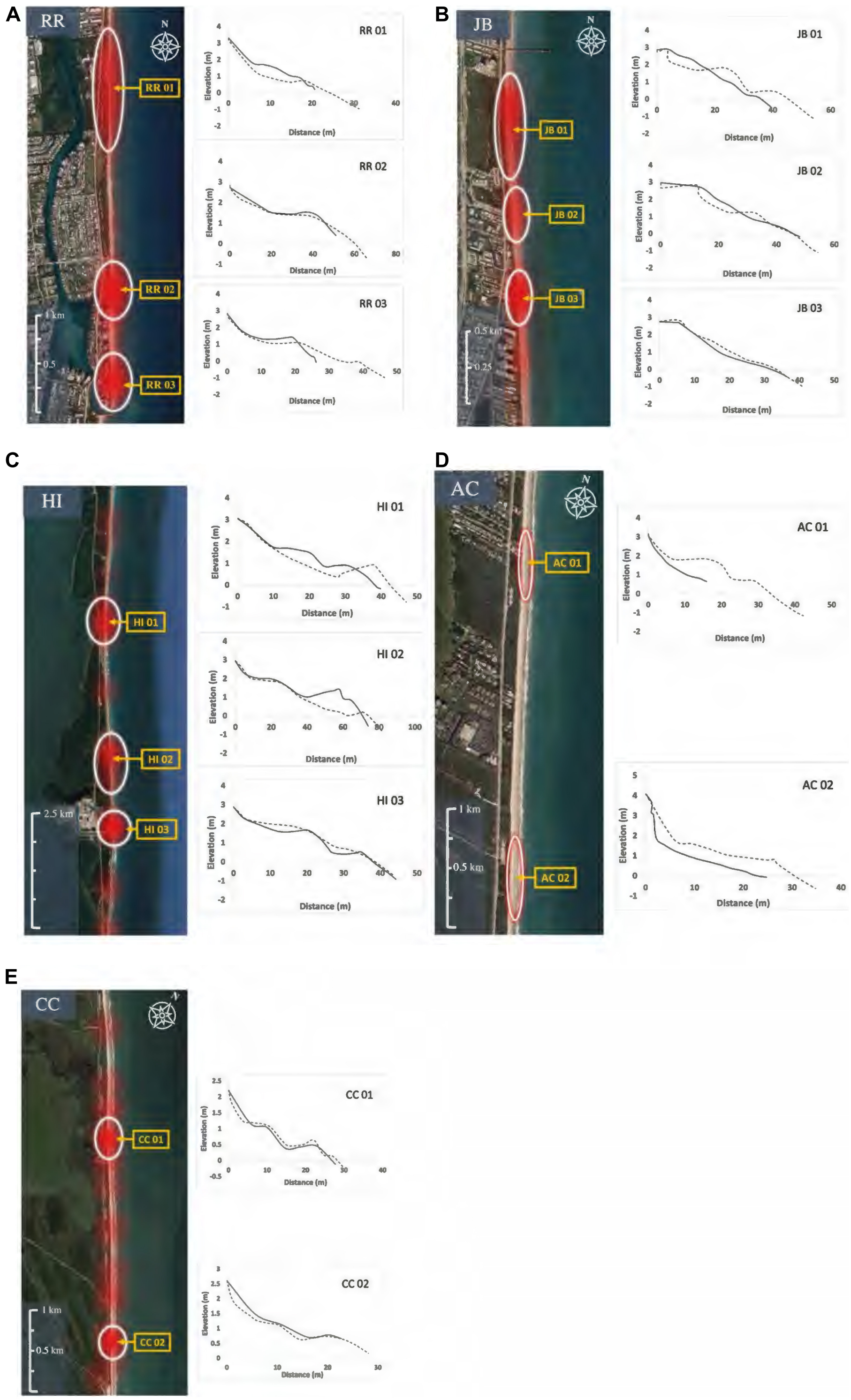

FIGURE 2 | (A–E) Heatmaps (QGIS 3.11) for highly successful loggerhead nesting areas. Hotspots are shown for Red Reef (RR), Juno Beach (JB), Hutchinson Island (HI), and Cape Canaveral (CC). Archie Carr National Wildlife Refuge (AC) hotspots were identified qualitatively through personal communication with long-term inventory staff. Loggerhead nests and sand were examined at two hotspots for each site. Transect locations for beach morphology measures are indicated with yellow arrows. Beach profiles corresponding with each transect are displayed to the right of each heatmap (June 2020 profiles—dashed line, September 2020 profile—solid line).

TABLE 2 | Descriptive statistics for hatching and emergence success at each nesting beach during the 2020 study period.

		RR (*n* = 10)	JB (*n* = 8)	HI (*n* = 8)	AC (*n* = 9)	CC (*n* = 9)
Hatching success	Mean	75.06	77.28	70.30	71.45	70.69
	(± 95% CI)	(± 1.34)	(± 3.32)	(± 3.15)	(± 7.20)	(± 2.66)
	Max	99.21	97.18	98.52	96.20	99.02
	Min	7.95	5.40	4.36	2.80	3.52
Emergence success	Mean	71.18	72.24	66.27	69.63	70.67
	(± 95% CI)	(± 1.51)	(± 2.92)	(± 2.71)	(± 7.47)	(± 2.66)
	Max	99.21	97.00	86.67	96.20	99.02
	Min	1.14	0.69	4.36	2.80	1.13

n, the number of nests inventoried per site.

TABLE 3 | Mean (± SD), minimum and maximum daily clutch temperatures and adjacent (control) sand temperature at 45 cm deep for the five study beaches during the 2020 nesting season.

		RR	JB	HI	AC	CC
Control sand temperature (°C)	Mean	32.1	32.3	32.8	29.7	30.4
	(± SD)	(± 1.1)	(± 1.2)	(± 0.8)	(± 0.9)	(± 1.0)
	Max	33.8	34.2	34.5	31.4	32.3
	Min	29.8	30.0	30.8	27.9	28.7
Internal clutch temperature (°C)	Mean	32.9	33.1	33.6	29.9	29.7
	(± SD)	(± 1.3)	(± 1.7)	(± 1.4)	(± 1.1)	(± 0.8)
	Max	35.6	35.9	36.2	32.7	31.2
	Min	30.0	30.3	30.6	27.4	28.5

The sites are designated as Red Reef Park, Boca Raton (RR), Juno Beach (JB), Hutchinson Island (HI), Archie Carr National Wildlife Refuge (AC), and Cape Canaveral National Seashore (CC). Note that minimum clutch temperatures usually were warmer than control sand, but maximum sand temperatures were cooler than clutches.

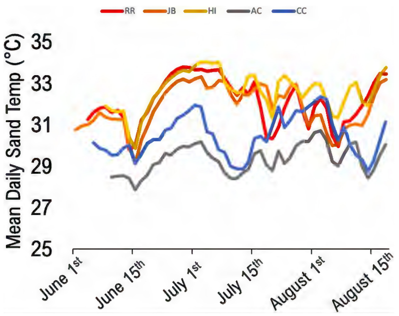

FIGURE 3 | Mean daily sand temperature profiles from 2020 control dataloggers. Gray and blue lines are north of the bioclimatic break in the warm-temperate regions. Red, orange, and yellow lines are south of the break (subtropical region) (*n* = 112 days).

drier and warm-temperate climate (**Figure 1**). Of the 44 nests analyzed, 60.5% were in the subtropical climate; the remainder were in the warm-temperate climate. Throughout the 2020 study period, clutches were warmer than the adjacent sand without eggs (**Table 3**). Daily mean sand temperatures of warm-temperate sites rarely exceeded 32°C, while subtropical regions often ranged from 32 to 35°C (**Figure 3**). Of the 44 analyzed clutches, 22 met or exceeded mean daily nest temperatures above 34°C and 12 of these nests experienced temperatures above 35°C. Generally, temperature profiles of the clutches were similar to those of each site's sand control during the first third of incubation. Some warming due to metabolic heating was evident by days 19–27 of incubation (**Figure 4**). During the last third of incubation when embryos are growing, metabolic heat generation is substantial (Hendrickson, 1958; Carr and Hirth, 1961) and clutch temperature surpasses the surrounding sand (**Figure 4**).

Sand moisture content differed between climates ($X^2 = 3.23$, $p = 0.001$); subtropical nests had a higher average sand-water content ($0.04 \pm 0.004\ \theta$) than warm-temperate nests ($0.03 \pm 0.0009\ \theta$).

Beach Morphology

The nesting beaches differed significantly in the physical beach characteristics. Warm-temperate sites were significantly narrower (Mann-Whitney $U = 9.96$, $n = 12$, $P = 0.008$) and had steeper beach slopes (**Table 4**) than the more southern subtropical sites. Overall beach slope was generally steeper in warm-temperate beaches than in subtropical beaches. The steepest slope was observed at AC along both hotspot transects during the late nesting season. In the warm-temperate region, beach slopes steepened June–September and slopes were more stable in the subtropical region (**Table 4**).

Egg Chamber Dimensions, Locations, and Clutch Sizes

Egg chamber dimensions differed between the regions. The mean nest depth of HI (top: 30.87 ± 8.11 cm, bottom: 52 ± 4.78 cm) was significantly shallower than JB (top: 44.35 ± 9.62 cm, bottom: 63.07 ± 4.28 cm) and CC (top: 46.89 ± 7.44 cm, bottom: 59.57 ± 11.2 cm; Kruskal-Wallis: 12.08, $P = 0.02$). Clutch size also varied among sites (Kruskal-Wallis: 13.55, $P = 0.008$). AC clutches (89.28 ± 10.59 eggs) were significantly smaller than RR (119.5 ± 33.58 eggs) and JB (117.1 ± 21.63). The distance between the nests and the dune toe was significantly greater at the subtropical sites (Mann-Whitney $U = 10.36$, $n = 43$, $P = 0.015$) so the nests were often in the middle of the open beach. In contrast, warm-temperate site nests tended to be clustered near the dune toe.

Environments of Highly Successful Nests

The first two principal components explained 96.2% of the variation in the loggerhead nest physical characteristics. The first component, PC1 (80.7% variance), represented nest microclimate, as it described an axis of variation in temperature, sand water content (moisture), bulk density, porosity, and $CaCO_3$ content. PC2 (15.5% variance), represented egg chamber characteristics, egg chamber depth, and clutch size. The biplots (**Figures 5A–C**) indicated the spread of successful nest characteristics into four quadrants. Nests ordered in quadrant I are large clutches, placed in loosely packed, moist sand with high carbonate content, and high clutch temperatures (negative PC1 coefficients and positive PC2 coefficients). Quadrant II nests have large clutches, placed deeply in densely packed sand, with low carbonate content, low moisture, and lower clutch temperatures (positive coefficients on PCs 1 and 2). Quadrant III describes small, shallowly placed clutches in loosely packed sand with high carbonate content and warm temperatures (negative coefficients on PCs 1 and 2). Quadrant IV nests are small, shallowly placed clutches in densely packed sand, with low carbonate content, low sand moisture, and lower nest temperatures (positive PC1 coefficients and negative PC2 coefficients). The nests grouped into two distinct environmental regimes with minimal overlap: the subtropical sites (RR, JB, and HI) in quadrants I and III, and the warm-temperate sites (AC and CC) in quadrants II and IV.

The model that explained the greatest variation in hatching and emergence success was a PC1 and PC2 interaction term (**Supplementary Tables 3**, **4**). The interaction between the principal components in the model indicates that the effects of nest microclimate and egg chamber dimensions are interdependent. At lower values of PC1, the higher values of PC2 (meaning deep, large clutches closer to the waterline) have a more important effect on hatching and emergence success.

DISCUSSION

Together, the interactions between nest microclimate and egg chamber characteristics highlight the diversity of environments in which loggerheads can successfully nest. The connections between sand and egg chamber characteristics are illustrated in the PCA analysis. Highly successful subtropical nests showed evidence that nest microclimate is strongly associated with success in this region. Key interactions were high calcium carbonate content, low bulk density along with high porosity (that allowed for high moisture to percolate through and still provide for robust gas exchange). The large variation in subtropical egg chamber characteristics supports previous evidence suggesting that nest depth is less likely to influence clutch temperatures in nesting areas that are closer to the equator (Van De Merwe et al., 2006). The highly successful

TABLE 4 | Beach morphology characteristics at all five study sites during 2020 nesting season.

	June 2020			September 2020		
	Beach slope	**Foreshore slope**	**Beach width (m)**	**Beach slope**	**Foreshore slope**	**Beach width (m)**
Red Reef						
Hotspot 1	1/14	1/13	31.24	1/15	1/12	20.62
Hotspot 2	1/6	1/11	51.12	1/5	1/12	66.37
Juno Beach						
Hotspot 1	1/7	1/11	56.24	1/8	1/8	46.15
Hotspot 2	1/8	1/11	49.12	1/9	1/12	39.02
Hotspot 3*	1/9	1/11	42.02	1/9	1/10	35.11
Hutchinson Island						
Hotspot 1	1/7	1/14	39.82	1/7	1/19	46.94
Hotspot 2	1/4	1/11	73.99	1/4	1/8	82.53
Hotspot 3*	1/7	1/11	44.27	1/7	1/12	43.35
Archie Carr						
Hotspot 1	1/9	1/13	42.19	1/14	1/11	21.61
Hotspot 2	1/13	1/16	34.81	1/18	1/4	24.75
Cape Canaveral						
Hotspot 1	1/8	1/13	29.93	1/8	1/12	27.74
Hotspot 2	1/9	1/11	28.11	1/13	1/12	22.71

*Each highly successful nest hotspot was measured in June and September 2020 to capture temporal variability as a snapshot. * Indicates hotspots that were identified and characterized but not used for nest sampling.*

warm-temperate nests were shallow with smaller clutches (e.g., restricted to negative PC2 values) which may reflect a different set of incubation compromises. Temperature differentials between the nest and surrounding sand differed between the warm-temperate sites, suggesting that warm-temperate nests not only warm differently, but the embryos of this subpopulation may differ on physiological and developmental levels.

For sea turtles, population recruitment occurs over a large spatial scope. Consequently, understanding the factors that lead to successful hatching and emergence can enable us to better direct conservation efforts (Mazor et al., 2013). Through a reconstructed time-series of loggerhead hatching and emergence success spanning 25 years (roughly a generation), we identified nesting beach sites that were consistently productive along Florida's east coast. Average hatching and emergence success in 2020 were considered as high relative to historical moving averages. Despite high variability in incubation environments among sites and climatic regions, hatching and emergence success were similar indicating that loggerhead eggs are resilient to diverse beach, sand, and nest environmental conditions (thermal, moisture, and porosity/potential for gas exchange) across time and space. The study spanned a subpopulation within the loggerhead turtle peninsular Florida Recovery Unit.

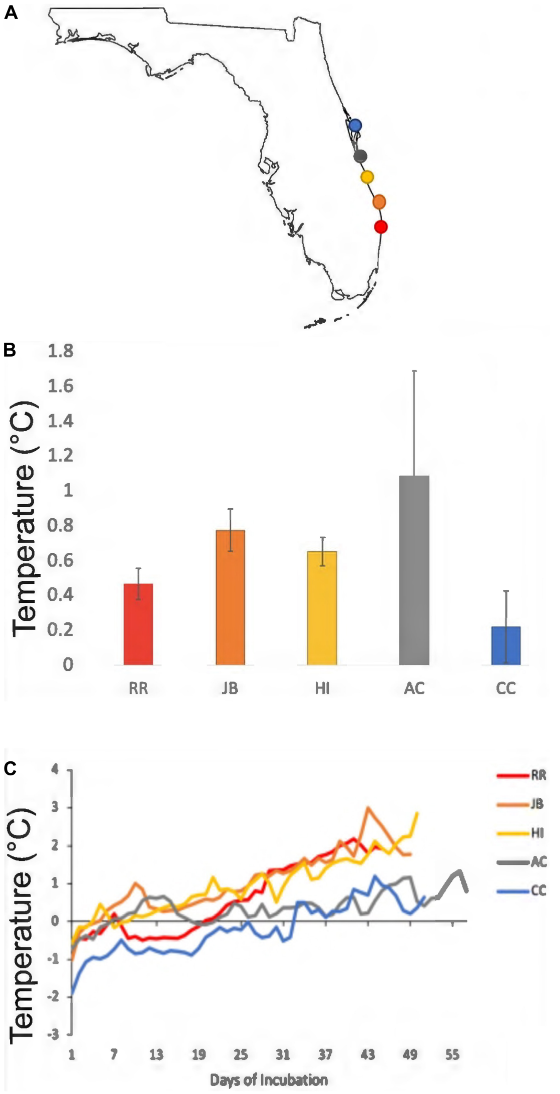

FIGURE 4 | Mean daily differences between internal clutch temperatures and control sand temperatures from the 2020 nesting season. **(A)** Gray and blue colors are the warm-temperate sites. Red, orange, and yellow colors are the subtropical sites. The colors are the same in **(B,C)**. **(B)** The mean temperature differentials between internal nest and control sand for each study site. Error bars represent 95% confidence intervals. **(C)** Differences between daily mean clutch and sand temperature throughout incubation durations (days). Note how nest temperatures exceed surrounding sand temperatures due to metabolic heating, and how metabolic heating is more pronounced in subtropical nests compared to warm-temperate nests.

While hatching and emergence success can be highly variable on both spatial and temporal scales (Brost et al., 2015), distinct hotspots of high success were identified at each of the five nesting beaches and the hotspot locations remained relatively stable across the 25 years of historical loggerhead nesting, indicating that nests are consistently more successful in certain locations in any given year. The apparent consistency of hotspot locations aligns with previous studies on loggerhead nesting in Florida (Weishampel et al., 2003), which showed that nest distributions were non-random across east-central beach sites. However, here we identified patch distributions of nests on multiple beaches. These patch distribution patterns suggest that the hotspots identified in this study have characteristics that are important in loggerhead nest site selection, yet the generalities differed between the management units and between the bioclimatic regions. Although this study did not manipulate specific environmental factors and did not track the nesting behavior of individual turtles, the approach illustrates the suites of environmental factors that together were associated with high nest success. Because hatching and emergence success are necessarily linked, the study results indicate that highly variable environments can allow for both complete development and the hatchlings' competence to escape the nest chamber.

This study allows for informed inferences about the characteristics of highly successful nests. In particular, success was not constrained within a narrow window of environmental conditions. There were variations in sand characteristics, nest locations, and egg chamber dimensions within and across climatic regions. This environmental variation is not surprising, as nesting beaches are dynamic environments that may change in width and slope within and among seasons. Individual nesting turtles spread their reproductive effort (as multiple clutches) over time and space within seasons and across years, via multi-year remigration intervals (Carthy et al., 2003; Schroeder et al., 2003). These life history traits, along with the wide latitudinal breadth of loggerhead nesting assemblages, suggest that the species has evolved to successfully nest under diverse combinations of conditions. Previous studies documented how critical tolerance limits nest success and can vary among sea turtle species, incubation scenarios, and populations (Drake and Spotila, 2002; Howard et al., 2014; Montero et al., 2018; Gatto et al., 2021).

The value of this evaluation of highly productive nesting sites identifies important locations used by southeastern management

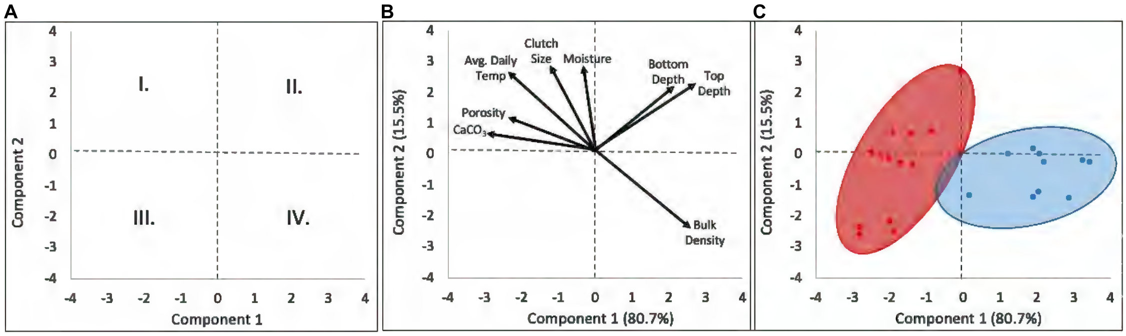

FIGURE 5 | The biplots indicated the spread of successful nest characteristics into four quadrants. **(A)** Quadrant I represents large clutches, placed in loosely packed, moist sand with high carbonate content, and high clutch temperatures (negative PC1 coefficients and positive PC2 coefficients). Quadrant II represents large clutches, placed deeply, in densely packed sand, low carbonate content, low moisture, and lower clutch temperatures (positive coefficients on PCs 1 and 2). Quadrant III describes small clutches, located shallowly, loosely packed sand, high carbonate content, warm temperatures (negative coefficients on PCs 1 and 2). Quadrant IV represents small clutches, placed shallowly, densely packed sand, low carbonate content, low sand moisture, and lower nest temperatures (positive PC1 coefficients and negative PC2 coefficients). **(B,C)** Note that the subtropical sites (red) were clustered in quadrants I and III (negative PC1), and the warm-temperate sites (blue) clustered in quadrants II and IV (positive PC1). See text for further discussion.

unit loggerheads (RR, JB, HI) and AC and CC provide similar information for the central eastern management unit. At a regional scale, there is a bioclimatic break at 28°N latitude (Troast et al., 2020). This climatic boundary is located between the HI and AC sites and is defined by large-scale temperature and precipitation regimes. Superimposed upon this bioclimatic separation are subtle but identifiable genetic differences. Shamblin et al. (2011) found genetic partitioning of the Peninsular Florida Recovery Unit into six regions that are treated as management units. Two of the management units: Southeast and Central East Shamblin et al., 2011; Ceriani et al., 2019). Perhaps each MU has functional adaptations to these bioclimatic regions as well.

The incubation temperatures at the different sites were among the most notable environmental differences. Nests in subtropical sites generally were 2–3°C warmer than at warm temperate sites (**Figure 4**). For loggerhead sea turtles in the southeastern United States, embryonic mortality tends to increase at incubation temperatures that reach 34–35°C (McGehee, 1979; Matsuzawa et al., 2002). McGehee (1979) worked with the Central East MU at a site located between the AC and CC and reported that loggerhead nests incubating at 32°C had a hatching success of 71%, while those incubating at 35°C had no surviving embryos. Several nests from the Southeast MU (which is in the subtropical regions) in our study experienced temperatures exceeding this 33–35°C range, with 33% of all nests warming beyond 35°C at some point during incubation. Differences between sand temperatures may be attributed to sand color, with dark beaches being warmer than light beaches (Hays et al., 2001; Laloë et al., 2014). Despite the differences in maximum temperatures, there were no significant differences in hatching and emergence success between warm-temperate and subtropical sites, implying that other factors, in concert, mediate the potential detrimental effects of high temperatures on embryos. Our analyses of nest traits alongside the other physical characteristics of the nest chamber, sand, and beach traits suggest that inclusion of multiple abiotic interactions more accurately characterizes suitable nesting habitat than individual ranges of "optimal" conditions.

Previous studies established clutch temperature, sand moisture, and sand physical characteristic thresholds beyond which embryonic development begins to deteriorate (Miller, 1985; Ackerman, 1997; Foley et al., 2006; Howard et al., 2014). However, the effects of these three variable types are interdependent along Florida's dynamic Atlantic coast. For example, high sand moisture was associated with lower nest temperatures; previous research identified that moisture mitigates high temperature through evaporative cooling of the nest substrate (Lolavar and Wyneken, 2017, 2020) or eggs (Tezak et al., 2018). RR, JB, and HI nests had higher levels of sand moisture than those in the warm temperate region. To the extent that moisture mitigates the deleterious effects of high incubation temperatures, hatching and emergence success from these nests that were few degrees warmer than published lethal limits for a cheloniid that nests on the same beaches (*Chelonia mydas*; Laloë et al., 2014). Nevertheless, the difference in water content in this study has two important limitations: (1) the sand moisture measured represent snapshots in time of how nest hydration varied throughout incubation, and (2) while water content provides an index of moisture availability to eggs, sand water potential, which indicates the tendency of water to move, was not measured. Despite these limitations, the greater sand moisture content found within the subtropical region suggests that nests incubating at or above upper thermal tolerance limits necessitate more moist conditions to survive.

The observed regional differences in egg chamber characteristics may reflect sand characteristics. Nest excavation and egg chamber integrity are highly dependent on sand compaction (such as porosity and bulk density) and water content (Magron, 2000). When a nesting female digs in dry, loose sand, lack of grain cohesiveness makes it difficult to shape an egg chamber (Milton and Lutz, 2003). Carthy (1996) found that loggerhead nests in highly compacted sand differed in egg chamber depths. These two characteristics, sand water content and compaction, are fundamental in preserving suitable nesting habitats.

Our results also illustrate how nest microclimate interacts with nest location. Those nests located within wide and flat beaches had the highest hatching and emergence success in the mid-beach zone and closer to the waterline than those on more narrow, steep beaches with nests clustered near the base of the dune at higher elevations. Differences in sea turtle nest distributions associated with differences in beach morphology have been documented in other studies (Fish et al., 2005; Cuevas et al., 2010; Fujisaki et al., 2018), suggesting that nest distributions are structured, in part, by the nesting turtle's responses to the beach morphology and potentially its physical limitations. For example, previous studies suggest that nest location represents a trade-off between inundation risk low on the beach (Mrosovsky, 1983; Hays and Speakman, 1993) and desiccation risk high on the beach (Witherington et al., 2009), such that predicted cross-shore clutch distributions are bell-shaped (Schoeman et al., 2014). Bladow and Briggs (2017) found significantly higher hatching success rates in nests at RR were within the upper limit of wave runup than nests closer to the dune. However, other cues seemingly unrelated to nest moisture and elevation can cause nesting turtles to select sites and cluster nests (e.g., horizon brightness, Price et al., 2018; and horizon elevation, Salmon et al., 1995). The mechanisms that explain the clustering and dispersion of nests across a continuum of beach types have not been fully explored and likely are multifactorial.

When multiple environmental factors were considered simultaneously, a pattern of resilience in loggerhead sea turtles became apparent. However, caution must be used in applying a principal components approach to environment and nest success data. While there is a clear pattern that suggests resilience in loggerhead sea turtles, the effects of any one incubation environment on hatching and emergence success were not directly measured but are inferred across the study nests. Studies which assess nest environments under controlled conditions or via split clutch manipulation experiments (Ratterman and Ackerman, 1989; Bodensteiner et al., 2015) are useful in decoupling the individual effects of environmental factors. Additionally, the major axis of variation extracted from the PCA should explain a substantial proportion of variation in the data. In our study PC1 and PC2 explained 96.5% of the total environmental variation. By collapsing multiple environmental factors into principal components, we assessed the breadth of factors in which loggerhead nests can successfully incubate. This result is informative even in highly dynamic environments of nesting beaches, but not specific to any cluster of nests.

Together, the results of this study indicate that Northwest Atlantic loggerhead clutches are resilient and successfully productive under a variety of incubation environments. What makes a highly successful beach is not one or two things. The eggs laid both in warm-temperate and subtropical climates successfully developed and hatchlings emerged without significant differences in survival. Nests in both bioclimatic zones differed in location, temperature, moisture levels, and clutch dimensions as well as the subtle genetic differences of the turtles nesting in these two regions. This examination of highly successful nesting beaches identified that loggerheads may be resilient to variable nest environments within the parameters examined in this study. Furthermore, because of the persistence of highly successful nest hotspots, there is potential for a simple and effective method for identifying high-priority conservation areas. What is clear is that the maintenance of these highly successful nest hotspots will be important for the recovery of imperiled loggerhead sea turtles and the management of essential habitats.

DATA AVAILABILITY STATEMENT

The original data contributions presented in the study are included in the article and **Supplementary Material**. Inquiries about historic data can be directed to the corresponding author or https://myfwc.com/research/wildlife/sea-turtles/nesting/beach-survey-totals/.

AUTHOR CONTRIBUTIONS

JG designed the study, collected the data, did the analyses, and wrote the initial manuscript. JW conceived of the project, helped design the study, and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.853835/full#supplementary-material

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Lost to the Sea: Predicted Climate Change Threats to Saltwater Crocodile Nesting Habitat

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Climate change is predicted to have devastating impacts on apex predators such as eliminating their required habitats. Crocodilians are no exception as most species require freshwater for nesting, and such freshwater habitats are particularly vulnerable to saltwater inundation (SWI) caused by the sea level rise (SLR) from global warming. Here, we examined the impacts of climate change on saltwater crocodiles *Crocodylus porosus* in terms of the potential loss of nesting habitat in the Northern Territory, Australia; an area that contains the world's most extensive nesting habitat for the species. Our spatial model, derived from 730 nest locations and selected environmental features, estimated a total of 32,306.91 km^2 of current suitable habitat across the study region. The most important variable was distance to perennial lakes (71.0% contribution, 87.5% permutation importance), which is negatively correlated with nesting habitat suitability. We found that projected changes in temperature and rainfall by 2100 could impact the area of suitable nesting habitat negatively or positively (0.33% decrease under low future emission climate scenario, and 32.30% increase under high emission scenario). Nevertheless, this can be canceled by the strong negative impact of SLR and concomitant SWI on nesting areas. A portion (16.40%) of the modeled suitable habitat for a subsection of our study area, the Kakadu Region, were already subject to > 0.25 m SWI in 2013. The suitable area for nesting in this region is predicted to be further reduced to 1775.70 km^2 with 1.1 m SLR predicted for 2100, representing 49.81% loss between 2013 and 2100. Although the estimates of habitat loss do not account for the potential creation of new habitat, nor for the uncertainty in the degree of future SLR, our results suggest that SLR driven by continuing global warming can be the major threat to mound-nest-building crocodilians including *C. porosus*, rather than direct impacts from changes in temperature and rainfall. The degree of impact on saltwater crocodiles will be determined by the interplay between the loss of nesting habitat, which would appear inevitable under current global warming, and the ability to expand into new areas created by the expansion of the tropics.

Keywords: climate change, sea level rise (SLR), crocodile, habitat suitability analysis, Maxent, saltwater incursion, Kakadu National Park, Australia

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INTRODUCTION

Apex predators include some of the world's most imperiled species and climate change is predicted to have devastating impacts on some members of this important trophic group. Perhaps the best publicized example of climate impacts on an apex predator is the polar bear (*Ursus maritimus*), which undergoes population decline in years with low levels of arctic ice coverage due to reduced reproduction and adult female survivorship (Hunter et al., 2010; Laidre et al., 2020). Projections of continued reductions in arctic ice area indicate that the polar bear will experience severe population declines by 2100 (Hunter et al., 2010; Molnár et al., 2011). Climate change is also predicted to impact tropical apex predators. For example, in the mangrove Sundarbans of southern Bangladesh, sea-level rise is predicted to eliminate all suitable habitat for the Bengal tiger (*Panthera tigris tigris*) by 2070 (Mukul et al., 2019). Climate change impacts are also likely to extend to reptile apex predators, including the world's largest lizard, the Komodo dragon (*Varanus komodoensis*). The Komodo dragon occurs on five islands in Indonesia and climate change under moderate emissions scenarios is predicted to reduce suitable habitat by ∼90% by 2050 (Jones et al., 2020). From these examples, it is apparent that climate change will impact apex predators via several pathways and across multiple biomes.

Crocodilians, with 25 extant species currently recognized, function as a crucial apex predator in semiaquatic ecosystems, and are also anticipated to be negatively impacted by climate change. For example, 37-year observations in Florida showed that *Crocodylus acutus* hatching shifted to earlier dates by 1.5 days, every 2 years with increased sea surface temperature (Cherkiss et al., 2020). Similar results show that increased temperature led to shorter incubation periods for the same species in Mexico (Charruau et al., 2017). Moreover, the sex ratio is determined, in crocodilian species, by incubation temperature, and climate warming is expected to interfere with this (Maciejewski, 2006; González et al., 2019; Bock et al., 2020). For *Alligator mississippiensis* in Florida, it is estimated that a temperature rise by 1.1–1.4°C in 2040–2050 may skew the sex ratio initially to 95.6% males and then to 97.8% females with a temperature rise by 1.6–3.2°C in 2090–2100 (Bock et al., 2020).

Global warming also can be a threat to crocodilians by destroying their habitat, in particular freshwater swamps or floodplains, through saltwater inundation (SWI) as a result of the sea level rise (SLR). Most species require freshwater habitat for breeding and nesting and such areas typically lie at a low elevation along coasts or rivers and are, therefore, vulnerable to imminent SWI (Pezeshki et al., 1990; Mulrennan and Woodroffe, 1998; Pettit et al., 2018). Despite the adaptation to the saline environment, as implied by their common name, saltwater crocodiles, *C. porosus* is one such species and requires constant or regular access to freshwater for breeding.

Here, we examine the impacts of climate change on *C. porosus*, the largest extant crocodilian species, and quantify the potential loss of nesting habitat through SWI and SLR in the Northern Territory (NT), Australia. This region is of global significance, as it supports the most extensive freshwater wetlands and floodplains and contains the largest population of this apex predator in the world (Webb et al., 2010; Fukuda et al., 2021).

MATERIALS AND METHODS

Study Area

The study area is the northern coastal region of the NT, Australia, within the latitude range −11.0 and −17.0°, called the Top End (**Figure 1**). The Top End includes the Kakadu Region, which largely consists of the Kakadu National Park. Four major tidal rivers (East, South, West Alligator Rivers and Wildman River) feed into extensive freshwater floodplains contained within the Kakadu National Park. The climate is tropical and monsoonal with distinct dry (May–October) and wet (November–April) seasons. In the coastal areas of the study region, the daily rainfall can exceed 200 mm, and averages approximately 25 mm daily at the peak wet season (Bureau of Meteorology, 2020). The annual rainfall typically ranges between 1,500 and 2,000 mm. The mean maximum and minimum monthly ambient temperature is approximately 17 and 34°C, respectively.

Study Species

C. porosus is the largest extant crocodilian species, with the largest individuals exceeding a total length of 6 m and weighing over 1,000 kg (Britton et al., 2012). The species is physiologically adapted to both the freshwater and saline environment (Grigg et al., 1980; Grigg, 1981; Taplin and Grigg, 1981) although they require freshwater for breeding (Webb et al., 1977, 1983). They are found in many different waterbodies including beaches, estuaries, lakes, rivers, and swamps. Some individuals have been reported in the sea far from the shore (Brackhane et al., 2018; Spennemann, 2021). Females build a mound-like nest from vegetation such as tall grasses in the freshwater floodplains or swamps and lay typically 40–55 eggs inside the nest (Webb et al., 1977, 1983; Fukuda and Cuff, 2013). Eggs are incubated by the heat generated by the decomposition of the vegetation material and hatch after approximately 75–95 days (Webb et al., 1983; Richardson et al., 2002). Their breeding is annual and highly seasonal, and is restricted to the wet season.

The species was heavily hunted for commercial use between the 1940s and 1960s, but since legislative protection in 1971, has substantially recovered (Fukuda et al., 2011). They are not considered threatened at any level in the NT and are categorized as Least Concern under the Territory Parks and Wildlife Conservation Act 1976. The Australian population is listed in Appendix II under the Convention on the International Trade in Endangered Species (CITES, IUCN, 2012). As part of the sustainable harvest program implemented in the NT, up to 70,000 eggs are collected annually across the study area for the commercial ranching program (Saalfeld et al., 2015, 2016).

Nesting Habitat Modeling

We used the software package Maxent version 3.4.4 (Phillips et al., 2021) to estimate the current and future areas of suitability for saltwater crocodile nesting in the Top End. Maxent is a presence-only model that minimizes the relative

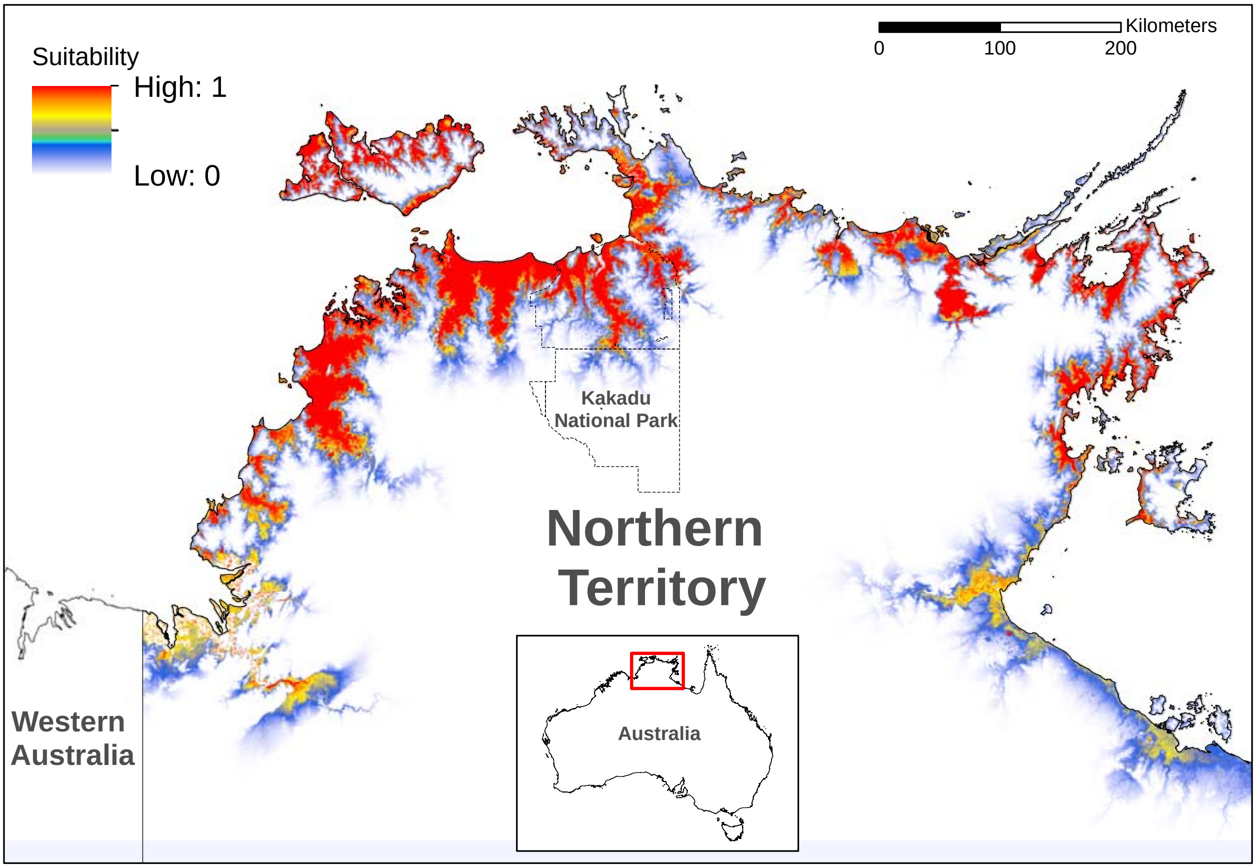

FIGURE 1 | Habitat suitability for nesting of *Crocodylus porosus*, predicted by the non-thresholded Maxent model incorporating eight environmental variables (**Table 1**) in the study area of the Top End, the NT, Australia.

entropy of estimated probability densities between presence points and the background landscape (Elith et al., 2011) and has frequently outperformed other distribution modeling techniques (Hernandez et al., 2006; Wisz et al., 2008).

For the model of suitable nesting habitat at the present time, we collated 730 individual nest locations from across the study area. The crocodile nests were located and harvested by multiple commercial operators during the wet season in 2019 (November–December) and 2020 (January–May) as part of the sustainable harvest program (Saalfeld et al., 2015). We started with an in initial list of 15 environmental variables anticipated to limit saltwater crocodile nesting habitat based on the literature (**Table 1**). We converted all vector topographic variables to rasters, matching the grain size of a 3 s (∼90 m) digital elevation model (DEM), and masked existing raster layers to the same grain size as the DEM, in ArcGIS version 10.6.1 (esri, 2021). We screened variables for collinearity using the correlation matrix in ArcMap, and excluded one of variables in a pair with a correlation coefficient of ≥ 0.7 (Merow et al., 2013). This left us with the following eight variables: (1) "elevation," 3 s (∼90 m) digital elevation model; three climate variables from WorldClim downloaded at 30 s resolution including (2) "BIO06," minimum temperature of coldest month; (3) "BIO15," precipitation seasonality (coefficient of variation); and (4) "early wet season rainfall," October–December rainfall (Booth et al., 2014; WorldClim, 2020); and five variables from a Northern Territory 1:250,000 topographic map (Geoscience Australia, 2006), including (5) "floodplain," distance to land subject to inundation; (6) "perennial lakes," distance to perennial lakes; (7) "perennial streams," distance to perennial watercourse; and (8) "freshwater swamp," categorical value of freshwater swamp (1) vs. other landform type (0) (**Table 1**). We made this variable categorical because, according to the definitions by Geoscience Australia (2006), the freshwater swamps are small and sparsely distributed in some catchments across the study area. We fitted a nesting habitat suitability model in Maxent with only the linear relationships feature option selected (Merow et al., 2013) and retained 20% of records for validation testing. In addition to the standard cloglog suitability raster output, which we interpreted as relative nesting habitat suitability (Phillips and Dudík, 2008), we also selected a threshold (Maximum training sensitivity plus specificity; Bean et al., 2012) to assign areas of the study area as suitable vs. unsuitable based on the fitted model. To predict suitable nesting habitat in the future, we applied a projection in Maxent using

TABLE 1 | Environmental variables and their attributes used for the Maxent models.

	Variable	Description	Source
1	Elevation	Meters above the average sea level	DEM 3 s (Geoscience Australia, 2010)
2	BIO06	Minimum temperature of the coldest month in a year	BIOCLIM (WorldClim, 2021)
3	BIO15	Coefficient of variation in monthly precipitation expressed as a percentage	BIOCLIM (WorldClim, 2021)
4	Early wet season rainfall	Mean total rainfall (mm) in October–December	BIOCLIM (WorldClim, 2021)
5	Floodplain	Distance (km) to the closest land subject to inundation	GEODATA TOPO 250 series 3 (Geoscience Australia, 2007)
6	Perennial lakes	Distance (km) to the closest perennial lakes	GEODATA TOPO 250 series 3 (Geoscience Australia, 2007)
7	Perennial streams	Distance (km) to the closest perennial watercourse	GEODATA TOPO 250 series 3 (Geoscience Australia, 2007)
8	Freshwater swamp	Categorical value of freshwater swamp vs. other landform	GEODATA TOPO 250 series 3 (Geoscience Australia, 2007)

	Biological relevance
1	Nesting habitat are found at low elevation. *,**
2	Distribution is limited by the minimum temperature.*
3	Distribution is limited to the monsoonal climate and breeding occurs in the wet season.*
4	Higher recruitment occurs after the west season with more rainfall in October–December.***
5	Freshwater floodplains are major nesting habitat. *
6	Perennial lakes are major residential habitat for females, connecting to breeding sites.*
7	Perennial watercourses are major residential habitat for females, connecting to breeding sites.*
8	Patchy freshwater swamps are residential and nesting habitat.*

*Fukuda et al. (2007), **Fukuda and Cuff (2013), ***Fukuda and Saalfeld (2014).

the WorldClim future weather climate projections for the three climate variables. We selected the period 2081–2100 using BCC-CSM2-MR, a medium-resolution global climate model developed by Wu et al. (2019) under two climate scenarios based on low Shared Socio-economic Pathways (SSP 126) and high (SSP 585) future emissions (Hausfather, 2018; WorldClim, 2020).

Sea Level-Rise in Kakadu

Detailed tidally driven, hydrodynamic models of SWI driven by SLR have previously been developed for the Kakadu region (Bayliss et al., 2016), enabling us to make detailed predictions for changes in habitat suitability in this important region. Our thresholded Maxent model produced a raster with binary nesting suitability (0 for unsuitable and 1 for suitable). We overlaid this raster of the thresholded model with the raster datasets of the coastal and river freshwater floodplains simulated by Bayliss et al. (2016), using ArcGIS version 10.6.1. The sea level around the Australian coastlines is expected to rise in a range of 0.75–1.90 m with the mid-range value of 1.1 by 2100 (Short and Woodroffe, 2009). We used the SWI simulation with 1.1 m SLR from Bayliss et al. (2016) to estimate how much of the suitable nesting habitat in the Kakadu Region would be affected by SWI in 2100. Although *C. porosus* prefers the freshwater environment for nesting, some areas with saline vegetation such as *Halosarcia*, *Tecticornia,* and *Suaeda* are sometimes used because they largely become freshwater in the breeding wet season due to the large input of flushing rainwater during monsoonal events (Fukuda and Cuff, 2013). Thus, we considered that floodplains with less than 0.25 m SWI would remain as habitat suitable for nesting and those areas with SWI of more than 0.25 m are unsuitable. We used the raster predictions from Bayliss et al. (2016) for > 0.25 m SWI to identify areas that will be lost from our thresholded model of current suitable nesting habitat by the year 2100.

RESULTS

Our model of current saltwater crocodile nesting habitat performed well (test AUC of 0.958 ± SD 0.003) and showed suitable areas around the coasts and floodplains of the Top End of the NT (**Figure 1**). The most important variable was distance to perennial lakes (71.0% contribution, 87.5% permutation importance), with a negative logistic relationship with nesting habitat suitability (**Figure 2A**). Early wet season rainfall was the next most important variable (12.9% contribution; 0.9% permutation importance), with a positive logistic relationship between Early wet season rainfall and nesting habitat suitability (**Figure 2B**). The freshwater swamp variable (8.3% contribution; 1.2% permutation importance) showed much higher suitability than other landform types (**Figure 2C**). Seasonality in rainfall (BIO15) showed 6.6% contribution; 3.0% permutation importance, with a negative logistic relationship to nesting habitat suitability (**Figure 2D**). All other variables contributed $< 5\%$ to the model.

For the whole of the Top End, our thresholded Maxent model predicted a total of 32,306.91 km^2 of suitable nesting habitat across the study area for the current time. For the period 2081–2100, using the predicted future temperature and rainfall patterns derived from the BCC-CSM2-MR climate model but retaining the other variables at their present values, our Maxent model predicted an area of suitable habitat of 32,199.32 km^2 under the low emissions scenario (SSP 126) and 42,740.78 km^2 under the high emissions scenario (SSP 585) across the study area.

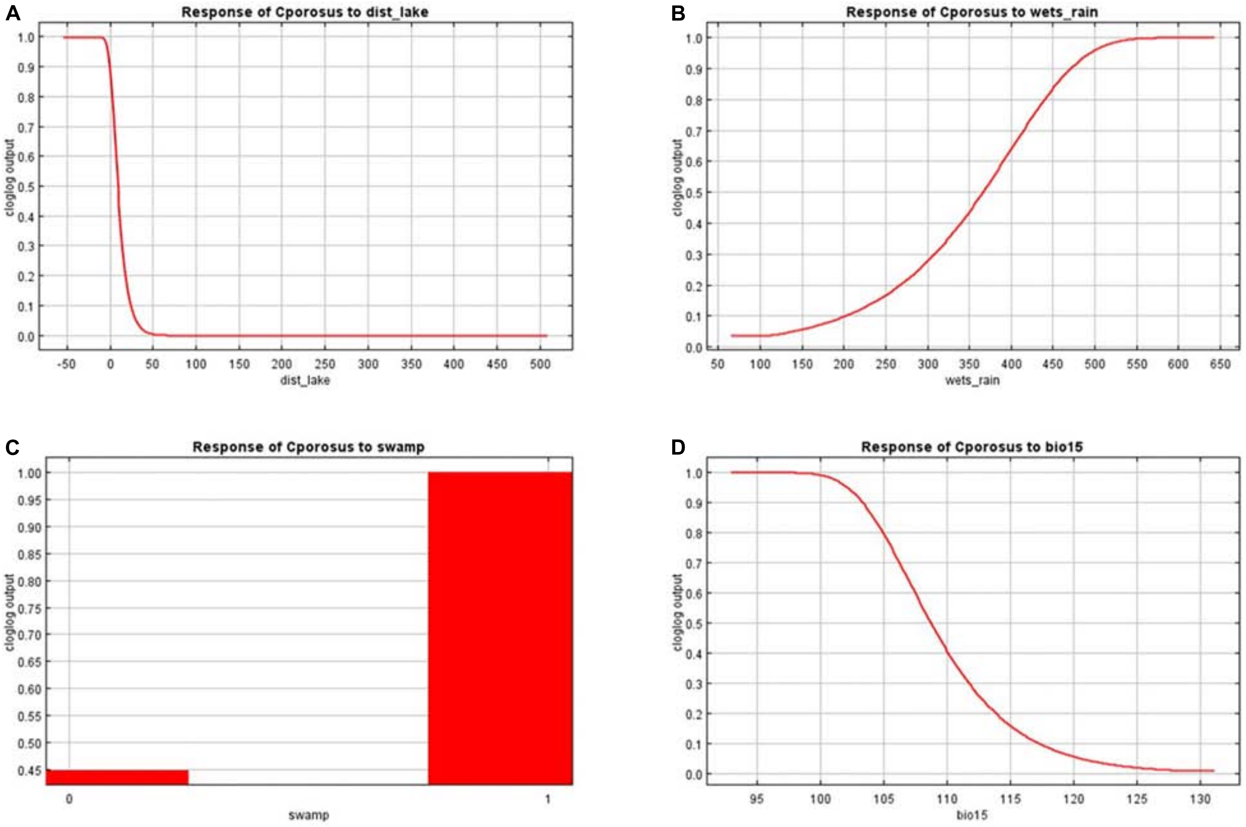

FIGURE 2 | Relationship plots between the nesting suitability and **(A)** distance to perennial lakes (71.0% contribution, 87.5% permutation importance), **(B)** early wet season rainfall (12.9% contribution; 0.9% permutation importance), **(C)** freshwater swamp (8.3% contribution; 1.2% permutation importance), and **(D)** seasonality in rain (6.6% contribution; 3.0% permutation importance) estimated by Maxent. These plots reflect the dependence of predicted suitability both on the selected variable and on dependencies induced by correlations between the selected variable and other variables (Phillips et al., 2021).

Within the Kakadu Region, a total area of 4232.08 km^2 was assigned as suitable nesting habitat by our current model (**Figure 3A**). However, the hydrodynamic models by Bayliss et al. (2016) show that as at 2013 sea level, approximately 16.40% of the suitable habitat was already affected by $<$ 0.25 m SWI, and 3538.10 km^2 remains suitable for nesting (**Figure 3B**). After 1.1 m SLR (that is, sea levels forecast for 2100), only 1775.70 km^2 remains suitable (**Figure 3C**). This represents a 58.04% reduction in predicted suitable nesting habitat over 87 years, between 2013 and 2100).

DISCUSSION

Our model of suitable nesting habitat for the saltwater crocodile revealed a dominant importance of abundant, perennial lakes in Australia's Top End. The estimate of nesting suitability rapidly dropping beyond a 50 m range from perennial lakes (**Figure 2A**) is consistent with field observations that a vast majority of nests are made around the edges of waterbodies during the breeding season (Webb et al., 1977, 1983; Fukuda and Cuff, 2013). The perennial lakes appear to be important as females guarding their nests during the incubation period, typically 2–3 months, require access to freshwater (Webb et al., 1977; Magnusson, 1980). The model also identified the importance of early rains in the wet season (**Figure 2B**), supporting previous findings that rains in early wet season (Oct–Dec) trigger the highly seasonal breeding of *C. porosus*, and higher precipitation during this period results in higher abundance of hatchlings in the following dry season (Fukuda and Saalfeld, 2014).

We found that projected changes in temperature and rainfall under climate change could impact the area of suitable nesting habitat negatively or positively (0.33% decrease under a low emission scenario SSP 126 and 32.30% increase under a high emission scenario SSP 585). It is worth mentioning that, apart from the adverse impact of SLR, the projected nesting habitat would otherwise be increased to some extent under the higher future emission scenario, because of some positive effects on the climate variables such as increased early rains and decreased seasonality. Nevertheless, these effects will be canceled by the much higher, negative impact by SLR.

Most importantly, our analysis for the Kakadu Region showed that almost 50% of the suitable nesting habitat in 2013 would be lost to the 1.1 m SLR by 2100 (**Figure 3**). While fine-scale SLR data and forecasts are not currently available outside

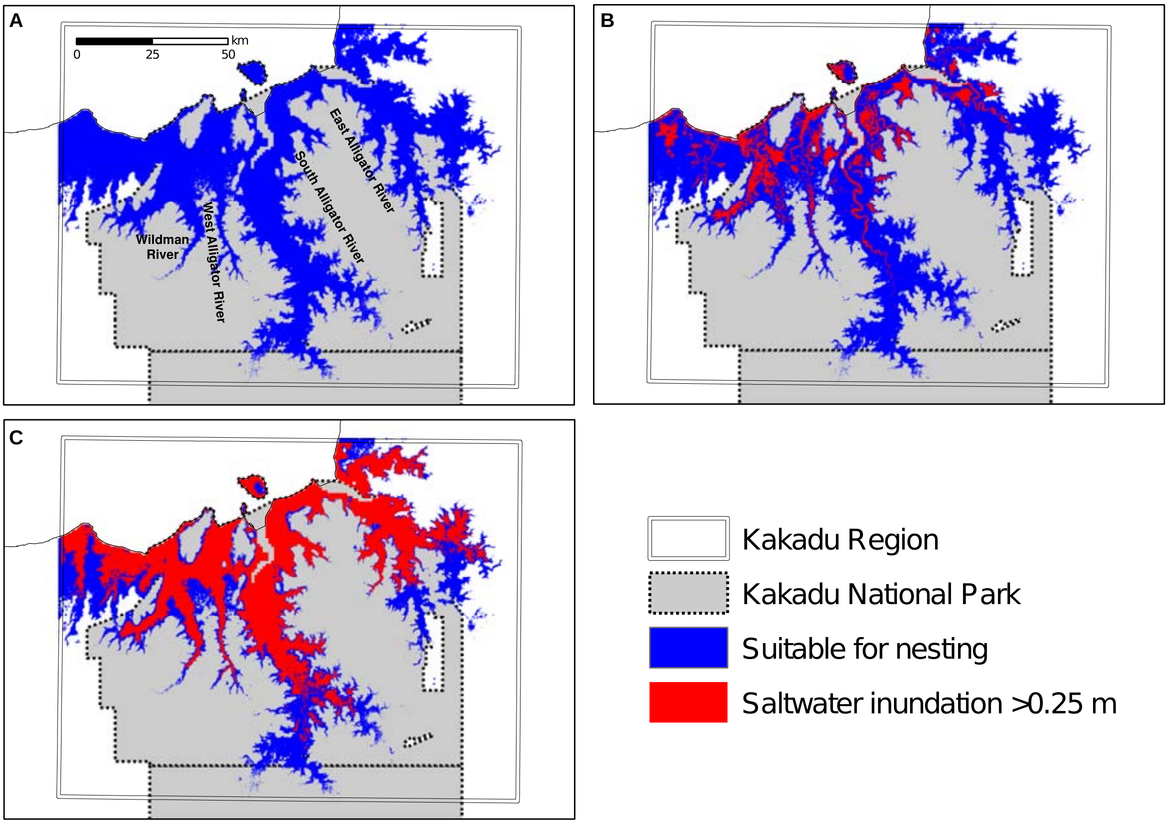

FIGURE 3 | Suitable nesting habitat for *Crocodylus porosus* in the Kakadu Region of the Northern Territory, predicted by the thresholded Maxent model, with **(A)** no sea level rise (SLR) and no saltwater inundation (SWI) incorporated, **(B)** 0 m SLR but excluding areas with > 0.25 m SWI as simulated for 2013 by Bayliss et al. (2016), and **(C)** 1.1 m SLR and excluding areas with > 0.25 m SWI as simulated for 2100 by Bayliss et al. (2016).

of the Kakadu region, if a similar proportion of the habitat is affected across the larger study area, 16,522 km^2 would be lost to SLR across the Top End. This is of significant concern as the majority of the Australian population of *C. porosus* resides in this area (Fukuda et al., 2007, 2021). This suggests that SLR by climate change may represent the major threat to the species. Other crocodilians that build mound nests in freshwater wetlands or floodplains, such as *A. mississippiensis* and *C. moreletti*, could also be at similar risk, as their nests are prone to flooding (Kushlan and Jacobsen, 1990; Platt et al., 2021).

One important caveat to the predicted loss of saltwater crocodile nesting habitat is that the creation of new freshwater habitats in the Kakadu region has not been accounted for. Bayliss et al. (2016) were unable to predict new areas of freshwater habitat created through SLR because the Lidar-derived fine-scale elevation data did not extend beyond the current floodplain. Undoubtedly, SLR will result in the formation of some areas of new habitat suitable for saltwater crocodile nesting which may somewhat offset the areas lost. However, the sandstone escarpments that occur further inland (Northern Territory and Geological Survey, 2006) form a physical barrier to the formation of extensive freshwater floodplains. In addition, while small billabongs or lakes may form on the escarpment, the steep and rocky terrain curtails the dispersal of saltwater crocodiles, and to date, they have never been observed in these areas (Letnic and Connors, 2006). For areas outside of the sandstone escarpments, the potential creation of new floodplain habitat is an important avenue for further research. Additional data such as LiDAR (Dong and Chen, 2017) would facilitate more accurate forecasts of potential future suitable habitat.

Another source of uncertainty is the degree of future SLR. Bayliss et al. (2016) adopted a value of 1.1 m in 2001 based on a range of published projections (Solomon et al., 2007; Jevrejeva et al., 2010). The IPCC recently released their 6th Assessment Report which includes predictions of 0.63–1.01 m by 2100 under the moderate emission scenario and 0.66–1.33 m by 2150 under the very high emissions scenario (Masson-Delmotte et al., 2021). Therefore, the 1.1 m SLR used in our study represents likely SLR between 2100 and 2150. However, several studies document the acceleration of SLR in recent decades (Nerem et al., 2018; Dangendorf et al., 2019), and in addition, the IPCC stated that due to the substantial uncertainty in global ice sheet dynamics, SLR approaching 2 m by 2100 and 5 m by 2150 cannot be ruled out (Masson-Delmotte et al., 2021). The substantial uncertainty in the potential creation of new habitat and the degree of SLR means that estimates of the loss of saltwater crocodile nesting habitat should be recalculated as new data become available.

It should be noted that the change from a freshwater to more saline habitat is not anticipated to be monotonic, and gradual replacement of freshwater plant species with those that are more saline tolerant is the likely scenario. While the floodplain vegetation is determined by fine-scale variation in topography (Finlayson et al., 2006) and the global mean SLR is 4.8 mm annually (Dangendorf et al., 2019), even infrequent SWI can lead to sudden changes in salinity (Finlayson et al., 2006; Bayliss et al., 2016). Freshwater plant taxa have a unique tolerance to salinity and water depth (Cowie, 2003), and most freshwater plant species are unable to tolerate salinity much in excess of 1 ppt (Pettit et al., 2018). Previous work has shown an association between crocodile nesting and *Oryza* dominated tall tussock and *Melaleuca* open grassland with an understory comprised of species also present in the *Oryza* dominated grasslands (Fukuda and Cuff, 2013), and *Oryza* species are unable to tolerate salinity above 1 ppt (Pettit et al., 2018). In contrast, mangrove species can tolerate salinity to 50 ppt (Ball, 1998), and are not obligate halophiles, that is, they are also able to live in freshwater conditions (Woodroffe, 1988).

Although fossil records and molecular analysis indicate that extant crocodilians were capable of tracking changes in their distribution in response to drastic climate changes in the last 100 million years (Roos et al., 2007; Brochu et al., 2009; Ryberg and Lawing, 2018), whether they can continue to track changes in the spatial distribution and quality of suitable nesting habitat is unknown. *Crocodylus porosus* is one of the oldest extant species in the world, little changed since dinosaurs roamed the planet, yet their survival during the Anthropocene may now depend on how quickly humanity can transition away from the burning of fossil fuels and thus stabilize global warming.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: Fukuda and McDonald (2022).

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this study did not involve live animals and only used the location data of crocodile nests that were reported to the Northern Territory Government, Australia.

AUTHOR CONTRIBUTIONS

YF, PM, and BC contributed to conception and design of the study, and wrote the manuscript. YF organized the data access. YF and PM performed the spatial analysis. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Nesting in *Anolis* Lizards: An Understudied Topic in a Well-Studied Clade

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Maternal nesting behavior in oviparous species strongly influences the environmental conditions their embryos experience during development. In turn, these early-life conditions have consequences for offspring phenotypes and many fitness components across an individual's lifespan. Thus, identifying the evolutionary and ecological causes and effects of nesting behavior is a key goal of behavioral ecology. Studies of reptiles have contributed greatly to our understanding of how nesting behavior shapes offspring phenotypes. While some taxonomic groups have been used extensively to provide insights into this important area of biology, many groups remain poorly studied. For example, the squamate genus *Anolis* has served as a model to study behavior, ecology, and evolution, but research focused on *Anolis* nesting behavior and developmental plasticity is comparatively scarce. This dearth of empirical research may be attributed to logistical challenges (e.g., difficulty locating nests), biological factors (e.g., their single-egg clutches may hinder some experimental designs), and a historical focus on males in *Anolis* research. Although there is a gap in the literature concerning *Anolis* nesting behavior, interest in nesting ecology and developmental plasticity in this group has grown in recent years. In this paper, we (1) review existing studies of anole nesting ecology and developmental plasticity; (2) highlight areas of anole nesting ecology that are currently understudied and discuss how research in these areas can contribute to broader topics (e.g., maternal effects and global change biology); and (3) provide guidelines for studying anole nesting in the field. Overall, this review provides a foundation for establishing anoles as models to study nesting ecology and developmental plasticity.

Keywords: nesting, *Anolis*, oviparous, development, plasticity

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INTRODUCTION

Nesting is an important reproductive behavior that can have lasting impacts on maternal and offspring fitness (Refsnider and Janzen, 2010). Because embryos are highly sensitive to environmental perturbations, even short-term exposure to different conditions can have far-reaching consequences across subsequent life stages (West-Eberhard, 2003; Uller, 2008; While et al., 2018). For example, brief exposure to temperature or pH during development can permanently affect offspring sex in some animals (Cook, 2002; Valenzuela, 2004) and have lasting individual- and population-level consequences (Warner and Shine, 2008a; Grayson et al., 2014). Because successful early-life development is a prerequisite for future reproduction, natural selection on maternal nesting behaviors should be particularly strong. These points illustrate the importance of nesting ecology, which is a topic that broadly incorporates aspects of maternal behaviors (e.g., choice of nest location and level of parental care), nest environment (e.g., biotic and abiotic variables around and within the nest), embryo or neonatal development (e.g., physiological tolerances and developmental plasticity), and a variety of other factors that influence the nest and its surrounding environment (e.g., soil composition, predators, and parasites) (Doody et al., 2021). Overall, understanding an organism's nesting ecology can provide insight into a range of behavioral and physiological traits (Angilletta et al., 2009; Hall and Warner, 2021), answer basic questions about ecology and evolution (Resetarits, 1996; Refsnider and Janzen, 2010), and solve problems in conservation and management (Hare et al., 2002, 2004; Mazaris et al., 2017).

Nest-site selection is particularly important for oviparous species that lack post-nesting parental care because developing offspring have little opportunity to adjust to changing environmental conditions (but see Warkentin, 2011; Shine and Du, 2018). For example, extended parental attendance in fish, amphibians and birds can reduce the risk of nest predation (e.g., Sargent, 1988; Komduer and Kats, 1999; Lehtinen et al., 2014; Schulte et al., 2020), whereas unattended eggs may be considerably more vulnerable in species without parental care depending on where mothers place their nests. Additionally, nest temperatures of some species (e.g., birds) are relatively stable and predictable due to parental incubation (Deeming and Reynolds, 2015; Refsnider, 2015), but temperatures fluctuate widely in nests of most non-avian reptiles (henceforth "reptiles") (Warner and Shine, 2008b; Du et al., 2010). Reptiles have been useful models for studies of nesting ecology, largely due to their considerable variation in nest environments across both large and small spatial and temporal scales. Indeed, maternal choice of microhabitats for nesting is well documented in reptiles, and nests can vary considerably in thermal and hydric conditions among and within species (Janzen and Morjan, 2001; Doody et al., 2006; Refsnider, 2015). As such, embryos vary across species with respect to environmental tolerances (Andrews and Schwarzkopf, 2012; Hall and Sun, 2021) and respond to incubation environments in ways that influence fitness-related morphological, physiological, behavioral, and life-history traits of offspring (i.e., developmental plasticity; Noble et al., 2018; Warner et al., 2018; While et al., 2018). The long-term effects of developmental plasticity on fitness and their potential adaptive significance are important components of nesting ecology, but this topic is poorly studied (Mitchell et al., 2018). Indeed, filling in this knowledge gap would provide important insight into the factors that shape maternal nesting behaviors.

While reptile nesting ecology and developmental plasticity have been active subjects of study for several decades (Warner et al., 2018), we have a poor understanding of these topics in many otherwise well-studied clades. The lizard genus *Anolis* is an excellent example. This group consists of approximately 400 species found across the Caribbean and the mainland from the southern United States through Central and South America. Additionally, many species are colonizers and have become invasive across the globe (Losos, 2009; Latella et al., 2011). Anoles have served as important models for testing foundational hypotheses in ecology, evolution, behavior, and developmental biology (Losos, 2009; Sanger and Kircher, 2017; Feiner, 2019). Yet relatively little is known about their nesting ecology despite its potential importance for individual fitness and population dynamics (Andrews, 1988). Several factors contribute to the deficiency of empirical work on anole nesting. For example, the single-egg clutch of anoles can complicate experimental designs for studies of egg incubation because eggs from a single clutch cannot be allocated among different treatments. Their single-egg clutch also makes observing nesting in the wild challenging because the time required for nesting is short. Relatedly, the small eggs and inconspicuous nesting behavior of anoles hinders our ability to detect nests in complex habitats. Finally, the historical precedent of research on male morphology and behavior (e.g., charismatic dewlaps and display behaviors) has diverted research attention away from important aspects of female biology (Kamath and Losos, 2017). These factors present roadblocks for empirical work on nesting ecology of anoles. Establishing methodologies that overcome these logistical difficulties is essential.

Studies on nesting in anoles can provide new insight into many well-studied aspects of their biology. For example, nest environments shape fitness-related phenotypes of offspring (e.g., body size and locomotor performance) that may relate to resource competition (Pearson and Warner, 2018); this could inform work on niche partitioning and community ecology (Roughgarden, 1995). Important eco-morphological traits that exhibit considerable convergence across species (e.g., limb length and body size) may be developmentally sensitive to early-life conditions (Losos et al., 2000; Downes and Hoefer, 2007; but see Warner et al., 2012); this could illuminate the role of developmental plasticity in convergent evolution and adaptive radiation (Losos, 2009; but see Feiner et al., 2020). Availability and location of suitable nest sites may influence the spatial distribution of females and nest predators in ways that influence mate competition among males and population dynamics (Andrews, 1988; Chalcraft and Andrews, 1999; Angilletta et al., 2009); this could inform studies of territorial behaviors, mating systems, and population biology. Lastly, methodology and protocols that are developed for studying *Anolis* nesting can be applied to other taxa, and therefore be useful tools for providing broader information about nesting ecology and reproductive behaviors in many other oviparous reptiles. These are just a few examples of how studies of nesting ecology in anoles could enhance their usefulness as models for a range of topics.

Anoles have several features that make them useful models for studies of nesting ecology. First, several species are conspicuous members of their ecological communities and occur in high densities. This facilitates large sample sizes for observational and experimental studies and indicates that nests/eggs are abundant in the field during the reproductive season. Second, anoles are somewhat unique among reptile clades because all species are constrained to produce a single egg-clutch every 1–3 weeks over a relatively long reproductive season; while a single-egg clutch could hinder experimental designs (noted above), this potentially facilitates repeated observations of nesting, which could benefit some laboratory experiments of nesting behavior. Third, many species have a relatively short life span (1–2 years), which makes studies of the life-long effects of developmental environments feasible. Fourth, captive husbandry and protocols for captive breeding are well established and logistically feasible (Sanger et al., 2008a). Moreover, the small body size of most species means that large numbers of captive individuals can be housed in relatively small areas; these are welcome features for most research programs where animal-housing space and funds are limited. Indeed, studies on anole nesting and development have even been conducted in a secondary school classroom (Reedy et al., 2013). Finally, because anoles have been studied extensively for other purposes, there is a wealth of knowledge concerning their life-history, reproductive physiology, evolutionary relationships, and general ecology (Losos, 2009). Such background knowledge provides critical context for observations of nesting behavior and its effects on lifetime fitness.

In this review, we aim to achieve three major goals. First, we provide an overview of the current state of knowledge on anole nesting ecology and developmental plasticity. Second, we identify knowledge gaps in anole nesting ecology and developmental plasticity and discuss how filling these gaps will contribute to broader topics. Third, we establish useful guidelines for finding nests in the field, which will help future anole biologists address some of the gaps we identify. Overall, by addressing these objectives, we hope to bring more attention to anoles as models for studying nesting ecology and developmental plasticity.

AN OVERVIEW OF NESTING ECOLOGY IN ANOLES

Most descriptions of anole nesting are based on anecdotal observations of nest microhabitats, but some laboratory and field experiments have provided key insights into nest-site selection and oviposition behavior (**Table 1** and **Supplementary Table 1**). Collectively, these anecdotes and experiments indicate that females nest in a wide range of microhabitats while seeking conditions that have important developmental consequences for offspring. Much of the variation in nest microhabitats is likely explained by differences among species, local environments, seasonal timing of nesting, and habitat availability. Here we summarize the broad range of behaviors associated with oviposition, as well as general trends concerning maternal choice of nest microhabitats in anoles.

Anecdotal Reports

Direct observations of nesting in the wild are rare, because nest sites and nesting behavior are inconspicuous. For example, *Anolis carolinensis* females dig a nest, oviposit, and cover the egg in 11–26 min (Propper et al., 1991), which is much shorter than in many reptiles (e.g., turtles – nesting can take several hours). Consequently, most reports of anole nest sites involve serendipitous discoveries of eggs in the field, and several researchers have made the most of these discoveries by describing various aspects of the immediate nest microenvironment (e.g., Allen and Slatten, 1945; Alfonso et al., 2012; Delaney et al., 2013; **Supplementary Table 1A**). These observations indicate that anoles typically nest beneath cover objects or within cryptic spaces (Rand, 1967; Tiatragul et al., 2019). For example, eggs of several species have been found under rocks (*A. carolinensis*, Carr, 1940; Allen and Slatten, 1945; *Anolis aquaticus*, Swierk et al., 2019; *Anolis sagrei*, Pruett et al., 2020), within leaf litter (*Anolis limifrons*, Andrews, 1988; *A. cristatellus*, Tiatragul et al., 2019), under or within plants (*Anolis argillaceus*, Alfonso et al., 2012; *Anolis lionotus*, Montgomery et al., 2011), and above ground within tree holes (*Anolis angusticeps*, Robinson et al., 2014; *A. limifrons*, Andrews, 1988). In some cases, eggs have been found buried in substrate underneath a cover object (Allen and Slatten, 1945; Delaney et al., 2013), but eggs are often unburied (Tiatragul et al., 2019). Interestingly, eggs of the Cuban anole, *Anolis lucius*, have been discovered stuck to the walls of caves (Dunn, 1926) with numerous eggshells on the cave floor, indicating that eggshells fall during or after hatching (Hardy, 1957); although eggs of *A. lucius* are exposed to ambient air, the humid cave environment might reduce the risk of egg desiccation. Similar observations have been made in several Asian geckos (Somaweera, 2009; Kalaimani, 2015), and this relatively unique choice of oviposition site warrants further investigation in *Anolis*.

Observations of multiple eggs within a single nest are common, indicating that females nest communally and/or return to the same site to oviposit. In some cases, anoles may use the same nest site as other species. For example, eggs of *A. argillaceus* were found with eggs of two gecko species (Alfonso et al., 2012), and *A. carolinensis* eggs have been found within alligator nests (Kushlan and Kushlan, 1980) and in the upper chambers of ant mounds (Kwapich, 2021). Evidence of communal nesting (i.e., between 4–24 eggs in a single nest) in the field has also been reported for *A. angusticeps* (Robinson et al., 2014), *A. lionotus* (Montgomery et al., 2011), *A. aquaticus* (Márquez and Márquez, 2009), *A. sagrei* (Pruett et al., 2020) and *A. carolinensis* (Godfrey et al., 2018; García-Padrón, 2021). Given these observations, communal nesting may be relatively widespread across anoles. Importantly, communal nesting behavior may be a function of several factors, such as limited suitable habitat for nest sites (resulting in aggregations of eggs in specific locations) or due to females actively seeking eggs as cues for nest-site choice. Additionally, given that many anoles continuously produce an egg almost every week, these communal nests may reflect the same female returning to a nest site to lay subsequent eggs. Little is known about the developmental consequences for eggs incubating in communal versus solitary environments; however, communal nesting is generally common across reptiles (Doody et al., 2009) and anoles could make excellent models to unearth the evolutionary impetus and ecological effects of this behavior (Warner and Chapman, 2011; Dees et al., 2020).

Although field observations of nest sites exist for a diversity of species, lab observations of oviposition behavior have been reported only for *A. carolinensis*. Captive *A. carolinensis* have been observed using their forelimbs and snout to create a nest hole in Spanish moss or in soil substrate

TABLE 1 | Summary of studies that provide information on nesting ecology for different *Anolis* species.

	Maternal behaviors		**Nest microhabitat variables**		**Egg incubation studies**	
Species	**Nest-site choice**	**Oviposition behaviors**	**General descriptions**	**Quantitative measurements**	**Field data**	**Lab data**
A. aeneus		FS (1)				
A. alutaceus			AO (2)			
A. angusticeps		AO (1)	AO (1)			
A. aquaticus			AO (1), FS (1)			
A. argillaceus			AO (1)			
A. auratus					EI (1)	
A. carolinensis		AO (1), FS (4)	AO (7)			EI (4)
A. cristatellus	FS (1)		AO (1)	FS (3)		EI (4)
A. equestris		AO (1)				
A. fraseri			AO (1)			
A. garmani		AO (1)				
A. grahami			AO (1)			
A. limifrons	FS (1)		AO (1), FS (1)	FS (1)	EI (4)	
A. lineatopus			AO (1)			
A. lionotus			FS (1)			
A. lucius			AO (2)			
A. polylepis	FS (1)			FS (1)	EI (1)	EI (1)
A. porcatus			AO (1)			
A. pulchellus			AO (1)			
A. sagrei	FS (5)	AO (1)	AO (2)	FS (4)	EI (2)	EI (19)
A. seminineatus			AO (1)			
A. smallwoodi		AO (1)				
A. valencienni			AO (1)			

These studies are classified as Anecdotal Observations (AO), Focused Studies (FS), or Egg Incubation studies (EI). Focused studies include observational and experimental approaches, and egg incubation studies are primarily experiments on the effects of incubation environments on eggs, embryos, and offspring traits. The numbers in parentheses refer to the number of studies within each category. ***Supplementary Table 1*** *provides details about the type of study (e.g., lab/field and experimental/observational) and brief summaries for each study included here.*

(Greenberg and Noble, 1944; Gordon, 1956). Females often place their cloaca directly over the hole for oviposition. If eggs do not fall directly into the hole, females will push the egg in with their snout. This process usually takes about 1 min (likely why nesting is rarely observed) but can last up to about 26 min. Females use their forelimbs to push substrate over the eggs, and the snout is used to pack the substrate around the egg (Greenberg and Noble, 1944). Detailed descriptions of these behaviors are in Tokarz and Jones (1979) and Propper et al. (1991). Intriguingly, several female *A. sagrei* have been observed in the laboratory and field carrying an egg in their mouth (Delaney et al., 2021); whether the egg was produced by that same female is unknown, but this behavior raises exciting questions about maternal care in anoles (e.g., do females move their eggs among different locations?).

These anecdotal observations are critical in understanding the types of habitat females use for nesting, and for generating new questions and hypotheses about nesting behavior. Generally, these reports suggest that females select nest microhabitats that are relatively cool, moist, and thermostable as well as sites that are hidden from predators. While these reports are largely descriptive and often do not provide quantitative microhabitat measurements (e.g., temperature and moisture levels), they can form the basis for focused observational and experimental work needed to obtain such data. Of course, conclusions about anole nesting that are drawn from anecdotal observations must be done cautiously, as these observations rarely involve systematic approaches to finding nests and can inevitably create a biased perspective on nesting ecology. Therefore, such observations are most useful when further tested by experimental studies.

Experimental Studies

While focused experimental studies of anole nesting are rare, these few laboratory and field studies provide critical insight into maternal nest-site choice and its adaptive significance (**Supplementary Table 1B**). Field studies have been particularly useful in assessing the fitness consequences of maternal nesting behavior under ecologically-meaningful conditions. Unlike field studies, however, laboratory experiments can hold specific variables constant so that we can pinpoint critical components of the micro-environment that females choose for nesting and their effects on development. Both laboratory and field studies can also be designed to examine the consequences of maternal nest site choice on egg survival. For example, laboratory studies can quantify egg survival across a range of incubation conditions that include and exceed those chosen by females. Whereas field studies can compare egg survival and environmental variables between actual nests (those chosen by females) and putative nests (those chosen and constructed by researchers in randomly or non-randomly selected locations or microhabitats). Overall, by integrating laboratory and field studies we can combine the power of controlled experiments with the realism of natural environments and make ecologically-relevant conclusions about nesting ecology. Here, we review the current state of knowledge about *Anolis* nesting ecology based on experimental approaches in the lab and field.

Focused studies of anole nesting aim primarily to quantify choice of nest microhabitat by females and consequences of the nest environment on offspring. In many cases, results are generally consistent with the anecdotal observations above: anoles seek relatively cool, moist microhabitats for nesting, and often lay eggs under some type of cover (e.g., leaf litter, rocks, and logs). Laboratory experiments on *Anolis polylepis* and *A. sagrei*, which provided females with nest pots that vary in moisture levels, demonstrate that females choose relatively moist substrates when nesting (Socci et al., 2005; Reedy et al., 2013). Moist nesting conditions facilitate egg hatching success because eggs must absorb water during incubation for proper development (Warner et al., 2011; Reedy et al., 2013). Moreover, *A. sagrei* choose relatively moist nest sites at the time of oviposition even when the hydric conditions of those nest sites fluctuate unpredictably toward lethally dry conditions (Warner et al., 2021). These lab-based results are consistent with observations where nesting behavior is stimulated by artificially watering substrate that simulates rainfall (*A. carolinensis*, Gordon, 1956; *Anolis aeneus*, Stamps, 1976; *A. sagrei*, Brown and Sexton, 1973), and parallels results of field studies showing that substrates of maternally-chosen nest sites have greater moisture than those of randomly-chosen sites (Pruett et al., 2020; Tiatragul et al., 2020).

Focused studies of anole nesting also corroborate the anecdotal reports concerning communal nesting. Searches for *A. lionotus* eggs along a river in Panama revealed that all nests were communal and always covered with vegetation on downstream sides of rocks surrounded by water (Montgomery et al., 2011). Communal nesting in this species was evident for active nests that contained unhatched eggs (mean egg number per nest was 11 eggs, range 4–24), and for inactive nests of hatched eggs (mean = 6.3 eggs, range 0–16). In a laboratory experiment with *A. sagrei*, Dees et al. (2020) examined nest-site choice based on the presence or absence of hatched eggshells, as well as the use of fresh soil versus soil previously used by nesting females to determine if these were important cues that facilitate communal nesting. Females chose nest sites that contained hatched eggshells more frequently than sites without eggshells. Moreover, females preferred nesting in previously-used soil to fresh, unused soil. These results indicate that (1) both visual and olfactory cues play a role in nest-site choice, and (2) communal nesting may be a function of females actively seeking cues of conspecifics, rather than just aggregating eggs in a limited number of suitable oviposition sites.

The thermal environment also correlates with nest-site choice, as successful development can only proceed over a limited range of temperatures (Sanger et al., 2018; Pruett and Warner, 2021). Because putative nest temperatures can vary considerably among microhabitats and across the nesting season (Schlaepfer, 2003; Pearson and Warner, 2016, 2018), gravid females face important challenges when choosing suitable nest habitats. Indeed, field data from an island population of *A. sagrei* in Florida, United States, show that females choose shadier nest microhabitats with temperatures that rarely reach lethal extremes compared to open sites that are also available for nesting (Pruett et al., 2020); this pattern remains consistent across the season even with temporal changes in air and ground temperatures. Similar trends were observed in a field study of *A. cristatellus* across suburban and forested locations in Miami, Florida (Tiatragul et al., 2019, 2020). In forested sites, nest temperatures are nearly identical to temperatures of randomly selected areas; thus, there is little opportunity for females to choose microhabitats based on temperature due to the high thermal homogeneity in this heavily shaded habitat. In contrast, females in suburban areas nest close to trees, and consequently, their nests have greater canopy cover and lower temperatures than what is generally available across the suburban landscape. These field studies also examined the consequences of maternally-chosen nest environments on egg survival by either placing eggs in putative nest sites in the field (Pruett et al., 2020) or by incubating eggs in the lab (Tiatragul et al., 2020). Both studies demonstrate that maternal choice of nest habitat is adaptive because eggs had greater survival under the relatively cool conditions chosen by females than under the warmer conditions readily available across the landscape. This tendency for anoles to select relatively cool sites is in contrast with other lizards which utilize warmer areas for nesting (e.g., Shine and Harlow, 1996; Warner and Shine, 2008a; Angilletta et al., 2009) and may relate to potentially high thermal variation of relatively shallow nests (discussed below).

Predators are also an important component of nesting ecology (Martin, 1993; Spencer and Thompson, 2003), and a few experiments demonstrate their influence on nest success, maternal behaviors, and population biology in anoles. Predation by invertebrates plays a major role in nest success of *A. limifrons* in Panama (Andrews, 1982); in this study, anole eggs produced by a breeding colony were placed in putative nest locations at two different sites and eggs were monitored for 50 days. Predation by *Solenopsis* ants and snails was the most common known source of egg mortality (57–77% egg mortality), and egg survival was positively associated with leaf litter abundance. Not surprisingly, after systematically searching for eggs across different habitat types (including several ground and above ground sites), Andrews (1988) estimated that 99.5% of eggs are laid on the ground under leaf litter. However, 60% of all eggs were depredated by *Solenopsis* ants regardless of whether nests were on the ground or above ground (Andrews, 1988). This predator-prey relationship between ants and anole eggs was further explored by simulating rainfall across different experimental plots and examining its impact on egg predation (Chalcraft and Andrews, 1999). This study revealed that ant density increased under conditions that simulated wet years, and in turn, resulted in greater egg predation than under conditions that simulated dry years. Similar interactions between predator density and microhabitat features have been shown for *A. sagrei* nests in Florida. In this study, DeSana et al. (2020) manipulated the density of terrestrial marsh crabs (*Armases cinereum*) and habitat type (open, leaf litter, palm fronds) within replicate experimental field enclosures. After placing eggs in these microhabitats, they found that egg predation was greatest in the high-density crab treatment, and significantly fewer eggs were depredated if they were buried under leaf litter compared to the other locations (DeSana et al., 2020). Together, results from these experiments in Panama and Florida indicate that the use of leaf litter for nesting would be favored by natural selection when invertebrate predators are abundant. Of course, the tendency to nest in cryptic spaces may benefit maternal survival as well as offspring.

Most studies of adaptive nest-site choice in reptiles focus on the fitness consequences of eggs/offspring rather than on the risks that mothers take to find suitable nesting habitat. However, theory predicts that natural selection will shape traits (e.g., nesting behavior) depending on how they influence maternal fitness, rather than that of individual offspring (Godfray and Parker, 1991; Roff, 1992; Delaney and Janzen, 2020). This issue was addressed in a recent study on the predator-prey relationship between the ground-dwelling curly-tailed lizard (*Leiocephalus carinatus*) and its arboreal prey, *A. sagrei*. In this study, Pruett (2021) provided female *A. sagrei* with ground and arboreal nest sites in large outdoor enclosures and showed that females prefer to nest on the ground. After 2 weeks, *L. carinatus* were introduced into half the enclosures, and females shifted to using arboreal nest sites, and did so more quickly than those in the non-predator control treatment. This pattern is consistent with field studies in the Bahamas where *A. sagrei* exhibits a shift toward high perches accompanied by a reduction in female survival when this natural predator is present (Lapiedra et al., 2018); although not acknowledged in that study, the reduction in female survival might be associated with maternal nesting behaviors and a lack of arboreal nest sites. Overall, given the diverse range of potential anole predators (e.g., birds, snakes, and lizards), this threat on maternal fitness likely plays an important role in shaping nesting behaviors as well as population dynamics (i.e., recruitment into juvenile or adult age classes). This understudied aspect of anole nesting ecology is ripe with questions waiting to be explored.

Egg Incubation Studies of Developmental Plasticity

The last decade has witnessed a proliferation of studies of reptile developmental plasticity that more closely replicate real-world nest environments in the laboratory (Carter and Janzen, 2021). *Anolis* lizards have been important models in this new frontier (Hall and Warner, 2018; Pearson and Warner, 2018; Sanger et al., 2018; Tiatragul et al., 2020; Hall et al., 2021) for several reasons. Methods for laboratory collection of anole eggs and embryos are established (Sanger et al., 2008a), and an embryo staging series is available (Sanger et al., 2008b). Such tools serve as a foundation for studies of development and are necessary to explore the underlying mechanisms that regulate embryo interactions with the environment (e.g., Sanger et al., 2018). Additionally, basic measures of ecological (egg survival and hatchling phenotypes) and physiological (water uptake, developmental rate, and oxygen consumption) responses to important nest variables like temperature and moisture are available for some species (e.g., Warner et al., 2012; Reedy et al., 2013; Sanger et al., 2018; Hall and Warner, 2019, 2021; Pruett and Warner, 2021). Thus, we have an understanding of embryo tolerances to nest conditions which is vital for designing ecologically relevant laboratory experiments and interpreting ecological data from nests in the wild (e.g., nest temperature; Hall and Warner, 2021). Finally, important environmental data, like canopy cover, temperature, and soil moisture, have been measured in and around nests (Sanger et al., 2018; Tiatragul et al., 2019; Pruett et al., 2020). Such studies provide ecological context to laboratory studies that assess how embryos respond to environmental conditions (e.g., Sanger et al., 2018; Hall and Warner, 2021). Although most of the aforementioned studies have been conducted on a few species (most commonly, *A. sagrei*), these studies can serve as models for other species, allowing for important cross-species comparisons. Ultimately, the unification of these tools and available data make anoles well positioned to serve as an important model for determining how natural nest environments influence embryo development, egg survival, and hatchling phenotypes (Hall et al., 2021).

Most studies of developmental plasticity in anoles focus on effects of temperature and moisture during development (**Supplementary Table 1C**), with comparatively few studies measuring the effects of other aspects of the nest environment (e.g., substrate type; Hall et al., 2021). Generally, studies of anoles are in congruence with those of other reptiles: a range of temperature and moisture allows for successful development and fitness-relevant phenotypes are compromised at extremes. Regarding moisture, all anoles studied have pliable-shelled eggs that must absorb water from the nest environment for successful development. As such, anoles have provided insight into relationships between water availability during development and important aspects of embryo physiology. Due to their small size and consequently large surface-area-to-volume ratio, anole eggs quickly desiccate in relatively dry incubation media (Socci et al., 2005; Hall et al., 2021; Warner et al., 2021). Some studies find that egg survival increases with moisture availability (e.g., Reedy et al., 2013) but most observe an optimum level of moisture for egg survival (Andrews and Sexton, 1981; Socci et al., 2005; Hall et al., 2021). Indeed, eggs rapidly desiccate in dry conditions, but excessively moist environments lead to fungal infection and embryo death (Andrews and Sexton, 1981; Socci et al., 2005). Additionally, greater water availability during development results in relatively large hatchling body size by enhancing the efficiency of conversion of yolk to hatchling somatic tissue (Warner et al., 2012; Hall et al., 2021). This water uptake by anole eggs is a passive process early in development but actively regulated later in development (Warner et al., 2011). Results from these studies have implications for maternal nesting behavior: wetter substrates enhance hatching success and hatchling body size, demonstrating the fitness benefits of anoles' tendency to nest in moist microhabitats.

The relationship between nesting behavior and results from incubation temperature experiments is less straightforward than those of moisture experiments. Warmer temperatures speed developmental rates and enhance locomotor performance for *A. sagrei* (Pearson and Warner, 2016, 2018; Hall and Warner, 2018), but cooler temperatures enhance growth rates in *A. carolinensis* (Goodman, 2008). In the wild, females typically select nest sites that are cooler than what is generally available for nesting (Pruett et al., 2020; Tiatragul et al., 2020). Therefore, nesting behavior related to temperature probably represents a trade-off between enhancing fitness-relevant traits of hatchings and maximizing egg hatching success. This is illustrated by a recent study demonstrating that embryos and hatchlings have different thermal optima with respect to incubation temperature (Pruett and Warner, 2021): egg survival is greatest at relatively cool temperatures while hatchling survival is greatest for those incubated at warmer temperatures. The different optimal incubation temperatures for embryos vs. hatchlings might be related to the relatively shallow nests that anoles construct. As a result, anole nests exhibit extreme, diurnal variation in temperature which often exposes eggs to stressfully warm temperatures for a few hours each day (Sanger et al., 2018; Hall and Warner, 2021). Therefore, nest sites must be warm enough to enhance important hatchling traits but cool enough to avoid lethal extremes. Many incubation experiments demonstrate that heat stress during incubation (even exposure for <1h) have morphological, physiological, and ecological consequences (Hall and Warner, 2018, 2019, 2021; Pearson and Warner, 2018; Sanger et al., 2018; Tiatragul et al., 2020). For example, eggs of the crested anole (*A. cristatellus*) have greater survival when incubated under relatively cool temperatures that females select compared to incubation temperatures that reach high extremes that are rarely experienced in maternally-selected nests (Tiatragul et al., 2020).

In addition to numerous effects of abiotic factors, two studies have considered the biotic factor of egg aggregation due to communal nesting on egg survival and embryo development (Warner and Chapman, 2011; Dees et al., 2020). Most reptiles lay multi-egg clutches potentially generating competition for moisture or oxygen among eggs or altering nest temperatures via metabolic heating. However, the single-egg clutch of anoles may eliminate such effects. Conversely, in the wild, anole eggs are often found in communal nests which may indicate a shortage of acceptable nest sites (i.e., constraint hypothesis) or some advantage to laying eggs together (i.e., adaptive hypothesis). Warner and Chapman (2011) incubated *A. sagrei* eggs alone, paired with a live egg, and paired with an artificial egg. They found no adverse effects on egg survival and embryo development when eggs incubated adjacent to a live egg or an artificial egg, but eggs that incubated next to an egg that eventually died experienced reduced water uptake and hatchling body size. Dees et al. (2020) incubated *A. sagrei* eggs alone or in groups of 4 or 9 eggs. Incubation in groups reduced water uptake by eggs and body condition of hatchings. Indeed, in the 9-egg aggregation, water uptake was negatively related to the number of eggs that each egg in the cluster was touching, implicating competition among eggs for water. These studies demonstrate there are potential costs for egg-aggregation in anoles, thus, communal nesting in this group may be due to constraint rather than some adaptive advantage.

Comparative studies of egg incubation across populations or species can provide important insights into the evolution of embryo tolerances and plasticity. However, despite a substantial upward trend in the number of incubation studies conducted with anoles (**Figure 1**), most recent studies focus on a single species, *A. sagrei*, likely due to its availability, high fecundity, and hardiness in captivity. Of the nearly 400 species of anoles only six have been used in incubation studies. Although studies abound comparing morphological, ecological, and physiological traits of adult anoles across species or populations, only four incubation studies have made similar comparisons. For example, latitudinal comparisons of the North American *A. carolinensis* show that the effects of egg incubation temperature on offspring growth varies among populations (Goodman, 2008), and that thermal plasticity of offspring cell size (erythrocytes and epithelial cells) is greater in southern vs. northern populations (Goodman and Heah, 2010). In studies of species comparisons, eggs of *A. auratus* exhibit greater water storage and desiccation resistance under dry incubation conditions compared to those of *A. limifrons,* which may relate to habitat-specific adaptations in eggshell morphology (Andrews and Sexton, 1981). Additionally, *A. sagrei* embryos are more tolerant of heat stress than those of *A. cristatellus*, potentially reflecting physiological adaptation to species-specific nesting habitats (Hall and Warner, 2019). Given the results of these studies, the diversity of nesting habitats used across anoles, and the general tendency for adult anoles to differ in morphology

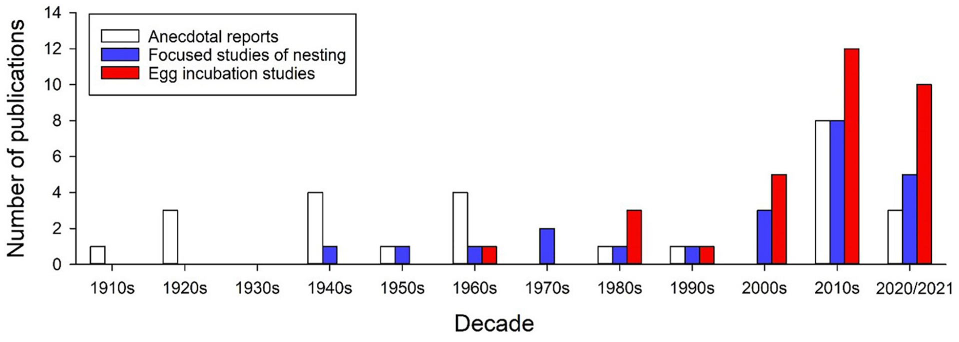

FIGURE 1 | Number of publications over the past century on aspects of nesting ecology in *Anolis* lizards. These publications include anecdotal reports, focused studies of maternal nesting behaviors and descriptions of nest environments (observational and experimental), and egg incubation studies (lab and field) of the effects of incubation environments on egg survival, embryo development and offspring phenotypes. Note that the upward trend will likely continue through the 2020 decade, which is currently represented by only 2 years. **Supplementary Table 1** provides details of each study in these categories.

and physiology, there is great potential for habitat-specific adaptations of embryo responses to nest conditions.

KNOWLEDGE GAPS AND FUTURE DIRECTIONS

The number of studies that have examined anole nesting and the effects of egg incubation environments has increased in the past decade (**Figure 1**), but there are several notable knowledge gaps. Research programs that address these topics would greatly advance our understanding of anole biology, and ecology and evolution more generally. Here, we have identified four topics that need more research attention and explain how addressing these knowledge gaps can contribute to broader areas of research.

Studying Developmental Plasticity in Real Nest Environments

While laboratory incubation studies are useful for quantifying developmental plasticity and for making predictions of microhabitats that females "should" use for nesting, there are several limitations concerning the utility of lab results. For example, treatments used in many incubation studies do not simulate the complex nest environment in the wild. That is, real nest environments are composed of a range of variables (e.g., substrate composition and chemistry, hydric and thermal conditions, oxygen, micro-organisms, and nest predators) that fluctuate over the incubation period. These variables can also include soil pollutants that are commonly used in ecotoxicology studies; to our knowledge, no studies have examined effects of chemical pollutants on embryo development in anoles. Decades of research demonstrate that variation in these incubation conditions results in a myriad of phenotypic effects on reptile embryos and hatchlings (Warner et al., 2018). Thus, while several authors of laboratory incubation studies make inferences about embryo plasticity and nesting behavior in the wild (including ourselves), we must be cautious in our interpretations of lab results. Indeed, most experiments poorly reflect natural nest environments but have served as a foundation for our current understanding and future work (Hall and Warner, 2020). These concerns highlight a major gap in our understanding of the effects of the multifaceted nest environment, and creative designs for field and laboratory studies are needed to quantify the interactive effects of multiple environmental factors (e.g., factorial experiments of moisture and temperature) to better understand the consequences of developmental plasticity and nesting behavior. Fortunately, because anoles have several characteristics that make them suitable models for studies of developmental plasticity (discussed above), filling this knowledge gap is achievable.

Predicting Effects of Global Change

Several attributes make anoles particularly useful to study effects of global change on nesting behavior and egg survival. Females construct relatively shallow nests which exhibit wide variation in temperature and moisture. For example, nest temperatures can vary greatly across spatial scales (e.g., differences in canopy cover; Pearson and Warner, 2016), across temporal scales during a long reproductive season (Pearson and Warner, 2018), or due to both spatiotemporal effects (Pruett et al., 2020). In particular, the relatively broad reproductive season of anoles and their propensity to colonize a diversity of habitats (e.g., urban vs. natural areas) means that nest conditions can change markedly throughout the breeding season and across habitats resulting in eggs experiencing an array of environmental conditions. As such, developmental tolerances of embryos are broad with respect to moisture and temperature (Reedy et al., 2013; Pruett and Warner, 2021) and nesting behavior is highly plastic, since females are capable of locating acceptable nest sites across a diversity of conditions (Hall et al., 2021).

Seasonal changes in precipitation due to climate change will likely influence nesting behavior and egg survival in complex ways. For example, Chalcraft and Andrews (1999) simulated rainfall in both wet and dry years and observed greater egg depredation of *A. limifrons* by ants in wet vs. dry plots, indicating an interaction between precipitation and depredation. This system could be used to understand how changing patterns of rainfall due to climate change will alter species interactions. Additionally, nests exhibit wide spatiotemporal variation in temperature, resulting in variable egg survival (Pruett et al., 2020). *Anolis* embryos routinely encounter stressful temperatures in the wild under current climate conditions (Sanger et al., 2018; Hall and Warner, 2021); therefore, future changes in mean and maximum nest temperatures due to climate warming will require mitigation via changes in nesting behavior, embryo thermal physiology, or both. Anoles could be useful models to understand how nesting behavior may mitigate adverse effects of climate change on offspring.

Finally, human-caused habitat changes (e.g., agriculture and urbanization) have context-dependent effects on embryo development and egg survival in anoles. For example, nests in urban and suburban habitats exhibit higher mean and maximum nest temperatures compared to forested areas (Tiatragul et al., 2017, 2019), potentially reducing hatching success and hatchling viability (Hall and Warner, 2018). However, the harmful effects of these extreme temperatures vary among species (Hall and Warner, 2019). Alternatively, Schlaepfer (2003) found that anole eggs incubating in agricultural fields exhibit faster development and higher survival than those in adjacent forest interior or edge habitat. Thus, habitat alteration may enhance survival for species whose eggs are well-adapted to disturbed conditions but reduce survival for others. Given the broad variation in habitat preference across *Anolis*, the high density of many species in both natural and disturbed areas, and the abundance of eggs due to high fecundity, research on anoles can provide novel insight into how nesting ecology relates to habitat disturbance and other aspects of global change.

Evolutionary Potential of Nesting Behavior and Developmental Plasticity

While several of the studies described above provide evidence of adaptive nesting behavior, we lack a strong grasp of the evolutionary potential of this important reproductive trait.

Quantifying the evolutionary potential of nesting behavior and embryo plasticity will provide insight into basic questions about the evolution of maternal effects and phenotypic plasticity. Moreover, nesting behavior may also place constraints on the evolution of embryo plasticity in that maternal nest-site choice could buffer embryos from environmental variation (Tiatragul et al., 2020) and reduce the strength of selection on embryo reaction norms. In addition to these important conceptual topics in ecology and evolution, an understanding of the evolutionary potential of these traits will inform predictions of how these animals respond to environmental change. Moreover, given the abundance of invasive anoles across the planet, research on this topic will provide key insight into their capacity to successfully establish in novel habitat outside their native range.

To address these knowledge gaps, we need to quantify the strength and form of natural selection acting on nesting behaviors and embryo reaction norms, as well as estimates of their heritability. Studies that address these topics are challenging, particularly in species that lay single egg clutches over long reproductive seasons, such as anoles. In particular, researchers would need to identify a suitable proxy for maternal fitness (i.e., hatching success of some or all the eggs produced by an individual) and examine its relationship with some aspect of nesting behavior (e.g., choice of nest habitat). Although this is inherently difficult with anoles due to the numerous nesting events of individuals throughout a reproductive season, these challenges can still be addressed with well-designed field or laboratory experiments that investigate selection on maternal nesting behaviors and embryo reaction norms for relevant traits. Additionally, lab-based quantitative genetics experiments that span generations could be designed to quantify the degree of heritability in maternal nest-site choice and embryo reaction norms. Such experiments have been conducted for thermal traits in anoles (Logan et al., 2018), and could be extended to traits associated with nesting ecology. Of course, such experiments are challenging, but creative experimental designs, dedicated research programs, and recent advances in quantitative genetic methods will help advance this poorly understood aspect of anole nesting ecology.

Comparative Studies of Embryo Physiology

Anolis has been used extensively in studies of comparative morphology and physiology; however, similar studies of embryos are lacking (but see Sanger et al., 2008b; Hall and Warner, 2019). Moreover, *Anolis* lizards are considered a model system for adaptive radiation, but studies never consider that embryo plasticity and egg survival may be an important driver of evolution (Kolbe et al., 2012a). This is important because egg survival can determine population cycles for anoles (Andrews, 1988), and likely plays a vital role in population viability, survival, and colonization success (Losos et al., 2003). Additionally, the anole radiation is characterized by multiple, independent dispersal events, often from and to small islands throughout the Caribbean (Poe et al., 2018; Huie et al., 2021). Although key innovations, phenotypic plasticity, niche expansion and other processes are considered important in such dispersals, these processes are typically evaluated from the perspective of adult phenotypes. Successful embryo development, however, is required for persistence in every environment. Comparative studies of embryo physiology and developmental plasticity would illuminate the importance of embryo adaptation in colonizing novel environments (e.g., urban landscapes) and responding to environmental perturbations caused by global change (e.g., climate change). Currently, data on nest conditions and embryo physiology are only available for a few species. More studies of nesting ecology and embryo development are needed for a variety of species from across the phylogenetic tree. Such comparative studies (which are currently underway, Muell et al., 2022) will enable phylogenetically-informed analyses of the macroevolution of developmental plasticity of anoles, and have the potential to provide novel insights into the role of nest environments in shaping the *Anolis* radiation.

METHODS FOR STUDYING ANOLE NESTING

Quantitative data on anole nests in the field are needed to design laboratory experiments and interpret their results in an ecological context. Yet, this information remains largely unavailable for most species. Perhaps the primary reason why *Anolis* nesting ecology is poorly studied is due to the difficulty of finding nests in the field. Here we describe methods we have used to find nests in habitats that are commonly used in evolutionary and ecological studies of anoles: urbanized habitat (Winchell et al., 2016; Battles et al., 2019), densely forested habitat (Leal and Fleishman, 2002, 2004), and small islands (Schoener and Schoener, 1980; Losos et al., 1997; Campbell and Echternacht, 2003; Calsbeek and Cox, 2010; Kolbe et al., 2012b; Stuart et al., 2014). Importantly, anole biologists could incorporate our methods into their work to enhance understanding of *Anolis* ecology and evolution more generally. Our approaches were developed for studies of invasive anoles in Florida (*A. sagrei* and *A. cristatellus*), but our methods could be applied or modified for other species or in different habitats. Searching for small eggs is a daunting task, but to make searches manageable we limited the coverage to several 1 m^2 quadrats at each location and used spoons to turn up 1–5 cm of soil and comb through the substrate. Although this protocol was used for both approaches, we describe below important differences in methodology in our different habitat types.

Nest Searching on Spoil Islands

Spoil islands have been important settings for *Anolis* research for several reasons (geographic separation, relatively easy population manipulations, etc.) (Campbell and Echternacht, 2003; Stuart et al., 2014; Pearson and Warner, 2018; Kahrl et al., 2021). Our study was conducted on a spoil island within the Guana Tolomato Matanzas National Estuarine Research Reserve in the Intracoastal Waterway near Palm Coast, Florida (**Figure 2A**). The island is approximately 90 m long and 60 m wide at its longest/widest points with a canopy of palm trees and red cedar trees (∼30 m

FIGURE 2 | (A) An aerial view of the spoil island used for studying nesting behavior. Each white dot represents a stake in the grid system. **(B–E)** Photos from the island field site where we searched for anole nests. The 1 m^2 quadrat that we used for searching **(B)**, a brown anole egg found under a rock **(C)**, a targeted search being conducted under rocks **(D)**, and a random search being conducted in dense vegetation **(E)**.

in diameter) in the central part of the island, surrounded by low brush and dirt patches, and a periphery of needlerush (*Juncus roemerianus*) near the shoreline. This island contains a dense population of *A. sagrei*, but *A. carolinensis* is present in lower numbers. We installed a permanent 9 × 11 grid system across the island using PVC stakes (spaced 5 m from each other), resulting in eighty 5 m × 5 m square grids (**Figure 2**). Stakes along the eastern shoreline were occasionally swept away when parts of the island eroded due to waves. Such a grid system is useful for studies of nesting in anoles as well as studies of population ecology (Andrews, 1988). We credit Robin Andrews for the original use of such a system to study anole nesting.

At this site, we used three separate search methods to reduce biased searching among microhabitats while ensuring sufficient sample sizes. These included selecting randomly-located quadrats (i.e., random searches), an intermediate method that combined random and non-random components (i.e., targeted-random searches), and non-randomly selecting quadrats (i.e., targeted searches). The random searches were performed to eliminate potential bias by covering as much of the island as possible and searching across all microhabitats. We used a random number generator to select a stake in the grid system, and then used the random number generator to select a distance (0–500 cm) and direction (0–359°) from each stake. We placed a 1 m^2 quadrat over the resulting location and searched the square thoroughly, removing all potential cover objects and leaf litter. While this method reduces bias and covers all microhabitats, much of the landscape is not used for nesting and this method resulted in finding few nests. Thus, additional search methods were used.

For the targeted-random search method, we randomly selected a grid and then placed the 1 m^2 quadrat in a location within the grid that we thought was most likely to contain an egg (based on *a priori* knowledge of where we find eggs; i.e., beneath cover objects in relatively shaded, moist areas). This method ensured that we covered a significant portion of the island and the various habitat types, but greatly increased sample size. For the targeted searches, we did not use the grid system, but searched the island for sites that looked suitable for egg incubation based on previous knowledge. The specific microhabitats that we targeted were based on anecdotal reports (e.g., in leaf litter, under cover objects, in moist areas, see review above and **Supplementary Table 1**). When an egg was located using the targeted search method, we placed the 1 m^2 quadrat over the nest (with the egg in the middle) and searched the entire quadrat for eggs. We conducted 20 of each search type in each sampling period to get a thorough sampling of the island.

This combination of approaches was necessary to successfully locate nests while minimizing biased search effort. For instance, had we used only the random and random-targeted approaches, we would have found only 7 and 19 nests, respectively; this low sample size would not have given an accurate assessment of the range of microhabitats used for nesting. However, by including the targeted searches (by definition, a biased method), we found 131 nests with comparatively little effort. Additionally, nests found via targeted searches can be compared to those found via randomized searches to ensure that targeted searching does not introduce substantial bias. Moreover, microhabitat data (e.g., temperature, moisture, and canopy cover) should be collected from all sites, so that nest microhabitats can be compared with what is generally available across the island (e.g., comparing sites with and without nests; Tiatragul et al., 2020) and across search methods. Thus, we advocate for using a combination of approaches to (1) quantify the general characteristics of microhabitats available for nesting and (2) determine how these microhabitats compare to those selected by females.

Nest Searching in Suburban and Forested Habitats

Anoles have been a model species for studying urban ecology and evolution (Kolbe et al., 2012b, 2016; Winchell et al., 2016, 2018; Chejanovski et al., 2017; Lapiedra et al., 2017; Battles and Kolbe, 2018; Campbell-Staton et al., 2020; Lailvaux, 2020; Narváez et al., 2020) but few studies address aspects of nesting ecology (but see Tiatragul et al., 2017, 2019, 2020). The following protocol for nest searches was used in suburban and forested habitat in Miami-Dade County, Florida, United States (**Figure 3**). Our two sites included several species of anoles, but *A. cristatellus* and *A. sagrei* were most abundant (Battles and Kolbe, 2018; Tiatragul et al., 2019). The suburban site was along a 1-km stretch of a two-lane road (State Road 959, also known as "Red Road") running parallel to a canal. The habitat consisted of mowed lawn, sparsely planted trees (including *Ficus citrifolia*, *Ficus aurea*, *Sabal* spp.) and many human-made structures like lamp posts, houses, bus stops, and guardrails. This area receives frequent vehicle and pedestrian traffic during the day and night. Searchable areas were also fragmented by roads, paved footpaths, and a canal. Additionally, because we were working next to a residential area, we limited our searches to public areas. As such, setting up a permanent grid system (as above) in this area was not preferable. The forested habitat was at Matheson Hammock Park which is a fragment of dense forest consisting of fig trees intermixed with smaller shrubs; this habitat had no human-made structures except for narrow walking trails. Since our studies (Tiatragul et al., 2019, 2020) aimed to compare characteristics of sites used by females for nesting to those not used by females, we decided that a limited random search was the best way to obtain an unbiased representation of maternal choice of nest microhabitats.

We chose four blocks within each habitat (suburban vs. forest) and searched 10 randomly selected 1 m^2 plots in each block (**Figure 4**). We used four blocks because this number captured a representative area (with statistical replication) that anoles use throughout our habitats, but researchers may increase the number of blocks depending on other factors (e.g., size of the field site; diversity of microhabitat across the study site). Additionally, researchers could also include targeted searches (as described above) to increase representation of sites used for nesting if nests are difficult to find in some habitats (see Tiatragul et al., 2020).

The location of each block was determined by the abundance of anoles to ensure that plots are sampled where nests are likely to occur. This was most critical in the suburban habitat where extensive areas were not suitable for nests (e.g., roads and buildings) or are not searchable (private property), but our block locations in the forest were not constrained because anoles were continuously abundant. Because *A. cristatellus* was the target species and they prefer relatively large, broad canopy trees, we chose four large trees in each habitat as the "anchor" point for each block. Thus, each block was a circle about the tree with radius of 20 m. Plots were selected using a random number generator to select a distance (1–20 m) and direction (0 – 359°) from the central tree. We created a 1 m^2 quadrat at each plot and searched it for eggs as described previously (i.e., removing all cover, sifting through the soil with a spoon). We counted hatched and unhatched eggs. Hatched eggs are a good proxy for where females nest, as heavy rain and flooding did not wash away hatched eggshells indicating that eggshells likely remain in the location where females oviposited. However, we still recommend observing or experimentally determining whether hatched eggs are good proxies at other field sites. Overall, we found 44 plots with eggs ($n = 31$ in forest, $n = 13$ in suburb) and 36 plots ($n = 9$ in forest, $n = 27$ in suburb) without eggs (Tiatragul et al., 2020).

FIGURE 3 | Photographs of suburban **(A,B)** and forested **(C,D)** habitats where anole nesting ecology was studied in Miami-Dade County, FL, United States. Note that the suburban site includes mowed lawn, roads, and a walking path. The same egg search methods were used in both habitats.

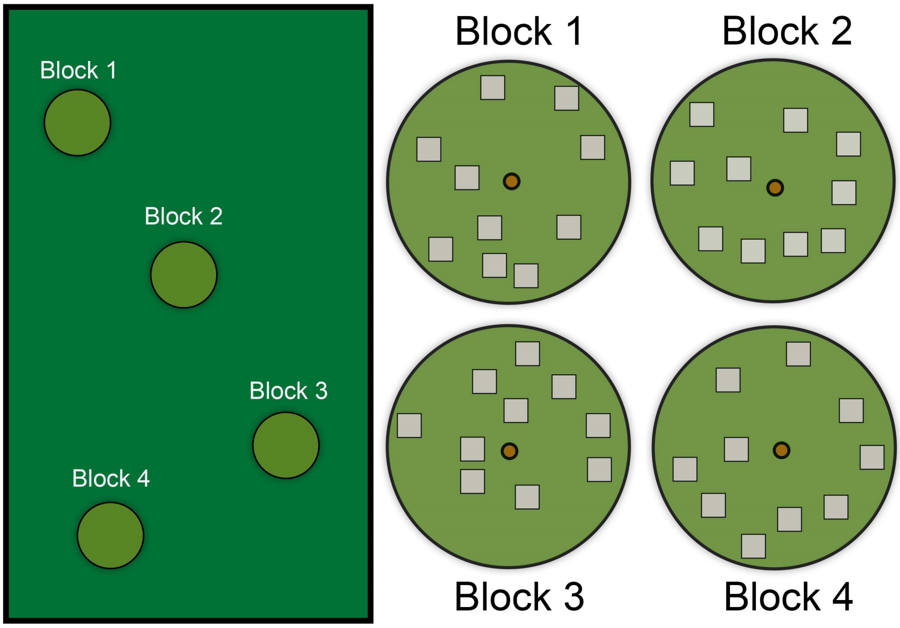

FIGURE 4 | Diagram showing four hypothetical random search "blocks" within a larger habitat. Each "block" contains ten randomly distributed 1 m^2 "plots." The location of each block is selected within the larger habitat area based on the presence of a tree inhabited by anoles. The location of each plot is determined by random distance and direction (with or without constraints) from the center anchor (i.e., small circle in the center, which was a tree in our study). In this scenario, the circular block represents the limit of the radius from the anchor. Each plot is then searched thoroughly for anole eggs.

These search methods provide a promising avenue for future research. Finding anole eggs in the field outside of chance encounters was previously considered logistically prohibitive, but the work outlined here shows otherwise. We were able to successfully locate anole nests in three different habitats, and our methods produced sample sizes large enough to perform informative statistical analyses.

Limitations and Recommendations

To maximize the chance of locating eggs, researchers should align searches with reproductive cycles since eggs are not found in equal frequencies across the year. Moreover, the random, targeted-random, and targeted searches were not equally productive, and the methods researchers employ will depend on the questions asked and may vary among species. We recommend using a randomized search method to accompany targeted searching when seeking to draw conclusions about nest site choice as this will reduce bias while allowing for sufficient sample sizes. However, if the goal is simply to locate as many eggs as possible (e.g., measuring specific habitat features like temperature or sourcing eggs for lab experiments), using targeted searches would be expedient. Additionally, because anoles occasionally nest above ground, a systematic method for searching above-ground nest sites may be required in some habitats (see Andrews, 1988). Finally, our methods are likely most effective for species that are highly fecund and occur in high densities (e.g., *A. sagrei*). Although eggs could still be found using these methods for species that occur in lower densities, sample sizes would likely be much smaller and effort much greater. For example, although the crown giant, *Anolis equestris*, is relatively abundant at Matheson Hammock, we only found one egg of this species during our study.

CONCLUSION

The environmental conditions that females select for nesting have important effects on embryo development, egg survival, and fitness-related phenotypes of hatchlings. These consequences of the nest environment indicate that maternal nesting behavior should be under strong selection, and likely varies across species and populations depending upon several factors (e.g., local habitat and evolutionary history). As such, nesting behavior is an important aspect of reptile ecology, evolution and natural history. While anole nesting ecology is relatively understudied compared to other aspects of their biology, we argue that this group of lizards has several features that could advance knowledge of nesting behavior and developmental plasticity. Indeed, we highlight several recent studies of anoles that make progress toward understanding their nesting ecology, but much work remains. We hope that future studies will consider this radiation of lizards as a useful taxonomic group for research related to nesting ecology and evolution as well as developmental plasticity. With established protocols for locating and monitoring nests in the wild, breeding anoles in captivity, and incubating eggs and staging embryos in the laboratory, we have a complete toolkit to illuminate broader trends in ecological developmental biology, global change research, comparative embryo physiology, and the evolution of nesting behavior in vertebrates.

AUTHOR CONTRIBUTIONS

JMH and DAW conducted comprehensive reviews of the literature on nesting and developmental plasticity. JEP, ST, and DAW developed nest searching methodology. JEP, JMH, and DAW contributed equally to writing the first draft of the manuscript. All authors contributed critically to the subsequent drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.821115/full#supplementary-material

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REVIEW
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Do Freshwater Turtles Use Rainfall to Increase Nest Success?

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Rainfall following turtle nest construction has long been believed to increase nest survival by its effects on reducing the location cues used by nest predators. However, it is unclear if this is generally the case and if nesting turtles actively use this mechanism to increase their reproductive fitness by deliberately timing nesting to occur before or during rainfall. To address this question, we reviewed studies that examined freshwater turtle nesting behavior and nest predation rates in relation to rainfall. We supplemented our review with data on rainfall and nesting patterns from a 12-year study of two nesting populations of Ouachita Map Turtles (*Graptemys ouachitensis*). Our review revealed a diversity of responses in rainfall effects on predation and in the propensity for turtles to nest in association with rain. Our mixed findings could reflect a diversity of species- or population-specific responses, local adaptations, species composition of predator community, confounding abiotic factors (e.g., temperature decreases after rainfall) or methodology (e.g., most studies did not quantify rainfall amounts). Our case study on map turtles found very high yearly predation rates (75–100%), precluding our ability to rigorously analyze the association between nest predation and rainfall. However, close examination of the exact timing of both rainfall and predation revealed significantly lower predation rates when rain fell within 24 h after nesting, indicating that rainfall during or after nesting may reduce nest predation. Despite this effect, the best fitted model explaining the propensity to nest found that map turtles were more likely to nest after dry days than after days with rainfall, suggesting that rainfall was not a major factor driving turtles to nest in our populations. In both our review and in our map turtle populations there was little evidence that turtles can anticipate rainfall and nest prior to it occurring (e.g., in response to falling barometric pressure).

Keywords: nest predation, predator cues, nesting, rain, barometric pressure, Reptilia, Testudines

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INTRODUCTION

Turtles in the modern world face mounting challenges to their continued existence. In addition to population losses due to habitat loss and degradation, reproductively valuable adults are exploited as commodities for human consumption and the international pet trade while being concurrently exposed to increased mortality from introduced predators, roadways, pollution, and disease (reviewed in Lovich et al., 2018). At the other end of the life cycle, eggs and hatchlings are threatened by elevated nest predation levels due to both anthropogenically increased densities of nest predators (Mitchell and Klemens, 2000; Prugh et al., 2009) and potentially increased nest-detection efficiencies of predators concentrating their efforts on nesting areas reduced in size from habitat loss and vegetational succession (Temple, 1987; Oehler and Litvaitis, 1996; Jackson and Walker, 1997; Marchand and Litvaitis, 2004).

Emerging threats from human-induced climate change present yet another threat to turtle populations on a pervasive, worldwide scale. Climate change is expected to bring changes to rainfall patterns due to higher global temperatures producing a more active hydrological cycle and increasing atmospheric water-holding capacities (Collins et al., 2013). Precipitation has increased about 2% since the beginning of the 20th century, although its distribution is neither spatially or temporally uniform (reviewed in Dore, 2005). As nest predation rates may be affected by rainfall and its timing relative to nesting events, this climatic variation may impact turtle populations in different ways: in some cases, potentially increasing nest success and hatching recruitment, and in others, reducing nest success (Czaja et al., 2018). Understanding how rainfall and correlated factors affect turtle reproduction is thus important in placing their potential impacts into an ecological context and in predicting how these effects may be altered by ongoing anthropogenic climate change.

Unlike mammals and birds which typically invest large amounts of time and resources in the protection and nurturing of their young, turtles lack direct knowledge of the eventual fate of their reproductive efforts, eliminating the chance of increasing nest success and offspring survival via adult learning and experience that may occur in other taxa (e.g., in some songbirds; Zanette, 2001). Nonetheless, mechanisms to increase nest and hatchling survival are presumed to be under strong selection pressure, including those which reduce nest predation (e.g., Spencer, 2002; Refsnider and Janzen, 2010; Schwanz et al., 2010; Bernstein et al., 2015; Czaja et al., 2020) and, thus, may be expected to evolve via natural selection (Spencer, 2002) given sufficient heritable variation in the relevant behavioral traits (Dochtermann et al., 2019). However, as nest location cues available to above-ground predators may be diverse and may arise as necessary components of nest construction (e.g., odors from recently disturbed soil; reviewed in Geller and Parker, 2022), turtles may be limited in their ability to significantly reduce the nest location signals of their subterranean nests (Voves et al., 2016). Nonetheless, the role of significant rainfall in reducing the signal strength of nest location cues and increasing nest survival has long been recognized (e.g., Legler, 1954), with some authors suggesting that, in some contexts, little hatchling recruitment would take place without it (Carr, 1952). Thus, one of the few ways turtles may be able to mitigate nest predation risks is by nesting during or just before significant rainfall (e.g., Burke et al., 1994; Bowen and Janzen, 2005).

Herein we test the hypothesis that turtles can use rainfall as a cue to reduce nest predation rates. Specifically, we surveyed the literature to synthesize our present understanding of (1) the propensity for turtles to preferentially nest either before or after significant rainfall; (2) how nest predation rates of freshwater turtle nests are affected by varying amounts of rainfall; and (3) the degree to which female turtles appear physiologically able to anticipate rainfall and accordingly time nest construction activities to enhance their own fitness. To supplement our literature review, we investigated associations of rainfall with nesting activity and nest survival using a 12-year data set from two populations of Ouachita Map Turtles (*Graptemys ouachitensis*) from Wisconsin, during 2008–2021. This data set, among the first to use trail cameras as primary data collection tools, provided fine-scale resolution on nesting, rainfall, and nest depredation timelines as well as on predator species involved and allowed us to assess nesting propensity and nest predation risk in the proper temporal contexts. We discuss our results within the context of expectations based on our literature review.

METHODS

Literature Reviews

We surveyed over 60 papers providing data or speculating on the relationship between rainfall and nest predation rates in freshwater turtles. The papers were found by searching the references sections of published work and by on-line searches using Google, Google Scholar, and the academic research databases of the University of Wisconsin Library System (>1100 e-collection content selections from Primo Central Index [PCI] from Ex Libris [ProQuest] including Web of Science and Scopus) using the keywords: "turtle:nest:predation:rain;precipitation." The papers were mostly peer-reviewed publications but some unpublished M.Sc. and Ph.D. theses were also included. Papers that only cited previous publications without providing new data were not included or are distinguished as such in this review.

Case Study From a Ouachita Map Turtle (*Graptemys ouachitensis*) Metapopulation

To supplement literature reviews, we also analyzed data on rainfall, nesting activity, and nest survivorship from two populations of Ouachita Map Turtles (*Graptemys ouachitensis*) located on the lower Wisconsin River within 10 km of Spring Green, WI, United States (43°10′38′′N and 90°04′02′′W). Both nesting sites are on sand terraces approximately 15 and 52 m from the main river channel, respectively, and are comprised of various xerophytic herbaceous vegetation covering approximately 20% of the surface, with the remainder being open sand (for a more complete description of these sites, see Geller, 2012a).

The study was carried out over 12 years from 2008 to 2021 excluding the years 2012 and 2018. Newly constructed turtle nests were located beginning in late May of each study year by review of images from pole-mounted trail cameras (RECONYX®, Inc.; Holmen, WI, United States) monitoring each nesting area. Cameras were programmed to take continuous time-lapse images at 1-min intervals to document nesting events along with motion-triggered series of more closely timed photographs to provide detailed documentation and timing of nest predator (all Northern raccoons, *Procyon lotor*) visits. Surveillance ended each year with either the documented predation or hatchling emergence of the last monitored nest in each year.

We obtained total daily rainfall amounts (in mm) from two sources: (1) data from the Lone Rock Tri-County Airport, Sauk County, Wisconsin, approximately 9.7 km northwest of the study sites (downloaded from NOAA National Climate Data Center[1], station id: GHCND:USW00014921); and (2) from an on-site rain gauge. Where the two sources differed, we used the rainfall amounts from the on-site gauge, although these were not always available due to logistic constraints. In some years, rainfall timing on the site was determined by use of a funnel-driven waterwheel device enclosed within a Lucite®-fronted housing placed within the peripheral camera field-of-view (Geller, 2012a). Date-stamped time-lapse images indicating waterwheel movement delineated rain event timelines. These units were accurate to within 0.2 h of rainfall duration (sprinkles to heavier amounts), as determined by field tests and camera-visible nocturnal rainfall. In all years, within-day rainfall event amounts and timing were estimated using hourly rainfall tracking charts from Lone Rock, Wisconsin via Weather Underground historical weather charts[2]. We also used this database to derive metrics on daily high, low, and mean temperatures; and daily high, low, and mean air pressures.

Statistical Analysis

Our unit of observation was a day on which turtle activity was monitored for which we had rainfall data. For modeling purposes, we defined the nesting season as starting one day prior to the first recorded turtle nest and ending one day after the last recorded turtle nest for each year (12 years from 2008-2021, excluding 2012 and 2018). We excluded 26 days when the sites were flooded so nesting could not take place and, to be conservative, also excluded 28 nests constructed within 5 days after flooding because the timing of these nests may have been affected by females retaining eggs during flooded periods. In some years, some nests were protected from predation using nest cages. Protected nests were included in the analysis of nesting behavior in relation to rainfall but not in examining how rainfall affects nest predation risk. Reported sample sizes reflect varying numbers of camera records available for analysis, as influenced by camera position, intervening vegetation, and other variables.

We modeled the number of nests constructed each day (counts from 0 to 6) as a function of four variables that capture how turtle nesting might respond to daily rainfall variation: (1) rain on the day of nesting or not; (2) rain on two consecutive days (high rain frequency periods); (3) no rain for two consecutive days (low rain frequency periods); and (4) no rain on the day and rain the next day (nesting in anticipation of oncoming rain). Although we had data on the amount of rainfall for each day, using rainfall presence/absence provided a better fit to the data as indicated by lower values of Akaike's Information Criteria (AIC).

We fitted four models to the data, with the number of nests constructed each day as the response variable and one of the four rainfall variables above as a univariate predictor in each model. We specified a Poisson error distribution (appropriate for count data) and included an observation level factor and year as random effects in each model. The observation level factor accounted for any overdispersion in the data, while the inclusion of year as a random effect accounted for the possibility that the probability of nesting per day was higher, on average, in some years than others. We compared the fit of the four models to the data using AIC, with better fitting models having lower AIC values. The models were fitted using the lme4 package (Bates et al., 2015) in the statistical analysis software R (R Core Team, 2017). We also tested for relationships the between number of nests constructed and the measures of daily temperature and air pressure by including these as univariate predictors as described above.

RESULTS

Literature Review of Association of Turtle Nesting Activity and Rainfall

We reviewed 42 studies that quantified or addressed the potential association of nesting with rainfall in 23 species of freshwater turtles. Of these, 29 (69%) studies demonstrated or suggested that the propensity to nest was associated with rainfall, compared to 13 (31%) studies that found no association (**Tables 1**, **2**). The research to-date is, thus, somewhat equivocal with regards to the association of rainfall and turtle nesting activity.

However, when associations have been found, our review revealed a general consensus for turtle nesting to occur during or after rainfall, rather than before it (**Table 1**). In addition, the few studies that systematically examined the effect of rainfall amount on nesting activity found larger rainfall amounts more likely to stimulate turtle nesting during or after the rain event than lesser amounts ($\geq$10 mm, Jackson and Walker, 1997; $>$2.5 cm, Tucker, 1997; see also Walde et al., 2007; Bernstein et al., 2015; **Table 1**). However, Buckardt et al. (2020) found no relationship between rainfall amount and the propensity to nest, while others noted that heavy rainfall suppresses nesting activity (Legler, 1954; Burger and Montevecchi, 1975), although in some cases this is likely due to a concurrent decrease in air temperatures to non-optimal levels rather than rainfall amount effects *per se* (e.g., Harding and Bloomer, 1979; Vogt, 1980).

Some early reports (e.g., Pallas, 1960) are primarily anecdotal observations about particular rainfall events and do not provide the ecological context in which to evaluate the uniqueness of the association, such as the numbers of turtles nesting in other conditions. Limitations in study designs or data acquisition have restricted some other studies to basic reports on the association of nesting with rainfall on a simple binary, rain present/absent basis within a given calendar day, and do not allow assessments of the impact of rainfall within different temporal periods (e.g., previous 24 h) on nesting propensity or, importantly, how rainfall amounts were quantitatively distributed in relation to the timing of nesting events. For example, 77% of the reviewed studies that failed to find an association of nesting activity and rainfall did not

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[1] https://www.ncdc.noaa.gov/cdo-web

[2] https://www.wunderground.com/history/daily/us/wi/spring-green/KLNR/date/2008-6-1

TABLE 1 | Papers suggesting freshwater turtles nest in association with rainfall.

Citations	Turtle species	Associated variables	Comments
Sexton, 1959	*Chrysemys picta*	Nesting periods were usually associated with rain	Generalized conclusion based on three nesting seasons
Pallas, 1960	*Glyptemys insculpta*	Observed nesting during a light drizzle	This single observation considered by Harding and Bloomer (1979) to be atypical for this species
Goode, 1965	*Chelodina longicollis, Chelodina expansa, Emydura macquarii*	Turtles tended to nest after heavy rains and during periods of high humidity	
Hammer, 1969	*Chelydra serpentina*	Light precipitation in association with rising nighttime temperatures increased nesting activity	Nesting activity was most likely on warm evenings coincident with or following precipitation, possibly because rainfall facilitated nest construction
Vestjens, 1969	*Chelodina longicollis*	Nesting activity followed rainfall	Believed rainfall facilitated nest construction (*in* Stott, 1988)
Thomas, 1972	*Pseudemys floridana*	Nesting activity is enhanced by rain (*in* Jackson and Walker, 1997)	
Burger and Montevecchi, 1975	*Malaclemys terrapin*	Nesting activity increased after rainfall, but was not observed during heavy or prolonged rain	
Congello, 1978	*Terrapene carolina*	Nesting occurred at night and was associated with periods of storm activity, but appeared independent of air temperature	
Clay, 1981	*Chelodina colliei*	Nesting associated with rain-bearing, low-pressure systems, falling barometric pressure, and air temperatures > 17°C	Turtle movements were believed triggered by barometric pressure changes in anticipation of rainfall
Thompson, 1983	*Emydura macquarii*	Nesting usually occurred during or just after rainfall	
Georges, 1984	*Chelodina longicollis*	Movements to nest sites coincided with rainfall	
Congdon et al., 1987	*Chelydra serpentina*	Nesting activity was usually associated with warm temperatures during or just after rainfall	Reported that nesting activity can occur during all hours of the day or night in association with periods of warm rain or warm periods following rainfall
Stott, 1988	*Chelodina longicollis*	Movements from ponds to nest sites were associated with rain and relatively warm temperatures	Although slightly more turtles began movements under falling barometric pressure conditions than rising conditions, this difference was not significant
Kuchling, 1993	*Pseudemydura umbrina*	Nesting typically occurred during rain-bearing, low-pressure weather fronts; nests were constructed on overcast days, during which rain fell approximately half of the time	
Burke et al., 1994	*Kinosternon subrubrum*	Most nesting forays and nest construction occurred during periods of rainfall	Study was conducted during a period when rain fell every few days, thus mud turtle nesting behavior during periods of infrequent rain during drought conditions may vary from that described; it is unlikely that mud turtles require wet soil for nest construction
Roosenburg, 1994	*Malaclemys terrapin*	Most nesting occurred after rainfall	
Jackson and Walker, 1997	*Pseudemys concinna*	Most nesting occurred after rainfall	Trace to heavy rainfall (≥ 5 cm) was associated with nesting activity; rainfall of 1 cm appeared to simulate most females holding adequately shelled eggs to nest; estimated that 83% of nesting events were in response to previous rain
Tucker, 1997	*Trachemys scripta*	Nesting activity generally appeared to increase after rain	Association of rainfall and its timing with nesting activity was complex; suggested that both rainfall and temperature were cues that initiated most nesting behavior; Figures 5, 6 show days with > 2.5 cm rain were often associated with peak nesting activity
Burke et al., 1998	*Kinosternon subrubrum, Pseudemys concinna, Trachemys scripta*	Nesting often occurred in bouts associated with rainfall	
Litzgus and Brooks, 1998	*Clemmys guttata*	Peak of nesting appeared to be delayed until rainfall following a 2-week dry period	
Wilson et al., 1999	*Kinosternon baurii*	Movements to and from nest sites coincided with rainfall	
Booth, 2002	*Chelodina expansa*	Movements to and from nest sites coincided with rainfall	
McCosker, 2002	*Chelodina expansa, Emydura signata*	Movements to and from nest sites coincided with rainfall	

(Continued)

TABLE 1 | (Continued)

Citations	Turtle species	Associated variables	Comments
Bowen et al., 2005	*Emydura macquarii*, *Chelodina expansa*	Nesting generally occurred during or after rain	Weather conditions differed significantly between nesting and non-nesting days; rainfall made the most impact on whether or not nesting occurred in *E. macquarii*; changes in air temperatures were most important for *C. expansa*. (Compare to Bowen et al., 2005, **Table 2** for *Chrysemys picta*.)
Najbar and Szuszkiewicz, 2005	*Emys orbicularis*	Yearly nesting activity began after a period of rainfall, usually on sunny days when temperatures exceed 20–25°C	A seasonal, not daily, assessment
Walde et al., 2007	*Glyptemys insculpta*	Nesting activity increased during heavy rainfall	38% of all nests were constructed during one heavy, mid-day rain event; turtles appeared and nested during rainfall
Bernstein et al., 2015	*Terrapene ornata*	Nesting activity increased during heavy rainfall (Bernstein, unpublished data)	Supporting metrics not reported
Espinoza et al., 2018	*Elusor macrurus*	Nesting peaked in October and November following > 10 mm rain	A seasonal, not daily, assessment
Muell et al., 2021	*Chrysemys picta*	Most (60%) rain events were associated with nesting activity within the same hour	Used marked turtles to allow for inter-clutch nesting availability; hours with higher temperatures and rainfall were associated with higher levels of nesting activity; did not discuss how this result may have differed from that if all nesting events were included

TABLE 2 | Papers suggesting freshwater turtles do not nest in association with rainfall.

Citations	Turtle species	Comments
Legler, 1954	*Chrysemys picta*	No turtles observed nesting during heavy rainfall; data observational, without supporting metrics
Plummer, 1976	*Apalone mutica*	Few nests (11%) constructed on overcast or rainy days; 72% of the nests constructed during fair weather were constructed during the first 3 days after overcast or rainy weather; effect of rainfall amount was not assessed
Harding and Bloomer, 1979	*Glyptemys insculpta*	Turtles delayed nesting in cold, rainy weather; believed temperatures were important in nest timing; data observational, without supporting metrics
Fahey, 1987	*Pseudemys concinna*	No association found between nesting and rainfall (*in* Jackson and Walker, 1997)
Feinberg and Burke, 2003	*Malaclemys terrapin*	Lowest percentage (15%, n = 86) of nesting took place on overcast or rainy days; suggested temperature effects were important in nesting propensity; effect of rainfall amount was not assessed
Aresco, 2004	*Pseudemys concinna*, *Trachemys scripta*	Chi-sq analysis found no association of nesting and rainfall on a given day; 31% of *P. floridana* nests and 34% of *T. scripta* nests were constructed on days with rain; noted that less direct associations (e.g., nesting propensity after prior day's rainfall) were not tested; effect of rainfall amount was not assessed
Bowen and Janzen, 2005	*Chrysemys picta*	Most nests (76.5%, n = 1674; from their Table 1) constructed on days without rain; were not able to determine when rainfall occurred within a given day; effect of rainfall amount not assessed
Bowen et al., 2005	*Chrysemys picta*	*Chrysemys picta* responded more to air and water temperatures than rainfall patterns. (Compare to Bowen et al., 2005, **Table 1** for *Emydura macquarii* and *Chelodina expansa*.)
Flitz and Mullin, 2006	*Terrapene carolina*	Chi-sq analysis found no association of nesting and rainfall on a given day; 50% of nests (n = 24) were constructed during or within 24 h of previous rainfall, but relationship was not statistically significant; effect of rainfall amount was not assessed
Geller, 2012a	*Graptemys ouachitensis*	Fisher's Exact Tests found no association of nesting and rain within previous 24 h; 53.9% (n = 102) of nests were constructed more than 24 h after previous rain; effect of rainfall amount not assessed
Escalona et al., 2019	*Podocnemis unifilis*	Linear regression found no association of nesting with daily rainfall amount; nesting activity was associated with intermediate air temperatures and full moon phases
Lazure et al., 2019	*Apalone spinifera*	Logistic Regression found no association of nesting and rainfall on a given day; air and water temperatures were the only meteorological variables tested that were associated with nesting activity; effect of rainfall amount not assessed
Buckardt et al., 2020	*Emydoidea blandingii*	ANOVA found no association of nesting and rainfall amount during the preceding day; nesting activity was most closely associated with air temperatures > 18.9°C, bright moon stages, and winds from the east and south

record and include rainfall amounts in their analyses (**Table 2**). These methodological differences may have contributed to some of the disparity in findings regarding the association of rainfall and freshwater turtle nesting activity.

However, even better-quantified studies sometimes report different responses of turtles to rainfall patterns. For example, Bowen et al. (2005) noted that *Chrysemys picta* nested on virtually every day during the nesting season and suggested that timing of nesting in that species was primarily a function of physiological readiness and sufficiently warm temperatures, rather than of rainfall or other environmental variables. However, Bowen et al. (2005) were unable to provide explanations for why this behavior contrasted with those for *Emydura macquarii* and *Chelodina expansa* in that same study, whose nesting was correlated with rainfall and associated changes in air temperature. Disparity in study findings sometimes appear even for the same species at the same nesting sites (e.g., for *Chrysemys picta* at the Thompson Causeway, Illinois [Bowen and Janzen, 2005; Muell et al., 2021]; see **Tables 1**, **2**).

Nonetheless, when associations have been found, many studies have concluded that rainfall on a given day is an important determinant of nesting propensity in freshwater turtles, either when considered as a single factor (Congello, 1978; Georges, 1984; Burke et al., 1994, 1998; Roosenburg, 1994; Wilson et al., 1999; Bowen et al., 2005, in part; Walde et al., 2007; Bernstein et al., 2015; Espinoza et al., 2018) or in conjunction with other meteorological variables such as rising or relatively high air temperatures and falling barometric pressures above certain minimums (Hammer, 1969; Burger and Montevecchi, 1975; Clay, 1981; Congdon et al., 1987; Stott, 1988; Kuchling, 1993; Tucker, 1997; Najbar and Szuszkiewicz, 2005; Muell et al., 2021).

Still other studies have found air and/or water temperatures alone to be principal cues to initiate nesting, with a preference for nesting on days with the relatively high daily temperatures that optimize locomotor performance and reduce the time spent nesting (e.g., for *Chrysemys picta*, Bowen et al., 2005; Frye et al., 2017) or that allow turtles that nest during the evening to maintain their body temperatures at functioning levels (e.g., for *Emydoidea blandingii*, Buckardt et al., 2020). Pig-nosed turtles (*Carettochelys insculpta*) ceased nesting for up to several days during cool periods in the tropical (winter) dry season (Doody et al., 2003). Rather than rainfall, additional studies have noted associations of nesting activity with bright moon phases, possibly a function of social facilitation and/or as a mechanism to reduce individual or nest predation (Escalona et al., 2019; Buckardt et al., 2020). These complexities show that single-factor analyses of rainfall frequencies alone (e.g., Aresco, 2004; Geller, 2012a) are not likely to be as informative for a given species and context as studies incorporating rainfall amounts and a broader array of meteorological parameters in their analyses, such as the concurrent effects of air and water temperatures, and time since last rainfall (noted by Jackson and Walker, 1997; Muell et al., 2021). Overall, the literature thus shows significant variation in the assessment of whether or not freshwater turtles appear to nest in greater numbers after rainfall, suggesting that this association is not a generalizable aspect of chelonian reproductive biology.

A Case Study of the Association of Rainfall and Nesting Activity With Ouachita Map Turtles (*Graptemys ouachitensis*)

A total of 245 *G. ouachitensis* nests were constructed on 147 of the 320 days on which activity was monitored, giving an average of 1.7 nests constructed on days when nesting occurred.

Rain fell on 142 of the 320 days (44.3%) when activity was monitored across all study years. Most nests (57.0%, $n = 139$) were constructed on calendar days without rainfall. Similarly, most nests were constructed more than 24 h after previous rainfall (54.9%, $n = 134$), more than 24 h before the next rainfall (63.1%, $n = 154$), and many (38.1%, $n = 93$) were without rain during both pre- and post-nest construction periods. A small number of nests (2.5%, $n = 6$) were constructed during rainfall itself. The all-year mean number of nests constructed on calendar days without rain (0.84 nests/d) did not differ from the mean number constructed on days with rain (Model 1 in **Table 3** and **Figure 1**). Similarly, there was no association between the number of nests constructed on a given day and (any) rainfall amount ($p = 0.81$) or on calendar days with $\geq$ 20 mm of rain ($p = 0.84$).

On a calendar day basis, Model 2 (rain on the day of nesting and rain on the previous day) provided the best fit to the data on the number of nests constructed per day (**Table 3**). The negative parameter estimate for this model indicated fewer nests were constructed when it had rained two days in a row. There was strong evidence that the parameter estimate for Model 2 was negative: the 95% confidence interval, given by ±2 times the standard error, did not include zero and the outcome was associated with a low *P*-value. **Figure 2** summarizes the pattern of turtle nesting in relation to whether it rained for two days or not. While there was a much lower probability of nesting given rain two days in a row (nesting probability = 0.31 relative to 0.51 if this did not occur), there was less of a difference in the mean number of nests constructed if nesting occurred on either occasion (1.7 versus 1.48, **Figure 2**). There were no significant effects of temperature or barometric pressure on the propensity to nest.

Literature Review of Effect of Rainfall on Turtle Nest Predation Rates

We reviewed 21 studies that quantified or addressed the potential for rainfall to increase nest survival in 7 turtle species. In our

TABLE 3 | Summary of the four regression models fitted to the data, each including a different rainfall variable as predictor (see section Methods).

Model	AIC	ΔAIC	Parameter estimate	Standard error of parameter estimate	*Z* value	*P*-value
Rain on two consecutive days (2)	765.7	0	−0.63	0.21	−3.03	0.002
No rain on the day and rain the next day (4)	774.0	8.4	0.23	0.19	1.21	0.23
Rain on the day of nesting (1)	774.5	8.8	−0.16	0.16	−0.99	0.32
No rain for two consecutive days (3)	775.4	9.6	0.03	0.17	0.22	0.82

The four models are ranked by Akaike's Information Criteria (AIC) from lowest (best-fitting) to highest. ΔAIC is the difference in AIC between the best-fitting model (which has ΔAIC = 0) and the other models. ΔAIC values of ∼10 are generally considered to imply that the model with the lowest AIC is clearly a better fit to the data. Also shown is the parameter estimate associated with each rainfall variable, the standard error of the parameter estimate, and the associated Z and P-values.

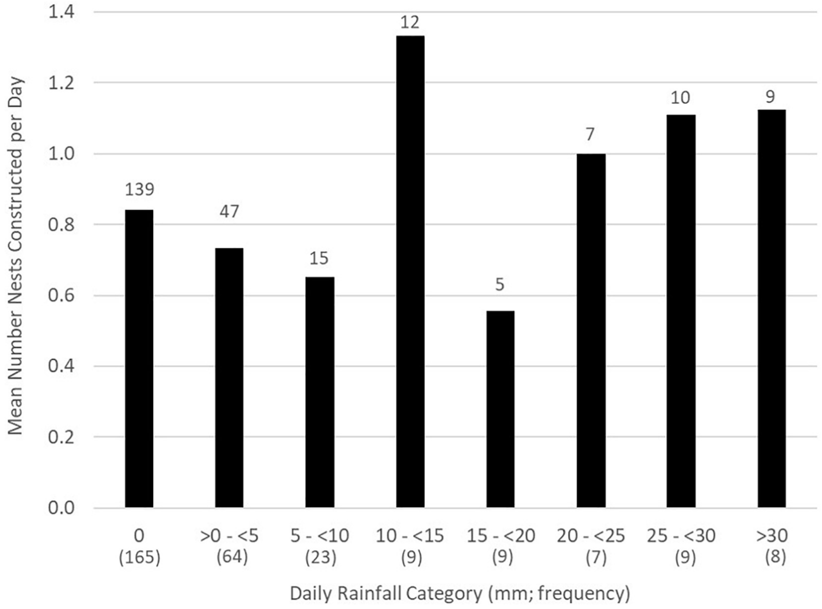

FIGURE 1 | All-year mean number of *Graptemys ouachitensis* nests constructed/d by daily rainfall category along the Wisconsin River, WI, United States, during 12 study years from 2008 to 2021 (*n* = 244 nests). Overall number of nests per rainfall category appear above each bar; number of days within each category appear below, in parentheses.

review, 11 (52%) of the studies demonstrated or suggested that rainfall increased nest survival, compared to 10 (48%) studies that found no effect (**Tables 4**, **5**).

The primary cues used by predators to locate newly constructed freshwater turtle nests are believed to be largely olfactory for mammalian predators, with varying degrees of visual use based on predator species (reviewed in Geller and Parker, 2022); largely olfactory for certain nest-depredating lizards (e.g., Soanes et al., 2015); and largely visual by bird predators (e.g., Jackson and Walker, 1997). Whether visual or olfactory, many researchers have suggested that rainfall may dilute these nest location cues (**Table 4**). One proposed mechanism by which rainfall diminishes olfactory cues suggests that water percolating through the soil column flushes out the odors produced by soil microbes (Lindbo et al., 2012), thereby reducing what were formerly point-source olfactory signals of disturbed soil at nest locations (Geller, 2015; see also, Buzuleciu et al., 2016). Greater rainfall amounts, percolating to greater soil depths (reviewed in Hess et al., 2018), are likely more thorough in aerosolizing the microbe-produced compounds within newly constructed nest cavities as well as from surrounding substrates, reducing the olfactory gradient. Both the amount of rain and its timing relative to nest construction are, thus, potentially important influences on the degree to which rain reduces nest predation. As a corollary, rainfall during and after nest construction is likely to be more effective in reducing both olfactory and visual nest location cues than when rainfall precedes nesting (Bowen and Janzen, 2005).

These inferences are supported our review, in that all of those which noted an effect of rain in reducing nest predation, either qualitatively or quantitatively, found larger rainfall amounts on the day of or soon after nest construction to be more effective in reducing nest predation than smaller amounts or none (**Table 4**); No studies suggested that rainfall in advance of nesting would decrease nest predation rates. In contrast, it is difficult to fully evaluate studies that did not show an enhancing effect of post-construction rainfall on nest survival (**Table 5**) because (like some that did show an effect) none reported the relevant rainfall amounts except for the 7.3 mm reported by Wilhoft et al. (1979) and the experimental work of Buzuleciu et al. (2016), wherein 2 cm of dechlorinated tap water was applied to individual artificial nests. However, as pointed out by Czaja et al. (2018), experimentally applied water at nests themselves does not scale in effect to that of larger areas affected during natural rainfall and possibly explains why these negative results differ from other research where the effects of natural rainfall were assessed.

Importantly, none of the studies finding no effect of rainfall on predation rates on newly constructed natural turtle nests with raccoons as predominant predators (Schwanz et al., 2010; Wirsing et al., 2012; Bougie et al., 2020) assessed the temporal association between rainfall and individual nesting events, which is likely central to the dynamics of how rainfall affects the strength of nest location signals (see above). Some other studies that discounted the role of rainfall in reducing turtle nest predation rates recorded red foxes (*Vulpes vulpes*) as nest predators (Congdon et al., 1983, 1987; Spencer, 2002; Dawson et al., 2014), which may utilize a wider array of nest location cues than some other predators (e.g., raccoons), including, potentially, the scent of eggs themselves (see Congdon et al., 1987; Geller and Parker, 2022). However, whether these differences in sensory abilities explain the apparent lack of rainfall effect on nest survival is unclear. Similarly unexplained are reported increases in nest

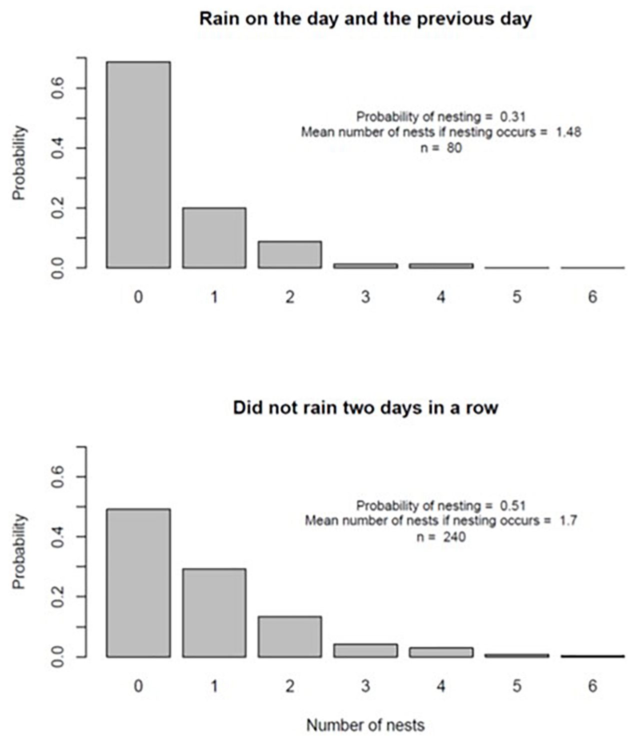

FIGURE 2 | Probability distributions for the number of nests constructed on days where it had rained two days in a row **(top panel)** and days where it had not **(bottom panel)**.

predation during or soon after rainfall for late-season turtle nests (Congdon et al., 1983; Brooks et al., 1992). However, late-season nests may have a different suite of nest location cues than those at recently constructed nests (see reviews in Riley and Litzgus, 2014; Geller and Parker, 2022), including, potentially, hatchling vocalizations (e.g., Ferrara et al., 2012; Geller and Casper, 2019). As hatchling emergence onto the surface is often associated with rainfall and warm or rising ambient temperatures (e.g., Tucker, 1997; Nagle et al., 2004; Geller et al., 2020), it is possible, although speculative, that rain during late, pre-emergence stages may increase overall hatchling activity, leading to increases in odors from disturbed soils or from the hatchlings themselves, hatchling-produced sounds, or other surface-detectable cues to nest locations.

A Case Study of the Interaction of Precipitation and Nest Predation With Ouachita Map Turtles (*Graptemys ouachitensis*)

Nest predation rates on our two nesting sites were high (overall 93.0%, yearly range 75–100%, n = 128) and only 9 unprotected (uncaged) nests escaped predation across all study years. All predation was from raccoons, which were present nearly every night during nesting periods (Geller, 2012b). Ninety-five percent of the nests depredated by raccoons were destroyed within 24 h of construction (mean nest survival = 11.8 h, range 0.0–7.6 days, n = 111), typically by the first raccoon within approximately 1 meter of a given nest (ca. 90% of the time; Geller, 2012b). Most depredated nests had no post-construction rain before predation (84.5%, n = 116 total depredated nests), and all of those without intervening rain were found by raccoons before or during the first night. Raccoons depredated 94.3% of the nests constructed without rain in the previous 24 h (n = 70) and 90.7% of the nests made when rain (median = 4.3 mm, mean = 9.5 mm, range 0.3–78.5 mm) did occur within 24 h before (n = 50) or during nest construction (n = 4) (Yate's χ^2 = 0.164, p = 0.685, n = 124), suggesting that rain falling before nest construction had little effect in reducing nest predation rates.

Given the high rate of predation and short nest survival timelines, we did not have sufficient data to reliably identify the characteristics of nests that escaped predation. However, surviving nests tended to have larger amounts of rainfall during both the first night after nest construction (mean = 7.8 mm, SD = 10.27, range 0.0–25.0, n = 9) and through the first four nights after nest construction (mean = 19.4 mm, SD = 14.63, range 0.8–35.1, n = 9) than did depredated nests (first night mean = 0.3 mm, SD = 1.23, range 0.0–10.4, n = 109) (**Figure 3**), and all surviving nests had either single or multiple rainfall bouts during the 4 days following nest construction. Moreover, raccoons depredated fewer nests having post-nest construction rain within 24 h (85.7%, n = 42 initial nests) than those without rainfall shortly after nest construction (96.4%, n = 83 initial nests), a difference unlikely to arise by chance alone (Fisher's exact test; p = 0.060, n = 125). Further review of the camera data showed that 58.3% (21 of 36) of the depredated nests constructed within 24 h of the next rain event were detected by raccoons before any of this rainfall occurred. When these nests are removed from the depredated nests within 24 h of post-construction rain counts, the depredation rate of nests actually exposed to post-construction rain decreases to 71.4% (n = 21), strengthening evidence for a non-random difference (Fisher's exact test; p = 0.002, n = 104). This result supports the idea that raccoons depredate a smaller proportion of nests when rainfall follows nest construction and highlights the importance of determining the exact timing of rainfall and nest predation in the field rather than using metrics based on the simple presence or absence of rain on a given day (see also Bowen and Janzen, 2005).

Literature Review and Case Study of Degree to Which Freshwater Turtles Appear Able to Predict Rainfall

To date, most research on the role of changing barometric pressure—declines in which are a key component of oncoming rainfall that could be a nest initiation cue to ovipositing turtles—has involved marine species. Drops in air pressure, in conjunction with other meteorological variables, have been suggested to be a cue to deteriorating habitat conditions for loggerhead sea turtles (*Caretta caretta*), resulting in movements away from nesting areas (Schofield et al., 2010). In other studies, higher barometric pressures were positively associated with higher rates of successful nesting (i.e., non-aborted attempts)

TABLE 4 | Papers suggesting freshwater turtle nest survival is enhanced by rainfall.

Citations	Turtle species	Mammalian predator species	Temporal association with rain	Comments
Carr, 1952	*Pseudemys floridana*	*Mephitis mephitis, Procyon lotor*	Survivorship enhanced for nests constructed before heavy rainfall	Suggested that most living *Pseudemys floridana* are a result of substantial post-nest-construction rain; based on observations of depredated nests at a well-used nesting site
Legler, 1954	*Chrysemys picta*	Unknown, but including *Procyon lotor*	Survivorship enhanced for nests constructed before heavy rainfall	Suggested that rain dissipated the odors of the nest and eroded the nest plug; without supporting metrics
Hammer, 1969	*Chelydra serpentina*	*Mephitis mephitis, Procyon lotor*	Not specified	Attributed greater nest survival in one study year was due to abnormally high rainfall levels inhibiting nest detection; comparative predation rates not presented
Shealy, 1976	*Graptemys ernsti*	*Procyon lotor*	Survivorship enhanced for nests constructed before rainfall	Suggested rain removed nest location cues of previously constructed nests; without supporting metrics
Burger, 1977	*Malaclemys terrapin*	*Procyon lotor*, *Vulpes vulpes*	Not specified	Noted that a particularly rainy week had both fewer numbers of nesting turtles and numbers of nests preyed on; comparative predation rates not presented
Burke et al., 1994	*Kinosternon subrubrum*	General	During rainfall	Suggested the benefits of nesting and moving during rainstorms might include disguising the scent of the nest or obliterating scent trails females made during nesting forays; without supporting metrics
Jackson and Walker, 1997	*Pseudemys concinna*	*Procyon lotor*	Survivorship enhanced for nests constructed before heavy rainfall	Suggested that substantial rainfall rapidly removes the physical and olfactory signs of nesting; comparative nest predation rates not presented
Bowen and Janzen, 2005	*Chrysemys picta*	*Procyon lotor, Mephitis mephitis*	Greater amounts of rainfall on the day of nesting were more effective than lesser amounts in enhancing nest survival	Rainfall amount, not simple presence/absence was influenced nest survivorship; suggested that rain reduced signal strength of both visual and olfactory nest location cues and that rainfall during or after nesting would be more effective than rain before nest construction
Rahman and Burke, 2010	*Malaclemys terrapin*	*Procyon lotor*	Not specified	Suggested that periods of heavy rainfall decreased signal strength of olfactory cues in one study year; comparative predation rates not presented
Geller, 2012a	*Graptemys ouachitensis*	*Procyon lotor*	Survivorship enhanced for nests constructed before heavy rainfall	Larger rainfall amounts ($\geq$20 mm) enhanced nest survival, but the association was not absolute
Czaja et al., 2018	*Malaclemys terrapin* and artificial nests	*Procyon lotor*	Greater amounts of rainfall on the day of nesting were more effective than lesser amounts in enhancing nest survival	Did not evaluate whether individual nests were constructed before or after rain events

by *C. caretta* and leatherback sea turtles (*Dermochelys coriacea*) (Pike, 2008; Palomino-González et al., 2020; respectively) and, although correlative in nature, were considered cues promoting nesting behavior. Within freshwater turtles, movements to nesting areas were believed triggered by falling barometric pressures in advance of rainfall by *Chelodina colliei* (then as *C. oblonga*) (Clay, 1981). In contrast, research on the allopatric congener, *C. longicollis*, indicated that, while there was a correlation of increased nesting-related movements out of ponds with falling barometric pressure, the association was not statistically significant and was weaker than that of rainfall itself, and was thus considered indirect and corollary (Stott, 1988, M.Sc. Thesis).

Our review of the literature reveals that air pressure data have rarely been collected in chelonian studies and we, thus, lack basic understanding of its potential role as a cue to nest initiation in freshwater turtles or how this environmental variable may affect turtles at different taxonomic and geographic scales. However, results from our case study indicated no relationship between decreasing barometric pressure or temperature and nesting activity, suggesting that turtles on our sites were not responding to these potential indicators of future rainfall.

DISCUSSION

Rainfall has been found to stimulate nesting behavior in a variety of taxa, mostly those inhabiting arid or semi-arid ecosystems, including birds (reviewed in Cavalcanti et al., 2016), and, within reptiles, in certain lizards (e.g., *Polychrus acutirostris*, Vitt and Lacher, 1981; *Oplurus cuvieri*, Randriamahazo and Mori, 2001). Similarly, rainfall is believed to trigger the onset of the nesting season for some freshwater turtles in regions that recurringly experience strong drought/monsoon cycles (e.g., in Australia, within genera *Chelodina* [Goode, 1965; Clay, 1981; Georges, 1984] and *Emydura* [Espinoza et al., 2018]; in India, for *Lissemys punctata* and *Melanochelys trijuga* [Premkishore and Chandran, 1996]) and may be a recurring aspect of the annual reproductive biology of turtles in certain regions. Doody et al. (2003) found that the onset of nesting, during the dry season, was related to the magnitude of rainfall during the previous wet season in *Carettochelys insculpta*. Even in parts of the world without pronounced seasonality in rainfall regimes, rainfall following drought periods within a nesting season sometimes stimulates nesting activity (e.g., in North America, for *Pseudemys concinna*

TABLE 5 | Papers suggesting freshwater turtle nest survival is not enhanced by rainfall.

Citations	Turtle species	Mammalian predator species	Temporal association with rain	Comments
Wilhoft et al., 1979	Artificial nests	*Procyon lotor*	Artificial nest excavation spiked after 0.73 cm of rainfall	Proposed that this small amount of rainfall was not enough to significantly reduce nest location cues
Congdon et al., 1983	*Emydoidea blandingii*	*Procyon lotor*, *Vulpes vulpes*	Noted late-season nest predation during or soon after rainfall	Relevant rainfall amounts not reported; does not refer to newly constructed turtle nests
Congdon et al., 1987	*Chelydra serpentina*	*Procyon lotor*, *Vulpes vulpes*	Noted predation on nests older than 6 days during or soon after rainfall	Relevant rainfall amounts not reported; noted that *Vulpes* were responsible for most nest depredation after 7 days
Brooks et al., 1992	*Glyptemys insculpta*	unspecified	Only nest predation was on late-season nests in a week in which rain occurred	Predators involved and relevant rainfall amounts not reported; does not refer to newly constructed turtle nests
Spencer, 2002	*Emydura macquarii*	*Vulpes vulpes*	Rainfall concurrent with nest construction did not influence nest predation risk	Red foxes may have heightened sensory abilities and use a wider array of nest location cues than some other predators
Wirsing et al., 2012	*Chelydra serpentina*, *Chrysemys picta*	mostly *Procyon lotor*	Rainfall presence/absence or amount did not significantly affect nest predation rates	Relevant rainfall amounts and within-day timing relative to nest construction and predation not assessed/reported
Schwanz et al., 2010	*Chrysemys picta*	*Procyon lotor*	May and June rainfall totals were not correlated with annual nest predation rates	Temporal relationships of nest construction, precipitation, and predator encounters were not assessed
Dawson et al., 2014	Artificial nests	*Vulpes vulpes*	Number of artificial nests excavated in a day was not significantly correlated with amount of rain in the preceding 24 hours or 7 days	Shows lack of effect of rain before nest construction; relevant rainfall amounts not reported
Buzuleciu et al., 2016	Artificial nests	*Procyon lotor*	2 cm of water applied to artificial nests did not reduce excavation rates	Treatment application was localized to the nest level, not over the larger areas that would be affected during natural rainfall
Bougie et al., 2020	*Glyptemys insculpta*	*Procyon lotor*, *Mephitis mephitis*	Rainfall on the day of nesting did not affect nest predation rates	Relevant rainfall amounts and within-day timing relative to nest construction not assessed/reported

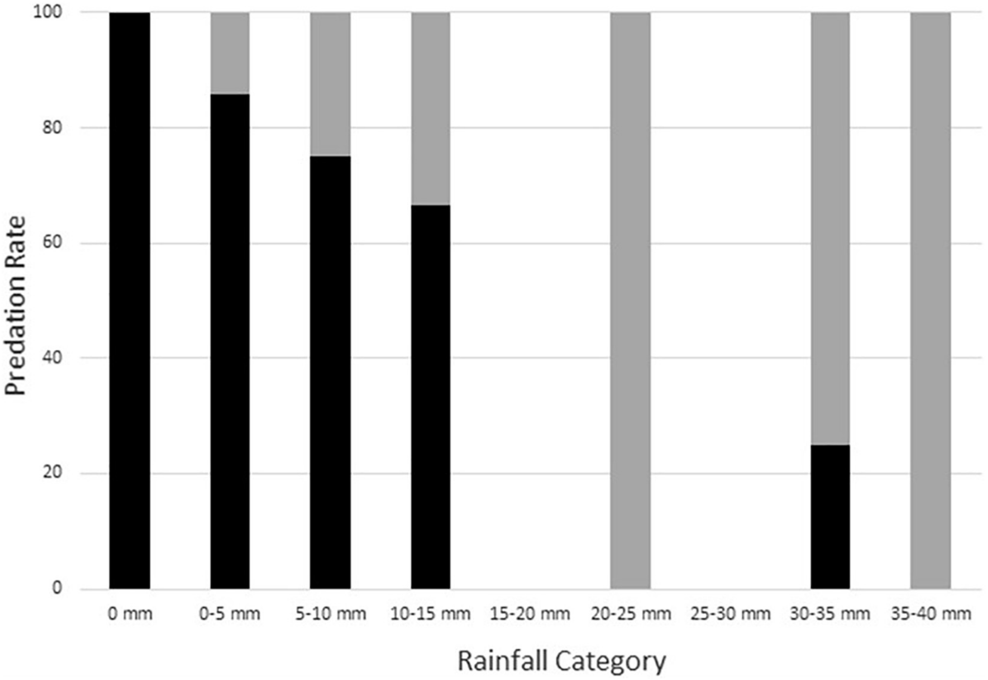

FIGURE 3 | Association of 4-day cumulative rainfall amount with rates of nest depredation (black bars; $n = 116$) and nest survival (gray bars; $n = 9$) for natural *Graptemys ouachitensis* nests along the Wisconsin River, WI, United States, during 12 study years from 2008 to 2021. Most nests depredated during the first night (see text for details).

suwanniensis, Jackson and Walker, 1997; for *Clemmys guttata*, Litzgus and Brooks, 1998).

On a within-season basis, however, the relationship between rainfall and nest initiation in turtles has been historically unclear, despite its potential effects on both nesting turtles themselves and on nest predation risk. Our review found a diversity of responses: while most (69%) studies found that the propensity to nest was positively associated with rainfall (**Table 1**), others

(31%) found either no association or a negative association (**Table 2**). Similarly, rainfall during or after nesting apparently increased nest survival by reducing predation in some species or populations (52%; **Table 4**), but not in others (48%; **Table 5**). Our mixed findings may reflect a diversity of species- or population-specific responses, local adaptations, confounding abiotic factors (e.g., temperature decreases after rainfall), and methodology (e.g., most studies did not quantify rainfall amounts). Disparate research findings resulting from differences in study designs or when relationships between interacting variables vary under different ecological and spatiotemporal contexts are widespread in ecological studies (Catford et al., 2022).

However, when sufficient data are available on rainfall amounts and its timing relative to nest construction, results reveal that predation rates on newly constructed turtle nests are typically reduced when significant rainfall occurs during or soon after nest construction, in accord with a long-suspected effect of post-nest-construction rain in reducing the olfactory/visual signals of nest presence (Carr, 1952; Legler, 1954; **Table 4**). In contrast, nest predation rates are maximal and unaffected when rainfall precedes nesting or when little or no rain occurs soon after nest construction. Our case study on map turtles found high predation rates and nest survival times were short, precluding our ability to rigorously analyze the association between nest predation and rainfall amounts. However, close examination of the exact timing of both rainfall and predation revealed significantly lower predation rates when rain fell within 24 h after nesting and before the first predator encounter, indicating that rainfall during or after nesting may reduce nest predation.

Despite potential benefits in reducing nest predation rates, we found no marked propensity of turtles to nest during or before oncoming rain, either in the reviewed literature or in our case study, where the best fitted model explaining the propensity to nest found that map turtles were more likely to nest after dry days and that rainfall was not a major factor driving turtles to nest in our populations. Previous authors (e.g., Aresco, 2004; Muell et al., 2021) have suggested that nesting associations with rainfall vary among turtle populations and are likely context specific. It is recognized that factors other than rain on the day of nesting are implicated in nest construction timing, as many turtles nest on days without rainfall. Other abiotic influences on nest timing, such as air and water temperatures and time since last rainfall, tend to obscure the role of rainfall on nesting propensity as a single variable (Jackson and Walker, 1997). Conditions on nesting substrates (e.g., soil type, compaction, and moisture retention), impacting the effort, time, and feasibility of making nest excavations (Doody et al., 2003) may also influence local associations between rainfall amounts and subsequent nest construction activity. For example, while the softening of nesting substrates by rain was believed important in nest timing by *Chelydra serpentina* (Hammer, 1969), the Ouachita map turtle study sites in our case study are comprised of easily worked sand and nesting durations are short (median = 34.0 min, $n = 221$) and diurnal, reducing the chances of direct predation by nocturnal mammals on nesting females.

Biotic factors also influence nest construction timing and further obscure the isolated effect of rainfall as a single variable. For example, multiple-clutching female turtles are physiologically unable to respond to appropriate nesting conditions for an interval of time after a prior nesting event, thus reducing an individual turtle's ability to time her nesting efforts to optimal conditions including, perhaps, periods before rainfall (Pike, 2008; Czaja et al., 2018; Muell et al., 2021). In a novel approach to isolate the impact of abiotic factors on nesting propensity in *Chrysemys picta*, Muell et al. (2021) attempted to control for this physiological variable by removing those turtles putatively unable to nest (individually marked turtles known to be within inter-nesting intervals) from various analyses. While this approach requires a population of marked turtles and may not be an option for some studies, it is a promising method to reduce some of the confounding variables underlying efforts to understand the responses of nesting turtles to meteorological conditions.

In studies where an association has been found, turtle nesting tends to occur not before rainfall, but during and after it, concurrent with relatively warm temperatures. Although nesting during rainfall also likely enhances nest survival, this timing may be due to factors more related to female turtle survivorship than to predation risk—such as reduced time on land, when freshwater turtles are most vulnerable to terrestrial predators (Spencer, 2002). A similar conclusion, based on the evolutionary trade-offs between adult and nest survival, was reached by Spencer (2002) for *Emydura macquarii*, in which nesting females appear to make nest location choices that maximize their own survival by nesting closer to water when they perceive the risk of fox predation to be high, at the cost of less-than-optimal incubation conditions for their eggs and greater levels of nest depredation. As long-lived, iteroparous adults, female turtles, maximizing their own survival, are likely to nest before significant rainfall at some point in their reproductive life by chance alone, and may have enhanced nest success as a result (Carr, 1952; Czaja et al., 2018).

To-date, research on the propensity of turtles to nest in temporal associations with rain and of the effect of rainfall on nest predation rates has involved only a small number of turtle species. Even within this small scope, these studies have sometimes produced conflicting results, potentially due to limitations in obtaining the necessary resolution on rainfall timing and amounts relative to individual nesting events (e.g., Aresco, 2004; Bowen and Janzen, 2005), variations in study rigor (from a few observed events to multi-year studies using statistical analyses), and other methodological differences. Comparative, interspecific studies using similar methodologies and analyses (time-lapse camera use and datasets amenable to meta-analysis), as well as intraspecific studies between populations of species with wide geographic ranges, would be especially useful in delineating the phylogenetic variation and factors underlying patterns of meteorological cueing in given contexts, especially when conducted over several years (Bowen and Janzen, 2005). However, the apparent variation in the association of nest timing and rainfall suggests that attempts to generalize its tendency or impacts on turtle hatchling recruitment will be problematic.

While the ability to sense barometric pressure change has been established for many organisms (e.g., for birds, Metcalfe et al., 2013; for anurans, Oseen and Wassersug, 2002), this review indicates a scarcity of related research for either marine or freshwater turtles. Several reviewed studies (almost all involving *Chelodina* and *Emydura* inhabiting hot and arid environments) reported turtle movements toward nesting areas before or concurrent with rainfall, suggesting the theoretical possibility of barometric changes in triggering the nesting response (e.g., Clay, 1981). However, barometric pressure changes preceding rainfall do not occur in isolation, but are accompanied by changes in air temperature, humidity levels, cloud cover, wind (Glickman, 2000), and perhaps even olfactory signals; all of which could be perceived by turtles, making the association of nesting behavior and barometric pressure indirect and correlative. In addition, many other studies, including ours, reported no relationship between nesting and decreasing barometric pressure (or with rainfall in general), suggesting that turtles were not responding to these potential indicators of future rainfall. Clearly more research—especially perhaps, laboratory-based studies where the behavioral responses of turtles to manipulated changes in air pressure can be isolated from the impacts of other meteorological variables that occur in nature—is needed before firm conclusions can be reached on the ability of turtles to detect changes in barometric pressure, including how this ability, or lack of it, may reflect phylogenic and genetic constraints (e.g., Oostra et al., 2018) and influence nest timing at differing taxonomic and geographic scales.

Ongoing anthropogenic climate change is predicted to introduce more stochastic variation into global temperature and precipitation patterns (Collins et al., 2013) and is expected to impact various parameters of turtle life history. For example, potential increases in nest success in areas with increased rainfall via reduced nest predation rates and decreases in nest success where rainfall frequencies and amounts decrease (Czaja et al., 2018), may continue to occur as functions of rainfall influences on nesting female behavior. However, changing rainfall patterns also impact air and substrate temperatures, nest site flooding potentials, and the vegetational cover on nesting areas, all of which affect hatchling recruitment. Whether turtles will be able to mount adaptive responses to changes in global precipitation patterns depends on rates of environmental and genomic change relative to inter-generation length, the amount of trait plasticity and heritable genetic variation within populations, and phylogenic constraints (e.g., Urban et al., 2014; Oostra et al., 2018; Dochtermann et al., 2019; Scheiner et al., 2020; Patrício et al., 2021). As animals of relatively low vagility, the potential for adaptive change in turtle populations also depends on the quality of available habitats and their distribution on the landscape (e.g., Valenzuela et al., 2019). While these variables are currently unknown for turtles overall, and will likely be highly context specific, their interplay and ultimate effects on turtle reproductive biology represent additional influences and challenges to chelonian persistence in the Anthropocene.

AUTHOR CONTRIBUTIONS

GG wrote the first draft, conducted the literature search, and provided the **Figures 1**, **3**, and **Tables 1**, **2**, **4**, **5**. JD edited the first draft, wrote parts of subsequent drafts, and provided additional citations. SC and RD provided statistical analysis, **Figure 2** and **Table 3**, and manuscript edits. All authors contributed to the article and approved the submitted version.

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Natural and Anthropogenic Factors Influencing Nesting Ecology of the American Crocodile in Florida, United States

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Nesting ecology of American crocodiles (*Crocodylus acutus*) in Florida has been both positively and negatively influenced by anthropogenic and natural factors since the species was placed on the federally endangered species list in 1975. This includes a shift in nesting sites and an expansion of nesting to anthropogenic habitat. Using a 50-year record of monitoring data (1970-2020), we assessed factors influencing nesting ecology (number of nests, nest morphology, success rate, and habitat use) from a total of 3,013 nests recorded across South Florida. We detected a change in nesting success rate, increasing from 61% in the 1970's to near 90% since 2010. Our hot spot analysis illustrates that nesting sites in northeastern Florida Bay and Flamingo/Cape Sable (Everglades National Park) were important for American crocodiles. Anthropogenic habitats, such as canals provided vital habitat nesting in areas such as Flamingo/Cape Sable (Everglades National Park), Turkey Point Power Plant, and Crocodile Lake National Wildlife Refuge for the current Florida population. Environmental parameters suspected to affect nesting success have shown an increasing trend over the past 50 years and minimum temperature and rainfall, during the summer season, are correlated with increased nesting success and temporal variation across South Florida. The adaptive capacity that American crocodiles exhibited in Florida gave the species advantages to face changes in climate and landscape over the last 50 years, however, it does not imply that the adaptive capacity of the species to face these changes (evolutionary potential) cannot reach a limit if changes continue. Here, we document *C. acutus* nesting ecology population responses to ecosystem restoration efforts in Florida; and further demonstrate the value of protecting and restoring habitat to support recovery of listed species.

Keywords: crocodylian, ecosystem restoration, nesting success, long-term monitoring, nesting ecology, Everglades

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INTRODUCTION

Successful nesting is a quantifiable metric of how well a species is doing in its environment (Ugarte et al., 2013). For crocodylians, development of the egg in the nest is a vulnerable period in life, for example, increased predation risk due to exposure in less secure, elevated, open areas such as beaches or increased risk of flooding due to nesting in wetlands (Platt et al., 2008; Balaguera-Reina et al., 2015) and therefore one of the best researched population traits (see: Webb et al., 1977; Ogden, 1978; Hall and Johnson, 1987; Mazzotti, 1989; Platt and Thorbjarnarson, 2000; Ugarte et al., 2013; Combrink et al., 2017).

The American crocodile (*Crocodylus acutus*) is the most widely distributed species of western hemisphere crocodiles and occurs at its northernmost distribution in South Florida, then across coastal Mexico, down into South America and along the Caribbean and Pacific coasts including insular areas (Cuba, Jamaica, and Hispaniola; Thorbjarnarson et al., 2006). The species has experienced severe declines due to overexploitation and habitat loss for nesting throughout its historical distribution (Thorbjarnarson, 2010). It is presently classified as Vulnerable across its range by the International Union for Conservation of Nature (IUCN) Red List (Rainwater et al., 2021) and locally in the United States as threatened by U.S. Federal Endangered Species List (USFWS, 2007). It is also listed on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora except for Mexico, Cuba, and Cispata Bay in Colombia (Convention on International Trade in Endangered Species of Wild Fauna and Flora [CITES], 2021).

In South Florida, nesting of American crocodiles was restricted to a small area of Northeastern Florida Bay (NEFB) in Everglades National Park (ENP) and northern Key Largo (NKL) by the early 1970s (Kushlan and Mazzotti, 1989a; **Figure 1**). In 1975, the species was placed on the Federal Endangered Species List (Federal Register 40). In 1978, the first nest at Turkey Point Power Plant (TP; Homestead, FL) was recorded and the first hatchlings captured, becoming since then a source of nesting for American crocodiles (Gaby et al., 1985). Expansion of crocodile nesting to the Flamingo/Cape Sable area (ENP) in the mid 1980's, concurrent with a local restoration project that plugged both Buttonwood and East Cape canals in those areas, provided a new location for nesting (Mazzotti et al., 2007a). Critical monitoring and management efforts of the Florida population of American crocodiles (see Brandt et al., 1995; Mazzotti et al., 2007b) provided valuable information to reclassify the species from endangered to threatened in 2007 (Federal Register 72; USFWS, 2007).

Nesting by American crocodiles in Florida historically has been along coastal areas where nearby freshwater or brackish habitats with low salinity and low wind and wave action provided critical nursery conditions for hatchlings (Mazzotti, 1983). In Florida, courtship is likely to occur in late January into February (Kushlan and Mazzotti, 1989b) and nesting season is from March to August, including nest preparation from March to May and egg laying in April and May (Mazzotti, 1989). The incubation period is around 90 days and is temperature dependent (Mazzotti, 1989; Thorbjarnarson, 1989). Hatching generally occurs in July and August (Mazzotti, 1989) though hatchlings have also been found in September (see Briggs-Gonzalez et al., 2021). More recently, Cherkiss et al. (2020), found that hatching is occurring earlier in Florida (late June) over a 37-year nesting period illustrating that increasing temperature could be influencing earlier hatching.

American crocodiles in Florida make two types of nests: a mound nest created when a crocodile first creates a mound and then digs a hole on top of the mound to deposit eggs, and a hole nest when a crocodile deposits eggs in a chamber below ground-level (Mazzotti, 1989). The former nests are most frequently associated with sandy beach sites and the latter with nests made on marl creek banks or on artificial surfaces such as canal banks (Mazzotti, 1989). Crocodiles nest in available substrate, for example sand, marl, or peat, and do not incorporate other nesting materials such as vegetation, into their nests, except incidentally (Mazzotti, 1989).

Mazzotti (1989) suggested that six major factors can adversely affect nesting success (percentage of nests that produce at least one hatchling) and hatching success (percentage of eggs in successful nests that produce hatchlings) of crocodylians: fertility, predation, extreme temperatures, moisture conditions, erosion of nest sites, and human disturbance to nest sites or breeding animals. The success of these two parameters (nesting and hatching) depends on when, where, and how females deposit eggs into nests and the environmental conditions present during egg incubation (Mazzotti et al., 1988; Mazzotti, 1989).

The aim of this study was to assess natural and anthropogenic factors influencing the nesting ecology (number of nests, nest morphology, success rate, and habitat use) of American crocodiles in Florida using long-term monitoring data (1970-2020). We focused on understanding the natural history of nesting across space and time as well as the ecological and evolutionary responses of *C. acutus* to anthropogenic loss and creation of habitat, and to changes in temperature and precipitation across South Florida. We used the theory of orientation ecology (Jander, 1975) to predict that spatial and temporal orientation of nesting behavior will maximize distance from stress sources and minimize distance from resources in both space and time, and importantly we recognized that tradeoffs and bet-hedging (Hairston and Fox, 2013; Rajon et al., 2014) may occur. We illustrate species adaptative capacity (Angeler et al., 2019) and population responses to ecosystem restoration efforts in Florida with nest numbers and nesting conditions.

MATERIALS AND METHODS

The study was conducted at the southern tip of mainland Florida from northern Biscayne Bay/Virginia Key, NEFB, and Cape Sable northwest through Marco Island and south into the Florida Keys, covering a total of 12 main areas (**Figure 1** and **Table 1**). We conducted surveys for crocodile nests by motorboat, jon boat, canoe, helicopter, and on foot of known and potential nesting habitat during April and May (egg laying period) and monitored historical (known) and potential nesting habitat from June through August (hatching period). We noted nesting activity (tail drags, digging, or scraping), the presence of eggs or hatchlings,

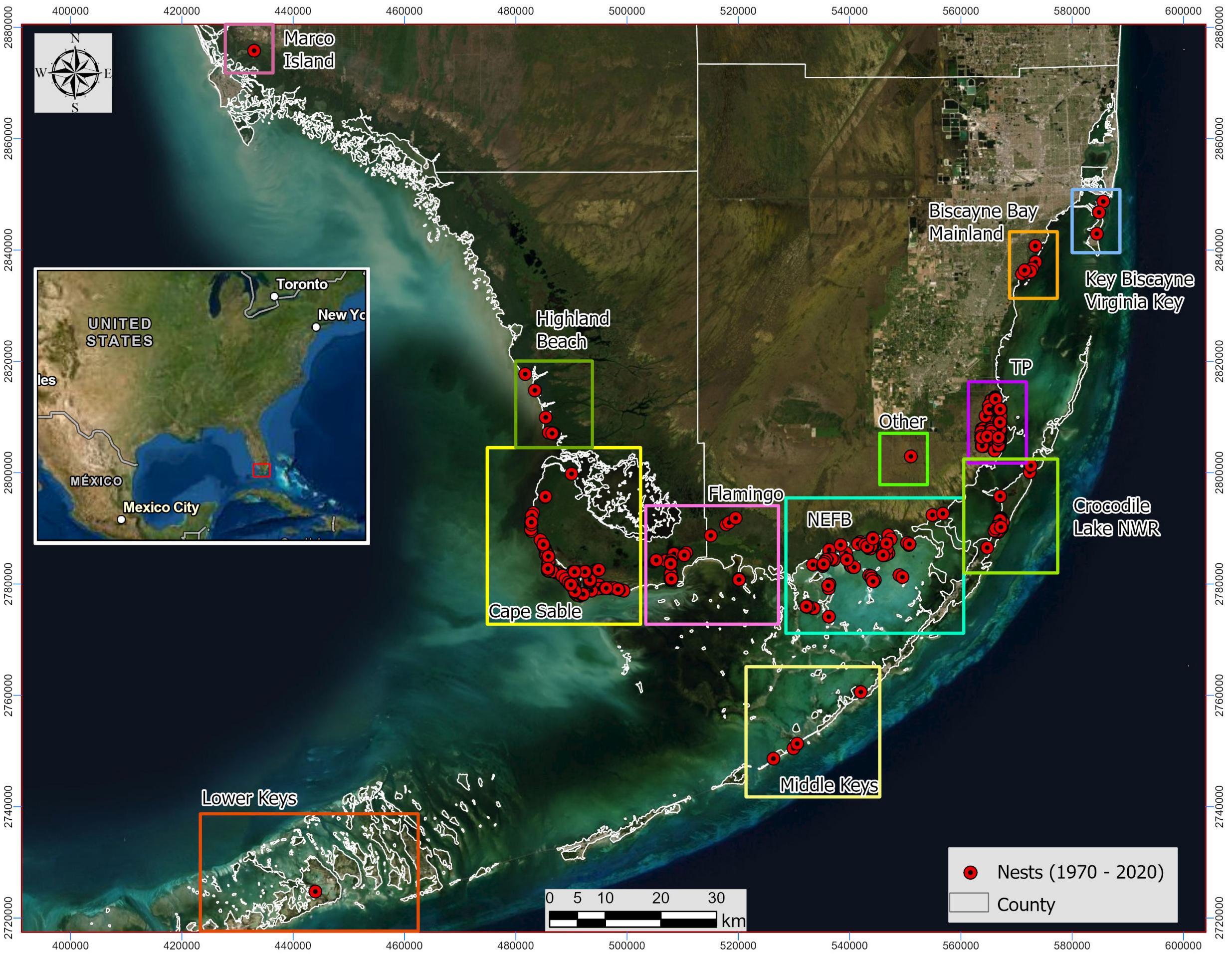

FIGURE 1 | Main nesting areas of American crocodiles (*Crocodylus acutus*) identified from 1970 through 2020 across South Florida, United States. The "Other" area refers to C-110 and MM113 Overseas Highway Southbound. Notice the most historically representative areas are within the Everglades National Park (ENP; Cape Sable, Flamingo, and Northeast Florida Bay –NEFB), Crocodile Lake National Wildlife Refuge (NWR), and Turkey Point Power Plant (TP)). However, areas around the Keys, the western coast (Marco Island and Broad River) and the eastern part of South Florida (Biscayne Bay and Virginia Key) are also nesting areas used by American crocodiles across this 50-year study.

and the number and causes of egg failure in both successful and failed nests whenever possible. We categorized nests as hole or mound construction (Mazzotti, 1989) and either isolated, near other nests, or within the same mound (see Cherkiss et al., 2020). Hatched nests were identified by the presence of an open hole, evidence of digging, or hatched shells. Shells of hatched eggs or hatchlings located at an open nest were considered evidence of successful nests and a nest was considered successful if at least one hatchling was produced. Hatching success could not be determined because some females moved some or all of the eggshells (hatched and unhatched) and hatchlings from the nest site. Once a nest had completed hatching, the egg chamber was inspected for any remaining unhatched eggs which were then counted; this process ensured that multiple nests in proximity that hatch in succession were not confused with each other. We defined failed nests when the nest passed hatching time (August) and no hatchlings emerged from it, and a depredated nest when we found any indication that at least one egg had been depredated. We also classified nest as "not determined" when no information could be recovered from the nest that allowed us to assign it to any of the categories described above. Hatch dates for all known nests were determined when possible. We recorded date, time, location (on a map or measured by global positioning system, GPS), and habitat type (categories = shoreline beach, pond, lagoon, creek, cove, roadside, and canal) of nests.

Data Analysis

We estimated the area used by American crocodiles for nesting in Florida based on a 100-m buffer calculated around nest geolocation (incident point data- IPD) and dissolved by year via ArcGIS Pro 2.9.1 (ESRI, 2021). We also assessed habitat type of nesting areas based on both first-hand observation from the field and the cooperative land cover map version 3.4 compiled by Florida Fish and Wildlife Conservation Commission (FWC) and Florida Natural Areas Inventory (FNAI, Kawula and Redner, 2018). We calculated success, predation, and

TABLE 1 | American crocodile (*Crocodylus acutus*) nesting summary recorded across the 12 main areas evaluated in South Florida from 1970 to 2020 analyzed by decades.

	1970s	1980s	1990s	2000s	2010s	Total
Number of nests	93	171	352	912	1485	3013
Successful nests	57	120	237	678	1333	2425
Success rate (%)	61.29	70.18	67.33	74.34	89.76	72.58
Predated nests	13	12	45	151	58	279
Predation rate (%)	13.98	7.02	12.78	16.56	3.91	10.85
Failed nests	23	37	47	69	81	257
Fail rate (%)	24.73	21.64	13.35	7.57	5.45	14.55
No determined	0	2	23	14	13	52
Nesting Area (km2)	0.05	1.53	2.24	6.4	8.22	11.9
Main Areas						
Biscayne Bay Mainland*	0	0	2	8	5	15
Cape Sable	0	3	13	337	827	1180
Crocodile Lake*	13	58	73	59	55	258
Flamingo	0	6	19	65	94	184
Highland Beach	0	0	0	1	12	13
Key Biscayne/Virginia Key	0	0	0	0	7	7
Lower Keys*	0	0	0	2	0	2
Marco Island*	0	0	0	20	0	20
Middle Keys*	0	0	0	7	5	12
Northeast Florida Bay	78	82	121	211	283	775
Turkey Point	2	22	124	202	192	542
Other	0	0	0	0	5	5

The main area called other refers to C-110 and MM113 Overseas Highway Southbound. Notice that the trend across these areas is an increasing number of nests across decades except for Biscayne Bay Mainland, Crocodile Lake, Lower and Middle Keys and Marco Island. Green colors are set up as positive gradient from 0 nest (no color) to 1485 (dark green).*

failure rates of nest based on the number of successful, predated, or failed nests divided by the total number of nest per decade.

We performed an optimized hot spot analysis (OHSA) based on IPD by decade and the whole dataset (50-year record) classifying areas based on statistical evidence [strong (p-value < 0.01), moderate (< 0.05), weak (< 0.10) or little-to-no evidence (> 0.10; Muff et al., 2022)] supporting high and low nest aggregations (hot and cold spots) using the Getis-Ord Gi statistic. For this analysis, IPD were aggregated and counted (weighted) based on the buffer layer previously calculated by decade, estimating the average nearest neighbor, and identifying via incremental spatial autocorrelation –Global Moran's I statistic method, the appropriate scale of analysis. Finally, we defined statistical evidence for IPD weighted based on the false discovery rate correction for multiple testing and spatial dependence (ESRI, 2021).

We analyzed minimum (min temp) and maximum (max temp) temperature as well as precipitation changes across nesting areas from 1970 through 2018 (due to environmental data availability) and the potential effect of these parameters in the number of nests found by year based on historical monthly weather data developed by Harris et al. (2014) downscaled to 2.5 min ($\sim$ 21 km^2) with WorldClim 2.1 (Fick and Hijmans, 2017). Environmental values were extracted monthly using coordinates from nest per year via ArcGIS Pro and analyzed in R version 4.0.3 (R Core Team, 2020) to assess trends across time. We included in this analysis data from spring (March, April, and May) and summer (June, July, and August) as the period where the nesting process occurs (from laying eggs to hatching) in South Florida (Mazzotti, 1989). We assessed relationships between environmental parameters and years and environmental parameters and number of nests reported by year via linear regression models using the *lm* function in R reporting 2.5 and 97.5 % confidence intervals (CI).

RESULTS

A total of 3,013 American crocodile nests were recorded in Florida from 1970 to 2020 with an overall increasing trend in number of nests recorded by decade (from 93 up to 1,485) as well as area used (100-m buffer around nest dissolved changed from 0.05 km^2 up to 11.9 km^2; **Table 1** and **Figure 1**). Nest success rate also showed an increasing trend from 61.3% in the 1970s up to 89.8% in the 2010s with an exception in the 1990's (67.3%). Most unsuccessful nests were either depredated ($N = 279$) or failed by unknown causes ($N = 257$). Failure rate showed a defined pattern across time with higher values in the 1970s and lower in the 2010s (24.7 and 5.5%, respectively). In contrast, predation rate showed a more variable pattern with the highest in the 2000s (16.6%) and the lowest in the 2010s (3.9%).

Nests recorded from the early 1970's came from NEFB (**Figures 1**, **2**). New nesting areas were recorded in early 1970's [Crocodile Lake National Wildlife Refuge (Crocodile

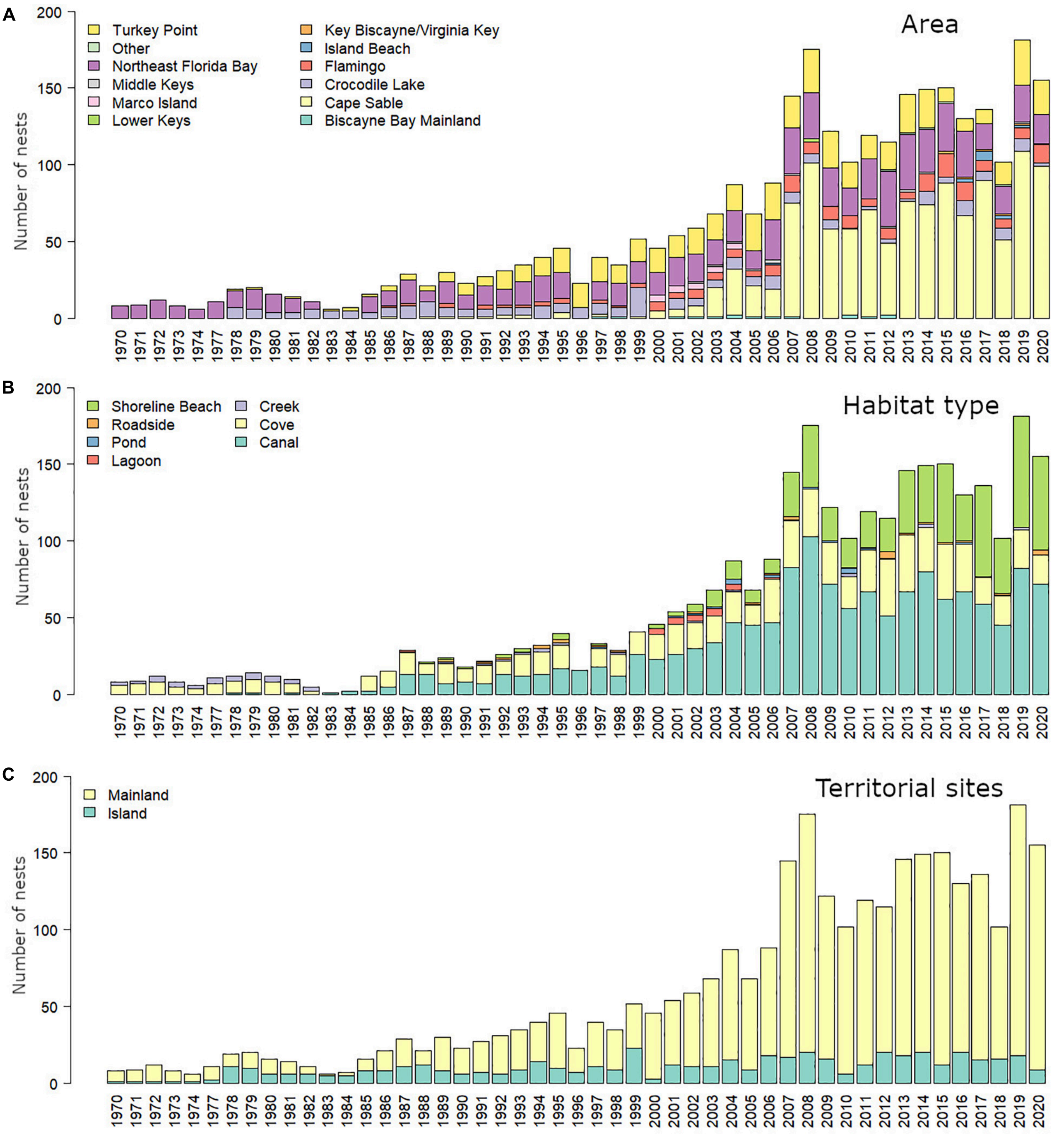

FIGURE 2 | Number of American crocodile (*Crocodylus acutus*) nests reported in South Florida, United States, from1970 to 2020. Nesting has been increasing across main areas **(A)** and habitat types **(B)** including manmade habitats. There has also been a greater increase in nest on mainland sites than island sites **(C)** likely related with space availability (see discussion).

Lake NWR)], 1978 TP], 1986 (Flamingo and Cape Sable), 1997 (Biscayne Bay Mainland), 2000 (Marco Island), 2002 (Middle Keys), 2006 (Highland Beach), 2008 (Lower Keys), 2012 (referred as other in the main areas: C-110 and MM113 Overseas Highway Southbound), and 2015 (Key Biscayne/Virginia Key). From those, Cape Sable and NEFB are the areas with the largest number of nests reported (1,180 and 775, respectively) followed by TP, Crocodile Lake NWR, and Flamingo (542, 258, and 184, respectively). Seven out of the 12 areas assessed showed an increasing trend in the number of nests reported by decade whereas areas such as Biscayne Bay Mainland, Crocodile Lake NWR, Lower and Middle Keys, and Marco Island showed a decreasing trend in the last two decades (**Table 1**). Most nests recorded in South Florida where nest type was described were hole nests (*N* = 1,008) but at least one third of the total historically recorded nests with a known type have been mound nests (*N* = 573). Northeastern Florida Bay is the area with the greatest number of mound nests historically recorded (*N* = 394) and Flamingo/Cape Sable and Crocodile Lake NWR are the areas with the greatest number of hole nests (*N* = 591 and 246, respectively). Mound nests predominate along the sandy beaches found in northeastern Florida Bay, whereas hole nests predominate on peat and marl anthropogenic substrates found along canals at Crocodile Lake NWR or in the Flamingo/Cape Sable area.

Nest have been consistently found in natural habitats such as coves, creeks, shoreline beaches, lagoons, and ponds across South Florida (*N* = 1,459; **Figure 2**). However, anthropogenic habitats such as canals have also played an important role in American crocodile nesting steadily increasing since recorded in 1978 at Crocodile Lake NWR and TP, representing almost half of the total nests reported across the last four decades (*N* = 1,406; **Figure 2**). Most nests have been reported in mainland (*N* = 2,510) rather than insular (*N* = 503) areas except in 1983 and 1984 where we reported larger number of nests on insular areas. Nests have been found across 22 types of habitats based on the Florida land cover classification system (Kawula and Redner, 2018), from which mangrove swamp (33.85%), cultural – estuarine (estuarine ditch/channel; 28.58%), marine (17.46%), and estuarine (4.50%) habitats were the most common used for nesting by American crocodiles across South Florida. Assuming no major land cover changes on the nesting areas where we found nests in the last 50 years, we observed an increasing number of habitat types being used by American crocodiles from the 1970s [mangrove swamp, marine, cultural – lacustrine, salt marsh, and tidal flat) to the 2010s (22 total, **Table 2**)]. Natural habitats (mangrove swamp – 48.53% and marine – 31.98%) were the most relevant in the 1970s. By the 1980s, manmade habitats such as cultural – lacustrine (27.57%; artificial impoundment/reservoir and industrial cooling pond) peak as important areas for nesting and by the 1990s this habitat turned out to be the most used for nesting (42.87%). After 2000, mangrove swamp (2000s – 32.72%, 2010s – 36.14%) and cultural – lacustrine (2000s – 30.98, 2010s – 21.21%) have been the most relevant habitats for nesting across South Florida.

Optimized hot spot analysis defined a total of 2,887 valid input features (nests with valid coordinates) based on the IPD and the buffer layer, from which 2,882 weighted IPD showed strong evidence of no spatial dependence (**Figure 3**). Incremental spatial autocorrelation analysis based on a peak clustering model

TABLE 2 | Types of habitats found across American crocodile (*Crocodylus acutus*) nesting areas in South Florida based on the Florida land cover classification system (Kawula and Redner, 2018).

	1970s		**1980s**		**1990s**		**2000s**		**2010s**		**All records**	
	A	**%**	**A**	**%**	**A**	**%**	**A**	**%**	**A**	**%**	**A**	**%**
Coastal Uplands							0.05	0.73	0.10	1.26	0.13	1.09
Cultural – Estuarine			0.02	1.56	0.04	1.59	0.07	1.15	0.12	1.43	0.13	1.05
Cultural – Lacustrine	0.01	12.48	0.42	27.54	0.96	42.87	1.98	30.98	1.74	21.21	3.28	27.53
Cultural – Riverine									0.00	0.06	0.00	0.04
Estuarine			0.09	5.69	0.06	2.65	0.26	4.03	0.34	4.14	0.53	4.50
Exotic Plants					0.01	0.41	0.01	0.14	0.02	0.26	0.03	0.22
High Intensity Urban							0.07	1.14	0.07	0.82	0.12	1.01
Low Intensity Urban			0.02	1.24	0.02	0.85	0.07	1.09	0.15	1.81	0.17	1.45
Mangrove Swamp	0.02	48.53	0.68	44.16	0.76	33.75	2.09	32.72	2.97	36.14	4.03	33.85
Marine	0.02	31.98	0.19	12.13	0.24	10.52	1.08	16.89	1.63	19.78	2.08	17.46
Maritime Hammock							0.08	1.27	0.15	1.88	0.20	1.72
Marshes							0.00	0.00	0.05	0.61	0.05	0.42
Mesic Hammock									0.01	0.12	0.01	0.08
Prairies and Bogs									0.00	0.03	0.00	0.02
Rockland Hammock			0.02	1.39	0.01	0.37	0.10	1.49	0.05	0.58	0.12	0.99
Salt Marsh	0.00	4.22	0.06	3.59	0.06	2.59	0.20	3.17	0.37	4.51	0.43	3.62
Sand Beach (Dry)					0.00	0.16	0.11	1.76	0.20	2.43	0.24	1.99
Scrub Mangrove			0.01	0.76	0.01	0.52	0.01	0.11	0.03	0.38	0.04	0.29
Shrub and Brushland					0.04	1.75	0.05	0.71	0.02	0.28	0.05	0.38
Tidal Flat	0.00	2.79	0.00	0.08	0.02	0.78	0.08	1.24	0.09	1.08	0.12	1.03
Transportation			0.01	0.39	0.00	0.15	0.04	0.66	0.06	0.77	0.09	0.77
Unconsolidated Substrate			0.02	1.47	0.02	1.02	0.05	0.71	0.03	0.42	0.06	0.49
Total (km^2)	**0.05**		**1.53**		**2.24**		**6.40**		**8.22**		**11.90**	

These data were extracted from the cooperative land cover map version 3.4 compiled by Florida Fish and Wildlife Conservation Commission and Florida Natural Areas Inventory based on a 100-m buffer calculated around nest geolocation. Blank cells indicates that no nests were reported on that specific type of land cover in that specific decade. A = Area (km^2), % = Percentage.

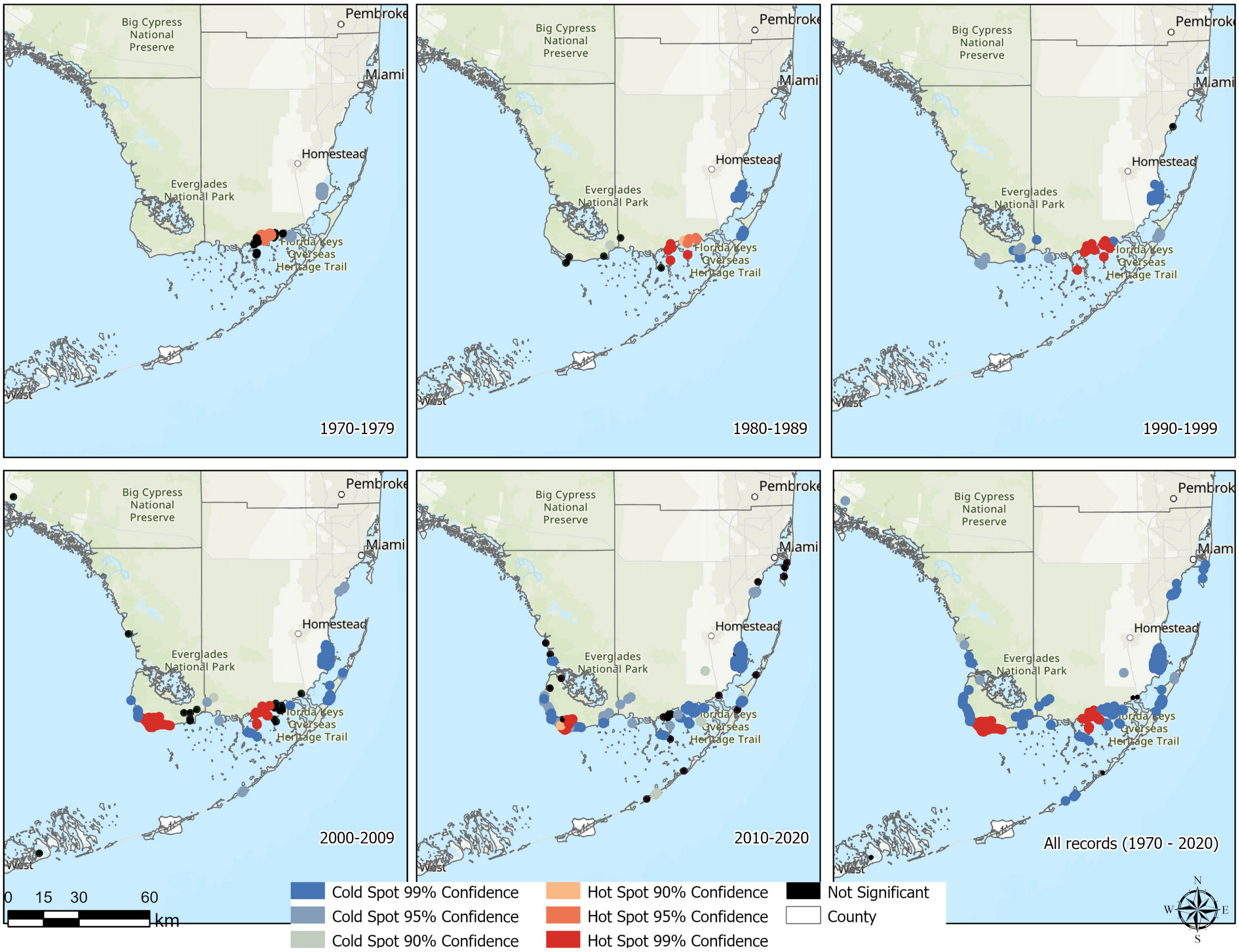

FIGURE 3 | Optimized hot spot analysis of American crocodile (*Crocodylus acutus*) nests registered across South Florida, United States from 1970 to 2020. Red dots represent hot spot areas with different levels of evidence (strong ≤ 99 %, moderate ≤ 95 %, and weak ≤ 90 %) where high incident counts (high and repeated number of nests) cluster based on a specific distance band (see **Table 3** for details). In contrast, blue-grayish dots represent cold areas with different levels of evidence where low incident counts (low but repeated number of nests) cluster based on a specific distance band.

defined an appropriate scale (distance band) for spatial analysis that ranges between 1.46 km for 2010s and 12.55 km for 1990s (**Table 3**). Northeastern Florida Bay and TP showed strong evidence for high and low values of nest spatial clusters (hot and cold spots, respectively) in the 1970s and 1980s due to a high number of nests and little variation in the former area, and lower but constant number of nests in the latter area. Crocodile Lake NWR in the 1980s and Cape Sable and Flamingo in the 1990s showed also strong evidence of nest spatial clusters although in low numbers (cold spots). By the 2000s, Cape Sable showed strong statistical evidence of high values of nest spatial clusters (hot spot) along with NEFB, with constant presence of reproductively active American crocodiles and a higher number of nests. Areas such as Biscayne Bay Mainland, Highland Beach, Middle keys, Marco Island, and Key Biscayne/Virginia Key, showed strong evidence of low values of nest spatial clusters after 2000s and are currently defined as cold spot nesting areas (**Figure 3**). In contrast, we found little to no evidence for hot/cold spot areas such as the lower Keys (i.e., Gi Z-score = -1.05, p-value = 0.29), such that no patterns could be identified in the number of nests registered and could be considered sporadic nesting areas.

Environmental parameters in nesting areas across South Florida ranged between 42.70 ± 23.21 mm (March) and 228.0 ± 74.0 mm (June) for precipitation, 17.9 ± 1.51°C (March) and 25.0 ± 0.45°C (August) for min temp, and 26.4 ± 1.27°C (March) and 32.6 ± 0.43°C (August) for max temp from 1970 through 2018. Data revealed weak-to-strong statistical evidence that these environmental parameters are positively associate with time in South Florida (**Figure 4**) except for mean spring (March, April, and May) precipitation where no evidence for a linear relationship was found (p-value = 0.79). Min and max temp in summer (June, July, and August) were the most relevant variables explaining at least 38% and 24% of the total variation of the

TABLE 3 | Results of the optimized hot spot analysis (OHSA) of American crocodile (*Crocodylus acutus*) nests identified across South Florida from 1970 to 2020.

	1970s	1980s	1990s	2000s	2010s	All records
WP	80	131	286	907	1483	2887
Outliers	0	0	1	3	10	3
Min	1	1	1	1	1	1
Max	11	20	40	52	96	128
Mean	7.2	7.59	12.06	16.52	26.72	39.44
SD	2.92	7.47	12.23	13.66	24.28	34.26
DB (km^2)	6.51	6.35	12.49	7.06	1.46	6.32
SE	18	118	284	727	1280	2882
Hot spot areas	NEFB	NEFB	NEFB	NEFB and Cape Sable	Cape Sable	NEFB and Cape Sable
$\overline{X}$ Gi Bin	2	2.36	3	3	2.8	3
$\overline{X}$ Gi Z-score	2.7	3.6	7.6	9	13.4	20.9
$\overline{X}$ p-value	0	0.01	0	0	0	0
Cold spot areas	TP and NEFB (Snipe Point)	TP, CL, and FL (Mrazek Pond)	TP, CL, FL, and Cape Sable	TP, CL, FL, and Middle Keys	BBM, TP, CL, FL, Middle Keys, and NEFB	BBM, TP, CL, FL, Middle Keys, NEFB, Island Beach, KB/VK, Marco Island, and Other
Mean Gi Bin	−2	−3	−2.9	−2.9	−2.9	−3
Mean Gi Z-score	−2.9	−3.7	−3.4	−6.8	−3.9	−8.8
p-value	0	0	0	0	0	0

Notice how as the number of nests analyzed (weighted polygons; WP) increase over time, the number of features showing weak-to-strong evidence (SE) increase as well, showing the consolidation of clusters (hot and cold spot areas) based on a defined distant band (DB). Min = minimum number of nest, Max = maximum number of nest grouped, TP = Turkey Point Power Plant, CL = Crocodile Lake National Wildlife Refuge, FL = Flamingo, BBM = Biscayne Bay mainland, KB/VK = Key Biscayne/Virginia Key.

number of nests. This trend was also identified when assessing the relationship between environmental parameters individually and number of nests reported, showing weak-to-strong evidence for a positive effect of environmental parameters on the number of nests over time and by season (spring and summer) except for mean precipitation in spring where we found little-to-no evidence (p-value = 0.86; **Figure 5**). Again, min and max temp in summer were the most relevant variables explaining at least 35% and 16% of the total variation of the number of nests reported in South Florida. However, a multiple linear regression analysis including all parameters showed that min temp (slope = 39.04, CI = 3.56 – 64.52, p-value = 0.004) in combination with precipitation (slope = 0.20, CI = -0.04 – 0.43, p-value = 0.094) in summer are the most relevant variables to describe the variation in the number of nests reported across South Florida (F = 6.926, df = 40, p-value < 0.001, adjusted $r^2 = 0.44$).

DISCUSSION

The increase in numbers and success of American crocodile nests in South Florida is attributable to recovery of a once endangered species (Mazzotti et al., 2007b), successful adaptation to a seasonable and variable environment (Mazzotti, 1989; Cherkiss et al., 2014), and underscores the value of providing, protecting, and restoring habitat to support recovery of listed species. In addition to protected crocodile nesting habitat that was provided in Everglades National Park, the U.S. Fish and Wildlife Service purchased Crocodile Lake National Wildlife Refuge to protect crocodile nesting habitat that had been created as a result of an illegal dredge and fill operation, and Florida Power and Light Co committed to managing American crocodiles at their Turkey Point Power Plant site in southern Miami-Dade County as part of its permitting and licensing process (Brandt et al., 1995). The rapid increase in nesting of American crocodiles during 1986 to 2008 is correlated with the plugging of canals in the Cape Sable area (Mazzotti et al., 2007b). We have no explanation, or even a good speculation, for why the increase in nesting leveled off during 2010 to 2020 as it does not appear that nesting or juvenile habitat had become saturated in the Cape Sable area.

Cape Sable has become a nesting hot spot (**Figure 3**) and the most important location for successful crocodile nests in Florida. The occurrence of new nests in a new area was primary impetus for reclassifying the American crocodiles from endangered to threatened (Mazzotti et al., 2007b). Increases in both numbers of nests and areas where nesting occurs were associated with crocodiles nesting on artificial substrates, especially along levees and berms of canals and ditches, which were dug for drainage, navigation, or cooling purposes (Mazzotti et al., 2007a; **Figure 2**). For instance, the expansion of crocodile nesting to the Flamingo/Cape Sable area in the mid 1980's concurrent with plugging of Buttonwood and East Cape canals, provided new locations as well as lower salinities in interior wetlands leading to increased growth and survival of hatchling crocodiles (Mazzotti et al., 2007a, 2009; Briggs-Gonzalez et al., 2021). That crocodiles found and nested in these new locations and habitats illustrates the dispersal and wandering instincts of this species (Kushlan and Mazzotti, 1989b; Cherkiss et al., 2014) and crocodylians in general (Read et al., 2007; Campbell et al., 2013; Calverley and Downs, 2015) as well as the willingness to nest in any diggable substrate at suitable elevation.

More crocodiles nesting in more places at least partially accounts for increase in habitats where crocodile nests occur (**Table 2**) and demonstrates that different substrates

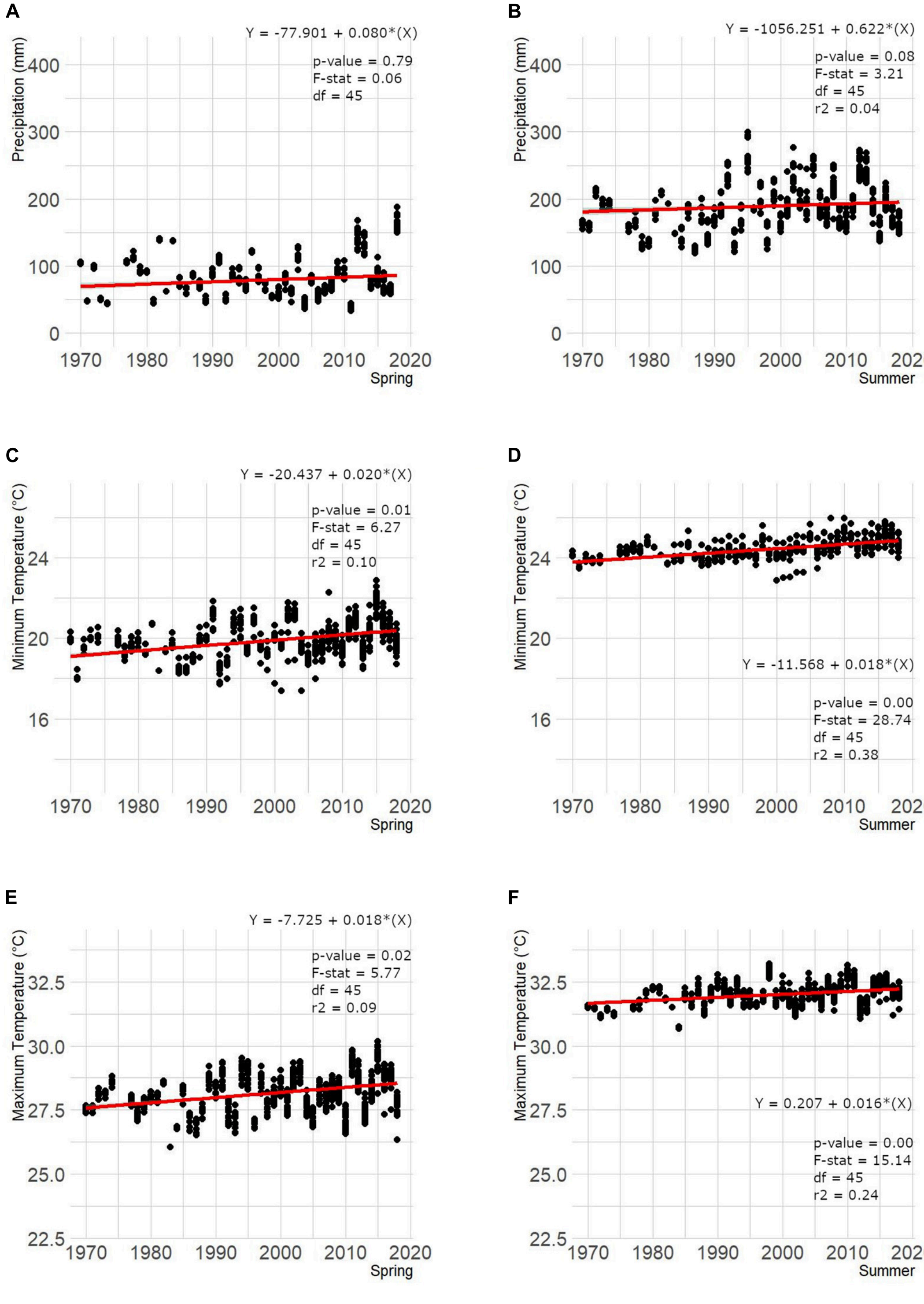

FIGURE 4 | Mean precipitation **(A,B)**, minimum **(C,D)** and maximum **(E,F)** temperature by season [spring (March, April, and May) and summer (June, July, and August)] from 1970 through 2018 reported across American crocodile (*Crocodylus acutus*) nesting areas in South Florida, United States. There is overall weak-to-strong evidence for a positive trend in all variables across time except for mean spring precipitation.

(e.g., marl, peat, sand) can provide suitable conditions for incubation of eggs and nest success (Swanepoel et al., 2000). However, impacts of different nesting substrates on temperature and moisture conditions of nest environments on sex, growth, and survival of hatchling American crocodiles remains unknown (Charruau, 2012).

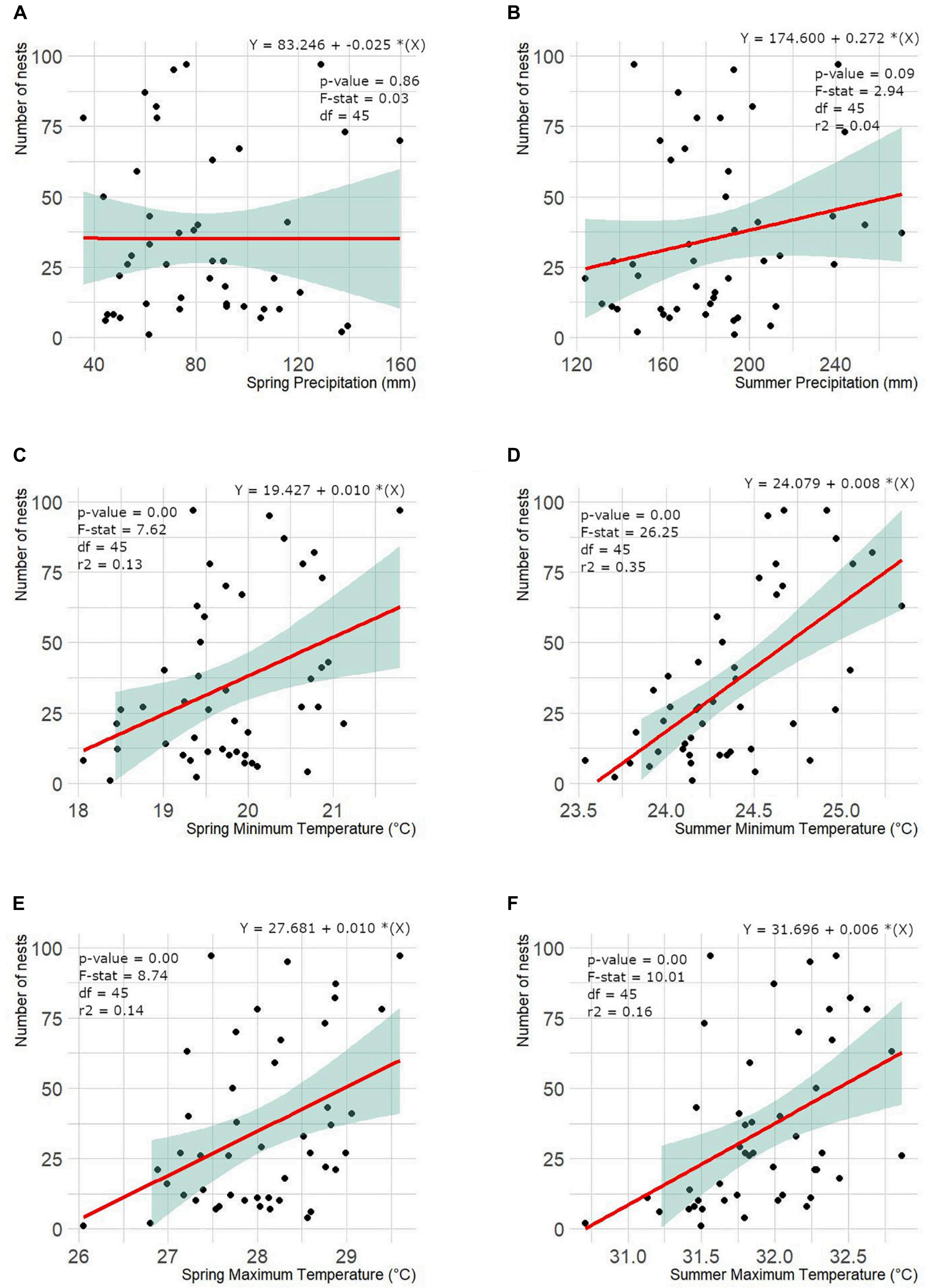

FIGURE 5 | Linear relationship between the number of nests found by year and the mean precipitation **(A,B)**, minimum **(C,D)** and maximum **(E,F)** temperature reported across South Florida from 1970 through 2018. Solid red line represents the linear model predicted and the shaded area represents the standard error of the model. All relationships showed weak-to-strong evidence for positive effect of environmental parameters on number of nests except for spring precipitation and number of nests.

Although substrate type varied, all nest sites were on elevated surfaces. It is common for crocodylian species to nest on drier, elevated surfaces (Metzen, 1977; Thorbjarnarson and Hernández, 1993; Platt and Thorbjarnarson, 2000; Platt et al., 2008) to avoid flooding conditions. However, nests at elevated sites, especially linear ones such as riverbanks, berms, levees, and beaches are at increased risk of predation (Metzen, 1977; Magnusson, 1982; Hall and Johnson, 1987; Platt et al., 2008). That American crocodiles can quickly find and use elevated sites is best illustrated by that a crocodile nested on a canal plug within hours of construction completion (Mazzotti et al., 2007a).

Nesting habitat on artificial substrate (peat deposited from dredging canals) at Crocodile Lake NWR has diminished in quality due to erosion and subsidence, and that the artificial peat substrate does not regenerate naturally (Mazzotti per. obs.). This is likely why Crocodile Lake NWR has exhibited a cooling trend for number of nests in the last decades (**Figure 3**). Follow up research on these aspects could help to elucidate the overall effect of artificial substrates on American crocodile nesting and the long-term effect on populations.

There are two components to the increasing trend in successful crocodile nests that require further consideration: that both the rates of failure of nests and rate of predation decreased (**Table 1**). Failure of American crocodile nests in Florida is primarily caused by flooding or desiccation which occurs in very wet or very dry years (Mazzotti et al., 1988; Mazzotti, 1989). Both flooding and desiccation are cryptic factors in American crocodile nests, and unless you know a nest was there, it would not be counted as a nest at all. We hypothesize that the decrease in failed nests could be a result of an increase in failure to find failed nests in the last decades, possibly due to the high number and spatial spread of nests checked every year, rather than a decrease in the rate of nests failing. An alternative hypothesis is that the success rate of nests is increasing concurrent with increasing summer rainfall and minimum summer temperature. In this study, the combination of summer rainfall and minimum temperature explained most of the variation in number of successful nests found and as described below, crocodile nesting behavior results in crocodiles avoiding cool, dry spring conditions. Similarly, nesting of alligators in the Everglades (Kushlan and Jacobsen, 1990) and Morelet's crocodile in Belize (Platt et al., 2008) is timed to avoid dry conditions during nesting.

Predation rate was variable through the 2000s and then declined in the 2010s, which interestingly relates with the diminished numbers of raccoons (*Procyon lotor*) across ENP and the concurrent increase in Burmese pythons (*Python bivittatus*; Dorcas et al., 2012). Raccoons have been documented as the primary predator of crocodile nests as well as other beach-nesting species such as marine turtles (Deitz and Hines, 1980; Mazzotti, 1989; Garmestani and Percival, 2005; Hénaut and Charruau, 2012; Wilson, 2017). We hypothesize that a decreasing population of raccoons influenced the predation rate historically recorded. This relationship (decreasing nest predation rate with increasing number of pythons) has been documented for turtles in southern Florida (Wilson, 2017).

American crocodiles have a suite of adaptations for orientation of nesting in time and space that help explain the rate of success of crocodile nests. These adaptations provide opportunities for successful nesting in an environment that is both seasonal and variable (Mazzotti and Brandt, 1994). For instance, South Florida is characterized by a warm wet season (May to October) followed by a cool dry season (November to April). However, conditions within a season can be variable (wet seasons can be extremely dry causing desiccating conditions in nests, or extremely wet, causing flooding). Both, desiccation and flooding, cause mortality of eggs, delay incubation, or result in deformed hatchlings (Mazzotti et al., 1988; Mazzotti, 1989). When and where crocodiles nest maximizes the distance in space and time from these sources of stress and minimizes the distance from resources such as suitable temperature and moisture conditions. Tradeoffs occur since not all resources or stress sources occur in the same place or at the same time and that sometimes a stress source and resource can occur together.

This adaptive capacity can be seen in NEFB where crocodile nests are located on sandy beaches surrounded by shoreline vegetation comprised primarily of red, black, and white mangrove (*Rhizophora mangle, Avicennia germinans*, and *Laguncularia racemosa*) interspersed with tropical hardwoods such as mahogany (*Swietenia mahagoni*) and gumbo limbo (*Bursera simaruba*; Olmsted et al., 1981), providing the well-drained elevated sites preferred by female crocodiles for their nest (Mazzotti, 1989; Mazzotti et al., 2007a). However, beach sites are in areas of higher salinity (> 30 psu) and greater exposure to wind and wave action which are stressful to crocodiles especially for hatchlings. Beach sites can also be further from nursery habitat and the need to travel further to protected areas could be an additional stress (Mazzotti, 1983, 1989; Mazzotti et al., 1986). In this case female crocodiles tradeoff putting eggs at a location with good conditions for incubation but stressful conditions for adult and hatchling crocodiles with little parental care of hatchlings evident, resulting in quick dispersal from nest sites.

Nesting behavior of American crocodiles in Florida exhibits a bet-hedging strategy which was defined by Hairston and Fox (2013) "as a trait of an organism, living in a variable environment, that provides greater net fitness over a range of environmental conditions than would a trait specialized for any single environment." In this case, the variable environment for American crocodiles is the variation in moisture and temperature conditions during incubation. In natural habitats, American crocodiles hedge their nesting bets by nesting in sand mound nests along beaches that are resistant to flooding but vulnerable to desiccation, and by nesting in marl hole nests which are resistant to desiccation but prone to flooding (Mazzotti et al., 1988; Mazzotti, 1989). This potentially guarantees that some nests will hatch even under extreme conditions. Climate change and sea level rise have the potential to impact crocodile nesting by changing the temperature during nesting and by flooding nests on low lying areas. However, manmade areas can play a role in securing some nest sites. Thus, whereas relatively low-lying nests on beaches and creek banks are vulnerable to flooding from sea level rise, nests on higher elevated sites along canals should prove less affected.

Timing of nesting is such that incubation is bracketed by cool-dry spring conditions not conducive to development of eggs and warm-wet conditions of late summer that can cause hatchling deformation and mortality of eggs (Mazzotti, 1989). As a result of cool spring temperatures, egg laying for American crocodiles in Florida occurs later than in other parts of its range (Charruau, 2012; Balaguera-Reina et al., 2015). The predictable nature of spring temperatures as a cue for crocodile nesting leads to a pulse nesting pattern (nests hatch within a few weeks of each other) rather than an extended nesting period as exemplified by other crocodylian species (Grigg and Kirshner, 2015). We hypothesize that pulse versus extended nesting periods is an adaptation to the predictability of seasonal patterns of temperature and rainfall. We predict that pulse nesting should occur when seasonal patterns of temperature and rainfall are predictable as for example in alligators (Kushlan and Jacobsen, 1990; Hall, 1991) and Morelet's crocodiles (*Crocodylus moreletii*) (Platt et al., 2008). Why hedge your bet when you have a sure thing? In contrast, we predict that extended nesting should occur when suitable conditions for nesting are not predictable temporally as for example for *Crocodylus porosus* (Magnusson, 1982) or for *Crocodylus novaeguineae* (Hall, 1991). In support of this, increased flooding of alligator nests in Everglades National Park was associated with changes in the predictable natural hydro-pattern caused by water management (Kushlan and Jacobsen, 1990).

Location of nests plays an important role in determination of incubation temperature (Maciejewski, 2006) and incubation temperature plays an important role in sex determination and survival of embryos (Charruau, 2012). Crocodiles have some plasticity in terms of where they deposit their nests with respect to distance from water, depth of the clutch, and whether the nest is in sun or shade (Platt and Thorbjarnarson, 2000; Charruau, 2012; Balaguera-Reina et al., 2015). However, this behavioral plasticity may not be enough to mitigate increases in temperature brought about by climate change (Murray et al., 2016). The only study done on nest temperatures in Florida was performed on sand beach nests and marl creek nests in northeastern Florida Bay by Lutz and Dunbar-Cooper (1984). Nothing is known about nest temperatures in anthropogenic substrates or about how nest temperatures may have changed over time. Since we cannot determine sex of hatchlings reliably, we also have no information on any changes in sex determination that may have occurred.

Cherkiss et al. (2020) found that the timing of nesting of American crocodiles in Florida has shifted in response to increasing temperatures which could have potential implications for the timing pattern displayed until now, showing that the variety of behaviors in timing and location of nests can mitigate some of the adverse effects of climate change. Nonetheless, we hypothesize that there is a limit to how much American crocodiles can shift the incubation period as conditions earlier in the season may be too dry to support incubation of eggs.

Overall, nesting ecology of American crocodiles in Florida has proven to be both positively and negatively influenced by anthropogenic factors, from the unintentional construction of nesting habitats through canals and ditches, and the introduction of invasive species that potentially reduced predation pressure on nests and hatchlings, to the unknown potential effects these events can have on the future of the community (i.e., diversity loss, bottom-up trophic effects). The bet-hedging strategy American crocodiles exhibit in Florida gives the species potential advantages to face the rapid (in evolutionary terms) changes in climate and landscape in the last 50 years, which can be seen in the increasing number of nests (and adults) reported in the last decades. However, it does not imply that the adaptive capacity of the species to face these changes (evolutionary potential) cannot reach a limit if changes continue. Careful consideration of the effects of climate change and sea level rise at local scales (i.e., temperature and moisture variation across nests, landscape modification, and prey availability) in the long term can help us further understand impacts on nesting ecology.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Florida IACUC # 202109072.

AUTHOR CONTRIBUTIONS

FM: initiated project, designed study, acquisition of funding, conceptualized, and contributed to manuscript. SB-R: statistical analysis, conceptualized, and contributed to manuscript. LB: conceptualized project, acquisition of funding, and manuscript review. VB-G: reviewed manuscript. MC, SF, and AG: data collection and management, reviewed manuscript. All authors contributed to the article and approved the submitted version.

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Negative Effects on Neurogenesis, Ovariogenesis, and Fitness in Sea Turtle Hatchlings Associated to *ex situ* Incubation Management

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Sea turtle egg relocation and hatchery incubation (hereafter termed *ex situ* incubation) is an effective strategy to protect clutches when *in situ* egg incubation is not viable. Nevertheless, it negatively affects the ontogenesis of male gonads and brain areas homologous to the mammalian hippocampus, as well as body size and fitness. Thus, it is imperative to analyze the effects of *ex situ* incubation on other developmental aspects and extend these observations to females. This work evaluated the effect of *ex situ* management on neurogenesis (cell proliferation in the dorsal and medial ventricular zones, neuronal integration in the dorsomedial and medial cortices), ovary cell proliferation, body size (mass and length) and self-righting ability. Additionally, this study examined if the incubation microenvironment is different between *in situ* and *ex situ* nests and whether it could contribute to explain the biological traits. An analysis of principal components showed differences in biological variables of hatchlings between *in situ* and *ex situ* clutches, driven by contrasting temperatures and silt composition. Each biological variable was also analyzed with linear mixed models using *in situ* vs. *ex situ* clutches, abiotic variables and their interaction. Turtles from *ex situ* clutches showed: (1) fewer proliferating cells in the dorsal and medial ventricular zones; (2) less mature neurons in the dorsomedial and medial cortices; (3) ovaries with a lesser number of proliferating cells; (4) lower body mass and length at emergence; and (5) slower self-righting time. Together, the results suggest that *ex situ* incubation in hatcheries is related to a slowing down of neurogenesis, ovariogenesis, body size and self-righting ability in hatchlings. Future studies should evaluate the effect of *ex situ* incubation on cognitive and reproductive performance to understand the long-term consequences of altered organogenesis. These studies should also disentangle the differential contribution of egg movement, reburial, nesting environment and parental origin to development. This information would likely result in better conservation strategies for sea turtles.

Keywords: *Lepidochelys olivacea*, brain development, ovarian development, neuronal integration, temperature, substrate composition, moisture

INTRODUCTION

Natural sea turtle populations are threatened globally (IUCN, 2020). Conservation efforts implemented in the last decades have gradually managed to mitigate the loss of some populations with scarce effect on others (Mazaris et al., 2017). One of the most broadly employed strategies is the relocation of endangered eggs to protected hatcheries. Eggs continue their development in *ex situ* nests constructed near the original site selected by the mother. This strategy has shown considerable success and may be the only chance for survival in some species of major concern (Blanck and Sawyer, 1981; Heppell et al., 2005, 2007; Mazaris et al., 2017).

However, several studies have reported negative effects associated to *ex situ* protection such as diminished hatching success, incomplete yolk resorption, sexual ratio bias, low body mass or length and reduced locomotor performance at emergence (Limpus et al., 1979; Eckert and Eckert, 1990; Pintus et al., 2009; Maulany et al., 2012; McElroy et al., 2015; Ahles and Milton, 2016; Tanabe et al., 2020). Recent studies evaluated the impact of the *ex situ* incubation strategy on organogenesis and proposed potential mechanisms of action for its negative effects (Sönmez et al., 2011; Herrera-Vargas et al., 2017).

Some studies explored the causal effect of abiotic variables on developmental traits (Sieg et al., 2011). A chief variable is nest temperature (Telemeco et al., 2013), which modifies incubation duration (Merchant-Larios et al., 1997; Booth and Astill, 2001a,b), sex determination (Reece et al., 2002), immune competence (Freedberg et al., 2008; Dang et al., 2015; Robledo-Avila et al., 2022) and brain development in reptiles (Amiel and Shine, 2012; Paredes et al., 2016; Amiel et al., 2017; Sanger et al., 2018). Another important factor is nest moisture, which alters sex ratio, incubation duration and embryo growth (Wallace et al., 2004; Patino-Martínez et al., 2012; Lolavar and Wyneken, 2020; Tezak et al., 2020). Successful sea turtle embryo development also depends on substrate composition, which could affect gas exchange and water retention, as well as the incubation period and sex-ratio (Ackerman, 1977; Lutcavage et al., 1997; Mitchell and Janzen, 2019).

The brain is a plastic organ; its early development is affected by external factors, such as temperature and moisture in ectotherms (Coomber et al., 1997; Beltrán et al., 2021; Sanger et al., 2021). This is especially true for the hippocampus, a structure that participates in cognitive tasks such as spatial learning and memory (Suh et al., 2011; Bannerman et al., 2014), as well as in the regulation of the hypothalamic-pituitary-adrenal axis (Sapolsky et al., 1983, 1984). Anatomical and genetic results suggest that the reptilian dorsomedial cortex is homologous to the mammalian CA1/CA3 hippocampal regions; whereas the medial cortex corresponds to the dentate gyrus (Medina et al., 2017; Tosches et al., 2018). Development of these cortices results from proliferation of neural precursors in the dorsal and medial walls of the lateral ventricles (Butler and Hodos, 2005; Medina et al., 2017; McDonald and Vickaryous, 2018; Tosches et al., 2018).

Proliferation is the earliest step in neurogenesis, which is followed by migration, differentiation, and integration of these new cells into the neural tissue (Altman and Das, 1965; Gage, 2002; Bayer and Altman, 2004; Kuhn et al., 2016). In mammals, early adverse environmental conditions can induce long-lasting brain alterations, including reduced hippocampal neurogenesis (Lajud and Torner, 2015; Vaiserman and Koliada, 2017). These alterations could result in lifelong cognitive and affective deficits, such as learning and memory disabilities, as well as anxiety-like or depressive-like behaviors (Heim and Nemeroff, 2002; McEwen, 2012; Lajud and Torner, 2015; Vaiserman and Koliada, 2017). Interestingly, species-specific non-optimal incubation temperatures decrease hippocampal neurogenesis in lizards, producing spatial learning disabilities (Amiel and Shine, 2012; Amiel et al., 2017; Dayananda and Webb, 2017). Likewise, early non-optimal conditions produce anxiety-like behaviors in lizards (Trnik et al., 2011), suggesting that reduced hippocampal neurogenesis during critical time windows may alter cognitive and behavioral traits in both mammals and reptiles.

Similarly, early environmental conditions also influence the development of the gonad. Its differentiation in reptiles displays the same progression as in other vertebrates and is highly sensitive to environmental variables such as temperature (Yntema and Mrosovsky, 1981; Ge et al., 2018; Weber et al., 2020). In humans and mice, altered seminiferous cord formation is associated with infertility, dysgenesis, and sexual development disorders (Chen and Liu, 2016). Likewise, disturbed oogenesis and folliculogenesis is related to reduced follicle reserves and infertility (Holm et al., 2016).

Motor abilities such as self-righting, crawling, and swimming speed constitute developmental traits associated with microenvironmental incubatory conditions like temperature and grain size. Better performance has been associated with cooler incubation temperatures and medium grain sand (Stewart et al., 2019; Reboul et al., 2021). Since reduced motor skills increase predation risk for newly emerged hatchlings during their displacement on land and sea, these traits constitute a good indicator of physical fitness and survival during this period. Indeed, these abilities may be good indicators of non-optimal incubation conditions in sea turtles (Fleming et al., 2020; Martins et al., 2020).

Ex situ incubation may alter neural and gonadal development in male *Lepidochelys olivacea* hatchlings by reducing the cell size and dendritic arborization of dorsomedial cortical neurons, as well as the number of epithelial cells per seminiferous cord (Herrera-Vargas et al., 2017). The functional relevance of these alterations is unknown, but results in mammals and lizards suggests that defective differentiation of hippocampal neurons and the seminiferous cord causes cognitive and reproductive problems (see above). Studies in rodents suggest that males are more vulnerable to developmental disruption (Dimatelis et al., 2015; Cowan and Richardson, 2018; de Melo et al., 2018), but in ectotherms some studies associate better phenotypes with male-producing environmental conditions (Freedberg et al., 2008).

Neurogenesis, ovariogenesis and motor performance are vital for the survival of sea turtle populations. These processes are highly sensitive to early adverse environmental stimuli. *Ex situ* incubation in hatcheries involves the early handling of eggs, as well their relocation to hatcheries. Moreover, nesting beaches include microenvironments that vary in moisture (depending on distance to the shoreline or vegetation), substrate composition, microbial load, and temperature (Stewart et al., 2019). All these variables could contribute to create non-optimal conditions for suitable development of turtle hatchlings. Thus, it is necessary to evaluate the effects of *ex situ* incubation in female turtles to determine if early adverse conditions have sex-specific effects on developmental traits. Moreover, it is imperative to identify the environmental variables likely modifying these developing traits in sea turtles. To test these hypotheses, this study evaluated the effects of *ex situ* incubation on hippocampal neurogenesis (cell proliferation in the dorsal and medial ventricular zones, as well as integration of newborn neurons into the dorsomedial and medial cortices), ovariogenesis (cell proliferation), body size (mass and length), and self-righting ability in *L. olivacea* females at emergence. Furthermore, this work analyzed the contribution of several nest-related abiotic variables (sand temperature, moisture, and grain size) to identify the most relevant variables for hatchling development. The results will contribute to determine the impact of *ex situ* incubation in hatcheries on female organogenesis and to identify some of the variables determining the altered phenotype observed in turtles incubated in hatcheries.

MATERIALS AND METHODS

Study Site and Nesting Conditions

This study was conducted at the *Centro de Protección de la Tortuga Marina* in Boca Seca beach, located in Lázaro Cárdenas, Michoacán, México (18° 04′ N, 102° 58′ W; **Figure 1**). Egg manipulation was kept to the bare minimum and done by the hatchery staff according to protocols stated in Mexican regulation (NOM-162-SEMARNAT, 2012) and a previous report (Herrera-Vargas et al., 2017). Briefly, beach patrolling during three consecutive nights (September 13–15, 2017) allowed identification of nesting females. Ten nests located approximately in the same beach zone (30–60 m away from the shoreline) were chosen and sheltered immediately after the female turtle covered the eggs and left the site. Five randomly selected natural nests remained undisturbed *in situ*, only fenced with cyclone mesh until hatchling emergence. The complete clutch of the other five nests was carefully collected as soon as the female left the nest, placed in individual plastic bags and transported to the hatchery. There, the eggs were immediately buried in previously built nests and remained undisturbed until emergence. This ensured that conditions related to clutch size (e.g., oxygen availability, temperature, metabolic heat, etc.) remained unaltered. The total time between laying and re-burial lasted less than 2 h. Efforts were made to avoid egg rotation and excessive handling, as well as to emulate natural nest architecture in hatcheries. *Ex situ* nests were constructed by the hatchery staff according to international norms for *L. olivacea*, with a narrow neck (20–25 cm) and a wider flask-shaped bottom, at a depth of 40–50 cm and 1 m separation between them (Kutzari, 2006). Nests from both conditions were not shaded or watered. Forty days after incubation started, the hatchery clutches were fenced with cyclone mesh until turtle emergence. This experimental design ensured that *in situ* nests were not disturbed and that clutches relocated to hatcheries only went through the routine procedures done by the hatchery staff. Egg handling was performed before organogenesis started (Miller, 1985).

Hatchling Collection

Animal sampling, handling and sacrifice protocols were approved by an Animal Rights Committee, under License Number SEMARNAT: SGPA/DGVS/10395/17; in accordance with Mexican regulation (NOM-033-SAG/ZOO, 2014). One hundred and fifty turtles were collected from five *in situ* and five *ex situ* nests (15 hatchlings/nest). Fifty hatchlings were used for histological and morphometric observations: for neurogenesis studies, 24 brains per nest type were evaluated, since one brain for each condition was damaged during dissection (48 brains in total); for ovarian cell proliferation quantification, at least two ovaries per nest were used (27 ovaries in total), while all fifty individuals were sexed. The other one hundred hatchlings were used to evaluate motor performance.

Hatchling collection was described by Herrera-Vargas et al. (2017) and Robledo-Avila et al. (2022). Briefly, fifteen emerging turtles from each nest were collected at 5-min intervals, as soon as they surfaced from each nest. Five randomly selected hatchlings per nest were weighted with a digital precision balance (OHAUS^{TM} Scout Pro Sp 602, Max 600 g, d = 0.01 g). Their straight carapace length was measured using a digital Vernier caliper (Mitutoyo^{TM}). These same five turtles per nest were used to investigate neurogenesis and gonadal cell proliferation.

Cell Proliferation and Neuronal Integration Studies

To evaluate the effect of *ex situ* incubation on brain and ovary cell proliferation, hatchlings received an intraperitoneal injection of the cell birth marker 5′-bromo-2′-deoxyuridine (BrdU, a thymidine analog incorporated in the S phase of the cell cycle. Sigma-Aldrich, 100 mg/Kg in 0.9% NaCl) immediately after morphometric data recording and a second injection 2 h after. Turtles were then placed in sand tubs and sacrificed 4 h after the last injection. This procedure (i.e., the timings) minimized the effect of factors other than the incubation condition on cell proliferation. The brain and gonad-mesonephros complex were dissected *in situ*, incubated in Bouin's solution (Sigma-Aldrich, Saint Louis, MO, United States) for 24 h and incubated in buffered paraformaldehyde (Sigma-Aldrich, Saint Louis, MO, United States, 4% in 0.1 M phosphate buffer) for 3 days at room temperature.

In the laboratory, brains were rinsed with 70% ethanol and transferred to buffered sucrose (Sigma-Aldrich, Saint Louis, MO, United States, 30% in 0.1 M phosphate buffer) at 4°C until they sank. Then they were frozen in the Peltier module of a cryostat (Microm) and sectioned coronally at 30 μm. Free-floating sections were collected in Tris-buffered saline (Sigma-Aldrich, Saint Louis, MO, United States, 50 mM Tris–HCl, 150 mM NaCl, pH 7.6) and processed for immunohistochemistry. Cell proliferation in neurogenic niches was evaluated by

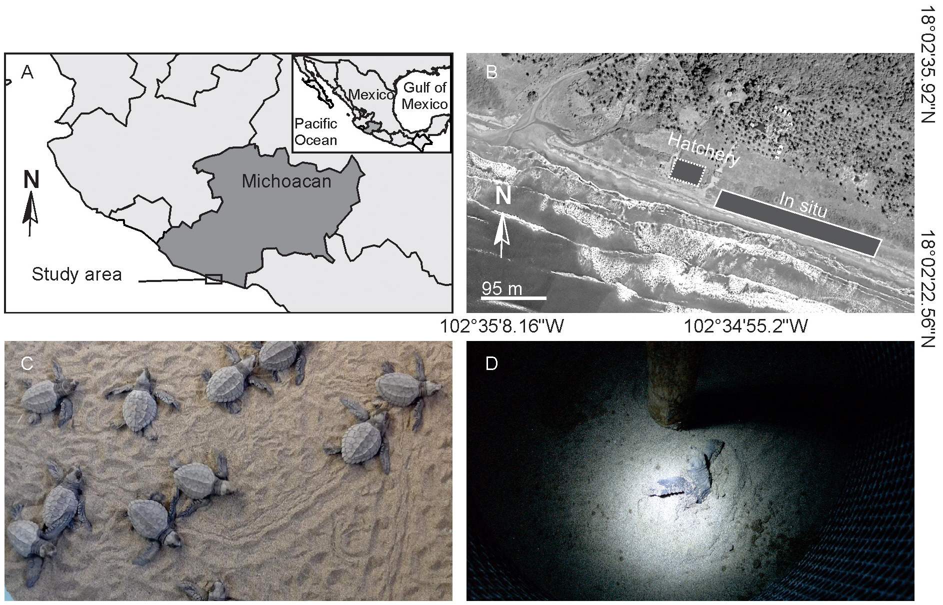

FIGURE 1 | Location of the study site and nesting conditions. **(A)** Map showing Lázaro Cárdenas nesting beaches in Michoacán, México. **(B)** Aerial photograph of *ex situ* (dotted line) and *in situ* nests (continuous line). **(C,D)** *Lepidochelys olivacea* hatchlings crawling to sea and emerging from nest, respectively.

immunoreactivity for BrdU and neuronal integration was evaluated by immunoreactivity for the anti-neuronal nuclear protein (NeuN) in separate brain sections. Briefly, the tissue was incubated in ImmunoDNA retriever 20× with citrate (Bio SB), then in 2 N HCl for 30 min at 65°C and finally in 0.1 M sodium borate buffer at room temperature. Blocking of non-specific binding was done by incubating the sections in 0.1% bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, United States) for 30 min. Sections including the dorsal and medial ventricular zones were incubated with mouse anti-BrdU monoclonal antibody (1: 500, Roche) and independent cortical sections were incubated with mouse anti-NeuN monoclonal antibody (1: 1000, Millipore) for 16 h at 4°C. Then they were incubated with a donkey anti-mouse biotin-conjugated secondary antibody (1:500, Vector Laboratories) for 2 h. Sections were incubated in avidin/biotin horseradish peroxidase (Vectastain Elite, PK-6100) for 2 h at room temperature and then incubated in diaminobenzidine as a chromogen, with peroxide and buffer for 10 min (Vector Staining Kit, SK-4100). Finally, the tissue was mounted onto gelatinized slides, dehydrated and cover-slipped using Cytoseal 60 (Richard Alan Scientific).

To evaluate cell proliferation in neurogenic niches, three equivalent non-adjacent brain sections containing the dorsal and medial ventricular zones were selected per turtle according morphological criteria (appearance of lateral ventricles) along the antero-posterior axis. BrdU + immunoreactive cells were quantified in two microphotographs per section per neurogenic zone at 1000× magnification. To evaluate neuronal integration, three equivalent non-adjacent brain sections including the dorsomedial and medial cortices were selected per turtle according morphological criteria (opening of the lateral ventricles). NeuN + immunoreactive cells in the cellular layer were quantified in three microphotographs per section per cortex at 1000× magnification. Microphotographs were captured with a Zeiss microscope using the Axio Vision 4.6 software and analyzed using NIH ImageJ software.

For gonadal sex determination, one gonad-mesonephros complex was dehydrated using increasing ethanol concentrations, embedded in paraffin, sliced transversally (7 μm) in a microtome (Leica) and stained with hematoxylin-eosin (Merck), as previously described (Herrera-Vargas et al., 2017). Gonadal histology showed that all fifty specimens were females, thus all gonadal analysis were performed in ovaries. To evaluate ovarian cell proliferation, at least two gonad-mesonephros complexes per nest were frozen, cryosectioned at 30 μm and immuno-stained for BrdU as described for the brain (the other gonad-mesonephros complexes were frozen for RNA quantification). Five adjacent gonadal sections per hatchling were selected from the central ovary. BrdU + immunoreactive germ cells were quantified in the cortex of ovaries throughout the whole section at 400 x magnification, as described for brain sections. After quantification, selected ovarian sections were stained with hematoxylin-eosin to observe cell density and cytoarchitecture.

Self-Righting

To evaluate the effect of *ex situ* incubation on motor skills, ten hatchlings per nest were randomly selected and separated in tubs with sand for 15 min, to prevent lethargy from interfering with performance (Booth et al., 2013). Turtles were placed upside-down on a tray full of dry sand and the time they took to self-right was recorded with a chronometer (Sper scientific 810015 5 channel timer). Hatchlings that exceeded 10 min to self-right were discarded from the analysis. Thus, 48 turtles from *in situ* nests and 31 *ex situ* hatchlings were analyzed.

Measurement of Abiotic Variables

To evaluate the contribution of abiotic variables to the developmental traits, nest temperature, moisture and sand grain size were determined. Nest temperature was recorded from developmental day 11 until emergence, since this period includes the bulk of hippocampal neurogenesis previously described for *Emys orbicularis* (Goffinet et al., 1986) and the critical time for gonadal development in *L. olivacea* (Merchant-Larios et al., 1997). Temperature was registered by data loggers (Onset HOBO® Bluetooth Pendant MX2202 series; accuracy ± 0.2°C) carefully located outside the nest to avoid disturbing the clutch. They were placed in sand 30 cm from the center of the nest and 50 cm deep, 11 days after the incubation period began. They were programmed to record the temperature every hour; results were averaged by nest.

Moisture and grain size were determined from 100 g of sand, sampled 10 cm deep inside the nests, immediately after hatchling emergence. The sand was placed in a sealed plastic bag, weighed, dried at 105–110°C in a standard oven and weighed again. Moisture content was calculated as the ratio of wet to dry sand mass (Head, 1992). Grain size analysis was performed by particle sieving, using international parameters (gravel: >2.0; coarse sand: 2.0 ± 0.2; fine sand: 0.2 ± 0.02; silt: 0.02–0.002 mm; Brady and Weil, 2008), and subsequent weighting with an analytical balance. Gravel, coarse- and fine-sand, as well as silt proportions were calculated dividing by the total dry mass (Gee and Or, 2002).

Statistical Analyses

Preliminary analysis showed that only one nest had a different gravel composition from the rest, thus gravel was discarded from further examination. Similarly, fine sand was collinear with coarse sand, hence only the latter was used. This was done because multivariate analyses are sensitive to collinearity between variables, which causes interpretation problems (Harrison et al., 2018). Accordingly, the selected parameters for analysis were abiotic variables within nests (temperature and moisture, coarse sand and silt), as well as turtle biological variables (cell proliferation in the dorsal and medial ventricular zones, as well as the ovary; neuronal integration in the cellular layer of the dorsomedial and medial cortices; body mass and length, as well as self-righting time).

Multivariate principal components analysis (PCA) was performed to reduce data dimensionality and investigate the distribution of samples in two-dimensions. This allowed the assessment of possible differences between conditions, based on the abiotic variables within nests and turtle biological data.

The outcomes of *in situ* ($n = 5$) vs. *ex situ* clutches ($n = 5$), abiotic variables and their interaction (*in situ* vs. *ex situ* condition interacting with each abiotic variable) were studied with linear mixed models to avoid violation of independence assumptions (turtles within clutches). Biological results were used as the response variables in these models; which included the following effects: *in situ* vs. *ex situ* clutches (main); abiotic variables and their interaction (fixed) plus turtles within clutches (random). All abiotic variables (temperature, moisture, coarse sand and silt) were standardized by subtracting the mean from every value and dividing by the standard deviation (Harrison et al., 2018), due to wide differences in their ranges. Outliers were removed from most turtle biological data (all except body mass and length). Ovarian cell proliferation and self-righting were also transformed to meet normality and homocedasticity assumptions; with square-root and natural logarithm, respectively.

Stepwise backward elimination followed by selection with the conditional Akaike information criterion (cAIC) were performed to obtain the best linear mixed model (**Supplementary Data 1**). The marginal R^2 for each model was determined as a measure of the proportion of the variance explained by the model. The effect size for *in situ* vs. *ex situ* clutches was evaluated by Glass's Δ (Sink and Mvududu, 2010; Sullivan and Feinn, 2012). Graphs presented in the results for each of the eight biological variables were obtained back-transforming the predictions of the final models. Residuals for each model were plotted to assess the distribution of the model fit (**Supplementary Data 1**).

Data analyses were done using R (R Core Team, 2020; version 4.1.1) and RStudio (RStudio Team, 2020; version 1.3.1073). Linear mixed models were performed using: readxl (Wickham and Bryan, 2019), lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), and cAIC (Säefken et al., 2018). Model parameters, including residuals, were evaluated with the performance package (Lüdecke et al., 2021). Principal component analysis results were graphed with ggbiplot (Vu, 2011).

RESULTS

Data provided by the national meteorological service during the entire incubation period (September 13–October 31, 2017) showed an average environmental temperature of 26.41°C ± 0.98 SD (mean minimum temperature = 20.53°C and mean maximum temperature = 32.27°C; **Supplementary Table 1**). Data loggers in sand near the nests recorded average maximum temperatures of 35.68°C ± 0.83 SD for *in situ* nests and 37.03°C ± 1.29 SD for *ex situ* nests, as well mean minimum temperatures of 26.28 ± 2.91 SD for *in situ* nests and 25.01 ± 0.14 SD for *ex situ* nests (**Table 1**). The average incubation period ± SD for hatchlings from *in situ* clutches was 46.40 ± 0.55 and 45.60 ± 1.82 days for hatchlings from *ex situ* clutches (**Table 1**). The mean moisture, coarse and fine sand, as well as silt proportions were: *in situ* (0.1180 ± 0.0936 SD; 0.9238 ± 0.0363 SD; 0.0643 ± 0.0384 SD; and 0.0005 ± 0.0008 SD, respectively) and *ex situ* clutches (0.0980 ± 0.0487 SD; 0.9011 ± 0.0176 SD; 0.0880 ± 0.0156 SD; and 0.0062 ± 0.0041 SD, respectively; **Supplementary Table 2**).

The PCA suggested that the synergic effect of some or all abiotic variables may be important to determine turtle phenotypes (see below). The first two principal components of the PCA, based on the biological and abiotic variables, explained 71.7% of the total data variance (**Figure 2** and **Supplementary Table 3**). Principal component 1 (52%) showed that coarse sand (r = -0.202) and moisture (r = -0.122) were higher for *in situ* nests, while temperature (r = 0.304) and silt (r = 0.295) were lower. In addition, all biological variables from hatchlings (except

TABLE 1 | Sand temperatures (°C) monitored by data loggers located 30 cm from the center of the clutch and 50 cm deep, as well as incubation periods registered for *L. olivacea* hatchlings at Boca Seca beach, Lázaro Cárdenas, Michoacán, México.

		Incubation period				
Nest no.	**Incubation condition**	**Mean temperature ± SEM**	**Maximum temperature**	**Minimum temperature**	**Incubation dates**	**Incubation days**
1	*in situ*	34.17 ± 0.75	35.44	31.47	15/09–30/10	46
2	*in situ*	33.19 ± 1.26	34.90	24.93	15/09–30/10	46
3	*in situ*	34.15 ± 1.50	36.51	25.22	15/09–30/10	46
4	*in situ*	34.24 ± 1.66	36.40	25.03	15/09–31/10	47
5	*in situ*	32.64 ± 1.21	34.69	24.74	15/09–31/10	47
6	*ex situ*	34.36 ± 1.49	36.30	24.93	13/09–30/10	48
7	*ex situ*	34.50 ± 1.68	36.95	25.22	14/09–31/10	47
8	*ex situ*	34.38 ± 1.47	36.30	25.03	15/09–29/10	44
9	*ex situ*	35.12 ± 2.18	39.28	24.84	15/09–29/10	44
10	*ex situ*	34.42 ± 1.49	36.30	25.03	15/09–30/10	45

		Thermosensitive period		
Nest no.	**Incubation condition**	**Mean temperature ± SEM**	**Maximum temperature**	**Minimum temperature**
1	*in situ*	32.98 ± 0.68	34.06	31.98
2	*in situ*	32.14 ± 1.22	33.95	24.93
3	*in situ*	32.96 ± 1.53	35.22	25.22
4	*in situ*	32.61 ± 1.34	34.59	25.03
5	*in situ*	31.69 ± 1.17	33.43	24.74
6	*ex situ*	33.49 ± 1.71	35.65	24.93
7	*ex situ*	33.41 ± 1.84	35.97	25.22
8	*ex situ*	33.20 ± 1.68	35.33	25.03
9	*ex situ*	33.04 ± 1.53	34.69	24.84
10	*ex situ*	33.38 ± 1.62	35.33	25.03

The table shows temperatures registered from incubation day 11 until emergence (incubation period) and during the thermosensitive period per clutch (in situ, n = 5; ex situ, n = 5).

self-righting) were higher for *in situ* conditions (**Figure 2** and **Supplementary Table 3**). Principal component 2 (20%) showed that moisture (r = -0.543) and coarse sand proportions (r = -0.300) most influenced environmental variation for *in situ* nests (**Figure 2** and **Supplementary Table 3**).

Principal components analysis results showed a clear difference between nest conditions. Thus, linear mixed models were performed to confirm these differences and explore their relationship to abiotic variables. Linear mixed models showed that *ex situ* nests were negatively related to *L. olivacea* development at nest emergence with moderate (Glass's $\Delta < 1$) to very large effect sizes (Glass's $\Delta > 1.3$; Sullivan and Feinn, 2012; **Table 2**). The simplest linear mixed models (*in situ* vs. *ex situ* nests and intercept) were the best fit for all biological variables (**Table 2**). Additionally, models with each abiotic variable alone (i.e., without *in situ-ex situ* factor) were performed for each biological variable. In all cases, the cAIC was better with the *in situ-ex situ* factor than with only one abiotic variable (**Supplementary Data 1**). Altogether, linear mixed models indicated that abiotic variables (temperature, moisture, and the proportion of different sediment sizes) did not individually affect any response variables, at least when using backward elimination and the lowest conditional AIC to select the best model.

In situ hatchlings showed on average 253.72 and 502.09 more proliferating cells in the dorsal (*in situ*: 491.04 ± 186.63 vs. *ex situ*: 237.32 ± 158.5 BrdU + cells/mm^2 mean ± SD; p = 0.014) and medial ventricular zones (*in situ*: 1005.45 ± 468.77 vs. *ex situ*: 503.36 ± 333.62 BrdU + cells/mm^2 mean ± SD; p = 0.031), respectively (**Figure 3** and **Table 2**) than *ex situ* incubated offspring. They also showed on average 148.26 and 664.82 more mature neurons in the dorsomedial (*in situ*: 2033.85 ± 151.42 vs. *ex situ*: 1885.59 ± 157.67 NeuN + cells/mm^2 mean ± SD; p = 0.041) and medial cortices (*in situ*: 2061.57 ± 283.11 vs. *ex situ*: 1396.75 ± 541.07 NeuN + cells/mm^2 mean ± SD; p = 0.001), respectively (**Figure 4** and **Table 2**) than *ex situ* hatchlings. The best models for dorsal and medial ventricular zone cell proliferation explained 33 and 28% of the data variation, while those for the dorsomedial and medial cortices explained 18 and 37% of the data variation (**Table 2**).

The ovaries from *in situ* incubated hatchlings showed on average 11.99 more proliferating cells (*in situ*: 13.28 ± 2.21 vs. *ex situ*: 1.29 ± 1.38 BrdU + cells/mm^2 mean ± SD; p < 0.001; **Figure 5** and **Table 2**) in comparison with ovaries from *ex situ* offspring. The best model for ovarian cell proliferation explained 89% of the data variation (**Table 2**). Cell density and architecture were evaluated in sections with hematoxylin/eosin

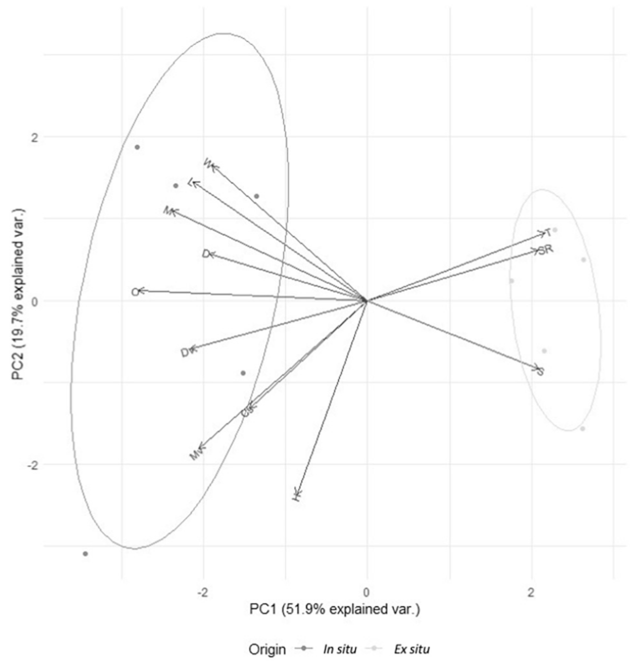

FIGURE 2 | Multivariate principal components analysis of developmental and abiotic variables from *in situ* (*n* = 5, dark gray, left) vs. *ex situ* clutches (*n* = 5, light gray, right). W, body weight/mass (gr); L, body length (mm); M, neuronal integration in the cellular layer of the medial cortex (number of NeuN + cells/mm); D, neuronal integration in the cellular layer of the dorsomedial cortex (number of NeuN + cells/mm); O, cell proliferation in the ovary (number of BrdU + cells/mm); Dv, cell proliferation in the dorsal ventricular zone (number of BrdU + cells/mm); Mv, cell proliferation in the medial ventricular zone (number of BrdU + cells/mm); Cs, coarse sand (proportion); H, humidty (proportion); T, temperature (°C); SR, self-righting time (s); S, silt (proportion); PC1, principal component 1 of PCA; PC2, principal component 2 of PCA.

staining after BrdU quantification, but it was not possible to quantify the number of ovarian cells due to section thickness (**Supplementary Figure 1**).

Turtles from *in situ* clutches were on average 1.66 g heavier (*in situ*: 17.65 ± 1.35 g vs. *ex situ*: 15.99 ± 1.1 g mean ± SD; $p = 0.041$; **Figure 6A** and **Table 2**), and 3.34 mm larger than *ex situ* offspring (*in situ*: 67.22 ± 2.50 mm vs. *ex situ*: 63.88 ± 2.33 mm mean ± SD; $p = 0.012$; **Figure 6B** and **Table 2**). Additionally, *in situ* incubated hatchlings were on average 5.49 s faster for self-righting (*in situ*: 2.56 ± 8.45 s vs. *ex situ*: 8.05 ± 6.90 s mean ± SD; $p = 0.007$; **Figure 6C** and **Table 2**) than *ex situ* hatchlings. The best model for body mass explained 31% of the data variation, while the model for body length explained 32% of the data variation and that for self-righting explained 26% of data variation (**Table 2**).

DISCUSSION

This work shows that *ex situ* protection of eggs in hatcheries can result in developmental deficits (compared to *in situ* nests) in female hatchlings, expanding our prior report in males (Herrera-Vargas et al., 2017). Herein, the *ex situ* incubation strategy was related to negative effects on neurogenesis, ovariogenesis, body size, and motor performance. Turtles from *ex situ* clutches showed lower cell proliferation in the ovary and neurogenic niches, as well as fewer differentiated neurons in brain areas homologous to the mammalian CA1/CA3 hippocampal regions and dentate gyrus. Additionally, newly hatched turtles from *ex situ* clutches showed lower body size and lesser motor abilities. Many factors could potentially account for the negative effects of *ex situ* incubation on offspring development. This study focused on the nest microenvironment (sand temperature, moisture, and sediment particle size). The PCA showed that this microenvironment was related to turtle phenotypes. However, the isolated contribution of the different abiotic variables to development could not be disentangled. A main limitation of this study was an impossibility to differentiate the effects of hatchery incubation from the effects of egg movement and reburial as well as genetic factors (i.e., parental origin). Of note, this study used a relatively small number of nests over a relatively narrow, but important, temperature range.

Ex situ Protection in Hatcheries Is Associated With a Lower Rate of Neurogenesis in Turtle Hatchlings

To evaluate the effect of *ex situ* incubation while minimizing the effect of turtle retention, two important events for early neurogenesis were evaluated 6 h after nest emergence: cell proliferation in neurogenic niches and neuron integration in cortices homologous to the mammalian hippocampus. The results showed that the *ex situ* incubation strategy is associated with a lower number of proliferating cells and differentiated neurons in turtle hatchlings. In vertebrates, hippocampal neurogenesis is a highly regulated process that includes cell proliferation, migration, differentiation and integration (Altman and Das, 1965; Gage, 2002; Bayer and Altman, 2004; Kuhn et al., 2016; McDonald and Vickaryous, 2018). Proper development of the mammalian hippocampus is required to achieve ecologically relevant cognitive tasks such as spatial learning and memory, as well as to regulate emotional responses (Gould et al., 1999; Deng et al., 2010).

In reptiles, newly born cells from the dorsal and lateral ventricular zones migrate radially for several days and mature as neurons as they approach the cortices. Neurogenic niches in the postnatal gecko brain produce neurons exclusively (McDonald and Vickaryous, 2018). Thus, it is highly likely that the BrdU + cells we observed herein were neuronal progenitors. Nonetheless, future cell-fate mapping studies should confirm the lineage of BrdU + cells produced in newly emerged sea turtles. In this study, BrdU + cells were only present lining the ventricular walls in both conditions, confirming the idea that proliferating neurons migrate several days after birth.

Offspring from *ex situ* clutches showed fewer BrdU + proliferating cells early after nest emergence, suggesting that a lower number of neural precursors will migrate to hippocampal cortices postnatally. Thus, less neurons (or glial cells) differentiate and integrate into functional circuits. Similarly, the lower density of NeuN + cells observed in the

TABLE 2 | Summary of the best linear mixed effect models for each variable, selected by cAIC, explaining biological variables of *Lepidochelys olivacea in situ* vs. *ex situ* hatchlings.

Best model	β ± SE	d.f.	*F*	*p*	ES	R^2
Dorsal ventricular zone-cell proliferation (# BrdU + cells/area)						0.33
Intercept	483.05 ± 56.13			<0.001		
in situ vs. *ex situ*	–244.92 ± 78.21	8.15	9.81	0.014	2.63	
*Medial ventricular zone-cell proliferation (# BrdU + cells/area)						0.28
Intercept	991.31 ± 144.94			<0.001		
in situ vs. *ex situ*	–534.39 ± 204.97	7.96	6.80	0.031	1.72	
Dorsomedial cortex-neuronal integration (# NeuN + cells/area)						0.18
Intercept	2031.77 ± 43.73			<0.001		
in situ vs. *ex situ*	–145.82 ± 60.89	8.78	5.73	0.041	3.25	
Medial cortex-neuronal integration (# NeuN + cells/area)						0.37
Intercept	2062.49 ± 97.28			<0.001		
in situ vs. *ex situ*	–665.58 ± 136.35	8.57	23.83	0.001	52.46	
Ovary-cell proliferation (# BrdU + cells/area)						0.89
Intercept	3.64 ± 0.18			<0.001		
in situ vs. *ex situ*	–2.56 ± 0.26	7.61	99.31	<0.001	24.71	
Body mass (g)						0.31
Intercept	17.72 ± 0.50			<0.001		
in situ vs. *ex situ*	–1.73 ± 0.71	7.96	5.97	0.041	1.48	
Body length (mm)						0.32
Intercept	67.27 ± 0.73			<0.001		
in situ vs. *ex situ*	–3.35 ± 1.04	7.90	10.39	0.012	3.09	
Self-righting (sec)						0.26
Intercept	0.94 ± 0.20			0.003		
in situ vs. *ex situ*	1.14 ± 0.31	7.42	13.99	0.007	–6.16	

cAIC, conditional Akaike information criteria; β ± SE, regression coefficient ± standard error; d.f., degrees of freedom calculated by Welch–Satterthwaite equation; F, F ratio; p, p-value; ES, effect size calculated by Glass's Δ; R^2, marginal coefficient of determination.

medial and dorsomedial cortices of hatchlings from *ex situ* clutches could reflect deficient prenatal cell proliferation or precursor migration. The difference between *in situ* and *ex situ* conditions for NeuN + cells was higher in the medial cortex, suggesting differential cortical sensitivity to early non-optimal conditions, as observed in mammals (Alkadhi, 2019). These findings, together with our prior work in male hatchlings (Herrera-Vargas et al., 2017), suggest that *ex situ* incubation alters neurogenic events during critical prenatal and early postnatal windows. The functional relevance of our findings for sea turtle cognitive and behavioral performance in the short and long-term are still unknown. However, recent evidence in lizards supports prior studies in mammals and birds, showing that a disturbance in neurogenesis during development could impair spatial memory and migration either during early life or adulthood (Amiel et al., 2017; Dayananda and Webb, 2017). Interestingly, these studies have related non-optimal incubation temperatures with decreased hippocampal neurogenesis in lizards (Amiel et al., 2017; Dayananda and Webb, 2017). Herein, nest temperature, moisture, and substrate composition differentiate *in situ* vs. *ex situ nests*; however, neither isolated abiotic variable was directly associated to altered neurogenesis in hatchling turtles. Follow-up studies should experimentally assess the effect of either variable separately or as a whole on hippocampal cell proliferation and neuronal integration early during ontogenesis in sea turtles.

Brain development begins at stage III (incubation day 4) in *Caretta caretta* turtles (García-Cerdá and López-Jurado, 2009). Thus, early relocation to hatcheries is not likely to account for the observed developmental alterations. However, future studies should be done to rule-out this possibility.

Ex situ Incubation Is Associated With a Lower Ovarian Cell Proliferation in *Lepidochelys olivacea* Hatchlings

The *ex situ* incubation strategy was associated with a dramatic reduction of ovarian cell proliferation in turtle hatchlings at nest emergence, evidenced by few BrdU + cells. In *L. olivacea*, gonadal development starts in the middle third of incubation (day 16 of development), when primordial germ cells accumulate at the base of gonadal ridge (Merchant-Larios et al., 1997). In this species, warm temperatures (32–33°C) promote ovary formation by stimulating the production of estradiol and aromatase from the undifferentiated gonad and adjacent tissues: the mesonephros and inter-renal glands (Wibbels et al., 1991, 1993; Ewert et al., 2004; Freedberg et al., 2006; Díaz-Hernández et al., 2015, 2017). The highest peak of ovarian cell proliferation is not known for *L. olivacea* hatchlings. However, folliculogenesis and oocyte entry into meiosis occur in the 3rd–5th post-hatching months (Merchant-Larios et al., 1989), suggesting that this peak occurs perinatally. The consequences of reduced germ

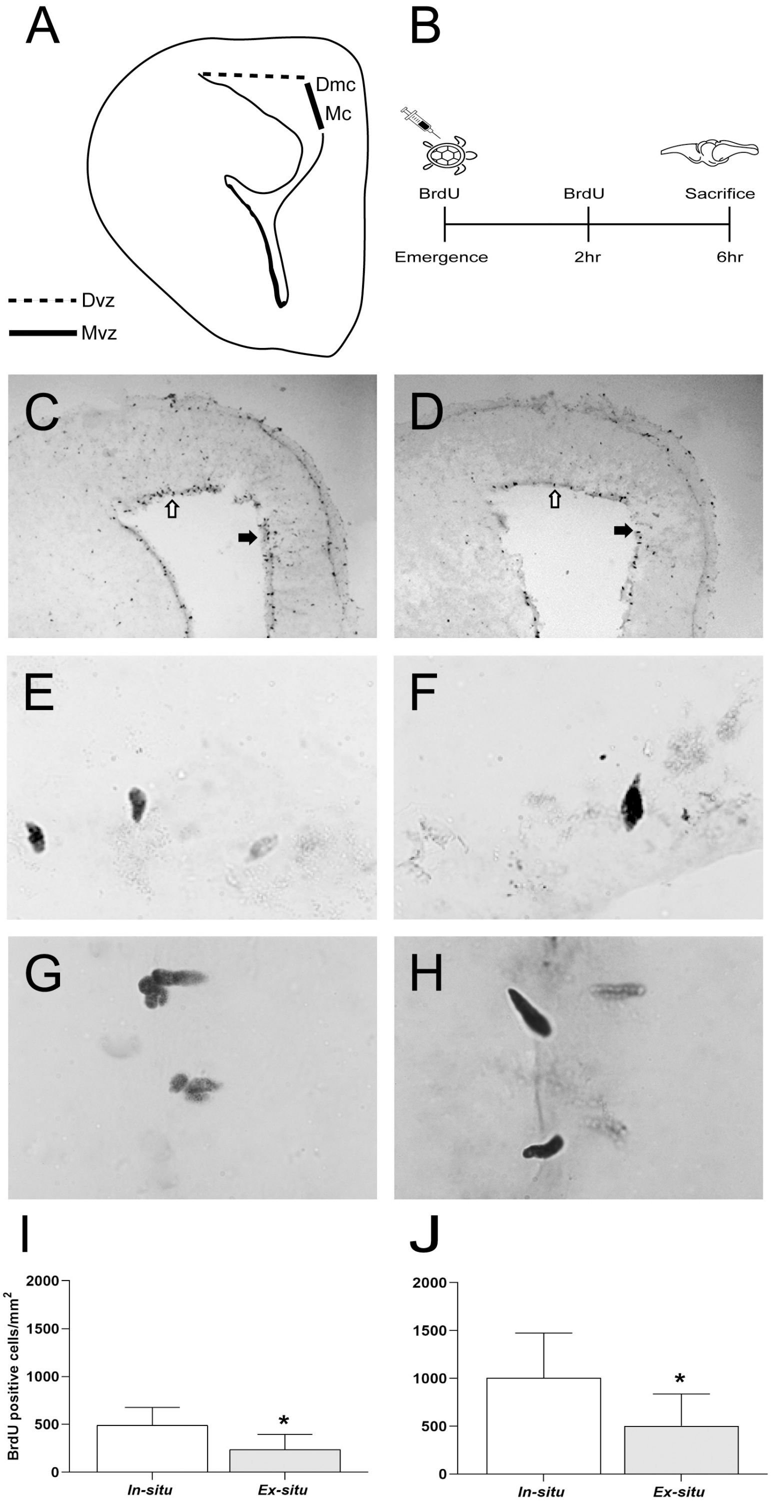

FIGURE 3 | Cell proliferation in neurogenic niches of *L. olivacea* hatchlings. **(A)** Schematic drawing of turtle brain hemi-section showing the dorsal (Dvz, dotted line) and medial ventricular zone (Mvz, continuous dark line), lining the lateral ventricle. **(B)** Timeline for 5′-bromo-2′-deoxyuridine (BrdU) pulse-chase protocol for cell proliferation analysis. **(C,D)** Representative brain sections showing BrdU + nuclei in the Dvz (open arrow) and Mvz (filled arrow) of one turtle from each condition (*in situ* and *ex situ*, respectively). **(E,F)** Representative high magnification photographs showing BrdU + nuclei in the Dvz of one turtle from each condition (*in situ* and *ex situ*, respectively). **(G,H)** Representative high magnification photographs showing BrdU + nuclei in the Mvz of one turtle from each condition (*in situ* and *ex situ*, respectively). **(I,J)** Graphs showing the density of BrdU + nuclei. *In situ* ($n = 5$), *ex situ* ($n = 5$). Mean ± SD, Type III Analysis of Variance $^*p \leq 0.05$, scale bar = 200 μm for panels **(C,D)** and 50 μm for panels **(E–H)**.

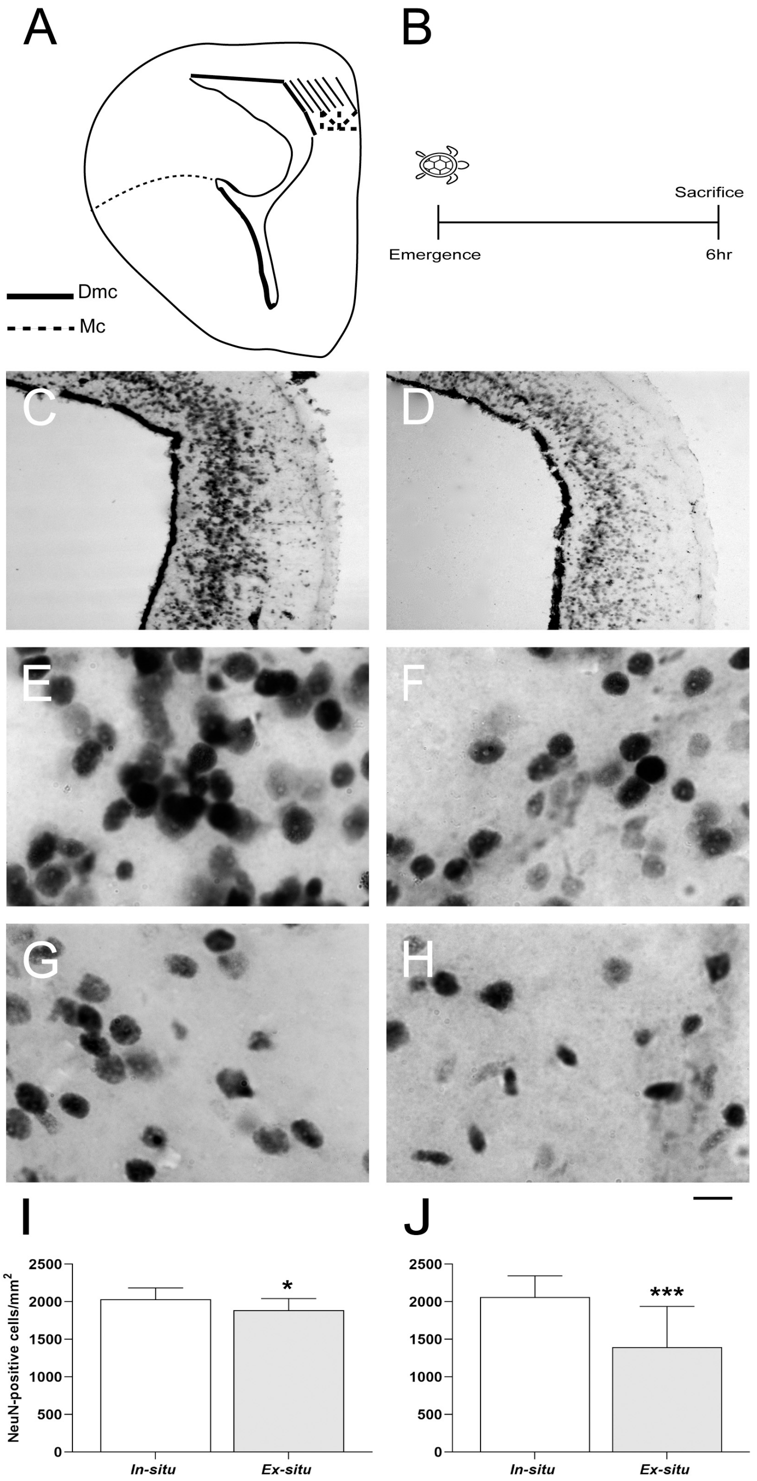

FIGURE 4 | Neuronal integration in brain areas homologous to the mammalian hippocampus in *L. olivacea* hatchlings. **(A)** Schematic drawing of turtle brain hemi-section showing the dorsomedial (Dmc, stripes) and medial (Mc, stippled) cortices. **(B)** Timeline for neuronal integration analysis. **(C,D)** Representative brain sections showing neuronal nuclear protein (NeuN) in the Dmc and Mc of one turtle from each condition (*in situ* and *ex situ*, respectively). **(E,F)** Representative high magnification photographs showing NeuN + nuclei in the Dmc of one turtle from each condition (*in situ* and *ex situ*, respectively). **(G,H)** Representative high magnification photographs showing NeuN + nuclei in the Mc of one turtle from each condition (*in situ* or *ex situ*, respectively). **(I,J)** Graphs showing the density of NeuN + nuclei. *In situ* ($n = 5$), *ex situ* ($n = 5$). Mean ± SD, Type III Analysis of Variance $^*p \leq 0.05$, $^{***}p \leq 0.001$, scale bar = 200 μm for panels **(C,D)** and 50 μm for panels **(E–H)**.

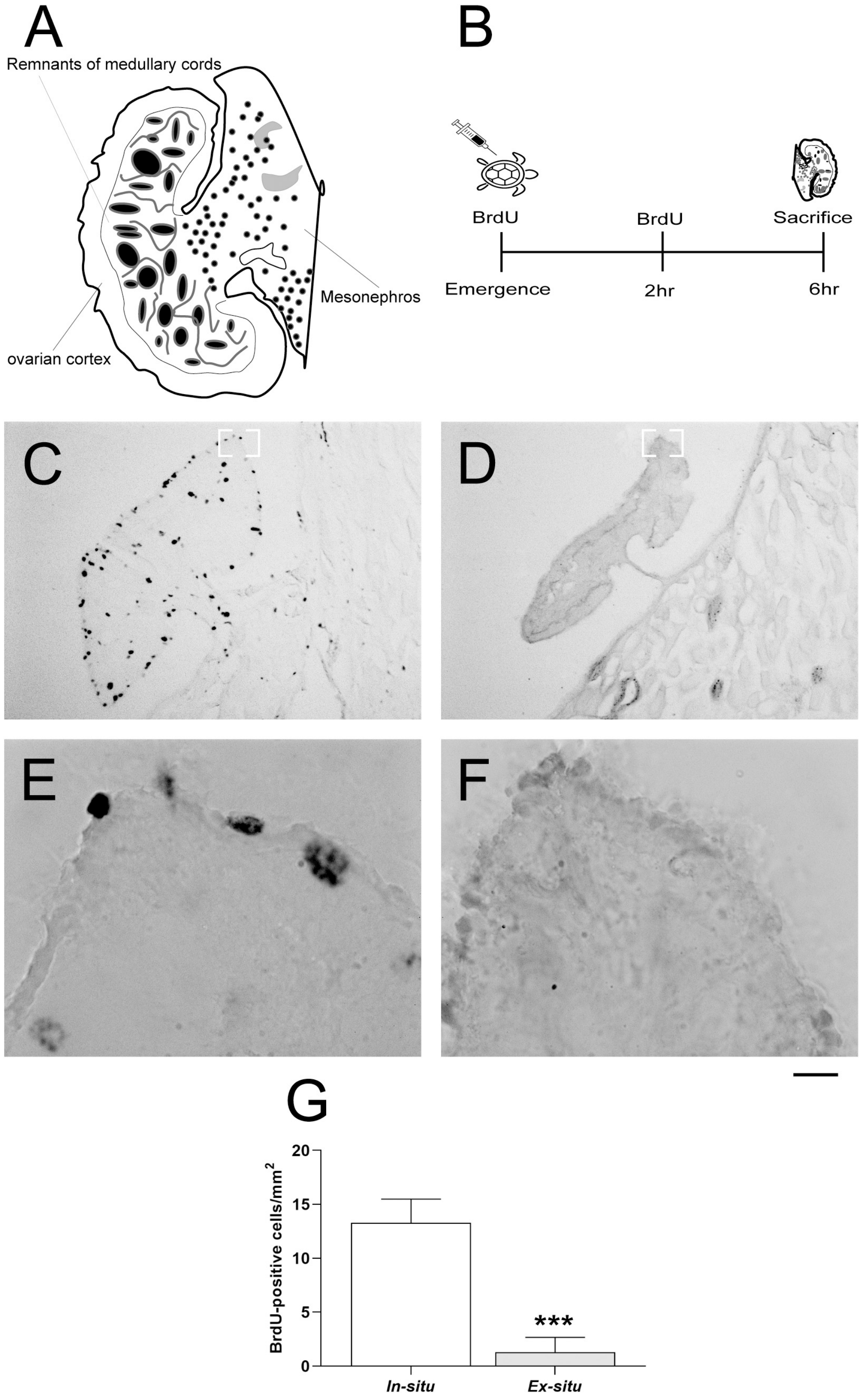

FIGURE 5 | Ovarian cell proliferation in *L. olivacea* females. **(A)** Schematic drawing of the turtle gonad-mesonephros complex. **(B)** Timeline for ovarian cell proliferation analysis. **(C,D)** Representative ovarian sections showing 5′-bromo-2′-deoxyuridine (BrdU) + nuclei in one turtle from each condition (*in situ* and *ex situ*, respectively). **(E,F)** Representative high magnification photographs showing BrdU + nuclei in the ovarian cortex of one turtle from each condition (*in situ* and *ex situ*, respectively). **(G)** Graph showing the density of ovarian BrdU + nuclei. *In situ* ($n = 5$), *ex situ* ($n = 5$). Mean ± SD, Type III Analysis of Variance ***$p \leq 0.001$, scale bar = 200 µm for panels **(C,D)** and 50 µm for panels **(E,F)**.

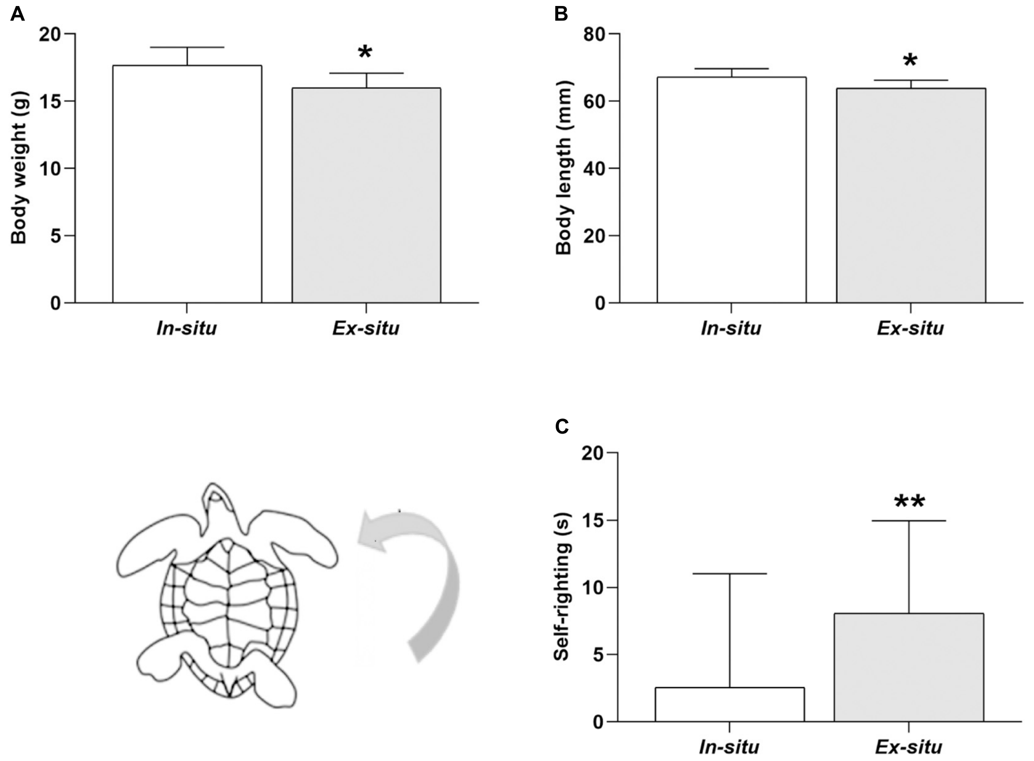

FIGURE 6 | Body size and self-righting time of *L. olivacea* hatchlings. **(A)** Graph showing body mass. **(B)** Graph of body length. **(C)** Graph of self-righting time. *In situ* (n = 5), *ex situ* (n = 5). Mean ± SD, Type III Analysis of Variance $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

cell proliferation in female juvenile or adult turtles have not been evaluated. In rodents, germ cell absence does not allow ovarian follicle development (McLaren et al., 1984) or results in follicle degeneration (Ray and Potu, 2010; Bishop et al., 2019). In mammals, reduced cell proliferation may result in delayed ovarian formation or even complete infertility (Monniaux, 2018).

In this study, ovarian weight was not formally evaluated, since each was dissected together with the mesonephros. Interestingly, qualitative histological observations of ovarian sections did not obviously evidence a reduction in cell density. Herein, the main variable explaining the diminished ovarian cell proliferation was the *ex situ* condition, which probably included the effect of incubation temperature plus moisture and substrate composition. Future studies are needed to identify the effects of *ex situ* incubation on ovarian cell density, evaluate the contribution of each abiotic variable on the ovarian phenotype and elucidate the long-term effects of poorly developed ovaries.

Ex situ Incubation Is Related to a Lower Body Size and Motor Performance at Nest Emergence

Hatchlings from *ex situ* clutches showed a lower body mass and straight carapace length than those from *in situ* nests. Linear mixed models for body mass and length suggested that the nest condition (*in situ* vs. *ex situ*) was an important factor influencing them. A direct contribution of isolated abiotic variables could not be identified. However, it is known that temperature plays a chief role determining reptile body size (Stewart et al., 2019). Although sand temperatures were above the threshold to promote female differentiation in both conditions, they likely differentially affected hatchling body size, as previously suggested (Robledo-Avila et al., 2022). Mean sand temperatures registered for *in situ* clutches were approximately 1°C below those in *ex situ* clutches, whereas mean maximum temperatures registered for both conditions showed broader ranges (35.68°C ± 0.83 SD for *in situ* nests and 37.03°C ± 1.29 SD for *ex situ* nests). Accordingly, the incubation duration was shorter by one day for *ex situ* clutches.

The mechanisms that may account for a differential temperature effect on the growth rate include a direct action on cell, tissue, or organ differentiation, as well as long-term neuroendocrine changes possibly *via* epigenetic alterations (Singh et al., 2020). Additionally, it is recognized that moisture also plays a role on body size, although the mechanisms are less well known. Modifications to gas exchange could explain the effects of moisture on development (Wallace et al., 2004). Other variables, such as nesting female size (Chatting et al., 2018), egg mass (Wallace et al., 2006), metabolic expenditure (Rusli et al., 2016; Gammon et al., 2020), or yolk absorption (Stand, 2002) also may contribute to determining body size in reptiles. A study showed an interaction between the maternal component, sand temperature, moisture, and body length in the loggerhead sea turtles. Moreover, it described differential effects of moisture on body length throughout development (i.e., a more prominent role of nest moisture on body mass during the last third of development; Tezak et al., 2020). Thus, the combined contribution of several abiotic and biotic variables could explain our results, as discussed below.

A larger body size has been consistently related with better motor performance and thus with better survival chances (Fleming et al., 2020; Martins et al., 2020). In this study, principal component 1 showed a negative relationship between body mass and length with self-righting ability, supporting prior observations. Moreover, self-righting has been associated with incubatory conditions like temperature and substrate composition, therefore constituting a good indicator of microenvironmental conditions (Stewart et al., 2019; Reboul et al., 2021). Herein, nest silt and temperature were related to increased turning time of hatchlings, in accordance with prior studies (Stewart et al., 2019; Reboul et al., 2021).

Impact of Abiotic Variables on Developmental Responses

Several nest-related abiotic variables were measured to determine their potential contribution to the observed developmental effects in *L. olivacea* hatchlings. The results showed that *in situ* clutch conditions were related to a better turtle phenotype (i.e., greater body size, higher neurogenesis, increased ovariogenesis, and lesser time to self-righting). *In situ* nests showed lower temperatures, were located higher on the beach and in coarse sand with lower silt levels, while *ex situ* clutches showed the opposite. Accordingly, sand composition, temperature, and moisture were important for differences between *in situ* and *ex situ* clutches. However, a differential contribution of each abiotic variable, in isolation, to the developmental traits could not be identified. One possible explanation is that emergent properties of microenvironment-associated abiotic variables affect the turtle hatchling development. Recent reports support this idea (Tezak et al., 2020; Tanabe et al., 2021).

Interestingly, although *in situ* nests showed lower temperatures than *ex situ* nests, the average temperatures for the incubation period, as well the mean maximum temperatures for both conditions were above the thermal tolerance reported for *L. olivacea* embryos (Valverde et al., 2010; Maulany et al., 2012). Temperature is a chief parameter that determines brain, gonadal and motor system development (Reece et al., 2002; Amiel and Shine, 2012; Paredes et al., 2016; Amiel et al., 2017; Fleming et al., 2020), as well embryo survival inside the nest (Robledo-Avila et al., 2022). Thus, it likely also plays a major role in explaining the observed phenotypes. However, its direct contribution could not be determined.

Ex situ clutches showed a higher silt proportion, which was negatively associated with hatchling development. Previous studies have shown that successful embryo development occurs in sandy substrates (grain diameter: 0.063–2 mm), whereas substrates with a high silt content (grain size < 0.063) cause mortality and diminished egg weight, reduced hatchling mass and size, as well as lower fitness (Sarmiento-Ramírez et al., 2014; Marco et al., 2017). The proportion of silt observed in both incubation conditions was below the value described as detrimental for embryo development (0.02; Abella-Pérez, 2011). Thus, although its effects on neurogenesis, ovariogenesis, body size, and motor abilities should not be ruled out, silt is unlikely to completely explain the observed phenotypes. The proportion of silt may affect moisture, gas exchange and/or microbial load (Marco et al., 2017).

Alternatively, the scarce contribution of sediment size, temperature, and moisture to completely explain the observed phenotypes could result from procedural details. Sand temperature was recorded before the thermosensitive period began (developmental day 11), until turtle emergence to avoid disturbing egg development. This interval includes the peak of hippocampal neurogenesis and the critical window for sex determination. Surface sand (10 cm deep) was sampled for moisture and particle size measurements right after turtles emerged. Future studies should record the temperature inside the nest during the entire incubation period and collect sand surrounding the eggs. This should verify the microenvironmental contribution to the effects of clutch relocation on development of sea turtle hatchlings. Moreover, future experiments should measure temperature inside the nest to consider the effect of metabolic heating on the observed phenotypes.

It must be noted that the *ex situ* incubation strategy, *per se* likely did not directly affect hatchling development. However, the combined effect of the incubation microenvironment was probably directly to blame for the phenotypic effects. This idea is supported by our prior study on the action of *ex situ* nests on the configuration of the immune system (Robledo-Avila et al., 2022).

Besides the measured abiotic factors, other variables such as micro-biological parameters (Patino-Martínez et al., 2012) may explain the developmental changes. Recent split-clutch designs have highlighted the maternal contribution to hatchling body size and self-righting response (Kobayashi et al., 2020; Tezak et al., 2020). This study did not consider the maternal component to privilege normal conditions (i.e., leaving natural nests undisturbed). However, parental origin, egg movement, and reburial should be studied to identify the factors that determine the altered phenotypes. These studies will result in recommendations for hatchery management to maximize the developmental potential of sea turtles.

CONCLUSION

Ex situ protection in hatcheries is undoubtedly the only choice if *in situ* incubation is not viable. However, our results suggest that this strategy can be associated with negative effects on sea turtle phenotypes. This include reduced neurogenesis, ovariogenesis, and motor performance during critical windows of development. Our results suggest a synergic effect of environmental variables on the observed phenotypes, but it was not possible to identify a differential contribution of each factor alone. More research should be done on the impact of *ex situ* incubation on cognitive, behavioral, and reproductive performance of juvenile or adult sea turtles experimentally manipulating abiotic variables.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Secretaría de Medio Ambiente y Recursos Naturales SGPA/DGVS/10395/17.

AUTHOR CONTRIBUTIONS

NU-D and CA: methodology and investigation. BP-F: formal analysis, writing – original draft, review, and editing. HN: formal analysis and writing – review and editing. LL-T and CM: methodology and formal analysis. NL and LT: visualization and supervision. MH-V: methodology. AF-F: conceptualization and funding acquisition. EM-H: conceptualization, data curation, writing – original draft, review, and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.850612/full#supplementary-material

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Hatching Failure in Wood Turtles (*Glyptemys insculpta*) Is Linked to Maternal Identity

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Wood turtles (*Glyptemys insculpta*) have been suffering range-wide population declines since the 1900s. Most monitoring efforts of these turtles involve population surveys to assess population size and viability but relatively few investigate rates of reproductive success. We collected four consecutive years (2013–2016) of wood turtle nesting data at a nesting site in northwestern New Jersey; population-level hatching success was unusually low. Furthermore, annual, intra-individual hatch rates and comparisons between natural and artificial incubation revealed that approximately half of all females usually produced clutches with low (<50%) hatch rates, regardless of incubation conditions. In contrast, the annual hatch rates of other females were either consistently high (>50%) or highly variable, ranging from 0 to 100%. Thus, some adult females are potentially making much larger contributions to the next generation than others. A repeatability analysis suggested that approximately 60% of the hatch rate variability observed in this population can be attributed to maternal identity. The remaining 40% may be attributed to the random environmental factors that are often theorized to be potential reasons for reduced hatch rates in turtle populations (e.g., unsuitable incubation conditions, flooding, desiccation, egg infertility, egg damage due to improper handling by researchers, root and insect predation, and microbial infection). The ultimate causes of this population's hatching success variability are uncertain, but maternally-linked hatching failure in turtle populations could be associated with inbreeding, infertility, senescence, inadequate maternal diets, or environmental contamination. This study indicates that commonly suggested hypotheses for hatching failure, such as unsuitable incubation conditions or infertility, are unlikely to explain all of the hatch rate variability in some turtle populations. This study also reveals a cryptic conservation implication for vulnerable turtle populations: that the presence of many nesting females and nests does not necessarily assure high or even sustainable reproductive rates. When coupled with the high rates of nest predation and low juvenile survival rates that are common in most turtle populations, the exceedingly low hatch rates observed in this population suggest that recruitment in some turtle populations could be severely hindered even when nests are protected in the field or incubated in laboratory settings.

Keywords: reproductive ecology, hatching success, multi-year turtle nesting study, repeatability, egg incompetence, maternal effects, egg infertility, ecotoxicology

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INTRODUCTION

In oviparous reptiles without parental care, the causes of nest failure and low hatching success are usually attributed to extrinsic (i.e., environmental) and/or intrinsic (i.e., genetic and non-genetic parental) factors (Bell et al., 2003; Rafferty et al., 2011; Noel et al., 2012). Extrinsic factors such as predation (e.g., Fowler, 1979; Congdon et al., 1983; Leslie et al., 1996; Kolbe and Janzen, 2002; Feinberg and Burke, 2003; Horne et al., 2003) and inundation by rising waters (e.g., Plummer, 1976; Leslie et al., 1996; Standing et al., 1999; Horne et al., 2003; Doody et al., 2004; Spradling et al., 2010; Pike et al., 2015) are major causes of visible nest failure and reduced hatching success in wild turtle populations. In studies involving protected or artificially-incubated nests, less conspicuous extrinsic factors, such as unfavorable thermal and hydric conditions, are often recognized as the primary reasons for reduced hatch rates (e.g., Packard et al., 1987; Spotila et al., 1994; Wilson, 1998; Standing et al., 1999; Congdon et al., 2000; Demuth, 2001). Even less perceptible than unfavorable incubation conditions are the various intrinsic reasons for nest failure and low hatching success; these include egg infertility (Blanck and Sawyer, 1981; Whitmore and Dutton, 1985; Wyneken et al., 1988), developmental failures attributed to maternal effects (i.e., non-genetic maternal influences on offspring phenotype; Wolf and Wade, 2009; Rafferty et al., 2011), and inbreeding depression (Ennen et al., 2010; Noel et al., 2012). Such factors are often overlooked, improperly diagnosed, and, for these reasons, poorly understood in wild reptile populations (Ewert, 1979).

Determining the true causes of hatching failure is vital to understanding reptile life histories and the development of conservation and management strategies for rare species, but making such determinations is difficult when the causes of hatching failure cannot be clearly linked to obvious extrinsic factors (e.g., predation, fungal infection, flooding, desiccation, unfavorable incubation temperatures, etc.). Intrinsic factors are almost always difficult to implicate as drivers of low hatch rates, especially in wild populations, but studying the inter- and intra-individual hatch rate variation within a population can provide insight into why some nests fail while others succeed (Bell et al., 2003; Wallace et al., 2007; Rafferty et al., 2011). To determine whether the reasons for hatching failure are primarily extrinsic or intrinsic, environmental/incubation conditions of nests and the annual hatch rates of multiple individual females can be investigated through multiple nesting seasons. Additionally, estimates of hatch rate repeatability can be compared among populations and used to formulate testable hypotheses for low hatch rates.

While overall, population-level hatch rates are often reported for multi-year turtle nesting studies, the variation among and within individuals is usually disregarded or simply not known (e.g., Congdon et al., 1983; Christens and Bider, 1987; Bobyn and Brooks, 1994; Standing et al., 1999; Horne et al., 2003; Litzgus and Mousseau, 2006; Walde et al., 2007; but see Buhlmann and Osborn, 2011). For any quantitative parameter (e.g., clutch size, hatching success, body mass, etc.), the total amount of variation that exists in a population is the sum of the parameter's inter- and intra-individual variation within the population. The relative amount of inter-individual variation alone, is known as repeatability (Wolak et al., 2012). Estimates of repeatability are useful for assessing the reliability of repeated measurements on more than one individual, as well as the consistency of individual performance seen in a group of individuals after multiple observations (Lessells and Boag, 1987). Additionally, repeatability estimates can be used to differentiate between the effects of individual identity and random environmental factors on quantitative parameters such as clutch size and hatch rates (Van Noordijk et al., 1980). Using nesting turtles as an example, higher repeatability estimates (i.e., closer to 1.0) indicate that more of the observed variation in hatching success is attributable to maternal identity, while lower repeatability estimates (i.e., closer to 0) indicate that more of the observed variation is attributable to random environmental factors that are independent of the mother. Because repeatability tests estimate a parameter's intra- and inter-individual variation, estimates of repeatability can only be obtained from repeated measurements on multiple individuals of known identity (Wolak et al., 2012). Consequently, obtaining repeatability estimates that pertain to the reproductive variability of turtle populations can be challenging because this requires collecting repetitive nesting data on the same individuals over multiple nesting runs or nesting seasons.

Wood turtles (*Glyptemys insculpta*) offer opportunities for obtaining hatch rate repeatability estimates and studying the causes of nest failure and low hatching success. Adults of this species are easily radio-tracked and females in some populations exhibit strong nesting site fidelity for easily monitored nesting sites that attract many turtles each year; therefore, several consecutive years of nesting data can be collected for individual females and both intra- and inter-individual variations in hatch rates can be observed.

Wood turtles currently face numerous conservation concerns; therefore, studies gathering critical details on wood turtle reproduction can inform strategies for their conservation and management. van Dijk and Harding (2013) estimated that wood turtles have experienced a range-wide population decline of more than 50% in the last century, warranting the current IUCN listing of "Endangered." Wood turtles have a geographic range coinciding with the highly developed northeastern region of the U.S., the upper Midwest U.S., and neighboring regions in Canada (Ernst and Lovich, 2009) and a unique habitat usage cycle that makes them susceptible to anthropogenic population decline and extirpation. From late fall to early spring, wood turtles use rivers and streams as breeding habitats and refuges from freezing temperatures. During warmer months, they disperse away from riparian corridors to a variety of habitats, including upland fields and forests and low-lying fens and meadows. These diverse habitat preferences and unique habitat-usage cycles increase the likelihood that wood turtle populations will be negatively impacted by habitat degradation, fragmentation, and destruction (Jones and Willey, 2015); automobiles (Gibbs and Shriver, 2002; Steen et al., 2006; *pers. obs.*) and agricultural machinery (Saumure et al., 2006; Tingley et al., 2009; *pers. obs.*); subsidized predators (Brooks et al., 1992; Buhlmann and Osborn, 2011); and illegal collection for the live animal trade (Garber and Burger, 1995; McCollough, 1997; Levell, 2000). In addition to anthropogenic pressures, most wood turtle populations experience exceptionally low recruitment due to low reproductive frequencies (i.e., on average females typically lay one or less than one clutch per year; Jones and Willey, 2015), lack of suitable nesting habitats (Buech et al., 1997), nest predation (Brooks et al., 1992; Buhlmann and Osborn, 2011), and high juvenile mortality (Paterson et al., 2012, 2014; Dragon, 2014). A high degree of inter-population variation in overall hatching success has also been documented in wood turtles, with some populations experiencing much lower hatch rates than others (**Table 1**).

In this study, our goal was to investigate the annual inter- and intra-individual hatch rate variation in wood turtle populations with different degrees of hatching success and to determine whether the hatch rates observed in these populations are primarily influenced by extrinsic or intrinsic factors. If annual hatch rates of individual females yielded relatively low repeatability estimates and improved under carefully monitored and controlled, artificial incubation conditions, then the hatch rates we observed were primarily influenced by extrinsic factors such as unfavorable thermal and hydric conditions. Alternatively, if annual hatch rates of individual females yielded relatively high repeatability estimates and remained relatively constant between natural and artificial incubation conditions, then the hatch rates we observed were primarily influenced by intrinsic factors and warrant further investigation.

MATERIALS AND METHODS

Nesting Site

We conducted most of this study at a turtle nesting site in a relatively undisturbed area of northern New Jersey (NNJ), with no known history of unusual chemical use or pollution. To protect this population from illegal collection, we refrain from disclosing this site's exact location and descriptive details of its surroundings. During the mid-1900s, the site was deforested and excavated for cobble, leaving a large patch of exposed, glacial till-laden sediment that has attracted nesting wood turtles, common snapping turtles (*Chelydra serpentina*) and painted turtles (*Chrysemys picta*) since at least the late 1990s. Prior to the 2013 nesting season, we remediated the site to facilitate turtle nesting and improve our ability to observe turtles from a distance. First, we tilled the entire nesting site to soften the ground and remove vegetation. Then, we excavated two 80 m^2 rectangular patches of the site's original, cobble-laden sediment and replaced them with sand from a nearby, eroding hillside. To minimize site disturbance, we left much of the site's original sediment in place, even though the cobble within it frequently precludes females from digging suitable nest cavities. In subsequent years, we repeated pre-nesting season tilling operations to delay succession and keep soft sediments exposed.

Nesting Site Monitoring

During the nesting seasons (i.e., 20 May–20 June) from 2013 to 2016, we monitored the nesting area for female wood turtles every morning from 6:00 to 12:00 and every evening from 16:00 to 21:00. We observed the area from a distance with binoculars or from a hunting blind to avoid disturbing nesting females. We often continued nesting site monitoring beyond these time intervals until all wood turtles either finished nesting or retreated into the vegetation surrounding the site. We retrieved all females immediately after staging or nesting behavior concluded to verify identity (i.e., notch code) and, if oviposition occurred, pinpoint nest locations.

This nesting study was part of a long-term mark-recapture study of the surrounding area's wood turtle population that we have been conducting since 1998. Throughout the study, we gave unique notch codes to most of the adult population and radio-tracked more than 20 adult turtles. Furthermore, an unpublished 2002 study conducted at the same nesting area revealed an exceedingly low overall hatch rate of 0% for six nests that were naturally-incubated *in situ* (i.e., where oviposited) and protected from predation with hardware cloth caging (Oorthuys, unpublished data; **Table 1**).

Egg Handling, Incubation, and Hatching

In 2013 and 2014, we incubated all nests *in situ* (i.e., where oviposited) under natural conditions and protected them from predation with hardware cloth caging; this type of predator excluder is unlikely to affect nest temperatures (Riley and Litzgus, 2013; Burke et al., unpublished data). Immediately after oviposition and prior to installing predator excluders, we carefully excavated all nests by hand, recorded clutch sizes, and promptly returned all eggs to their nest cavities. To ensure that all nests received similar treatment and to limit the possibility of embryonic mortality due to careless handling, we took great care when excavating and handling each egg; throughout the entire study, no eggs were handled or moved 6 h past oviposition, and no eggs were jarred, shaken, inverted, dropped, or visibly damaged in any way. Before reburying each nest, we programmed temperature loggers (Thermochron® iButtons) for hourly readings, sealed them in duct tape to waterproof them, and placed them among the eggs of each nest to record natural incubation temperatures.

During the 2013 and 2014 hatching seasons (1–20 August), we checked nest cages for hatchlings at least three times daily in the morning, afternoon, and evening to document hatchling emergence and prevent hatchling mortality from intense sun exposure. Since the objectives of this study were primarily concerned with hatch rates, recording hatching success (i.e., the percentage of hatchlings that hatched from each nest) took precedence over recording emergence success (i.e., the percentage of hatchlings that emerged from each nest). To obtain accurate records of hatching success, we excavated nests one day after hatchling emergence to recover unhatched eggs as well as any live hatchlings that failed to emerge.

In 2015 and 2016, we carefully excavated all nests by hand immediately after oviposition, recorded clutch sizes, and transported all clutches to a nearby laboratory where we incubated them at a constant temperature of 30°C. To prepare clutches for incubation and for transport from the nesting site to the laboratory, we equidistantly spaced the eggs of each clutch

TABLE 1 | Annual overall hatching success of naturally and artificially-incubated wood turtle (*Glyptemys insculpta*) nests in 10 nesting studies (excluding depredated nests). Overall hatching success and sums of nests, eggs, and hatchlings are shown in bold for studies that reported multi-year data.

Source and location	Year	Incubation	Nests	Eggs	Hatchlings	Hatching Success (%)
Duchak and Burke, 2022: New Jersey (present NNJ study)	2013	Natural	14	124	34	27.42
	2014	Natural	17	140	61	43.57
	2015	Artificial	21	173	60	34.68
	2016	Artificial	16	123	39	31.71
			68	**560**	**194**	**34.64**
Parren, 2013: Vermont	1998	Natural	1	8	1	12.5
	2003	Natural	3*	24	19	79.17
			4	**32**	**20**	**62.5**
Buhlmann and Osborn, 2011: New Jersey	2007	Natural	3	31	25	80.65
	2008	Natural	3	19	11	57.89
	2009	Natural	8	85	62	72.94
	2010	Natural	6	65	53	81.54
			20	**200**	**151**	**75.5**
Castellano, 2007: New Jersey	2001	Natural	15	113	71	62.83
	2002	Natural	16	115	59	51.3
			31	**228**	**130**	**57.02**
Walde et al., 2007: Quebec	1996	Natural	27	253	148	58.5
	1997	Natural	30	337	175	51.93
			57	**590**	**323**	**54.75**
Tuttle and Carroll, 1997: New Hampshire	1993	Natural	9	70	54	77.14
Farrell and Graham, 1991: New Jersey	NA	Artificial	NA*	161	119	73.91
Akre (unpublished data): Virginia (GWNF study)	2010	Natural	18	193	126	65.28
	2011	Natural	22	212	126	59.43
	2012	Natural	41	428	278	64.95
	2013	Natural	17	184	106	57.61
	2014	Natural	20	212	83	39.15
			118	**1229**	**719**	**58.5**
Hernandez and Hernandez (unpublished data): New Jersey	2009	Natural	5	39	17	43.58
	2010	Natural	3	22	4	18.18
	2011	Natural	4	32	3	9.375
	2012	Natural	3	25	8	32.00
	2013	Natural	1	8	8	100
			16	**126**	**40**	**31.75**
Oorthuys (unpublished data): New Jersey (past NNJ study; same location as present NNJ study)	2002	Natural	6	44	0	0

**Nests of captive animals in outdoor pens.*

on beds of moistened vermiculite within separate "incubation containers" (i.e., plastic food storage containers). As in the previous years of this study (2013 and 2014), we took great care when excavating and handling all eggs. We also took great care when transporting all clutches via motor vehicle; transportation time never exceeded 15 min, and we held all incubation containers aloft to reduce vibrations during transport.

The care we took in excavating, handling, and transporting eggs may have been excessive as we always finished moving eggs within 6 h of oviposition. Although the sensitivity of turtle eggs to mechanical disturbance is poorly known for most species (Booth, 2004), it is generally accepted that inversion during the first few hours after oviposition and even during the first 12 h of incubation is unlikely to negatively affect embryonic survival in most species (Deeming, 1991). Furthermore, after inverting a total of 22 eggs belonging to four different species during the second to fourth day of incubation, Ewert (1979) reported both hatching success and failure in unspecified numbers of eggs, thus demonstrating that egg inversions do not always result in embryonic mortality, even when inversions occur days after oviposition. Additionally, Ewert (1979) also mentioned that eggs are rather tolerant of the vibrations they experience in natural nests positioned along active railroad tracks and during transportation in vehicles on rough roads. Lastly, a study of painted turtle (*C. picta*) nests found no difference in hatching success between eggs that were left completely undisturbed in natural nests throughout incubation and eggs that were excavated, transported via motor vehicle for an hour, cleaned, weighed, measured, and reburied within 24 h of oviposition (Samson et al., 2007). Despite all of the information suggesting that freshly laid eggs are relatively tolerant of moderate mechanical disturbances, we still handled all eggs with the utmost level of care to ensure that improper handling did not affect hatching success.

Two weeks after collecting the final clutches in 2015 and 2016, we visually examined all eggs for signs of "chalking" (i.e., eggshell whitening). To provide an initial conservative estimate of fertility, we divided the number of eggs that showed evidence of chalking by the number of eggs that showed no evidence of chalking. Chalking in turtle eggs has long been regarded as direct evidence of fertilization and subsequent embryonic development (Ewert, 1979, 1985; Deeming, 1991); however, the absence of chalking is an unreliable indicator of egg infertility because external examinations of eggs cannot distinguish between true infertility and early embryonic mortality (Croyle et al., 2016). Consequently, while external examinations of eggs may confirm fertility in eggs that show clear signs of development, they cannot confirm infertility in eggs that show no evidence of development.

We determined hatching success for each nest by dividing the number of pipped live hatchlings by the clutch size. Similarly, we determined overall hatching success for each year by dividing the total number of pipped live hatchlings counted in all nests during a given year by the total number of eggs counted in all nests during the same year. We released all hatchlings at their respective nest sites immediately after hatching/emergence (naturally-incubated nests from 2013 to 2014) or as soon as they absorbed their external yolk sacs (lab-incubated nests from 2015 to 2016).

Comparison Data

We obtained hatching success data from a multi-year wood turtle nesting study conducted within George Washington National Forest (GWNF) in northern Virginia. GWNF is the southern limit of the wood turtle's range and is approximately 400 km southwest of the NNJ site. The GWNF dataset contained hatching success data from 118 nests laid by 55 females over five consecutive nesting seasons (2010–2014). All nests included in the GWNF dataset were incubated *in situ* under natural conditions and either unpredated or protected from predation with hardware cloth caging (Akre, unpublished data).

Data Analysis

To determine whether the NNJ hatch rates were different among years, we used a Kruskal Wallis test to compare the individual hatch rates of all nests from 2013 to 2016. To determine whether the NNJ hatch rates of eggs that were naturally-incubated *in situ* were different from the NNJ hatch rates of eggs that were artificially-incubated in the laboratory, we combined the hatch rates from 2013 to 2014 and compared them with the combined hatch rates of 2015 and 2016 using a two-tailed Mann–Whitney *U* test. We conducted this analysis once for all nests, then again including only "repeat nesters" (i.e., females who had more than one annual nest represented in the data) to determine whether the presence of multiple "single nesters" (i.e., females who only had one nest represented in the data) during some years influenced overall annual hatch rates.

To determine whether hatch rates were different between the NNJ and GWNF populations, we converted the overall hatch rates of all repeat nesters into counts in four categories: 0–24.99, 25–49.99, 50–74.99, and 75–100%. We then ran a Freeman-Halton extension of the Fisher's exact probability test for a two-rows by four-columns contingency table comparing the overall hatch rates of repeat nesters between the NNJ and GWNF populations. To determine whether the overall NNJ hatch rates of repeat nesters were significantly lower than the overall GWNF hatch rates of repeat nesters, we used a two-tailed Mann–Whitney *U* test.

To determine the proportion of reproductive variability that is due to inter-individual variation within each population, we estimated hatching success repeatability for all repeat nesters in the NNJ and GWNF populations with the following equations from Wolak et al. (2012):

$$s_A{}^2 = \left(MS_{groups} - MS_{error}\right)/n \tag{1}$$

where MS_{groups} = mean sum of squares for groups in a random effects (Model 2) ANOVA and MS_{error} = mean sum of squares for error in a random effects (Model 2) ANOVA.

$$\text{Repeatability} = \left(s_A{}^2\right)/(sA^2 + MS_{error}) \tag{2}$$

We obtained repeatability estimates and their associated 95% confidence intervals using the R package "ICC" developed by Wolak et al. (2012).

To investigate the possible effects of body size on hatching success in the NNJ population, we conducted a linear regression for each repeat nester's straight line plastron length (SLPL) versus each repeat nester's overall hatch rate. We also conducted linear regressions for each repeat nester's SLPL and each repeat nester's mean and maximum clutch sizes to determine whether body size was correlated with clutch size in the NNJ population. Lastly, to investigate the possible effects of clutch size on hatching success in the NNJ and GWNF populations, we conducted linear regressions for each repeat nester's mean and maximum clutch sizes versus each repeat nester's overall hatch rate.

RESULTS

From 2013 to 2016, we recorded the hatch rates of 68 nests laid by 27 females at the NNJ site. The annual overall hatching success rates for 2013, 2014, 2015, and 2016 were 27.42, 43.57, 34.68, and 31.71%, respectively, and the hatching success rate for all 4 years combined was 34.64% (**Table 1**). We found that 70.92% of all NNJ eggs collected for incubation in 2015 and 2016 showed evidence of chalking. At the GWNF site, the annual, overall hatching success rates for 2010, 2011, 2012, 2013, and 2014 were 65.28, 59.43, 64.95, 57.61, and 39.15%, respectively, the overall hatching success rate for all 5 years combined was 58.5% (Akre, unpublished data; **Table 1**).

We found no significant differences among the 2013, 2014, 2015, and 2016 NNJ hatch rates (Kruskal Wallis test: $H = 1.56$, DF = 3, $P = 0.716$). Additionally, we found no significant difference between the hatch rates of all nests that were naturally-incubated *in situ* during 2013 and 2014 (N females = 19, N nests = 31, mean = 0.32) and the hatch rates of all nests that were artificially-incubated in the laboratory during 2015 and 2016 (N females = 25, N nests = 37, mean = 0.33; two-tailed Mann–Whitney U test: $U_A = 563.5$, $Z = 0.12$, $P = 0.9045$). Similarly, we found no significant difference between the "natural" (N females = 17, N nests = 29, mean = 0.34) and "artificial" hatch rates (N females = 18, N nests = 31, mean = 0.38) of all repeat nests at the NNJ site (two-tailed Mann–Whitney U test: $U_A = 411.5$, $Z = 0.35$, $P = 0.7263$).

Throughout the entire four-year NNJ study (2013–2016), 27.78% of repeat nesters failed to produce more than 1 hatchling, 38.89% produced at least 10 hatchlings, and 22.22% produced at least 20 hatchlings (**Figure 1**). Throughout the entire five-year GWNF study (2010–2014), all repeat nesters produced at least 3 hatchlings, 86.11% produced at least 10 hatchlings, and 33.33% produced at least 20 hatchlings (**Figure 2**). Fifty percent of all repeat nesters from NNJ had overall hatching success rates between 0 and 24.99%, 5.56% between 25 and 49.99%, 27.78% between 50 and 74.99%, and 16.67% between 75 and 100%. Approximately six percent of GWNF repeat nesters had overall hatching success rates between 0 and 24.99%, 27.78% between 24 and 49.99%, 38.89% between 50 and 74.99%, and 27.78% between 75 and 100%. We found that the overall NNJ hatch rates of repeat nesters (N females = 18, N nests = 59, mean = 0.37) were significantly different (Fisher's exact probability test: DF = 4, $P = 0.00466$) and lower (two-tailed Mann–Whitney U test: $U_A = 194.5$, $Z = 2.37$, $P = 0.0178$) than the overall GWNF hatch rates of repeat nesters (N females = 36, N nests = 99, mean = 0.60; **Figure 3**). Additionally, 33.33% of repeat nesters from NNJ always had annual hatch rates of 50% or greater, 22.22% had annual hatch rates that varied from less than 50 to 50% or greater, and 44.44% consistently produced hatch rates below 50%, whereas 36.11% of repeat nesters from GWNF always had annual hatch rates of 50% or greater, 58.33% had annual hatch rates that varied from less than 50 to 50% or greater, and only 5.56% consistently produced hatch rates below 50% (**Figure 4**).

Our estimates of hatching success repeatability from the NNJ and GWNF datasets were 0.580 ($N = 18$; $K = 3.268$; lower CI: 0.328; upper CI: 0.792; CIW: 0.464) and 0.056 ($N = 36$; $K = 2.771$; lower CI: −0.162; upper CI: 0.274; CIW: 0.436), respectively. The 95% confidence intervals about our estimates do not overlap, indicating that the NNJ and GWNF hatching success repeatability estimates are significantly different from each other (**Figure 5**).

Linear regression revealed no significant association between SLPL and overall hatch rates among repeat nesters in the NNJ population ($T = 0.889$, DF = 16, $R^2 = 0.217$, $P = 0.3871$); however, significant associations between SLPL and both mean ($T = 3.782$, DF = 16, $R^2 = 0.472$, $P = 0.0016$) and maximum clutch sizes ($T = 4.068$, DF = 16, $R^2 = 0.508$, $P = 0.0009$) were found among repeat nesters in the NNJ population. No significant effects of either mean ($T = 0.062$, DF = 16, $R^2 = 0$, $P = 0.9517$) or maximum clutch sizes ($T = -0.896$, DF = 16, $R^2 = 0.048$, $P = 0.3836$) were found on the overall hatch rates of repeat nesters in the NNJ population. Likewise, no significant effects of either mean ($T = -0.304$, DF = 34, $R^2 = 0.003$, $P = 0.7633$) or maximum clutch sizes ($T = -0.267$, DF = 34, $R^2 = 0.002$, $P = 0.7913$) were found on the overall hatch rates of repeat nesters in the GWNF population.

DISCUSSION

In this study, our goal was to investigate the annual inter- and intra-individual hatch rate variation in wood turtle populations with different degrees of hatching success and to determine whether the hatch rates observed in these populations are primarily influenced by extrinsic or intrinsic factors. We initially revealed potential reproductive issues at the NNJ site when we found the population's 2013 and 2014 hatch rates to be considerably lower than the hatch rates reported for seven other wood turtle populations (**Table 1**). As an initial attempt to increase hatching success and determine the cause of the low NNJ hatch rates, we artificially-incubated all NNJ clutches under identical conditions during 2015 and 2016. When extrinsic factors like predation, microbial infection, flooding, desiccation, and unfavorable incubation conditions are primarily responsible for low hatch rates in wild turtle populations, carefully-monitored and controlled, artificial incubation should improve overall hatching success rates and the hatching success rates of individual females (Noel et al., 2012); however, we found that the NNJ hatch rates of nests that were naturally-incubated *in situ* (2013–2014) were similar to the NNJ hatch rates of nests that were artificially-incubated in the laboratory (2015–2016). This indicates that unknown intrinsic factors were reducing the hatch rates of this population. Additionally, we found the overall NNJ hatch rates of repeat nesters to be significantly lower than the overall GWNF hatch rates of repeat nesters (**Table 1**), indicating that the unknown factors that reduced the reproductive potential of the NNJ population are not present in all wood turtle populations.

Our NNJ hatching success repeatability estimate indicated that approximately 60% of the observed variation in hatching success is attributed to maternal identity while the remaining 40% is attributed to random environmental factors. In contrast, our GWNF hatching success repeatability estimate was significantly lower than the NNJ estimate and indicated that approximately 5% of the observed variation in hatching success is attributed

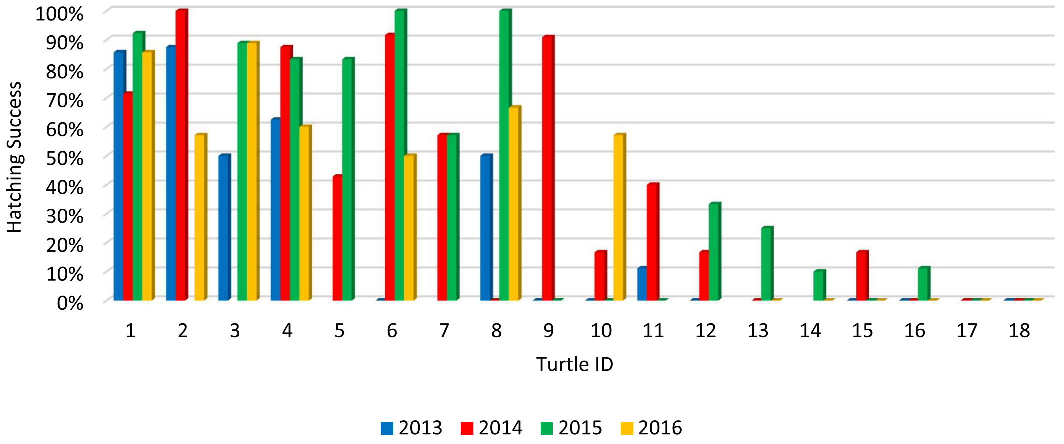

FIGURE 1 | Annual hatching success rates of all "repeat nester" female wood turtles (*Glyptemys insculpta*) at the northern New Jersey (NNJ) site from 2013 to 2016. Individual females are ranked from 1 to 18 according to their average hatching success rates.

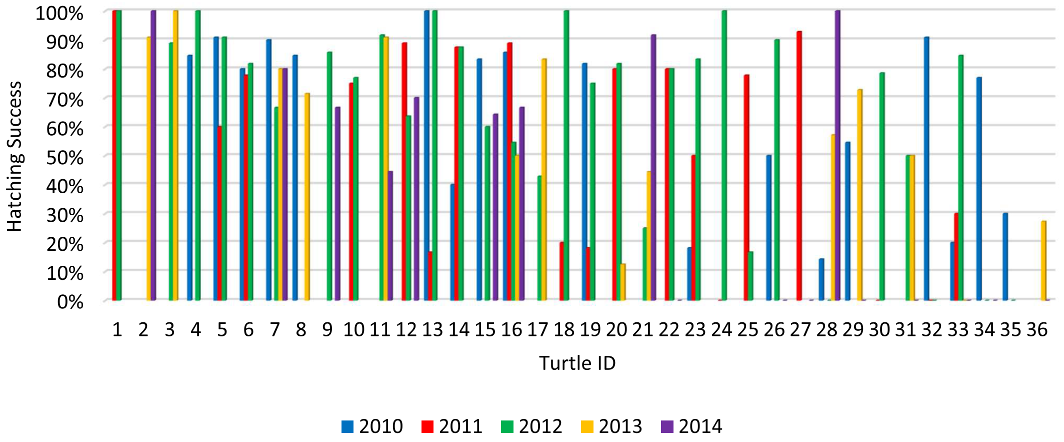

FIGURE 2 | Annual hatching success rates of all "repeat nester" female wood turtles (*Glyptemys insculpta*) at the George Washington National Forest (GWNF) site from 2010 to 2014. Individual females are ranked from 1 to 36 according to their average hatching success rates.

to maternal identity while the remaining 95% is attributed to random environmental factors. This low range repeatability estimate suggests that environmental factors influence hatching success variation more than maternal identity in GWNF (**Figure 5**). The higher NNJ hatching success repeatability estimate is certainly due to the many repeat nesters who consistently produced low hatch rates and the relatively smaller numbers of repeat nesters that produced either consistently high hatch rates or hatch rates that varied from year to year. Forty-four percent of repeat nesters in the NNJ study never had a clutch with a hatch rate of 50% or greater (i.e., "low hatch rate females"), 33.33% always had annual hatch rates of 50% or greater (i.e., "high hatch rate females"), and 22.22% had annual hatch rates that varied from less than 50 to 50% or greater (i.e., "variable hatch rate females"). In comparison, only 5.56% of repeat nesters in the GWNF study could be qualified as low hatch rate females, while the majority of repeat nesters could be qualified as either high hatch rate females (36.11%) or variable hatch rate females (58.33%; **Figure 4**). These contrasting results suggest that the reproductive capacity of the NNJ population is much more limited than that of the GWNF population and that this limitation is primarily due to a higher proportion of low hatch rate females in the NNJ population.

Similar to other wood turtle populations, we found significant associations between body size and clutch size in the NNJ population (e.g., Brooks et al., 1992; Walde et al., 2007; Jones, 2009); however, we found no significant associations between body size and hatching success. We also found no significant associations between clutch size and hatching success in either NNJ or GWNF populations. This indicates that parameters like body size and clutch size are not always reliable predictors of hatching success in turtle populations and that other factors are most likely causing maternally-linked hatching failure in the NNJ population.

It is difficult to conclude whether maternally-linked hatching failure is an abnormal feature of turtle populations because

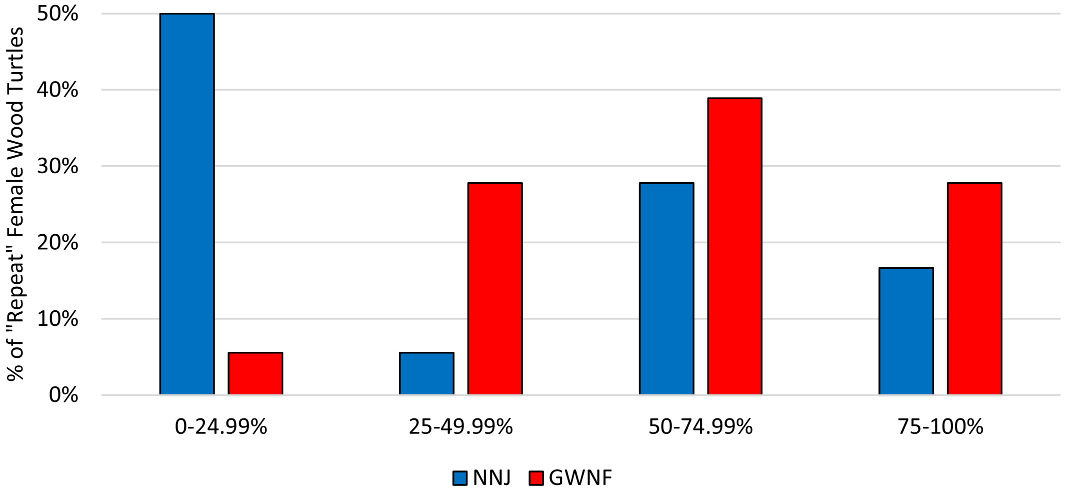

FIGURE 3 | Percentages of "repeat nester" wood turtles (*Glyptemys insculpta*) with overall hatching success rates between 0 and 24.99%, 25 and 49.99%, 50 and 74.99%, 75 and 100% in the northern New Jersey (NNJ) and George Washington National Forest (GWNF) studies. The overall NNJ hatch rates of repeat nesters (*N* females = 18, *N* nests = 59, mean = 0.37) were significantly different (Fisher's exact probability test: DF = 4, $P = 0.00466$) and lower (two-tailed Mann–Whitney *U* test: $U_A = 194.5$, $Z = 2.37$, $P = 0.0178$) than the overall GWNF hatch rates of repeat nesters (*N* females = 36, *N* nests = 99, mean = 0.60).

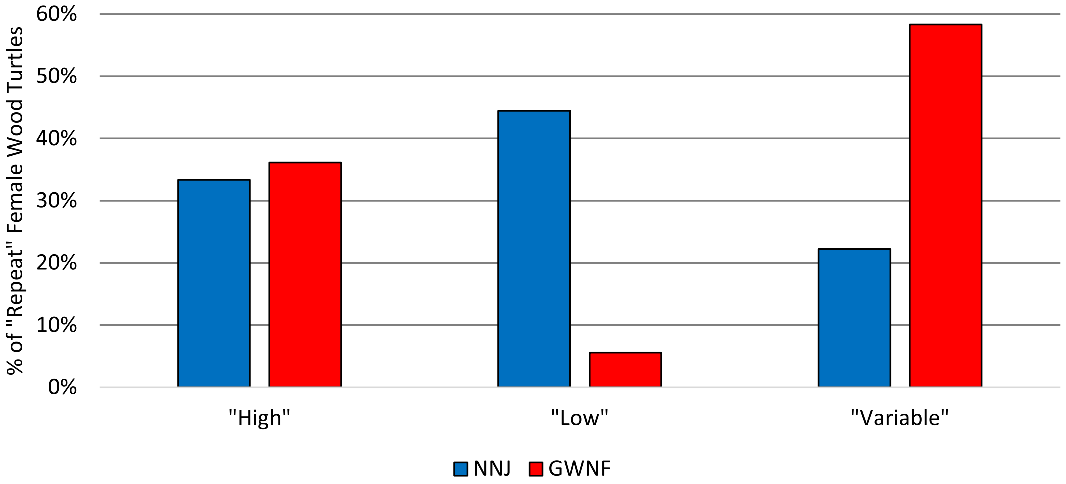

FIGURE 4 | Proportions of "high hatch rate," "low hatch rate," and "variable hatch rate," "repeat nester" wood turtles (*Glyptemys insculpta*) in the northern New Jersey (NNJ) and George Washington National Forest (GWNF) studies. "High hatch rate females" always had annual hatch rates of 50% or greater, "variable hatch rate females" had annual hatch rates that varied from less than 50 to 50% or greater, and "low hatch rate females" consistently produced hatch rates below 50%.

repeat nesting data are seldom reported or analyzed in scientific studies. Nevertheless, some studies documented maternally-linked hatching failure in turtles and crocodilians and suggested several hypotheses for its occurrence; among these are egg infertility (Heinz et al., 1991; Kuchling and Shunqing, 2015), inbreeding depression (Ennen et al., 2010), maternal senescence (Warner et al., 2016), inadequate maternal nutrition (Noble et al., 1993; Craven et al., 2008) and environmental contamination (Perrault et al., 2011).

Egg infertility is an unlikely explanation for maternally-linked hatching failure in NNJ wood turtles simply because 70.92% of all wood turtle eggs collected for incubation during 2015 and 2016 showed at least some evidence of "chalking" (i.e., eggshell whitening) and chalking in turtle eggs has long been regarded as direct evidence of fertilization and subsequent embryonic development (Ewert, 1979, 1985; Deeming, 1991). Furthermore, our long-term mark-recapture data indicate that there is no shortage of reproductive males in the NNJ population (Duchak and Burke, unpublished data). An abundance of males combined with the occurrence of female sperm storage (Figueras and Burke, 2017) and multiple paternity (Galbraith, 1991; Bouchard et al., 2016) in wood turtles make even sporadic occurrences of infertile nests especially unlikely in the NNJ and any other wild wood turtle populations of considerable size;

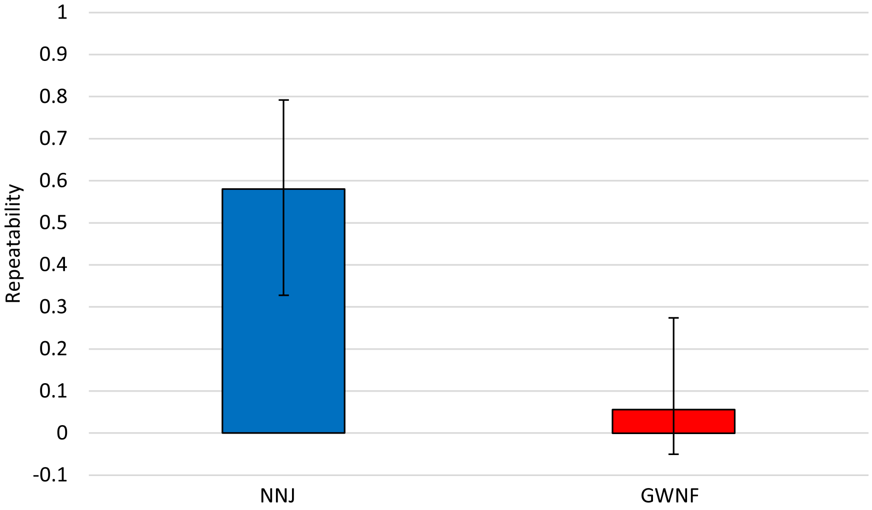

FIGURE 5 | Wood turtle (*Glyptemys insculpta*) hatching success repeatability estimates with corresponding 95% confidence intervals for the northern New Jersey (NNJ) and George Washington National Forest (GWNF) studies. Estimates of hatching success repeatability from the NNJ and GWNF datasets were 0.580 ($N = 18$; $K = 3.268$; lower CI: 0.328; upper CI: 0.792; CIW: 0.464) and 0.056 ($N = 36$; $K = 2.771$; lower CI: -0.162; upper CI: 0.274; CIW: 0.436), respectively.

unless contaminants that cause male infertility are prevalent (Heinz et al., 1991).

Maternally-linked hatching failure as a consequence of inbreeding is also unlikely at the NNJ site. Our mark-recapture efforts since 2012 have confirmed the existence of over 110 uniquely marked adult wood turtles that are not geographically isolated from neighboring wood turtle populations; relatively undisturbed stream corridors connect the NNJ population to adjacent upstream and downstream wood turtle populations. Moreover, Spradling et al. (2010) showed that an even smaller, geographically disjunct Iowa population was not inbred and actually exhibited a substantial amount of genetic diversity despite having significantly lower allelic richness and heterozygosity than a larger population in West Virginia. Additionally, Tessier et al. (2005) found high degrees of genetic variability among six Canadian wood turtle populations, some of which had experienced drastic declines in recent history. Tessier et al. (2005) and Spradling et al. (2010) theorized that declining turtle populations may be buffered from the effects of genetic drift by the presence of long-lived adults that maintain much of the population's original genetic variability when they breed with each other or individuals of younger age classes. Considering these studies and the NNJ population's size and connections to neighboring populations, the possibility of inbreeding reducing NNJ hatch rates seems implausible.

Maternal senescence is also an unlikely cause of the low NNJ hatch rates. Unlike many classes of vertebrates, female reptiles are not oocyte-limited and appear capable of reproduction until death (Kuchling, 1999; Jones, 2011); accordingly, chelonians were traditionally thought to exhibit only negligible senescence (Congdon et al., 2001, 2003; Miller, 2001). In contrast, Warner et al. (2016) showed that adult survivorship and hatching success decline with old age in painted turtles (*C. picta*); however, our long-term mark-recapture and morphometric datasets suggest that a diversity of age classes exist at the NNJ site and that some NNJ repeat nesters with consistently poor hatch rates are unlikely to be old. For example, the most unsuccessful repeat nester in the NNJ study laid four annual nests totaling 37 eggs from 2013 to 2016 but failed to produce a single hatchling (female #18; **Figure 1**). This turtle was first encountered in 1999 as a non-reproductive juvenile with a straight-line plastron length (SLPL) of 74 mm, indicating that this turtle was approximately 20 years old during the last year of the present NNJ study (2016). Twenty years is a relatively young age for a turtle species that reaches reproductive maturity during its teenage years and lives 50 years or more in the wild (Jones and Willey, 2015). Thus, it is unlikely that senescence accounts for all of the maternally-linked hatching failure observed in the NNJ population.

In contrast to egg infertility, inbreeding depression, and maternal senescence, differences in food resources and maternal diets may be a slightly more convincing hypothesis as to why some NNJ females have consistently low hatching success; however, this hypothesis may still be implausible, especially if the foraging sites of all NNJ females yield the necessary nutrients for producing healthy eggs. Turtle hatchlings receive either very limited or no parental care; therefore, maternal reproductive investment in turtles is largely represented by the allocation of dietary nutrients like lipids to the yolks of eggs (Congdon, 1989; Harms et al., 2005). Some of the fatty acid constituents of these lipids may be vital to embryonic development (Noble et al., 1993), and studies have shown associations of inadequate maternal diets with fatty acid deficiencies and reduced hatch rates in captive birds and reptiles (Noble et al., 1993, 1996; Craven et al., 2008). It may be possible that individuals of some species can exhibit hatch rate impairing dietary deficiencies in the wild, especially if a high degree of variation exists in a species' habitat/resource use and feeding ecology (Graveland and Drent, 1997).

Wood turtles are opportunistic omnivores that forage in diverse habitats (see Jones and Willey, 2015 for reviews of diet and habitat use). Like many other wood turtle sites, the NNJ site is a highly heterogeneous mix of habitats (e.g., streams, riparian floodplains, marshes, swamps, fens, mixed hardwood forests, hemlock groves, pine forests, thickets, old fields, agricultural fields, etc.), and we observed a high degree of inter-individual habitat use variation and foraging site fidelity among a sample of more than 20 NNJ females during a multiyear radio-tracking study (Duchak and Burke, unpublished data). Because some females spent the majority of their activity seasons foraging in old fields while others spent much of their time foraging in hardwood forests or various wetlands, diets and possibly nutritional statuses of these females could differ markedly. Given the wood turtle's highly variable ecology, one might expect that inter-individual variations in habitat use and, consequently, maternal diet could conceivably influence inter-individual variations in hatching success. Although, while dietary differences probably exist in wild wood turtle populations, it may be especially unlikely that any wild diets or foraging grounds are so nutritionally limited that they would repeatedly impair the annual hatch rates of multiple females in a given population; otherwise, we might expect maternally-linked hatching failure to be a common feature in many wood turtle populations. In any event, the extent to which maternal diet and the availability of key nutrients vary across wild wood turtle populations is not known, nor is how different diets affect hatching success.

Radio-tracking repeat nesters to determine whether major differences in summer foraging habitats exist between high and low hatch rate females would be an appropriate first step in examining what effect, if any, maternal diet has on hatching success. Tracking these females would also offer opportunities to collect regular fecal samples for dietary analyses and blood samples for health assessments that could examine the potential differences between high and low hatch rate females. If high and low hatch rate females are found to have dissimilar diets and blood chemistries, then assessing the lipid and fatty acid compositions of their freshly laid eggs might reveal different quantities of vital nutrients which could explain why some females consistently have less successful hatch rates than others. Alternatively, if no differences in diet or blood chemistry are found between high and low hatch rate females, then inadequate maternal diets would be an unlikely explanation for maternally-linked hatching failure in NNJ wood turtles.

While inadequate maternal diets may be a possible, yet perhaps unlikely hypothesis for maternally-linked hatching failure in NNJ wood turtles, environmental contamination is certainly a much more plausible one. Numerous studies link maternal accumulations of contaminants with reduced hatch rates or hatchling deformities in turtle populations (e.g., Bishop et al., 1998; Nagle et al., 2001; Bell et al., 2006; Perrault et al., 2011; Hopkins et al., 2013). At first glance, the NNJ site appears relatively free of environmental contamination, aside from a presumed history of past agricultural chemical use; however, closer investigation reveals that lead (Pb) from a long history (approximately 100 years or more) of intensive upland gamebird hunting has been and continues to be deposited at the NNJ site. Similar areas managed for upland gamebird hunting were estimated to hold several thousand lead pellets per hectare in the most superficial soil layers (Lewis and Legler, 1968; Esslinger and Klimstra, 1983; Keel et al., 2002). Spent lead pellets can take decades to completely disintegrate (Thomas, 1997), but they corrode rapidly and release particulate compounds, especially in acidic soils that are subjected to agricultural treatments like plowing or tilling (Jørgensen and Willems, 1987). These lead compounds tend to adsorb to detritus, making major wood turtle prey species such as earthworms and possibly slugs, carriers of high lead burdens at contaminated sites (Ma, 1982; Niederberger and Seidel, 1999; Jones and Sievert, 2009). Although no studies have investigated the impacts of lead shot deposition on turtles, many have documented the effects of lead shot deposition on upland birds (for reviews see Kendall et al., 1996; Fisher et al., 2006). In a controlled experiment, Edens et al. (1976) demonstrated that chronic dietary lead exposures can adversely affect hatching success rates in quail; the same could be true for terrestrial turtles. Given their small home range sizes, foraging site fidelity, and tendency to feed on invertebrates that can carry high lead loads, some wood turtles could be especially likely to accumulate lead concentrations high enough to affect the hatch rates of their clutches. Furthermore, the presumably heterogeneous distribution of lead shot throughout the landscape could explain why some female wood turtles always have lower hatch rates than others.

Measuring lead concentrations in the eggs of repeat nesters could potentially determine whether lead is influencing NNJ wood turtle hatch rates, but unfortunately, egg contaminant tests require the sacrifice of eggs and can also present analytical complications for researchers. Although less invasive than testing egg contents, testing eggshells alone and comparing lead levels between the shells of eggs that hatch and the shells of eggs that fail to hatch would still be problematic. In a study of slider turtles (*Trachemys scripta*), Burger and Gibbons (1998) found no correlation in lead concentrations, nor any other heavy metal concentrations, between egg contents and eggshells except for manganese. Lead concentrations were also significantly higher and, presumably, more readily detectable in egg contents than eggshells (Burger and Gibbons, 1998). In addition to correlations between egg contents and eggshells proving elusive, chemical compositions of eggshells and egg contents change throughout incubation with many elements decreasing in eggshells and increasing in egg contents as they are absorbed from eggshells by growing embryos (Yalçin-Ödilek et al., 2011; Orłowski et al., 2019). Moreover, eggshells and contents of embryonated eggs (i.e., fertile eggs with visible embryos) may have significantly higher heavy metal concentrations than eggshells and contents of non-embryonated eggs (i.e., presumably infertile eggs with no visible embryos). Therefore, to obtain objective comparisons of egg lead concentrations between high and low hatch rate females, it is advisable to: (1) measure lead concentrations in whole fresh eggs (i.e., undecomposed, homogenized eggshell and egg contents) of the same age or developmental stage and, if possible, (2) avoid including embryonated and non-embryonated eggs in the same analysis (Orłowski et al., 2016); however, effectively differentiating truly non-embryonated eggs (i.e., infertile eggs) from embryonated eggs that suffered early embryonic mortality is only possible via perivitelline membrane-bound sperm detection (Croyle et al., 2016), a technique that is not readily available to most turtle researchers and could potentially complicate contaminant analyses. Furthermore, any comparisons of lead concentrations between high and low hatch rate females would require adequate sample sizes to be conclusive, and sacrificing multiple eggs from multiple nesting females may not be permitted nor viewed as ethical in studies involving protected species. Therefore, prior to negotiating the complications associated with measuring lead concentrations in eggs, a much less invasive preliminary analysis should be performed to determine whether NNJ wood turtles even carry lead burdens high enough to justify testing their eggs.

Measuring lead concentrations in the scutes of repeat nester NNJ wood turtles would be the most conservative starting point for determining whether lead could be influencing their hatch rates. Since turtle shell scutes are composed of keratin, they provide a non-lethal, minimally invasive way to test turtles for heavy metal exposure (Overmann and Krajicek, 1995; Presti et al., 1999; Sakai et al., 2000; Blanvillain et al., 2007). Lead concentrations in hard, calcified materials like bone and keratin are good indicators of long-term accumulation (Overmann and Krajicek, 1995; Sakai et al., 2000) and should also be higher and more readily detectable than lead concentrations in eggs (Sakai et al., 2000), which represent short-term accumulation (Orłowski et al., 2016). Although it is entirely unknown whether wood turtle scute lead concentrations correlate with wood turtle egg lead concentrations, finding higher scute lead concentrations in NNJ wood turtles than those of other populations or other species would confirm that NNJ wood turtles indeed have a history of lead exposure that could be responsible for their low hatch rates. Because scute lead concentrations are better indicators of long-term rather than short-term exposure, such a result may not be sufficient to fully implicate lead as the main cause of the NNJ population's low hatch rates, but it would certainly warrant sacrificing a limited number of eggs from each repeat nester in order to measure their egg lead concentrations. As egg lead concentrations are indicators of recent exposure, they may be better suited to directly determine whether lead could be impacting the hatch rates of NNJ wood turtles but only if the previously mentioned complications associated with measuring heavy metal concentrations in eggs are mitigated. If females with consistently lower hatch rates bear significantly higher lead burdens in their eggs than females with consistently higher or variable hatch rates, it is very likely that lead contamination is responsible for the reduced hatch rates in the NNJ population. However, additional tests will be necessary to determine the possible source(s) and pathway(s) of lead exposure. Radio-tracking females with high and low hatching success records to their summer foraging habitats and quantifying lead levels in the soils and food items of these areas will be necessary to implicate recreational hunting as a major source of contamination.

CONCLUSION

The potential causes of hatching failure are important, yet often overlooked aspects of reptile ecology and conservation. The commonly suggested hypotheses for hatching failure, such as unsuitable incubation conditions or infertility, are unlikely to explain all of the hatch rate variability in some populations. This study found that approximately 60% of the hatch rate variability observed in a wood turtle population with low hatching success can be attributed to maternal identity. The remaining 40% can be attributed to the random environmental factors that are usually theorized to be major reasons for reduced hatch rates in many turtle populations (e.g., unsuitable incubation conditions, flooding, desiccation, egg infertility, egg damage due to improper handling by researchers, root and insect predation, and fungal/microbial infection). We hypothesize that maternally-linked hatching failure in seemingly healthy wild turtle populations is most likely an indicator of environmental contamination.

This study reveals a cryptic but critical conservation concern for vulnerable turtle populations: that the presence of many nesting females does not necessarily guarantee high or even sustainable reproductive rates. The exceedingly low hatch rates we observed suggest that recruitment in some turtle populations could be severely hindered even when managers attempt to mitigate well-known factors that decrease recruitment such as nest predation and low juvenile survival. Although time-consuming and labor intensive, we recommend that all turtle population studies investigate hatching success and document at least one year of overall, population-level hatch rate data as hatching success is an important indicator of every population's reproductive potential.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Hofstra University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

TD performed all field and lab work, performed all literature reviews, wrote most of the abstract, introduction, materials and methods, result, and discussion sections, and prepared all figures and tables. RB proposed and performed most analyses, contributed to portions of the materials and methods and result sections that are relevant to data analysis, and provided helpful edits and suggestions. Both authors contributed to the article and approved the submitted version.

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Nest-site choice by loggerhead sea turtles as a risk-management adaptation to offset hatching failure by unpredictable storms and predators

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Introduction: Along the coasts of Florida, United States, the nesting season of the loggerhead sea turtle, *Caretta caretta*, overlaps with the hurricane season. Nesting loggerhead females do not extend parental protection beyond depositing eggs in sandy, excavated nests in locations that provide a viable range of temperatures, moisture, and respiratory gas exchange. Thereafter, a female's clutches are subjected to the uncertainties of desiccation, predation, flooding, or beach erosion.

Methods: Here, we used data from a 1996-2004 study of 94 tagged loggerhead females nesting on a small barrier island off the Gulf Coast of south Florida, United States. We tested the hypothesis that the distribution of nest sites by loggerhead females was a randomized response to unpredictable hatching failure.

Results: We show that nest site choice accounted for 19.2% of variation in hatching success whereas breeding year and breeding month accounted for the remaining 81.8% of variation in hatching success. We show that the emergence site along the beach-length axis, nest site choice along the beach-width axis, and distances between nest locations did not fit a uniform-random distribution or a normal distribution. Instead, we show that loggerhead females employed a "Goldilocks" distribution in which nest sites were "neither too clustered nor too dispersed." Moreover, loggerhead females selected nest sites with limited overlap with nest sites from previous breeding seasons.

Discussion: We propose that nest site choice by this population of loggerhead females constitutes a significant maternal risk-management adaptation that deserves thoughtful consideration as we continue to assess the impacts of climate change on the future of loggerhead sea turtles.

KEYWORDS

behavioral plasticity, spatial navigation, maternal investment, *Caretta caretta*, parental care, lifetime replacement fitness, climate change

Introduction

Mated females of the loggerhead sea turtle, *Caretta caretta*, engage in transoceanic migrations every 2–3 years to oviposit egg clutches on their natal beaches (Whitmore and Dutton, 1985; Mortimer, 1990; Hays et al., 1995; Miller et al., 2003; Pfaller et al., 2009; Tucker, 2010). Because loggerhead females return to land to oviposit their eggs, the survival of hatchlings based on nest site choice has become a primary focus of research exploring the impact of climate change on sea turtles (Esteban et al., 2017).

All populations of loggerhead sea turtles share a core set of nesting behaviors. After nesting females emerge from the surf, they ascend the beach in a straight-line perpendicular to the water line and toward the dune line, also called the vegetative line (Wood and Bjorndal, 2000). However, the details of nest site choice vary depending on the ecological characteristics of a beach. On the beaches of Sanibel and Captiva, Keewaydin Island, and the 10,000 Islands of southwest Florida, loggerhead females spread nests along the beach in close proximity to the vegetative line (Hays et al., 1995; Garmestani et al., 2000; Cassill, 2021). On the beaches of Arembepe Beach in the State of Bahia, Northeastern Brazil, and the Yakushima Island, Japan, loggerhead females nested on wider beaches with less slope, preferentially nesting on open sand, equidistance between the high water line and the vegetative line (Serafini et al., 2009; Hatase and Omuta, 2018). On the sandy beaches of Zakynthos Island, Greece, beach width, emergence site, organic content, sand texture, and clay layers were important factors affecting nest site selection by loggerhead females and hatching success after clutches are abandoned (Mazaris et al., 2006).

The spring-to-fall nesting and hatching season of the loggerhead sea turtle, *Caretta caretta*, overlaps with the spring-to-fall monsoon and severe storm seasons (Ross, 2005). Torrential thunderstorms and tidal surges suffocate eggs in flooded sediment (Kraemer and Bell, 1980). During hurricanes or cyclones, extreme tides, and currents erode beaches, washing clutches out to sea (van Houtan and Bass, 2007). Severe storms also expose clutches to desiccation and predators (Matsuzawa et al., 2002; Stewart and Wyneken, 2004). Nests located too close to the high water line are at risk of being flooded or washed out by storms (Papi et al., 1997; Ross, 2005; van Houtan and Bass, 2007; Ackerman, 2017; Cassill, 2021). Nests located too far from the high water line are at risk of predation or desiccation.

After a female leaves an egg clutch behind, offspring survival is a game-of-chance. To increase the probability of meeting replacement fitness, the survival of two sexually mature offspring to replace the female and her mate (Cassill, 2019), loggerhead females employ a game-of-numbers. Over her lifetime, each female will produce 4,000–5,000 eggs, divided among 40 clutches with each containing an average of 105 eggs. Clutches are spread over 10–12 breeding seasons (Cassill, 2021). Ambient temperature, moisture, and respiration gas concentrations within a subterranean nest have profound effects on the survival of hatchlings (Mortimer, 1990). Because loggerhead females do not extend parental care beyond burying eggs in excavated sand pits, clutches deposited close to the sea have a greater likelihood of hatching failure by flooding and erosion, whereas clutches deposited farther inland have a greater likelihood of desiccation, hatchling disorientation, and predation (Wood and Bjorndal, 2000; Hughes and Brooks, 2006). Breeding loggerhead females must locate nests at sites that are suitable for incubating eggs and for mitigating the loss of eggs to unpredictable storms and predators.

Current consensus is that the site of emergence on the beach fits a random distribution (Hays et al., 1995), suggesting that females select a "good" beach in which any nest site will produce a successful hatch (Wood and Bjorndal, 2000). Others suggest that patterns of nest site selection are nonrandom (Miller et al., 2003), suggesting that females select a "good" site on an ecologically diverse beach to ensure hatching success (Wood and Bjorndal, 2000).

Here, we compared the distribution of nest sites by loggerhead females along the beach-length and beach-width axes to normal and uniform-random distributions. If nest sites fit a normal distribution, then loggerhead females have a fine-tuned preference for selecting "good" areas on a beach for egg deposition. If nest sites fit a uniform random distribution, then loggerhead females have a broadly tuned ability to select a "good" beach with suitable sites anywhere on the beach. We tested three null hypotheses. (1) The emergence site of females along the beach-length axis is random. (2) The nest site along the beach-width axis is random. (3) The distances between and across nests within and over multiple breeding seasons are random.

In the following sections, we present our findings on the impact of breeding year, breeding month, and nest site choice on hatching success. We then present the pattern of nest-site choice by loggerhead females at population and individual levels. Lastly, we frame our findings as risk-management adaptations in light of the spatiotemporal unpredictability of floods, storms, desiccation, and predation.

Materials and methods

Data collection

Keewaydin Island is a primary barrier island located off the coast of Naples, in Collier County, Florida, United States (Figure 1). Keewaydin Island is managed by staff from the State of Florida's Coastal Office, in cooperation with staff from NOAA, and the Rookery Bay National Estuarine Research Reserve. During the annual loggerhead sea turtle nesting season, staff and volunteers from The Conservancy of Southwest Florida Keewaydin Island monitor nesting females nightly. Staff and volunteers patrol the island nightly from 21:00 to 5:00 to record or tag new females and record information on the location and hatching success of their nest sites. Data included the female's ID number, the location of nest sites along the beach-length axis from a permanent marker and along the beach-width axis from the high

FIGURE 1
Keewaydin Island Preserve, Collier County, Florida, United States. Photographs are copyright free under the website https://linkrelated.com/terms-of-use/. This website is an integral part of Florida Media's digital internet media assets and solutions.

water line and relative to the vegetative line. The width of barrier-island beaches vary considerably along the beach length and are subject to dramatic change each year, depending on the frequency, intensity, and duration of storms and currents. Our analyses reflected nest site choice by loggerhead females against local conditions such as the high water line and the vegetative line, that were independent of past historical changes in the beach morphology. Each night, staff recorded evidence for predation, flooding, and partial or total washout. After the emergence of hatchlings, staff recorded the number of eggshells, the number of failed eggs, and the number of remaining hatchlings in the nest. Staff carried viable hatchlings that had not yet crawled out of the nest to the Gulf water. Conservancy staff provided data on 112 loggerhead females and their clutches nesting on Keewaydin Island in the southern Gulf coast of Florida from 1988 to 2004. From this information, we analyzed data on 389 nests produced by 94 females from 1996 through 2004. For a detailed analysis of individuals, we also selected six nesting females that produced 81 clutches over multiple breeding seasons.

Simulation

The simulation was undertaken using the following software: RStudio 2021.09.2 + 382 "Ghost Orchid" for Windows with "R-4.1.2 for Windows" using the function runif() from the package "stats" version 4.1.2 for uniform-random number generation. We produced a uniform-random distribution for a total of 400 nests by 10 simulated females. We compared the simulated distribution of nest sites to nests by loggerhead females. Variables were: percent hatching success per nest; emergence of females along the beach-length axis (11,000 m); nest site along the beach-width axis (40 m); distance between nearest-neighbor nest within a breeding season per female; longest distance across nests within a breeding season per female; and longest distance across all nests over multiple breeding seasons per female.

Analyses

To compare distances between and among nests within and over multiple breeding seasons per loggerhead female, we used the Shapiro–Wilk test to check for normality. Because our data did not meet the assumptions for normality and homogeneity, we used the nonparametric Kruskal-Wallis test and the Wilcoxon Each Pair. To compare nest site distance by loggerhead females versus simulated nest site distances, we used a nonparametric one-way test, Chi square approximation. We used a mixed model to analyze hatching success per nest (percent of hatched eggs) by five independent variables: breeding year, breeding month, nest site distance from the vegetative line, nest site along the beach-width axis from the high water line to the vegetative line, and emergence sites along the beach-length axis. Data analyses and graphics were generated using JMP Pro Statistical Software (16.1) and

PowerPoint. Data from this study are available in Excel format upon request.

Results

During the 1996–2004 period of this study, Hurricane George struck in September 1998; Irene struck in October 1999; and Gabrielle struck in September 2001. Hurricanes Charley, Frances, Ivan, and Jeanne struck in August and September 2004. During this period, mean hatching success per nest for loggerhead females was 65.3% (SE = 1.9; $n = 389$). Uniform-random hatching success for simulated females as 50.4%. Did nest-site choice by loggerhead females mitigate hatching failure? By modeling breeding year and breeding month as "confounding variables," we show that the closeness of nest sites to the vegetative line and the female's emergence sites along the beach-length axis were significant predictors of hatching success per nest, but not beach width (Figure 2; Mixed Model: $R^2 = 0.44$; $p < 0.0001$). Breeding year accounted for 71.4% of explained variation in hatching success; breeding month accounted for 10.4%; nest distance from the vegetative line accounted for 9.7%; and distance along the beach-length axis accounted for 9.1%. Beach width accounted for 0.4% of explained variation.

We graphically illustrate how the impact of nest site choice by breeding loggerhead females on hatching success per nest differed depending on the breeding year (Figure 3). In 1997, the impact of nest site choice on hatching success was insignificant. Hatching success was high for most nests. In 1998, emergence sites by loggerhead females at the far end of the island improved hatching success. In 2001, nest site choices closer to the vegetative line and further from the high water line improved hatching success. In 2002, nest site choices closer to the vegetative line, closer to the high water line, and furthest from the end of the island improved hatching success (see Appendix A for interaction effects).

Do loggerhead females randomize nest site selection to offset hatching failure by unpredictable storms and predators? To address this question, we plotted the distribution of 389 nest sites by a population of 94 nesting loggerhead females on the beach of Keewaydin Island from 1996 through 2004 (Figures 4A,B). We also plotted the uniform-random distribution of 400 nests divided among simulated females (Figure 4C). The vast majority of nests (94%) were located between 6,000 and 12,000 m along the 13,000 m beach-length axis. Beach width was a significant predictor of nest site choice for loggerhead females, but not for simulated females (Figures 4D–F; Regression: $R^2_{\text{vegetative line}} = 0.16$; $p < 0.0001$; $R^2_{\text{high water line}} = 0.62$; $p < 0.0001$; $R^2_{\text{uniform-random simulation}} = 0.0004$; $p = 0.712$). The majority (69%) of nest sites were located on narrow beach areas (<20 m) such that clutches were closer to both the vegetative line and the high water line that nests on wider beach areas (Figures 4A,B).

We show that nest site choice for loggerhead females relative to the vegetative line fits a leptokurtic distribution with double-exponential skew. Hence, nest site choice relative to the vegetative line differed significantly from a normal distribution and from a uniform random distribution (Figure 5A; Shapiro–Wilk: $W_{\text{normal}} = 0.84$; $p < 0.0001$; $W_{\text{uniform-random}} = 0.94$; $p < 0.0001$; $n = 100$; one-way Chi Square Approximation: $\chi^2 = 119.75$; DF = 1; $p < 0.0001$). Nest site choice for loggerhead females relative to the high water line fits a platykurtic distribution with moderate skew. Thus, nest site choice relative to the high water line differed significantly from a normal distribution and from a uniform-random distance (Figure 5B; Shapiro–Wilk:

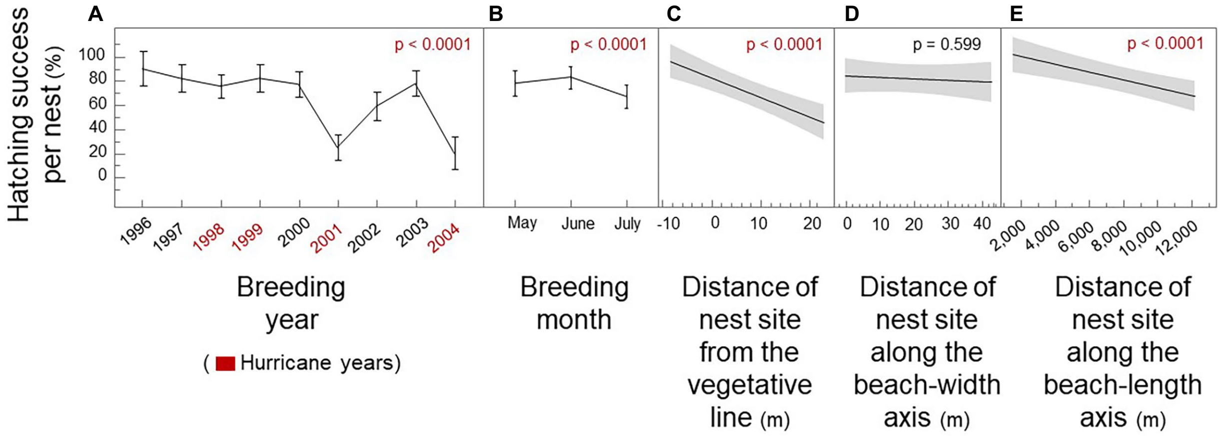

FIGURE 2
Hatching success per nest by spatiotemporal nest site choice of loggerhead females. Breeding year, breeding month, nest sites, and emergence sites. **(A)** Hatching success by breeding year **(B)** Hatching success by breeding month, independent of year. **(C)** Hatching success by the distance of the nest site from the line of vegetation. **(D)** Hatching success by the distance of the nest site along the beach-width axis starting from the high water line. **(E)** Hatching success by the distance of the nest site along the beach-length axis from a permanent marker. Hurricane years are shown as blue; mean shows as breaks in the line; SE shows as whiskers; regression line with 95% confidence intervals are shown in gray.

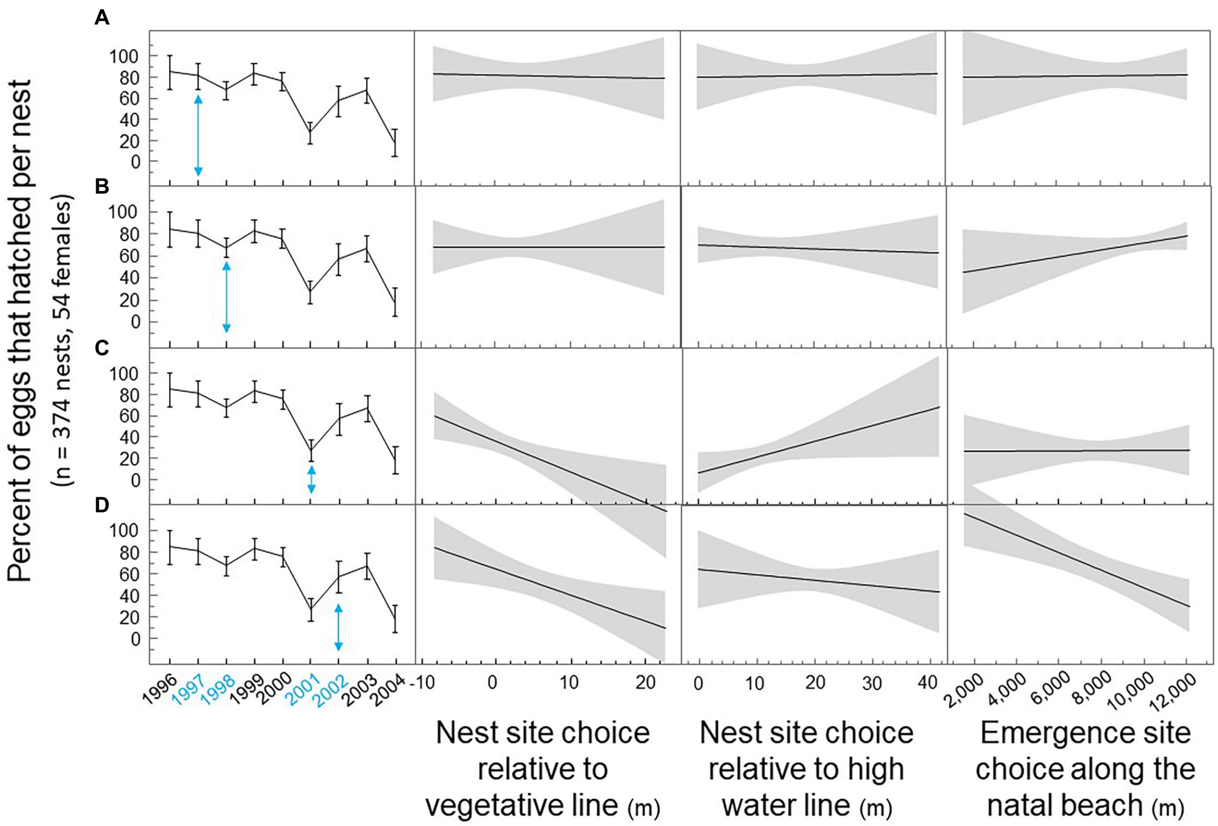

FIGURE 3
Nest site choice on Keewaydin Island by loggerhead females during multiple breeding seasons from 1996 through 2004. **(A)** Hatching success by nest site choice in 1997. **(B)** Hatching success by nest site choice in 1998. **(C)** Hatching success by nest site choice in 2001. **(D)** Hatching success by nest site choice in 2002.

$W_{\text{normal}} = 0.95$; $p = 0.010$; $n = 71$; $W_{\text{uniform-random}} = 0.94$; $p < 0.0001$; and one-way Chi Square Approximation: $\chi^2 = 20.31$; DF = 1; $p < 0.0001$). The emergence sites by loggerhead females along the beach-length axis fit a platykurtic distribution with moderate skew. Thus, emergence sites along the beach-length axis differed significantly from a normal distribution and from a uniform-random distribution (Figure 5C; Shapiro–Wilk: $W_{\text{normal}} = 0.97$; $p = 0.016$; $n = 389$; $W_{\text{uniform-random}} = 0.95$; $p < 0.0001$; and one-way Chi Square Approximation: $\chi^2 = 63.98$; DF = 1; $p < 0.0001$).

The distance among nest sites along the beach-length axis differed significantly within and across breeding seasons for loggerhead females (Figure 6A; Wilcoxon Kruskal-Wallas: $\chi^2 = 25.12$; DF = 2, $p < 0.0001$). Relative to a uniform random distribution of nest sites by simulated females, the distance between neighboring nest sites within a breeding season per loggerhead female was significantly smaller (Figure 6B; one-way test, Chi Square Approximation: $\chi^2 = 46.53$; DF = 1, $p < 0.0001$; median: 989 vs. 3,069 m). The longest distance among nests within a breeding season for loggerhead females was significantly smaller than the longest distance for simulated females (Figure 6C: $\chi^2 = 13.13$; DF = 1, $p = 0.0003$; medium: 2,315 vs. 5,943 m). The longest distance among all nests over multiple breeding seasons per loggerhead females was significantly smaller than the longest distance per simulated female (Figure 6D: $\chi^2 = 4.25$; DF = 1, $p = 0.039$; medium: 5,169 vs. 8,518 m). In short, loggerhead females selected nest sites that were one-third the distance to the nearest neighbor, 40% the distance across nests within a breeding season, and 60% the distance across all breeding seasons.

Because not every female in the current population of breeders will achieve lifetime replacement fitness (Cassill, 2019), we detailed hatching success and failure individually for six loggerhead females who produced at least nine nests over at least two breeding seasons on Keewaydin Island beach from 1996 through 2004. Mean hatching success was 68.3% for Female I with a range of 0–99% (Figure 7A; $n = 18$ nests). Mean hatching success was 66.4% for Female II with a range of 0–100% (Figure 7B; $n = 10$ nests). Hatching success was 63.1% for Female III (Figure 7C; range = 0–98%; $n = 16$ nests). Hatching success was 72.0% for Female IV (Figure 7D; range = 0–99%; $n = 12$ nests). Mean hatching success was 61.1% for Female V (Figure 7E; range = 0–100%; $n = 9$ nests); and mean hatching success was 84.8% for Female VI (Figure 7F; range = 32–98%; $n = 10$ nests).

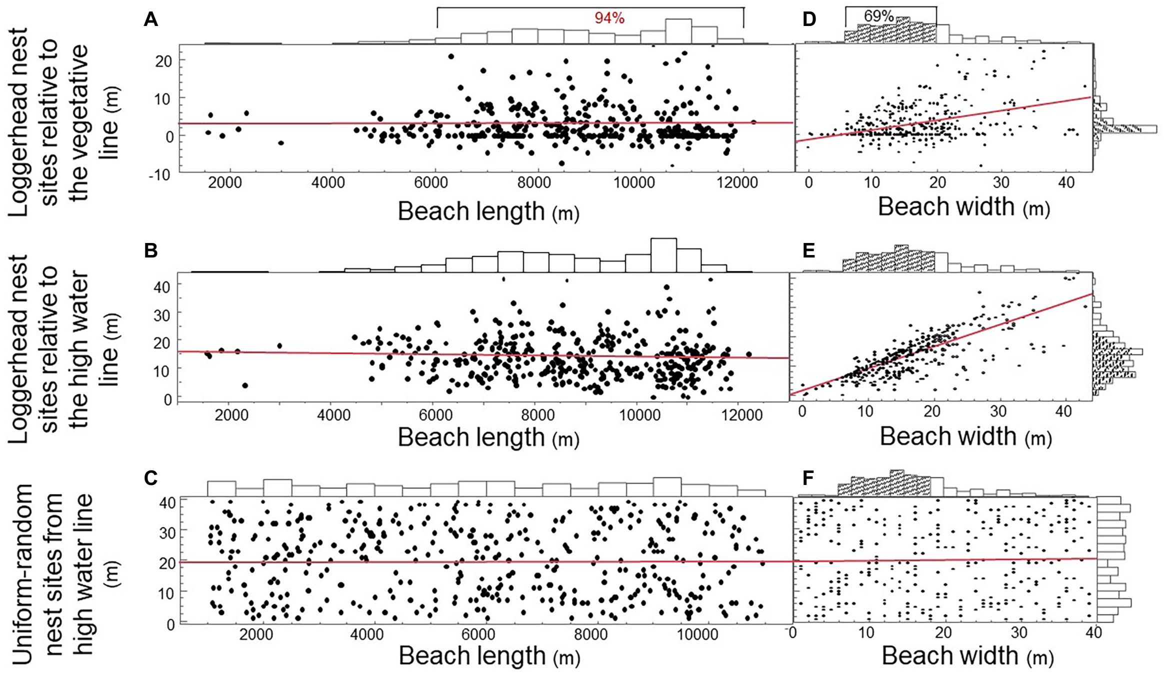

FIGURE 4
Distributions and predictors of loggerhead sea turtle nest sites on Keewaydin Island beach, Naples Florida, United States. **(A)** Emergence sites of loggerhead females along the beach-length axis by the distance of nest sites from the vegetative line. **(B)** Emergence sites along the beach-length axis by the distance of nest sites from the high water line. **(C)** Uniform-random emergence sites along the beach-length axis and nest site locations along the beach-width axis. **(D)** Nest site location by loggerhead females along the beach-width axis by the distance of nest sites from the vegetative line. **(E)** Nest sites along the beach-width axis by the distance of nest sites from the high water line. **(F)** Uniform-random nest sites along the beach-length axis by the beach-width axis.

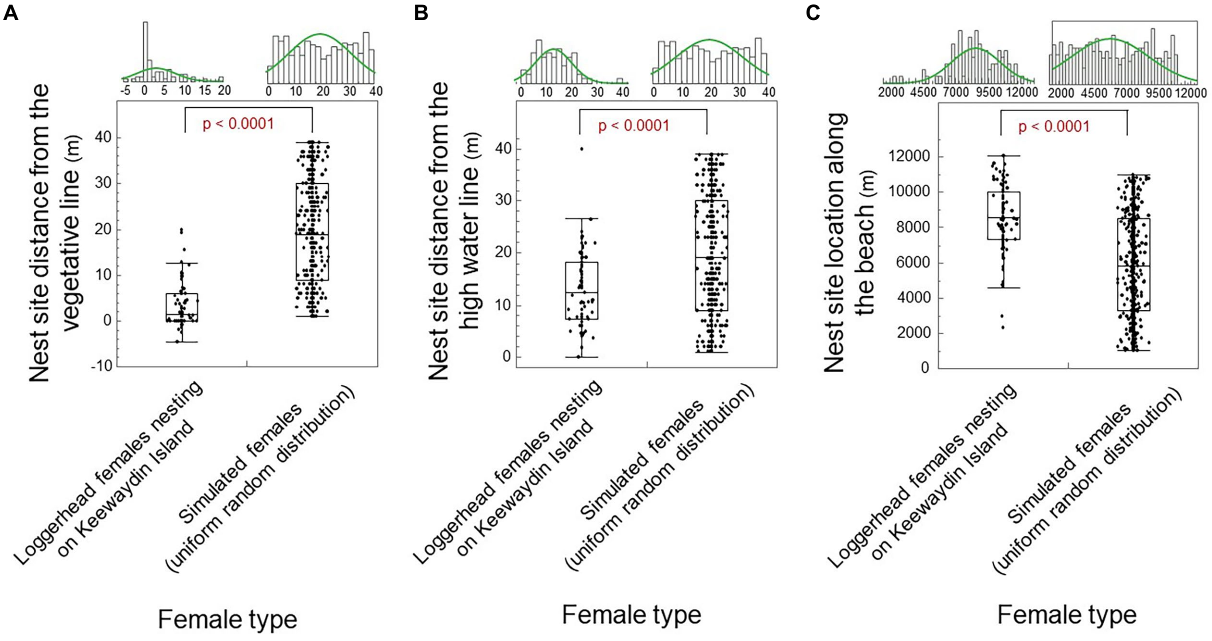

FIGURE 5
Nest site choice by female type (loggerhead females versus simulated females). **(A)** Nest site distance from the vegetative line by female type. **(B)** Nest site distance from the high water line by female type. **(C)** Nest site distance along the beach-length axis by female type. The median shows as a line in the box. The box ends are the 25 and 75th quantiles. Whiskers enclose all data except outliers. Significant differences show as red.

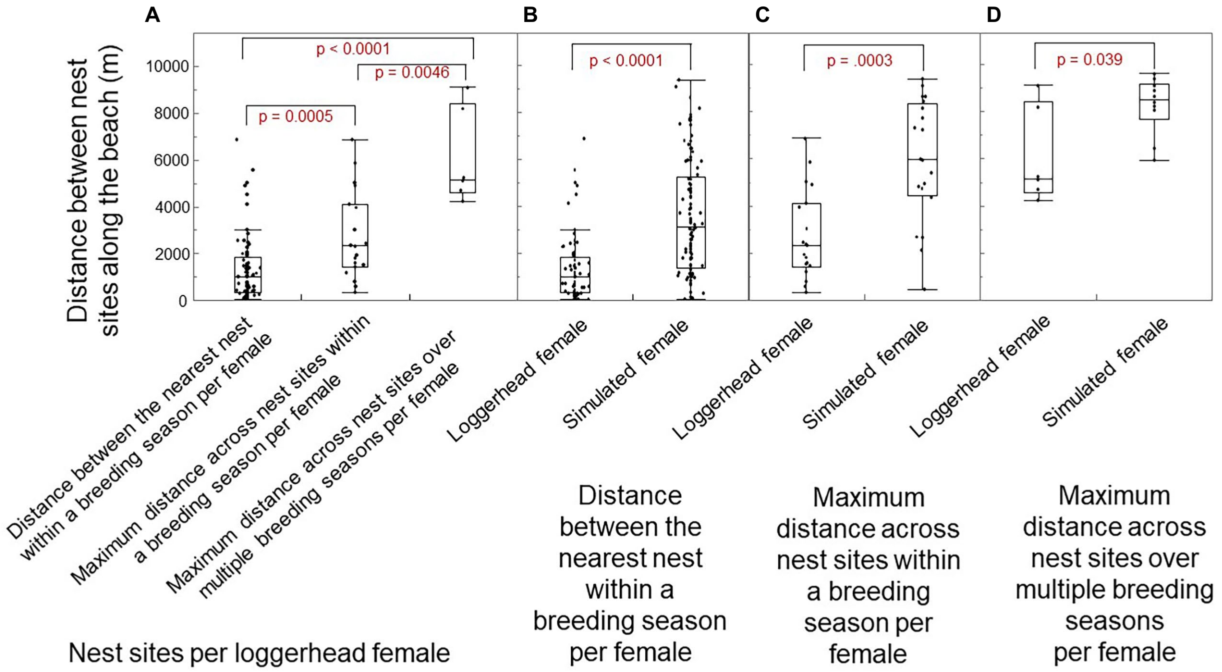

FIGURE 6

Distances among nests along the beach-length axis of Keewaydin Island for loggerhead females and simulated females. **(A)** Distance among nests per loggerhead female within and across breeding seasons. **(B)** Distance between the nearest-neighbor nests within a breeding season by female type. **(C)** Longest distance across nests within a breeding season by female type. **(D)** Longest distance across all nests over multiple breeding seasons by female type. The median shows as a line across the box. The ends of the box are the 25 and 75th quantiles (i.e., quartiles). Whiskers enclose all data except outliers. Significant differences show as red.

Discussion

In our 1996–2004 study of tagged loggerhead females nesting on Keewaydin Island's beach, hatching success per nest averaged 65.3%. Although nest site choice accounted for 19.2% of explained variation in hatching success, breeding year and breeding month accounted for 81.8% of explained variation. We hypothesized that a simple random distribution of nest sites by loggerhead females could provide significant mitigation of hatching failure. However, our study did not support our null hypothesis. The distribution of nest site choices along the beach-length and beach-width axes did not fit a uniform-random distribution. We show that loggerhead females preferentially deposited 94% of clutches in nest sites over 6,000 m of the 13,000 m beach. Moreover, females deposited 69% of clutches on narrow sections of the beach ranging from 8 to 22 m wide. On narrow section, nests were close to the vegetative line, and at the same time, close to the high water line. On wider areas of the beach, females spread clutches in open-sand sites between the vegetative line and the high water line. By "voting" with their feet, loggerhead females indicated that half of Keewaydin Island was a "good" beach area. Females also preferred to nest on narrower beaches such that their clutches had the benefit of higher elevation near the vegetative line and hatchlings had the benefit of shorter distances to the sea. Occasionally, females selected a nest site far outside the preferred beach length and beach width areas, perhaps on the chance that a high-risk site might pay off.

In short, nest site choice by the population of Keewaydin Island loggerhead females followed a "Goldilocks" distribution strategy that fell between a normal and random distribution. Within breeding seasons, nest sites were not too clustered or too dispersed.

How do females manage to prevent future nest sites from overlapping with nest sites from current or previous breeding seasons? Recent studies show that geomagnetic navigational signatures and local cues such as wind currents, tidal currents, taste, and smell are imprinted in the brains of hatchlings and might continue to be imprinted on the brains of juveniles and adults as they migrate to and from foraging and breeding areas (Papi et al., 2000; Lohmann et al., 2008a,b, 2017; Brothers and Lohmann, 2015; Kishkinev, 2015; Lohmann and Lohmann, 2019). The frontal brain of migratory sea turtles (i.e., the medial cortex) plays a key role in spatial cognition without extensive training (Striedter, 2016; Reiter et al., 2017). In vertebrates, the hippocampus and other homologous brain structures encode and consolidate spatial information into short-term and long-term memory. These findings present an exciting possibility for explaining the mechanisms by which females distribute nest sites that rarely overlap within and among breeding seasons. As they crawl up the beach, secure a nest site, excavate a pit, oviposit eggs, and then bury

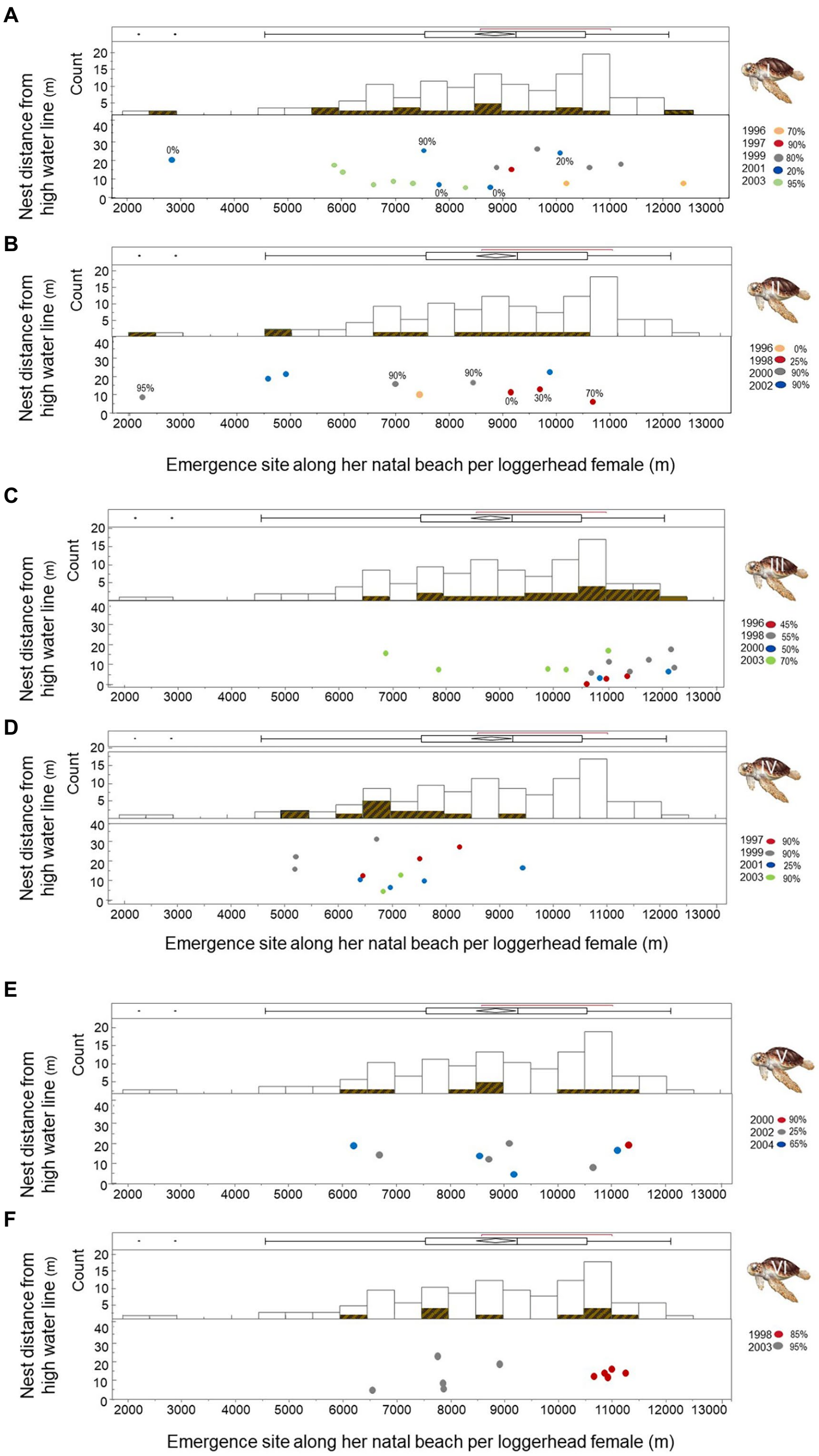

FIGURE 7
Nest site choice on Keewaydin Island by six loggerhead females during multiple breeding seasons from 1996 through 2004. Histograms represent the location of nest sites by each female relative to the combined nest site choices by the group of females. Dots show the years and location of nest sites for each female **(A-F)**.

and camouflage the site, females might imprint the geomagnetic signature of each nest on the medial cortex and at the same time, encode and consolidate local ecological conditions of the beach in their hippocampus homolog.

Do loggerhead females choose a "good" beach or do they select "good" sites on a low-quality beach? Our study suggests that females do both. It is possible that the presence of human activity and dwellings along the first half of Keewaydin Island beach explains their preference for selecting nest sites along the more distant half of the beach (Arianoutsou, 1988; Salmon et al., 1995; Witherington, 1997; Margaritoulis, 2005; Mazaris et al., 2009; Lutcavage, 2017). The steeper slope on the narrow beach areas might explain their preference for nest sites that were closer to the dune and, at the same time, the sea (Hays et al., 1995; Wood and Bjorndal, 2000). Certainly, sand temperature and sand quality played a role in nest site choice (Garmestani et al., 2000; Matsuzawa et al., 2002) as would female size (Hatase and Omuta, 2018) and their immediate energy budget (Hays and Speakman, 1991; Hatase and Tsukamoto, 2008; Perrault and Stacy, 2018).

In conclusion, evidence accumulated over recent decades supports the hypothesis that loggerhead sea turtles use multiple sensory cues when selecting nest sites (Mazaris et al., 2006). With increasing anthropogenic challenges, including rapid climate change, our hope is that this study on nest site choice by loggerhead females nesting on Keewaydin Island encourages other long-term studies of small populations with the aim of improving the management and conservation of sea turtles.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

DC analyzed and authored the manuscript. AW contributed the simulation and edited all versions of the manuscript including the final.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.850091/full#supplementary-material

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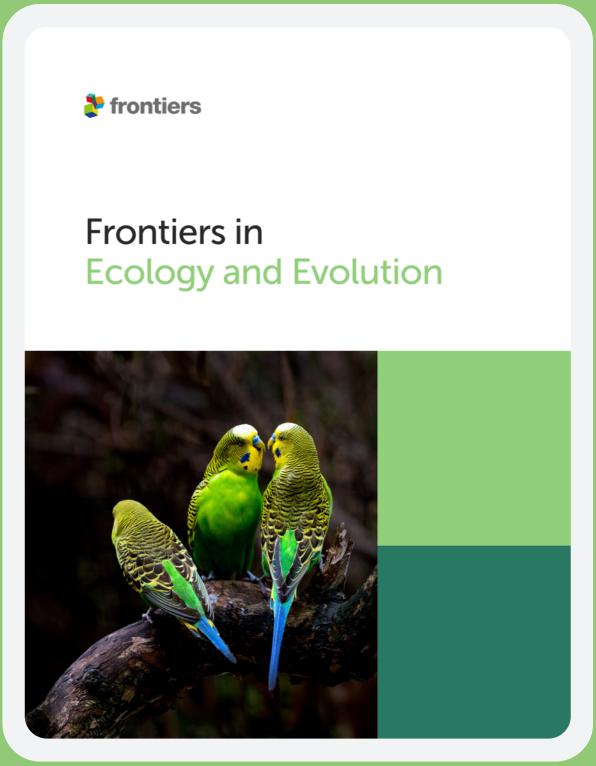

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