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DOI: 10.1016/j.zool.2017.06.007

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# Experimental assessment of the effects of moisture on loggerhead sea turtle hatchling sex ratios



Alexandra Lolavar\*, Jeanette Wyneken

Department of Biological Sciences, Florida Atlantic University, 777 Glades Rd, Boca Raton, FL 33431-0991, USA

## ARTICLE INFO

### Article history:

Received 10 February 2017

Received in revised form 15 June 2017

Accepted 15 June 2017

Available online 17 June 2017

### Keywords:

Temperature-dependent sex determination

Environmental sex determination

*Caretta caretta*

Moisture

Climate

## ABSTRACT

Many reptiles have temperature-dependent sex determination (TSD). Sex determination in marine turtles is described by a cool–male, warm–female pattern. Nest sand temperature strongly influences sea turtle embryo development and sex differentiation. Yet, variation in hatchling sex ratios is explained only partially by nest temperature and can be predicted only at the warmest and coolest temperatures. Hence, other factors during development influence sex determination. Rainfall is a common environmental variable that may impact development and sex determination. We experimentally evaluated bias in sex ratio production associated with nest moisture. Conditions tested in surrogate nests were sand moisture in combination with (i) very restricted evaporation, (ii) moderate evaporation (allowing evaporative cooling), and (iii) evaporative cooling plus cooling from rain-temperature water. We collected eggs from 32 unique loggerhead (*Caretta caretta* L.) turtle clutches, distributed them among the three different conditions, and incubated the eggs until they hatched. All hatchlings were raised for several months and sex was verified laparoscopically to establish sex ratios for each treatment. The nests were expected to produce 50:50 sex ratios or a moderate female bias (~70%) based on incubation temperatures. All experimental treatments produced high male bias (87–96%). These results support the hypothesis that moisture impacts sex ratios through evaporation and rainfall-based cooling. High male bias was observed in nests with and without restricted evaporative cooling and no direct cooling due to watering as well as those nests hydrated via cool (rainwater temperature) water. High moisture conditions may produce males through thermal or other mechanisms, highlighting the importance of examining other nest environmental factors on sex determination.

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## 1. Introduction

Vertebrate sex can be determined in development either genetically (genetic or gametic sex determination, GSD) or environmentally (environmental sex determination, ESD). In GSD, the presence of inherited chromosomes and key genes initiates the pathway directing molecular and morphological signals so that male or female sex-specific characteristics develop. Environmental sex determination is characterized by environmental factors directing the molecular and morphological signals and establishing the sex of developing embryos or changing the sex of fully developed adults.

Environmental factors affect sex in a variety of vertebrates. In the silverside fishes, *Odontesthes regia* and *Menidia menidia*, increased temperature and cortisol levels result in a greater pro-

duction of males (Strüssmann et al., 1997; Hattori et al., 2009). In some cichlid species (*Apistogramma* spp.) and a molly (*Poecilia melanogaster*), increased temperature produces more males and increased pH produces more females (Rubin, 1985; Römer et al., 1996; Beisenherz, 1996). In reptiles, temperature has been the primary factor identified as affecting sex determination. Temperature-dependent sex determination (TSD), a form of ESD, is found among many turtle species, crocodylians, some lizard species, and tuataras (Bull, 1980; Valenzuela, 2004). Several patterns of TSD characterize these taxa and differ from one another based on which sex is produced at higher and lower temperatures. All sea turtle species have a cooler male/warmer female TSD system (Bull, 1980; Miller and Limpus, 1981; Standora and Spotila, 1985; Pieau et al., 1995). Environmental factors, such as sand albedo (Hays et al., 2001), shading and sun exposure (Standora and Spotila, 1985), and moisture as rainfall (Godfrey et al., 1996; Houghton et al., 2007) may modify incubation temperatures. While biologists remain confident of temperature's major role in sex determination, several studies have explored if other environmental factors

\* Corresponding author.

E-mail address: [alolavar@fau.edu](mailto:alolavar@fau.edu) (A. Lolavar).

direct development and sex differentiation in sea turtles. Moisture as rainfall may impact the nest environment, affecting hatchling size (McGehee, 1990) and influencing hatchling sex (reviewed by Wibbels, 2003; Lolavar and Wyneken, 2015; Wyneken and Lolavar, 2015).

Several field studies suggest that increased nest moisture, from rainfall during incubation, influences hatchling sex ratios in turtles. Godfrey et al. (1996) found increased production of male green turtle (*Chelonia mydas* L.) and leatherback turtle (*Dermochelys coriacea* V.) hatchlings in Suriname during April and May, months with the most rainfall. Matsuzawa et al. (2002) examined loggerhead (*Caretta caretta* L.) nests in Japan and found sand temperature increased as the rainy season ended, suggesting an increase in female hatchlings. Houghton et al. (2007) found high rainfall in Granada produced unseasonably cool nest temperatures and interpreted the impact as shifting leatherback sex ratios toward male bias although no hatchlings were sampled. Together these studies suggest that rain influences nest temperature and sex ratios but none measured how rain acts. Does rain cool the nest directly or does increased nest moisture increase evaporative cooling of the eggs?

Experimental studies of moisture and sex ratios in two closely related freshwater turtles produced conflicting results. Gutzke and Paukstis (1983) incubated painted turtle (*Chrysemys picta* S.) eggs at constant temperatures in vermiculite and found more males were produced under wetter conditions. Another study of the same species found equal sex ratios were produced with varying incubation temperatures and wetter substrates; eggs in drier substrates produced mostly males (Paukstis et al., 1984). Packard et al. (1989, 1991) found no substrate moisture effects on *C. picta* sex ratios with either constant or variable incubation temperatures. LeBlanc and Wibbels (2009) incubated red-eared slider (*Trachemys scripta* S.) eggs at a constant temperature and 100% humidity without an incubation medium. Eggs that were misted daily with water at incubator temperature produced a greater proportion of males compared to eggs that were not misted. However, the proportion of males produced varied between study years. Together these studies suggest that moisture may impact sex ratios in some species and under some conditions. Yet, very few studies systematically address moisture's effects on sex ratios from eggs incubated in natural substrates (Bodensteiner et al., 2015). No study addressed if moisture impacts sex determination through evaporative cooling, direct cooling of the eggs, or if the effect is independent of temperature.

To better understand the effects of moisture on hatchling sea turtle sex ratios, we separated the possible mechanisms of cooling: normal evaporation vs. direct cooling in native sand substrates. We also investigated potential hydric effects on sex ratios beyond cooling. Male-biased sex ratios occurred when loggerhead (*C. caretta*) eggs were incubated at and above pivotal temperatures under several high moisture conditions. By applying water treatments under controlled laboratory conditions, we found direct relationships between high sand moisture levels and loggerhead sex ratios; these results suggest possible mechanisms by which sex ratio may be modulated by rainfall in nature.

## 2. Materials and methods

### 2.1. Eggs and incubation

We collected 21 eggs from each of 32 separate loggerhead (*C. caretta*) clutches within 12 h of nest deposition ( $n = 672$  eggs) from July to August 2012 in Boca Raton, Florida, USA. The eggs were moved in nesting beach sand to the Florida Atlantic University laboratory located 8 km (<15 min) away. There, eggs were brushed

clean of as much sand as possible and the eggs from one nest were transferred into three styrofoam boxes (20 cm × 15 cm × 23 cm; hereafter termed “nest boxes”) filled with sterilized nesting beach sand from Boca Raton. Each nest box contained 14 eggs; half of the eggs ( $n = 7$ ) came from one clutch and half from another clutch laid the same night. This approach was chosen to minimize potential loss of a replicate's sex ratio to factors we could not detect at egg collection, such as clutch-specific differences in embryo viability or fragility. Each of the three resulting nest boxes was placed in one of three incubators; 16 nest boxes were evenly distributed in each incubator ( $n = 48$  nest boxes total).

### 2.2. Incubation conditions

Each incubator provided treatment conditions that differed from the other two in their combination of air relative humidity, evaporative cooling potential, and the temperature of the DIH<sub>2</sub>O used to moisten the sand throughout incubation. All incubators were maintained at  $29 \pm 0.2^\circ\text{C}$ . Incubator 1 had high humidity (air 95–100% RH), which allowed very limited to no evaporative cooling of nest boxes; DIH<sub>2</sub>O for misting nest boxes was maintained inside the incubator at  $29^\circ\text{C}$ . Incubator 2 was maintained at 75–80% humidity, the average relative humidity on the nesting beach, allowing evaporative cooling of the sand. DIH<sub>2</sub>O used to mist the nest box sand was maintained in the incubator at  $29^\circ\text{C}$ . Incubator 3 was also maintained at 75–80% humidity to allow evaporative cooling of the sand. The incubator 3 nest boxes received water treatments from water maintained at  $25^\circ\text{C}$ , which is the most common rainwater temperature measured at the sand surface on the Boca Raton beach during the summer months. Both incubator humidity and water treatment temperature in incubator 3 simulated natural beach conditions in having both evaporative cooling of the surface sand and potential direct cooling from the  $25^\circ\text{C}$  DIH<sub>2</sub>O simulating rainwater. Desired levels of evaporative cooling were verified by monitoring changes in sand volumetric moisture content and in sand weight. Volumetric moisture content and sand weight in incubator 1 changed minimally over the course of several days, suggesting very little evaporation. Sand in both incubators 2 and 3 decreased in volumetric moisture content and weight after a day and water was added more frequently in order to maintain normal moisture levels.

The pivotal temperature, defined as the consistent incubation temperature that results in a 1:1 sex ratio (Mrosovsky and Yntema, 1980), is estimated to be  $\sim 29.0^\circ\text{C}$  for loggerhead turtles (Mrosovsky, 1988; Wibbels, 2003; Blair, 2005). All three incubators were set to  $29.0^\circ\text{C}$  for the experiment. Each incubator was equipped with an Omega General Purpose RTD probe (Model PR-10) with a temperature control device (Omega Temperature Controller Model CNI58C accuracy  $\pm 0.5^\circ\text{C}$ , resolution  $\pm 0.2^\circ\text{C}$ ; OMEGA Engineering, Inc., Stamford, CT, USA). The device controlled a bank of two incandescent light bulbs that warmed the incubator when air temperatures dropped to  $28.8^\circ\text{C}$  and then turned off once the incubator air reached  $29.2^\circ\text{C}$ . This regime kept the nest boxes at  $29 \pm 0.2^\circ\text{C}$ . The temperature and relative humidity of the air in each incubator was monitored and recorded every 15 min using HOBO U12-012 data loggers (temperature: accuracy  $\pm 0.35^\circ\text{C}$ , resolution:  $0.03^\circ\text{C}$ ; humidity: accuracy  $\pm 2.5\%$ , resolution  $0.03\%$  per manufacturer's specification and verified empirically; Onset Computer Corp., Bourne, MA, USA). Several nest boxes in each incubator were equipped with HOBO U22-001 temperature data loggers (accuracy  $\pm 0.2^\circ\text{C}$ , resolution  $0.02^\circ\text{C}$ , per manufacturer's specification and verified empirically) so sand temperatures were recorded simultaneously in nest boxes every 15 min throughout incubation. Mist humidifiers were placed in each incubator and set at either “max” or “sleep mode” (intermittent) depending on the desired humidity level. Two fans were placed in each incubator to keep temperatures

and humidity levels uniform throughout the incubators. Uniformity in the incubators was established prior to the start of the experiment.

The volumetric moisture content of each nest box was recorded daily at egg level to ensure normal moisture levels using a HOBO Micro Station data logger (H21-002) with Smart Soil Moisture Sensors (Model S-SMC-M005; accuracy  $\pm 0.031 \text{ m}^3 \text{ m}^{-3}$ , resolution  $0.0007 \text{ m}^3 \text{ m}^{-3}$ ; Onset Computer Corp.). The average volumetric moisture content of nest sand is  $\sim 6\%$  ( $0.06 \text{ m}^3 \text{ m}^{-3}$ ) (Ackerman, 1997). In the present study, sand moisture content was maintained between  $0.06$  and  $0.10 \text{ m}^3 \text{ m}^{-3}$ . All treatment groups received daily  $\text{DIH}_2\text{O}$  treatments, as needed, sprinkled on the sand above the eggs and allowed to percolate through the sand. The water for all incubators was collected at the same time in 4 l carboys; water was maintained within incubators 1 and 2 to insure that the water was the same temperature as the air and sand. A container of the same volume was maintained in incubator 3 but the water source for incubator 3 nest boxes was maintained at  $25^\circ\text{C}$  then taken immediately into the incubator that was located approximately 4 m away to mist the nest boxes.

### 2.3. Hatchlings and sex identification

Once the hatchlings emerged from the nest boxes, they were marked with their nest and incubator ID and transported to the Florida Atlantic University Marine Laboratory (4 km,  $\sim 15$  min drive) where they were raised for 2–3 months until they reached 120 g (standard carapace length:  $\sim 90$  mm). All turtles underwent laparoscopic examination of the gonads and gonadal ducts to identify sex (Wyneken et al., 2007). All turtles were released offshore in the Florida Current no less than 1 week following laparoscopy. Turtles that died while in the laboratory ( $n=6$ ) were dissected to identify their sex (Ceriani and Wyneken, 2008).

### 2.4. Data analyses

To assess the combined effects of moisture and thermal conditions, we compared observed sex ratios with two different expected sex ratios that differed in the method of estimation: (i) loggerhead pivotal temperature during the middle third of incubation when sex is determined and (ii) nest incubation duration as described by Godfrey and Mrosovsky (1997). Expected sex ratios estimated by nest temperature and by incubation durations both reflect the role of temperature on developmental rates. Each should predict sex ratios without consideration of potential effects of normal vs. increased nest moisture. Observed sex ratios were calculated for each nest box and compared to the two expected sex ratios by Mann–Whitney tests. Sex ratios, nest box hatch success, and incubation durations among incubators were also compared to identify effects of the different treatment conditions by Kruskal–Wallis tests. After verifying that the data were normally distributed and variances were similar, environmental conditions were compared by one-way ANOVA (Zar, 2010).

## 3. Results

The overall hatch success of all incubators ( $[\# \text{ live hatchlings}/\# \text{ eggs}] \times 100$ ) was 88.5% (Table 1) and did not differ among incubators (ANOVA  $F_{2,45} = 0.90$ ,  $p > 0.05$ ). Dead embryos ranged from gastrula to stage 29, but only eight embryos exceeded stage 23.

Incubation durations ranged from 52 to 68 d (Table 1) and differed among incubators (ANOVA  $F_{2,45} = 57.61$ ,  $p < 0.001$ ). Incubator 1 ( $29^\circ\text{C}$  water, 97–100% humidity) had the shortest mean incubation duration, while incubator 3 ( $29^\circ\text{C}$  water, modal RH: 76% range:

75–80% humidity,  $25^\circ\text{C}$  simulated rainwater treatment) had the longest mean incubation duration (Table 1).

### 3.1. Temperature and moisture

Incubator temperatures remained consistent throughout the study but averaged slightly above the published pivotal temperature (Wibbels, 2003). All incubators remained at set temperatures. Air temperatures in incubator 1, 2, and 3 averaged at  $29.4^\circ\text{C}$  (range: 28.9–30.5),  $29.5^\circ\text{C}$  (range: 29.1–30.3), and  $29.4^\circ\text{C}$  (range: 29.0–30.6), respectively. Brief increases or decreases in air temperatures beyond target temperatures were due to the investigator moving in or out of the incubators and were not reflected in sand temperatures. Incubator humidity varied within the expected ranges (Table 2) so that evaporative cooling was minimal in incubator 1 and similar to field conditions in incubators 2 and 3. Cooling in incubator 2 was limited to evaporative cooling while nest boxes in incubator 3 experienced both evaporative cooling and cooling from rainwater-temperature  $\text{DIH}_2\text{O}$ . Nest temperatures in incubator 3 decreased approximately  $0.5^\circ\text{C}$  immediately after water treatments. Mean nest sand temperatures and air humidity are summarized in Table 2. Nest box temperatures did not differ among the three incubators (ANOVA  $F_{2,45} = 2.42$ ,  $p > 0.05$ ). Volumetric water content remained within normal nest moisture levels ( $0.06$ – $0.10 \text{ m}^3 \text{ m}^{-3}$ ) in all nest boxes (Table 2). There were slight differences in volumetric water content among incubators. Incubator 1 had nests with the highest average moisture content ( $\sim 0.10 \text{ m}^3 \text{ m}^{-3}$ ) and nests in incubator 2 had the lowest ( $\sim 0.06 \text{ m}^3 \text{ m}^{-3}$ ).

### 3.2. Sex ratio

Strongly male-biased sex ratios were found for all nest boxes and all three treatments (Table 3). Sex ratios did not differ significantly among incubators (Kruskal–Wallis,  $H = 1.86$ ,  $df = 2$ ,  $p > 0.05$ ). Although there was a higher production of male hatchlings in incubators with evaporative cooling, with or without cool water treatments, the sex ratios in different incubators were statistically the same (Table 3). Observed sex ratios were compared with the two kinds of expected mean sex ratios based on (i) mean nest temperatures during the middle third of incubation, when sex is determined (Ackerman, 1997) and (ii) by incubation duration (Godfrey and Mrosovsky, 1997). Table 3 summarizes these sex ratio comparisons for each incubator; observed sex ratios differed significantly from each expected sex ratio and the direction of the sex ratio bias was toward more extreme male bias than any predicted sex ratio.

## 4. Discussion

In all treatments, observed sex ratios were moderately to highly male-biased and differed significantly from expected sex ratios. The results are consistent with the hypothesis that high sand moisture increases male hatchling production. Surprisingly, nest boxes experiencing little evaporative cooling and no water-based cooling also produced male-biased sex ratios at temperatures that should have produced even or slightly female-biased sex ratios. Even when evaporation is severely restricted (incubator 1), the majority of the limited evaporation is from the sand surface not the deeper sand surrounding the clutch; thus net evaporative cooling of the clutches likely did not occur or was so limited it was outside the measurement resolution of the current study. Nest boxes in incubator 2 (limited evaporative cooling, water treatments with incubator-temperature water) likely lost some moisture and heat via evaporation from the surface of the sand and via evaporation from some of the deeper sand. Nests in incubator 3 experienced

**Table 1**

Incubation durations of all nest boxes by treatment. Nest boxes with the same date laid had eggs from the same clutches; when 4 clutches were obtained (e.g., 16 Jul, 18 Jul), two were used in one set of nest boxes and two were used in the other set of nest boxes. Incubation durations (d) differed statistically among incubators. Nests in incubator 1 had the shortest incubation durations while nests in incubator 3 incubated the longest. Hatch success did not statistically differ among incubators. Most dead embryos could not be staged due to decomposition or death at very early stages. Dead embryos that could be sexed were identified as one female in incubator 1, two females and two males in incubator 2, and one female and two males in incubator 3. Those results were incorporated into sex ratios for their respective nest boxes.

Date Laid	Incubator 1 (no/minimal evap. cooling)	Incubator 2 (limited evap. cooling)	Incubator 3 (limited evap. cooling/cool H <sub>2</sub> O)
12-Jul	57	60	62
13-Jul	56	57	60
15-Jul	56	60	63
16-Jul	55	60	60
16-Jul	55	61	63
17-Jul	54	59	65
18-Jul	55	61	64
18-Jul	55	62	64
19-Jul	54	61	65
19-Jul	54	64	68
21-Jul	53	63	62
27-Jul	52	63	61
28-Jul	52	60	59
29-Jul	54	59	64
31-Jul	59	63	64
02-Aug	60	62	61
Inc. duration (d) mean ± SD	55.1 ± 2.21	60.9 ± 1.84	62.8 ± 2.32
Mean hatch success ± SD	90.2 % ± 8.99	90.2 % ± 11.33	85.3 % ± 14.86

**Table 2**

Summary of incubator and nest box conditions. All summary statistics are for the thermosensitive period estimated from incubation duration. Incubator air temperatures exceeded presumed pivotal temperature and nest temperatures remained close to the presumed pivotal temperature used to predict a 1:1 sex ratio or female bias. Incubator relative humidity (RH) and nest moisture remained within the target range values.

	Incubator 1 (no/min. evap. cooling)	Incubator 2 (limited evap. cooling)	Incubator 3 (limited evap. cooling/cool H <sub>2</sub> O)
Incubator air temp. (°C) mean ± SD	29.3 ± 0.34	29.4 ± 0.25	29.3 ± 0.20
Incubator % RH mean ± SD	94.8 ± 1.96	76.5 ± 5.20	76.0 ± 4.80
Nest temp. (°C) mean ± SD	29.1 ± 0.24	29.1 ± 0.15	29.0 ± 0.34
Nest moisture (m <sup>3</sup> m <sup>-3</sup> ) mean ± SD	0.10 ± 0.03	0.06 ± 0.02	0.08 ± 0.04

**Table 3**

Sex ratios by incubator across all nest boxes by treatment. Proportions of female:male hatchlings per nest box are given for each nest box (rows) and incubator (columns). Total hatchlings by nest box contributing to each sex ratio are in parentheses. In all incubators, the sex ratios were strongly male-biased. Incubator 1, with high humidity and no to minimal evaporative cooling, produced the highest mean proportion of females (just 9 completely male nest boxes), while incubator 3 had the lowest (11 completely male nest boxes). However, sex ratios among incubators were not statistically different. Note that observed proportions of females (\*) were significantly lower than either expected proportion. In all incubators, observed sex ratios differed from expected values derived from incubation duration (Inc Dur) (Mann-Whitney *U*-Test:  $H_{Inc1} = 21.00$ ;  $H_{Inc2} = 11.77$ ;  $H_{Inc3} = 14.55$ ;  $p < 0.01$ ) and from mean nest temperatures (Nest Temp) (Mann-Whitney *U*-Test:  $H_{Inc1} = 27.27$ ;  $H_{Inc2} = 27.87$ ;  $H_{Inc3} = 27.88$ ,  $p < 0.01$ ).

Date Laid	Incubator 1 (no/minimal evap. cooling)	Incubator 2 (limited evap. cooling)	Incubator 3 (limited evap. cooling & cool H <sub>2</sub> O)
12-Jul	0:1 (11)	0:1 (14)	0:1 (12)
13-Jul	0:1 (13)	0:1 (14)	0:1 (7)
15-Jul	0.31:0.69 (13)	0.23:0.77 (13)	0.27:0.73 (13)
16-Jul (A)	0:1 (14)	0:1 (10)	0:1 (12)
16-Jul (B)	0:1 (12)	0:1 (14)	0:1 (11)
17-Jul	0:1 (13)	0:1 (13)	0:1 (8)
18-Jul (A)	0.46:0.54 (13)	0.29:0.71 (13)	0.07:0.93 (12)
18-Jul (B)	0.08:0.92 (13)	0:1 (14)	0.07:0.93 (14)
19-Jul	0:1 (13)	0.09:0.91 (14)	0:1 (14)
21-Jul (A)	0.33:0.67 (9)	0.29:0.71 (11)	0.17:0.83 (13)
21-Jul (B)	0.27:0.73 (12)	0.15:0.85 (14)	0.09:0.91 (13)
27-Jul	0:1 (12)	0:1 (12)	0:1 (14)
28-Jul	0:1 (14)	0:1 (13)	0:1 (12)
29-Jul	0:1 (13)	0:1 (11)	0:1 (10)
31-Jul	0.43:0.57 (14)	0:1 (13)	0:1 (12)
2-Aug	0.21:0.79 (13)	0:1 (9)	0:1 (14)
Expected sex ratio <sub>IncDur</sub>	0.55	0.23	0.14
Expected sex ratio <sub>NestTemp</sub>	0.53	0.50	0.47
Obs. mean prop. female ± SE	0.13 ± 0.17*	0.07 ± 0.11*	0.04 ± 0.08*

the same conditions as in incubator 2 plus had the potential for the eggs to be cooled by 25 °C water treatments (average local rainfall temperature at the beach surface).

Differences in incubator evaporative cooling and water treatment temperature did not affect average nest temperatures. Nest box temperatures in all three incubators were approximately 0.3 °C lower than their respective incubator's air temperatures, which may be due to continuous evaporation of moisture from the sand surface. Moist sand may also buffer temperature changes so variation is less than in air. In natural nests, temperature fluctuations decreased in amplitude at greater sand depths (Lolavar and Wyneken, 2015). Eggs in the present study were encased in sand and ~15 cm below the surface. However, moisture enters or leaves the egg as water vapor (Ackerman, 1997) so we hypothesize that nest temperatures were necessarily cooler due to the state change of water in the sand to water vapor, thus lowering the clutch temperature slightly below that of air temperatures.

Our hatching success was high in all treatments and there was no evidence of differential mortality of female embryos in our study. Dead embryos that could be sexed were included in their nest box sex ratios (Tables 1 and 3).

Typically, incubation duration relates negatively to nest temperature. Incubation duration differed among the incubators, reflecting the developmental rate; yet, incubation temperatures (air and sand temperatures) were statistically identical among all incubators. What might account for the difference in incubation duration? Incubator 1 had the least variable environment, which likely promoted the most steady embryo growth. While overall average nest temperatures did not vary among incubators, we hypothesize that relative fluctuations in evaporation (incubators 2 and 3) and direct cooling (incubator 3) may have influenced embryo developmental rate. Differences in temperature variation among incubators may have also affected incubation duration. Ashmore and Janzen (2003) incubated smooth softshell turtle (*Apalone mutica* L.) eggs at fluctuating temperatures, which resulted in longer incubation durations compared to eggs incubated at constant temperatures.

Incubation durations can be an indicator of hatchling sex ratio trends because warmer (feminizing) temperatures cause more rapid embryonic development (Godfrey and Mrosovsky, 1997). Although we detected differences in incubation durations, sex ratios among incubators did not differ statistically and all were male-biased. Our results indicate that sex ratio proxies based on nest temperatures or incubation durations alone without verification (e.g., Hanson et al., 1998; Glen and Mrosovsky, 2004; Zbinden et al., 2007) may not provide accurate estimates of sex ratios when other environmental factors such as high moisture conditions (e.g., high rainfall) occur.

In the field, increased rainfall is associated with higher proportion of males. Godfrey et al. (1996) found increased production of male *D. coriacea* and *C. mydas* hatchlings in months with more rain in Suriname; these results were associated with cooler temperatures due to rainfall. Wyneken and Lolavar (2015) found higher male proportions in *C. caretta* nest samples in both wet years and normal years compared to dry years; in most cases, these natural nest samples incubated at higher incubation temperatures than would be predicted to produce males (Lolavar and Wyneken, 2015). It is theoretically possible that both evaporative cooling and cool rain contributed to the increased number of males in these field studies. However, sand temperature measurements at the level of the nest showed rain percolating through sand equilibrates to ambient sand temperatures and is no longer cool at even the upper-most loggerhead egg depths (Lolavar and Wyneken, 2015). Additionally, Ackerman (1997) states that similar to other flexible-shelled reptile eggs, loggerhead eggs have a high water vapor conductance, resulting in a fast rate of water loss at environments

only slightly drier than the egg itself. The natural nest environment of sea turtle eggs is very humid; eggs are turgid until just before pipping (except under extreme drought conditions; unpublished data), consequently the natural state likely minimizes evaporation and subsequent cooling. Together these lines of evidence suggest the potential for effects of increased sand moisture on sex ratios beyond simply a cooling effect.

One explanation for the similarity of sex ratios in our incubators with and without normal evaporative cooling of the surface sand may be that moisture impacts sex ratios by altering other aspects of the incubation environment. Previous studies of freshwater and sea turtle species noted that the hydric environment may affect various aspects of embryonic development including incubation duration (Steyermark, 1999), hatching success, residual yolk, and hatchling size (Paukstis et al., 1984; Packard et al., 1989; McGehee, 1990; Brooks et al., 1991; Cagle et al., 1993; Spotila et al., 1994). Tucker et al. (1998) found that even relatively small changes in moisture produced measurable effects on embryonic development, specifically hatchling size, residual yolk size, and incubation period. Other studies document the impact of the embryo's thermal environment on embryonic growth rate and on the developing gonads (Pieau and Dorizzi, 2004; Delmas et al., 2008).

We cannot rule out the possibility that the overwhelming and unexpected male bias in our study may also be a result of inaccurate pivotal temperature estimates for the loggerhead population on Florida's east coast. The previously established laboratory-based curve sets the pivotal temperature (1:1 sex ratio) at ~29 °C with some indication of occasional clutch-specific differences (Mrosovsky, 1988; Wibbels, 2003). The temperature range at which both sexes may be produced (transitional range of temperatures) for loggerheads in Florida is thought to span 26.5–32.0 °C based on laboratory and field calculations (Blair, 2005). Clearly, the currently established laboratory temperature–sex ratio response curves (Mrosovsky, 1988; Mrosovsky and Godfrey, 2010) do not provide accurate sex ratio predictions for loggerhead nests sampled in Boca Raton, Florida. Our experimental result may indicate that the temperature response curve is in fact shifted to the right, resulting in a higher pivotal temperature than previously thought. A right-shifted curve is consistent with 29 °C yielding a male bias rather than a 1:1 sex ratio and temperatures required to obtain a female bias could then be higher than predicted. This hypothesis may gain support because nest temperatures did not differ among treatments and all remained above 29.0 °C; yet, the majority of hatchlings were male. A future study to refine or confirm the pivotal and transitional ranges of temperatures will allow us to address the 29 °C pivotal temperature questions for the eastern Florida loggerhead population. The accuracy of primary sex ratio predictions for Florida loggerheads will be affected if the current temperature–sex ratio curve differs with nest moisture. Consequently, including moisture's impact on sex ratios is important to accurately predict sex ratios, particularly during periods of high nest moisture from rainfall.

Past studies focused on freshwater turtle eggs and differed in their incubation methods; LeBlanc and Wibbels (2009) applied liquid water treatments directly to the eggs incubating on racks; other studies incubated eggs in vermiculite at different moisture levels and temperature regimes (Gutzke and Paukstis, 1983; Paukstis et al., 1984; Packard et al., 1989). Few studies have examined the cooling or hydric effect of moisture on sex determination in sea turtles (Godfrey et al., 1996); most did not incubate eggs in nest-like conditions (eggs in contact with each other), natural substrates, and none were comparative experimental approaches representing many different mothers. The present experimental study is one of the first to use natural nesting beach sand as an incubation substrate, to incubate clusters of eggs together (similar to natu-

ral incubation conditions), and to allow DIH<sub>2</sub>O (simulated rain) to percolate through the sand to egg level.

Future comparative studies are warranted based on the findings of this study and because different species use different parts of the beach (and depths) to nest and so likely experience different thermal, moisture and evaporative cooling conditions (Whitmore and Dutton, 1985; Zavaleta-Lizárraga and Morales-Mávil, 2013; Ehrhart et al., 2014). Future laboratory studies are needed to quantify the amount of evaporation at different humidity levels occurring in standardized nests (such as nest boxes) in order to identify if or how much it may contribute to nest sand cooling.

Changes in precipitation will contribute to changes in nest moisture, so a clear understanding of moisture's role is essential in assessing possible impacts on sex ratios. Global climate change is considered a threat to sea turtle sex ratios (Hawkes et al., 2009; Hulin et al., 2009; Poloczanska et al., 2009; Jourdan and Fuentes, 2015). The impacts on Florida's climate are highly dependent on geographic location and also on season (Florida Oceans and Coastal Council, 2010; Misra et al., 2011). In the 21st century, peninsular Florida is predicted to experience overall drier conditions during June, July, and August, which coincides with peak loggerhead nesting. Climate models for Florida project an 8% decrease in average rain during these months (Misra et al., 2011). Southern Florida likely will experience drier conditions compared to northern Florida. Florida can expect increased frequency of heavy rainfall and intense storms (Goldenberg et al., 2001; Webster et al., 2005; Misra et al., 2011). Thus, nests may experience sudden large amounts of rain in a short span of time. How such episodic events will affect developing embryos is unclear, assuming they do not submerge eggs and result in suffocation (or nest washout). Changing precipitation will impact the several geographically distinct sea turtle populations differently. While we can predict changes in overall rainfall and total number of storms, the gaps in our understanding of TSD in natural systems weakens the predictive nature of temperature and sex ratio prediction.

## Acknowledgments

This study would not have been possible without the generous help of Gumbo Limbo Nature Center's sea turtle specialists, K. Rusenko, N. Warraich, M. Young, and the FAU sea turtle lab students. Earlier drafts of the manuscript were improved by S. Epperly, E. Frazier, K. Lohmann, J. Nambu, A. Rivera, M. Salmon and two anonymous reviewers. The study was conducted under permit MTP-073 and approved by the FAU IACUC protocol A12-07. Funding for this project was provided by The Disney Worldwide Conservation Fund (to J.W.), SaveOurSeas Foundation (J.W.), National Save the Sea Turtle Foundation (J.W.), the Nelligan Sea Turtle Fund (J.W.) and personal funds. A.L. received funds from an Undergraduate Research Grant at Florida Atlantic University and the National Science Foundation Undergraduate Research and Mentoring Program (#0839250) to E. Frazier and R. Murphey.

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