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Seawater irrigation on nests can increase male marine turtle production

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ABSTRACT: Global warming is increasing marine turtle nesting beach sand temperatures throughout the world. All marine turtles have temperature-dependent sex determination, with female hatchlings produced at warmer incubation temperatures. These warmer sand temperatures are causing a scarcity of male hatchlings at many nesting beaches. A range of mitigation strategies including shading and freshwater irrigation are being trialled at marine turtle nesting beaches around the world to address this issue. Because seawater is always abundant at marine turtle nesting beaches, we trialled a number of intense, one-off seawater irrigation experiments (equivalent to 100 and 200 mm rainfall) to test if male green turtle *Chelonia mydas* hatchling production could be increased without decreasing overall hatching success at Heron Island, southern Great Barrier Reef, Australia. We found that different combinations of seawater volume and temperature could produce a short-term drop in nest temperature by 2°C. When applied during the middle of embryonic development, these irrigation treatments could increase the proportion of male hatchlings compared to non-irrigated control nests, with less than a 10% decrease in hatching success. Hence, seawater irrigation has the potential to be a viable management strategy to increase the proportion of male marine turtle hatchlings at beaches that produce all, or nearly all, female hatchlings.

KEY WORDS: Green turtle · *Chelonia mydas* · Seawater · Irrigation · Sea turtle · Nest · Hatching success · Sex-ratio · Temperature-dependent sex determination

1. INTRODUCTION

Anthropogenic influence on global temperatures is threatening biodiversity worldwide (McCarty 2001, Descamps et al. 2017). Atmospheric temperatures have already increased by 1.3°C over the past 140 yr and are expected to increase to 2–4°C by 2100 (IPCC 2018). This rapidly changing climate may put animals into a state of physiological stress (Cooke et al. 2013). In response, some animals are changing geographical distribution, behaviours and life history traits (Visser 2008, Chen et al. 2011). Species need to keep up with, or outpace, these climate-induced environmental changes, and failure to do so leaves animals increasingly vulnerable to population decline and potential extinction (Gomulkiewicz & Holt 1995, Hoffmann & Sgró 2011, Refsnider & Janzen 2016).

Specifically, there is increasing concern for species that rely heavily on environmental temperature for important life-history processes, such as sex determination (Janzen & Paukstis 1991, Janzen 1994, Valenzuela & Lance 2004). Temperature-dependent sex determination (TSD) is a process by which environmental temperature, during a specific period of embryonic development, determines the sex of the offspring. TSD is believed to be the ancestral form of sex determination in vertebrates (Marshall Graves & Shetty 2001), with all crocodilians, tuatara, marine and many freshwater turtles, and lizards experiencing

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varied forms (Lance 2009). In marine turtles, the nest temperature during the middle third of constant temperature incubation (MTI), when the gonads are developing, determines the sex of hatchlings (Yntema & Mrosovsky 1980, 1982, Wibbels 2003). Low incubation temperatures produce males, high temperatures produce females, and intermediate temperatures produce a mixture of sexes. For the southern Great Barrier Reef (sGBR) green turtle *Chelonia mydas* population, the theoretical temperature that produces a 1:1 sex ratio is 28.1°C (Miller & Limpus 1981).

Due to this male–female pattern of sex determination and the rise in nest temperatures associated with recent climate warming, marine turtle primary sex ratios (i.e. the sex ratios at hatching) have tended to skew towards a strong female bias (Poloczanska et al. 2009, Hays et al. 2017). For example, at Australia's northern Great Barrier Reef (nGBR) on Raine Island, the largest green turtle rookery in the world, nest temperatures have been near or above all female-producing temperatures for the last 30 yr, resulting in 86.8% of adults, 99.8% of sub-adults and 99.1% of juveniles being female (Jensen et al. 2018).

To counter this trend towards highly skewed female primary sex ratios, marine turtles may shift the timing of their nesting to a cooler time of year (Laloë & Hays 2023). However, modelling has suggested that this strategy has limited ability to counter the increasing global warming trend (Laloë & Hays 2023). Another strategy might be to shift nesting location to a cooler beach. However, because such shifts would take multiple generations to occur, requiring hundreds of years, whereas climate warming is occurring in tens of years, this change cannot occur quickly enough (Fuentes et al. 2012). For these reasons, managers are actively seeking methods that can cool sand temperatures at current marine turtle nesting beaches. It is now well documented that both shade (Patino-Martinez et al. 2012, Hill et al. 2015, Jourdan & Fuentes 2015, Esteban et al. 2016, Staines et al. 2020, Reboul et al. 2021) and rainfall (Houghton et al. 2007, Lolavar & Wyneken 2015, Laloë et al. 2020, Staines et al. 2020) can cool sand temperatures at marine turtle nest depth. Moving clutches of eggs under shade structures in designated hatcheries can reduce nest temperatures by 0.6–2.0°C (Hill et al. 2015, Esteban et al. 2016, Staines et al. 2020, Reboul et al. 2021), but this can be logistically difficult because of the lack of personnel to move nests. Similarly, artificial rainfall (irrigation) can decrease nest temperatures by 0.6–2.2°C depending upon the frequency, volume and temperature of the water applied (Jourdan & Fuentes 2015, Staines et al. 2020, Lolavar & Wyneken 2021, Smith et al. 2021, Gatto et al. 2023). Typically, freshwater irrigation is used as a nest coolant, whereas seawater irrigation was recently trialled with promising outputs (Smith et al. 2021).

Irrigation cools nests by 2 mechanisms: (1) direct cooling due to cool water penetrating down to nest depth and (2) cooling the surface sand. Cooling the surface sand slows down heat transfer from surface sand to deeper sand, or may even reverse this trend if the deeper sand is cooler than the surface sand. Surface sand cooling occurs by a combination of direct temperature drop (when the water is cooler than the surface sand) and evaporation of water from the sand's surface. Small volumes of water (i.e. <30 mm rainfall) do not penetrate to nest depth, so surface sand cooling is the only mechanism operating when using small volumes of water and results in moderate 0.2–0.3°C cooling (Lolavar & Wyneken 2021). Larger volumes of water do penetrate to nest depth, and clearly, a greater nest temperature cooling effect will result with cooler irrigation water; but to date, only Jourdan & Fuentes (2015) and Smith et al. (2021) have reported the temperature of water used for irrigation.

Freshwater irrigation is benign to reptilian embryos, as long as the clutch is not immersed in water, because it helps embryos maintain a hydrated state and any excess water absorbed can be stored in the allantois and shed to the surrounding environment at hatching (Packard 1991). However, freshwater is frequently a precious commodity at marine turtle nesting beaches, so irrigation with freshwater may not be a viable management strategy at such beaches. On the other hand, seawater is always abundant at marine turtle nesting beaches, and therefore a relatively inexpensive resource for irrigation purposes. Despite this, seawater irrigation of marine turtle nests is not routinely used due to concerns that it may increase embryonic death caused by osmotic water loss from eggs exposed to seawater (Bustard & Greenham 1968). Indeed, green turtle eggs incubated in sand moistened with 75 and 100% seawater resulted in 0% hatching success (Bustard & Greenham 1968) and embryo deaths from tidal inundation and storm surges have also been reported (Foley et al. 2006, Pike & Stiner 2007). However, recent studies have found that marine turtle embryos could tolerate seawater for up to 6 h if embryos were exposed beyond the first week, and before the last week of incubation (Limpus et al. 2021), and that one-off exposure to an intense seawater irrigation event (equivalent to >100 mm rainfall) did not decrease hatching success of eggs incubated in clutches (Smith et al. 2021).

A one-off intense seawater irrigation event applied one-third through incubation has shown that it may not unduly stress embryos developing within a nest (Smith et al. 2021). This is likely because osmotic stress exposure is of shorter duration, and the eggs that surround the periphery of the nest (that are in continuous contact with sand and exposed to the greatest stress) can have the dehydrating effect buffered by exchanging water with eggs more centrally located within the nest. That is, the large volume of eggs within a clutch probably can act as a water reservoir for the outer eggs, and this may be why one-off exposure to seawater does not result in increased embryo mortality (Smith et al. 2021). A one-off intense seawater irrigation event can decrease nest temperature by up to 1.5°C, with the cooling event lasting several days (Smith et al. 2021). If timed to occur during the sex-determining period, short-term cooling events have the potential to increase male hatchling production (Woolgar et al. 2013, Porter et al. 2021).

The aims of our study were to confirm that a one-off intense seawater irrigation event does not increase marine turtle embryonic mortality, and to determine whether the proportion of male hatchlings can be increased if such an event is conducted during the sex-determining period. The latter aim was assessed by sampling hatchlings emerging from nests and directly determining their sex via histological examination of gonads.

2. MATERIALS AND METHODS

2.1. Study site

Our study used clutches of the sGBR population of green turtles nesting on Heron Island during the 2020–2021 nesting season. Heron Island is a vegetated, coral-sand cay located 80 km off the east coast of Australia (23° 26.588′ S, 151° 55.086′ E) within the Capricornia Bunker group of islands and is considered a medium-density nesting site for green turtles (Limpus 2008). The study site was located behind the first dune on the eastern side of the island at Shark Bay. Female tracks and nests indicated that green turtles nest in this area of the island. Grass and leaf debris were removed from a 4 × 4 m plot, and the site was barricaded with fallen trees and branches to stop females from nesting in the study site while experiments were in progress but allowed hatchlings to escape the site. Setting up of experimental nests, irrigation application and hatchling collection occurred during 11–14 December 2020, 11–14 January 2021 and 8–19 February 2021, respectively. All research procedures were approved by The University of Queensland's animal ethics committee (SBS/237/20). All egg and hatchling collection was completed under Queensland Parks and Wildlife Service (QPWS) Scientific Purposes Permit number PTU19-002377-1.

2.2. Experimental design

At the study site, 16 locations for nests were marked with wooden stakes in a 4 × 4 m array, following a Latin square design. The centre of each nest was 1 m from the next. The Latin square design randomised treatment position and controlled for any row or column effects on embryonic survival and sex ratios. There were 5 treatments: no irrigation (control), 4 replicates; 100 mm of simulated rainfall using freshwater (FW), 3 replicates; 100 mm of simulated rainfall using seawater (SW), 3 replicates; 100 mm of simulated rainfall using chilled seawater (CSW), 3 replicates; and 200 mm of simulated rainfall using seawater (SW200), 3 replicates. Daily rainfall data were obtained from the Heron Island Research Station (HIRS) weather station.

2.3. Field procedures

Over 4 consecutive nights, 11–14 December 2020, beaches were patrolled and 4 clutches were collected per night from nesting females. During oviposition, eggs were collected as they were laid and placed into a plastic bucket and once laying ceased, the entire clutch was transported to the study site, a 5–10 min walk away. At the study site, artificial nests were dug at the stake locations, with the bottom of the nest 65 cm below the sand surface (the average nest depth for this nesting beach, Limpus 2008). Eggs were counted, and after half the clutch was transferred into the artificial egg chamber, a previously calibrated (Staines et al. 2022) iButton temperature logger (Maxim Integrated model DS1922L-F5) sealed inside a 70 ml plastic specimen jar along with 1 probe from a dual probe HOBO temperature logger (ONSET model MX2303) was placed on top of the eggs. Both loggers were programmed to log temperature once per hour. The remainder of the clutch was then placed into the nest and sand backfilled on top of the entire clutch to the level of the surrounding sand surface.

In laboratory experiments, brief decreases in incubation temperature around the half-way point of em-

bryonic development can increase male hatchling production (Porter et al. 2021); therefore, we targeted the half-way point of embryonic development to determine the time of water application to the irrigated nests. To estimate the half-way point of development in nests, we periodically downloaded nest temperature data as incubation progressed. These temperature data were then imported into an algorithm that calculates embryo development stage from nest temperature traces (Booth et al. 2022). Embryos are between Phases 3 and 4 of development as described by a 1- to 6-phase visual staging guide (Booth et al. 2020) half-way through development. Therefore, we assessed embryo mortality during Phases 3 and 4, as well as embryo mortality post Phase 3 to discover if irrigation immediately affected embryo mortality, and mortality post irrigation, respectively. On the day before the one-off irrigation event occurred, seawater was collected adjacent to the shore in the afternoon (between 15:00 and 17:00 h) and was kept in the shade overnight to cool in 52 l closed plastic bins (n = 5). An additional 9 l of seawater were cooled in a −18°C freezer at HIRS overnight to reduce the temperature of the CSW treatment. Freshwater was collected from taps at HIRS the afternoon before application and transported to the study site, placed in 52 l covered plastic bins in the shade in an identical manner to seawater. Between 11 and 14 January, the assigned irrigation treatment was applied to nests before sunrise (between 04:30 and 05:10 h), when the sand surface and water were at their coolest. Water temperature was measured immediately before application using a digital thermocouple. The assigned volume of water — 54 l per nest for 100 mm treatments (FW, SW and CSW) and 108 l per nest for 200 mm treatments (SW200) — was applied using 9 l watering cans over a 5027 cm^2 area of sand around the centre of each nest as marked by an 80 cm diameter ring using continuous circular motions with the watering can until all water was applied. Two rain gauges within the watered area were used to measure the rainfall equivalent. Irrigation took no longer than 5 min per nest. Following irrigation, the clutches were left to develop without further intervention until hatching.

On 8 February 2021, each nest was gently excavated by hand until the top of the eggs was reached, and a hatching detector was placed on top of the eggs (Booth et al. 2022). If hatchlings were felt or seen, or movement within the chamber was detected, the nest was refilled without setting up a hatch detector. In nests fitted with hatching detectors, the voltage was tested 4–6 times per day, and hatching was indicated by a drop in voltage below 9 V (Booth et al. 2022). Once hatching was detected, plastic 'gutter guard' mesh corrals (diameter = 48 cm, height = 18 cm) were placed on top of the nests every afternoon (~15:00–17:00 h) and removed every morning at ~06:00 h until the clutch emerged. Corralled nests were visited at ~2 h intervals throughout the night to check for emergent hatchlings. When at least 30 hatchlings emerged, a random sample of 10 hatchlings was collected and transported to HIRS, which was a 15 min walk away. Once a sample of hatchlings was collected, the corrals were removed to allow the remaining hatchlings to make their own way to the sea.

Two days after the first hatchling emergence from a nest, the nest was excavated by hand. The number of empty shells (successful hatchings) and unhatched eggs (deceased embryos) were counted. Unhatched eggs were opened and the phase of embryonic development was assessed visually using a Phase 1 to 6 staging guide (Booth et al. 2020). These data were used to calculate hatching success and the proportion of embryos that died during different phases (Booth et al. 2020).

2.4. Laboratory procedures

At HIRS, each hatchling was assigned a unique number ('nest number' – 'hatchling number') which was written on their plastron with a black marker pen. A blood sample for an adjunct study was taken from either the external jugular vein or transverse cervical vein on the back of the neck before the hatchlings were humanely euthanised via isoflurane overdose for adrenal–kidney–gonad (AKG) complex extraction and subsequent sex identification from gonad features (Miller & Limpus 2003). Hatchlings were euthanised by placing them in a sealed glass jar with isoflurane anaesthetic. After at least 30 min in the jar, which was about 20 min after the last movement was noted, hatchlings were removed and the AKG complexes removed by making an incision near the cloaca on the ventral surface of the hatchling and cutting along the carapace–plastron junction on both sides of the hatchling. The plastron was then pulled back, exposing the body cavity, and the intestines were removed to expose the AKG complexes. The right AKG complex was carefully removed from the surrounding connective tissue and placed into a 10 ml sample vial with 10% neutral buffered formalin solution before being transferred to 70% ethanol 2 wk later at the University of Queensland St Lucia campus for storage until histological preparation.

At The University of Queensland's School of Biomedical Sciences histology laboratory, the preserved AKGs were placed into a histology cassette, dehydrated and exposed to a treatment of paraffin wax in an automated Leica ASP300S tissue processor following tissue embedding in a block of paraffin wax using a Medite TES Valida Embedding Station arranged for transverse sectioning of the tissue. Once the wax blocks cooled, a Leica rotary microtome (HistoCore MULTICUT) was used to section the tissue at 6 µm. Approximately 300 µm of the embedded AKG was cut and discarded, and then a ribbon of approximately 102 µm was cut and set aside. This process was repeated until 6 ribbons were set aside. The first 6 µm section of each ribbon was placed onto a glass test slide and viewed under a compound microscope. Where the gonad was visible, 3 serial sections were fixed to a glass slide for later identification of ovarian or testicular tissue. This sectioning protocol produced 3 slides of various sections along the length of each AKG, ideally including the beginning, middle and end of each gonad. Specific locations were not essential. The slides were left to air dry for a minimum of 12 h and then placed in an oven at 38°C for 15 min before staining. All slides were stained using Mayer's haematoxylin and eosin staining procedure (Feldman & Wolfe 2014). Once stained, slides were mounted with a coverslip using Depex and left in a 38°C oven to set for a minimum of 30 min. Slides were then relabelled for blinded gonad identification. The 6 µm transverse sections (n = 3) from each AKG were examined under a Nikon Eclipse Ci microscope at 10× magnification for differences in cortex, medulla and Müllerian ducts (paramesonephric ducts) as described by Miller & Limpus (2003). Photos of each slide were captured using a Nikon DS-Fi1c camera attached to the Nikon Eclipse Ci microscope and were assessed by 3 independent experts for gonad identification. The samples were then un-blinded and hatchling sex ratios for each nest were calculated.

2.5. Quantifying the amount of cooling due to irrigation

At the time irrigation water was applied to nests, each nest was at a slightly different temperature compared to the other nests. Therefore, simply comparing the nest temperatures across different treatments would not give an accurate measure of the effectiveness of the water application on cooling nests. To overcome this problem, the amount of cooling was quantified by calculating the degree-day temperature cooling effect for each nest. First, the nest temperature in the hour immediately prior to application of water was assigned as the starting point temperature, and the individual nest temperature traces were examined to determine how long it took nests to return to the starting point temperature after water application. This period of time was termed the 'cooling interval'. Second, the difference in temperature between the starting point temperature and the nest temperature for each hour during the cooling interval was calculated. The sum of these hourly differences in temperature throughout the cooling interval was then divided by 24 (the number of hours in a day) to calculate the degree-day cooling effect for each nest.

2.6. Statistical analysis

R statistical software (R Core Team 2020) was used for all statistical analyses. Separate generalised linear mixed-effects models (GLMMs) were performed for all hatching success (%), embryonic survival (%) and sex-ratio (%) analyses under a binomial distribution, with irrigation treatment as the fixed effect, and row and column as random effects. A 2-way ANOVA determined the significance of treatment on hatching success and sex ratios, followed by Tukey post hoc tests that identified which treatments were significantly different from each other. Row and column as random effects were included in the models when they had a significant effect on the dependent variable to form a linear mixed-effects model (LMM); otherwise, a linear model (LM) was created. All models were tested for normality, and results are presented as means ± SE. Results were considered statistically significant at $p \leq 0.05$. A Spearman rank-order correlation was used to test for a relationship between the average nest temperature during the MTI and the proportion of male hatchlings produced.

3. RESULTS

3.1. Nest temperature

Clutch size averaged 116 and did not differ between treatments (Table 1). From oviposition until the time of irrigation, all nests experienced similar temperature profiles (Fig. 1), with an average incubation temperature of 27.4 ± 0.1°C, regardless of treatment (Table 1). The amount of 'rainfall' received

Table 1. Green turtle clutch sizes, nest temperatures during different stages of incubation, incubation periods, degree-day cooling after water application, proportion of male hatchlings, hatching success, embryo mortality during Phases 3 and 4 of development, and embryo mortality post Phase 3 of development from the different irrigation treatments. The proportion of males was determined by histological examination of hatchling gonads. Sample size was 4 for control clutches, and 3 clutches for all irrigation treatments. Data are presented as means ± SE. For statistical analyses with a significant row and/or column effect from the irrigation treatment on the response variable, linear mixed-effects models were fitted to the data (*F*-values). When there was no significant row or column effect on the response variable, the model was simplified to a linear model (χ^2 values). Control: no irrigation; FW: 100 mm rainfall equivalent with freshwater; SW: 100 mm rainfall equivalent with seawater; CSW: 100 mm rainfall equivalent with chilled seawater; SW200: 200 mm rainfall equivalent with seawater; MTI: middle third of incubation; NA: not applicable

	Control	FW	SW	CSW	SW200	df	Probability statistics	p	Post hoc analysis
Clutch size (n)	123 ± 6	108 ± 17	121 ± 13	111 ± 5	117 ± 8	4, 11	$F = 1.16$	0.38	Control = FW = SW = CSW = SW200
Entire incubation nest temperature (°C)	29.7 ± 0.2	29 ± 0.3	29.6 ± 0.2	29.1 ± 0.5	28.7 ± 0.2	4, 11	$F = 2.34$	0.12	Control = FW = SW = CSW = SW200
Incubation temperature before irrigation (°C)	27.3 ± 0.1	27.3 ± 0.1	27.7 ± 0.1	27.4 ± 0.2	27.3 ± 0.1	4	χ^2 (N = 12) = 5.079	0.171	Control = FW = SW = CSW = SW200
MTI nest temperature (°C)	28.8 ± 0.1	28.3 ± 0.1	28.9 ± 0.1	28.4 ± 0.1	28.3 ± 0.1	4, 11	$F = 8.17$	0.003	Control > CSW, FW, SW200 Control = SW SW200 = CSW = FW = SW
MTI duration (d)	19.6 ± 0.1	20.6 ± 0.1	19.5 ± 0.1	20.4 ± 0.1	20.5 ± 0.1	4, 11	$F = 7.1$	0.009	Control = SW < FW = CSW = SW200
Temperature drop after irrigation (°C)	NA	−0.8 ± 0.2	−1.0 ± 0.2	−1.7 ± 0.7	−2.0 ± 0.2	3, 8	$F = 1.08$	0.41	FW = SW = CSW = SW200
Temperature drop duration after irrigation (d)	NA	4.08 ± 0.7	3.6 ± 0.3	4.03 ± 0.6	4.3 ± 0.1	3, 8	$F = 0.41$	0.75	FW = SW = CSW = SW200
Degree-day cooling (°C d^{-1}) after irrigation	NA	1.2 ± 0.2	1.2 ± 0.2	2.4 ± 0.9	2.7 ± 0.3	3, 8	$F = 4.21$	0.048	FW = SW < CSW = SW200
Incubation period (d)	62.8 ± 0.6	64.7 ± 0.5	63.7 ± 1.5	63.5 ± 0.6	62.8 ± 1.6	4, 11	$F = 0.61$	0.66	Control = FW = SW = CSW = SW200
Proportion of males (%)	12.5 ± 7.5	81.2 ± 10.5	46.7 ± 14.5	23.3 ± 15.3	63.3 ± 12.0	4	χ^2 (N = 16) = 32.24	<0.001	FW > SW, CSW, control SW200 > CSW, control control = CSW = SW SW200 = FW, SW
Hatching success (%)	77.6 ± 6.8	68.2 ± 13.8	69.6 ± 7.6	85.0 ± 5.0	67.2 ± 1.1	4	χ^2 (N = 16) = 28.79	<0.001	SW200 < CSW, control Control = FW = SW = CSW FW = SW = SW200
Mortality during Phases 3 and 4 (%)	3.1 ± 1.9	5.5 ± 4.8	8.0 ± 3.2	2.3 ± 1.2	1.6 ± 1.0	4	χ^2 (N = 16) = 5.123	0.192	Control = FW = SW = CSW = SW200
Mortality post Phase 3 (%)	13.2 ± 6.6	17.5 ± 13.5	19.4 ± 5.8	10.8 ± 3.5	23.0 ± 2.1	4	χ^2 (N = 16) = 72.98	<0.001	SW200 > FW, SW, CSW, control CSW > control, FW control = FW, SW SW = CSW

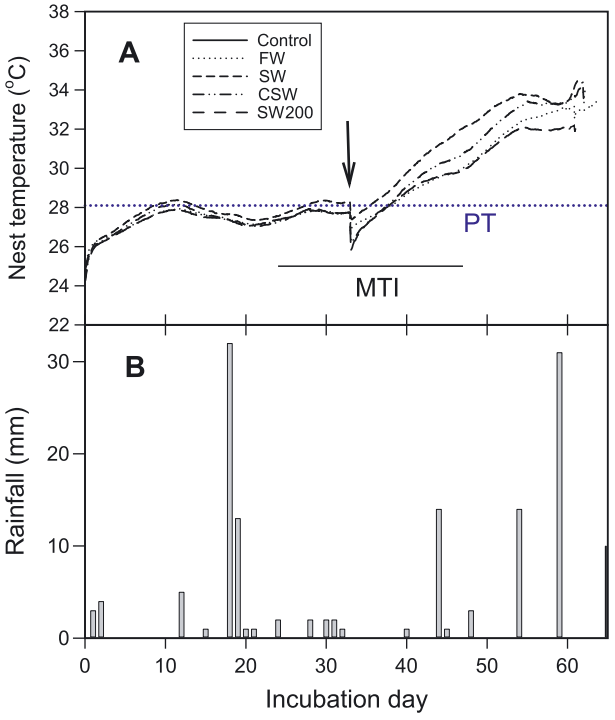

Fig. 1. (A) Mean nest temperatures for the various treatments throughout incubation. Control: no irrigation; FW: 100 mm rainfall equivalent with freshwater; SW: 100 mm rainfall equivalent with seawater; CSW: 100 mm rainfall equivalent with chilled seawater; SW200: 200 mm rainfall equivalent with seawater; PT: pivotal temperature; MTI: middle third of incubation. Vertical arrow = time when irrigation water was applied. Sample size was 3 nests except for the control where the sample size was 4. (B) Daily rainfall totals throughout incubation

on nests during irrigation as recorded in rain gauges was 115 ± 17 mm in the 100 mm treatments (FW, SW and CSW) and 242 ± 15 mm in the 200 mm treatments (SW200). The temperature of water in the freshwater and seawater treatments averaged 24.3 ± 0.2°C, and 19.7 ± 0.2°C for the CSW treatment. Immediately after irrigation, all irrigated nests experienced a sharp decrease in temperature, and after this, all nests, including control non-irrigated nests, experienced an increase in temperature, but the pattern of this increase varied across treatments (Fig. 1A). Despite this variation, the average temperature across the entire incubation period was similar (~28.8°C, Table 1). As a consequence, there was no difference in the incubation period between treatments (Table 1).

The magnitude of the decrease in temperature after the application of water was calculated as the difference in nest temperature 1 h prior to irrigation and the lowest nest temperature reached after irrigation. All irrigation treatments reduced the nest temperature below the population's pivotal temperature (28.1°C) for varying durations within the MTI (Fig. 1A, Table 1). There was no difference in the duration of the nest temperature reduction after irrigation between irrigation treatments, with this duration averaging 4 d (Table 1). Average nest temperature during the MTI varied between treatments (Fig. 2). All irrigation treatments with the exception of SW were cooler than control nests (Table 1). Duration of the MTI varied across treatments (Table 1), with controls and SW treatments being similar, but shorter than the other irrigation treatments (Table 1). The amount of cooling as indicated by degree-day analysis varied between irrigation treatments (Fig. 3) with the amount of cooling being similar in the FW and SW treatments but greater cooling occurring in the CSW and SW200 treatments (Table 1). Daily rainfall totals up to 32 mm were recorded during the nest-monitoring period (Fig. 1B), but these rainfall events did not result in conspicuous changes in nest temperature (Fig. 1A).

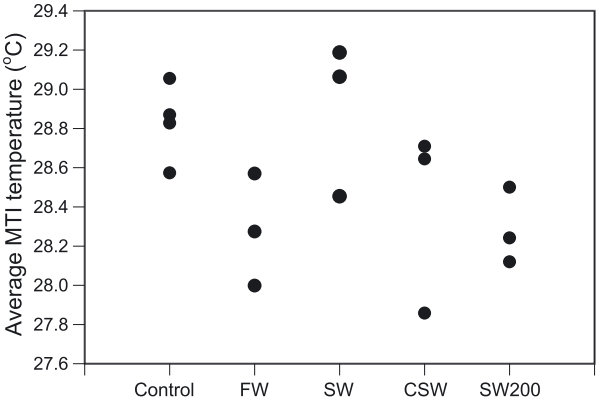

Fig. 2. Average temperature during the middle third of incubation (MTI) for the various treatments. Sample size was 3 clutches except for the control where the sample size was 4. Treatments as in Fig. 1

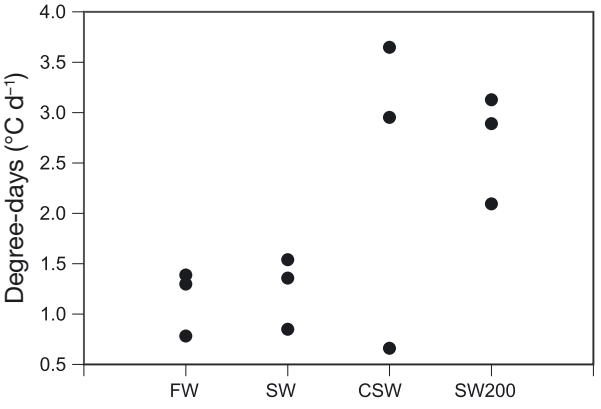

Fig. 3. Degree-day cooling immediately after application of irrigation water to the various treatments. Sample size was 3 clutches for all treatments. Treatments as in Fig. 1

3.2. Hatching success and embryo death

Hatching success varied across treatments (Fig. 4), with clutches in the SW200 treatments having lower hatching success compared to control and CSW treatments (Table 1). Hatching success was similar for all treatments except SW200 (Table 1). Embryonic mortality during Phases 1 and 2 occurred before irrigation so are not attributable to treatment effects. Embryo mortality during Phases 3 and 4 varied between treatments, but post hoc analysis indicated that the only difference was between the SW and CSW treatments (Table 1). Overall, there was less than 8% mortality during development Phases 3 and 4 (Table 1). Embryo mortality post Phase 3 varied between treatments, with embryo mortality being greater in the SW200 treatment compared to all other treatments, and the CSW treatment had greater mortality compared to the control and FW treatments (Table 1).

3.3. Male production

Histology of gonads unequivocally identified the sex of all hatchlings sampled. Irrigation treatments influenced the proportion of male hatchlings produced (Fig. 5, Table 1). The FW treatment produced a greater proportion of males than the SW, CSW and control treatments. The SW200 treatment also produced more males than CSW and control treatments. The control treatment had 2 nests that produced no male hatchlings, and within the FW treatment, 1 nest produced 100% male hatchlings. The proportion of male hatchlings produced was correlated with the average temperature during the MTI (Fig. 6).

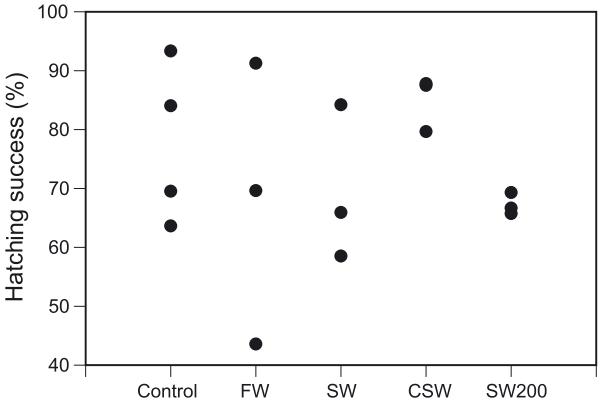

Fig. 4. Hatching success for the various treatments. Sample size was 3 clutches except for the control where the sample size was 4. Treatments as in Fig. 1. Note that 2 clutches in the CSW treatment had 87% hatching success

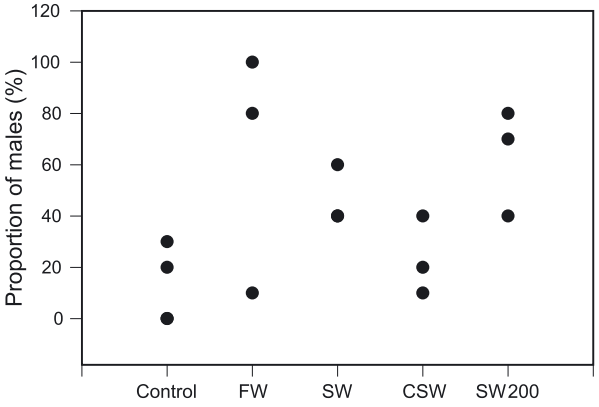

Fig. 5. Proportion of male hatchlings for the various treatments. Sample size was 3 clutches except for the control where the sample size was 4. Each nest had 10 hatchlings sampled for sex determination. Treatments as in Fig. 1. Note 2 control nests produced no males, and 2 SW clutches produced 40% males

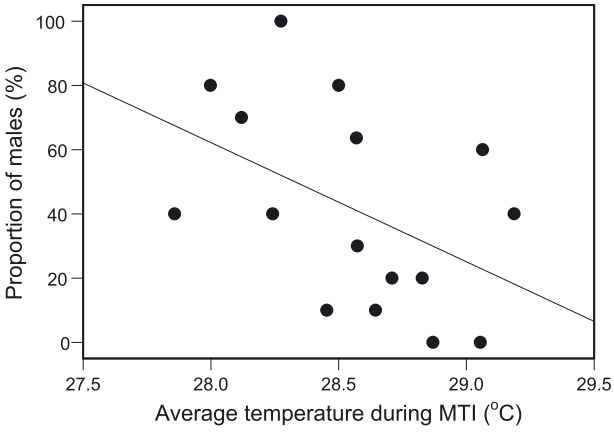

Fig. 6. Proportion of male hatchlings against average temperature during the middle third of incubation (MTI). A Spearman rank-order correlation was significant (Spearman r = –0.504, t(N–2) = –2.186, p = 0.046, N = 16)

4. DISCUSSION

4.1. Cooling effect of irrigation water

There is no doubt that a one-off irrigation event can cool sand temperatures in a similar manner to natural rainfall events (Houghton et al. 2007, Lolavar & Wyneken 2015, 2021, Laloë et al. 2016, 2021, Staines et al. 2020, Smith et al. 2021, Gatto et al. 2023, this study). Our one-off irrigation protocol induced a direct cooling event, as indicated by an immediate decrease in nest temperature, and stayed cool for 4 d, similar to the one-off irrigation events tri-

alled by Smith et al. (2021). The volume of water applied (equivalent to 100–200 mm of rainfall) was enough to absorb the heat stored in the column of sand to the nest depth and beyond. The lowest temperature a nest can reach in this circumstance is the temperature of the water applied, but may not reach this temperature if the volume of water applied is insufficient to absorb all of the heat from the sand column above the nest. In our case, the volume of water applied was not enough to absorb all the heat from the sand because even when 200 mm of equivalent rainfall was applied, nest temperature did not fall to the temperature of the water applied. Similar results were observed by Smith et al. (2021) when they used the equivalent of 100 mm of rainfall. Other studies have used more frequent (usually daily) irrigation events which also cooled sand by 2–3°C, but the cooling was sustained for the duration of irrigation (Hill et al. 2015, Jourdan & Fuentes 2015, Erb et al. 2018, Lolavar & Wyneken 2021). When using irrigation events to cool sand at nest depth, applying greater volumes of water has the potential to decrease nest temperature to a greater extent. In our study, the minimum temperature obtainable would be ~20°C if using chilled water and ~24°C if using air-temperature water. The temperature of the water we applied in the air-temperature water treatments (24.3°C) was within the range of rainfall water temperature (23.8–26.9°C) recorded during 3 separate rainfall events on Heron Island during February 2023 (D. T. Booth unpubl. data), but occasional tropical storms can include hail, so such events would have a greater cooling potential. For instance, heavy rainfall events have been noted to drop nest temperatures, or sand temperature at nest depth, by 2–4°C (Godfrey et al. 1996, Matsuzawa et al. 2002, Houghton et al. 2007, Lolavar & Wyneken 2015, Laloë et al. 2016, 2021, Rivas et al. 2018, Staines et al. 2020). On Heron Island, 'light' rainfall events (<32 mm) do not result in a detectable decrease in sand temperature at green turtle nest depth (Fig. 1). Light rainfall was also found not to affect sand temperature at nest depth in experiments conducted by Jourdan & Fuentes (2015). Light rain probably does not affect sand temperature at nest depth because bulk water flow fails to reach nest depth during these events.

As the amount of cooling achieved depends on several factors including volume of water applied and the temperature of water applied (reviewed by Gatto et al. 2023), the CSW and SW200 treatments were expected to result in a greater cooling effect than the FW and SW treatments, which is what was recorded. Nest temperatures were able to cool by 2°C, from 28 to 26°C, by applying either the equivalent of 100 mm rainfall with chilled water (CSW) at 19.7 ± 0.2°C or 200 mm rainfall of air temperature water (SW200) at 24.3 ± 0.2°C. This 2°C drop in temperature was enough to bring nests into the male-dominated temperature range. However, these cooling events were short-lived, only lasting 4 d on average. Longer cooling periods would probably occur if the area of the irrigated sand was increased to cover the entire plot, such as might occur in a beach-management situation where mechanical sprinklers could be used for irrigation (Gatto et al. 2023). In our trials, only a limited area immediately above the nest was irrigated, and heat from the surrounding non-irrigated sand would have been conducted laterally inwards to the nest, causing a shortening of the cooling event. If all of the sand in the area surrounding nests was cooled by irrigation, this lateral movement of heat would be minimal, and heating would occur almost exclusively from the sand surface downwards, prolonging the nest re-heating process.

4.2. Hatching success

Theory suggests that because seawater has a greater osmolarity than sea turtle embryo fluids, sea turtle eggs incubating in seawater-saturated sand should lose water to the surrounding sand and become dehydrated, which would cause embryo death. Indeed, when eggs are incubated individually completely surrounded by sand and watered with seawater, they rapidly dehydrate and die (Bustard & Greenham 1968). For this reason, using seawater irrigation as a management strategy to cool incubating sea turtle nests has not been seriously considered until recently. We found that our SW200 treatment was the only seawater irrigation treatment to show a decrease in hatching success compared to non-irrigated nests. However, this conclusion needs to be treated with caution because a power analysis indicated that a sample size of 10 nests in each treatment would be needed to be 95% confident of no difference between groups. Examination of eggs that died during incubation revealed that there was no spike in embryo death in the few days after irrigation and that with the exception of the SW200 treatment, there was no elevation in embryo mortality compared to control nests. The greatest mortality in the SW200 treatment occurred during development Phases 5 and 6, when metabolic heat production and nest temperatures were highest. The combination of high nest temperature and osmotic

stress may have caused the observed elevation in embryo mortality in this treatment. Even in the SW200 seawater irrigation treatment, the increase in embryo mortality we observed was small (<10%). Although our sample sizes are small, our results are similar to those of Smith et al. (2021), who also found that one-off intense irrigation of green turtle clutches with seawater did not result in increased embryo mortality when 100 mm rainfall equivalent of seawater was added. The combination of results from our study and that of Smith et al. (2021) clearly indicate that one-off intense irrigation of entire marine turtle clutches does not cause a decrease in hatching success, although the sample size is still relatively small (15 nests). The probable explanation for this finding is that the entire clutch of eggs with its large combined volume of water acts as a buffer to the peripherally located eggs within the clutch that are in direct contact with the seawater-saturated sand surrounding the nest (Smith et al. 2021).

The possible direct effect of heavy rainfall events on sea turtle hatching success has not been reported. However, because beach sands are usually well drained, it is unlikely that eggs would be immersed in water for very long, and so a decrease in hatching success would not be expected. Indeed, short-term total immersion in both freshwater and seawater have little effect on egg mortality, except at the very beginning and very end of incubation (Limpus et al. 2021).

4.3. Male hatchling production

We sampled 10 hatchlings from the first cohort of hatchlings to reach the surface for sex determination in each nest. This first cohort varied in number from 30 to 80 hatchlings, and in some cases, hatchlings continued to emerge in small cohorts for up to 24 h after the first emergence event. It is possible that one sex has a propensity to emerge from nests earlier than the other sex in nests with multiple emergence events. However, we are not aware of any evidence for such a phenomenon, so until proven overwise, we assume that the 10 hatchings we sampled reflect the sex ratio of the entire clutch within a nest. The brief cooling period that intense irrigation provided midway through incubation increased the production of male hatchlings above that of the non-irrigated control nests. Controlled laboratory experiments have previously demonstrated that brief exposure (<5 d) to male-producing temperatures at otherwise all-female producing incubation temperatures can cause some male hatchling production (Woolgar et al. 2013, Porter et al. 2021), but our study is the first to demonstrate this *in situ*. We also found that the average temperature during the MTI was correlated with the proportion of male hatchlings, with lower temperatures tending to produce more males. However, this relationship was not at all similar to the classic sigmoid-shaped curve describing the relationship between incubation temperature and hatchling sex ratios obtained through constant temperature laboratory data (Hays et al. 2017). This difference might be explained by 2 phenomena. First, it is known that there can be considerable inter-clutch variation in marine turtle hatchling sex ratios even when the clutches are incubated under identical incubation temperature regimes when incubation temperatures are within the variable sex ratio zone (Porter et al. 2021), so the scatter in data points might reflect this fact. Second, in our nests, temperature was continuously changing, and varied from highly male-skewed temperatures to highly female-skewed temperatures for different periods of time. Under such circumstances, the driving forces behind sex determination are complicated and difficult to predict even if the exact temperature profiles are known, and a simple average temperature is a poor predictor of hatchling sex ratios (Monsinjon et al. 2022).

4.4. Conservation management implications

One of the major issues faced by marine turtle population managers is the increasing trend of extreme female hatchling bias at major sea turtle nesting beaches due to an increase in global temperatures (Poloczanska et al. 2009, Hays et al. 2017). A possible management strategy is irrigation with freshwater on a regular basis (Hill et al. 2015, Lolavar & Wyneken 2021), but such strategies would use large volumes of freshwater, a resource that is scarce or expensive at many nesting beaches. We have demonstrated that using targeted one-off intense seawater irrigation can increase the proportion of male hatchlings by decreasing nest temperature, the degree of the temperature decrease being controlled by a combination of water temperature and volume of water applied. Because seawater is always available in abundance at marine turtle nesting beaches, this method has the potential to be broadly applied. However, there are some considerations that need to be thought through before its implementation (Gatto et al. 2023). First, this sand cooling strategy is most effective when the seawater being applied is considerably cooler than

the sand temperature. For example, intensely irrigating 30°C sand with 29°C seawater will not decrease nest temperatures significantly. Next-to-shore seawater at marine turtle nesting beaches during the nesting season can be relatively warm, typically 27–30°C, and this is why we collected our water the afternoon before application (collection temperature was 28–29°C) and had it cool overnight before being applied. Cooler water may need to be pumped from deeper water further away from the shore in order to provide effective cooling. Secondly, intense irrigation with seawater cannot be repeated frequently across a nesting season because salt could accumulate in the sand to high levels, which would be detrimental to the development of eggs. Seawater irrigation could be easily instigated at intensely managed marine turtle nesting beaches where freshly laid clutches are routinely moved into protected hatcheries, a situation where multiple clutches of similar age can be incubated close together. In this situation, the intense one-off seawater irrigation can be targeted to the mid-incubation period (Porter et al. 2021) to ensure the cooling event occurs during the MTI. One-off intense seawater irrigation could also be used at high-density nesting beaches to produce a 'pulse' of increased male production by irrigating stretches of the beach a few weeks after the peak in nesting activity (i.e. when many nests will be withing the MTI). At medium- to low-density nesting beaches that are intensively patrolled on a nightly basis (even if only during the peak nesting period), individual nests could be marked when laying occurs and then re-found midway through incubation (i.e. during the MTI) and intensely irrigated with seawater. To conclude, our trials have demonstrated that an intense one-off irrigation with seawater midway through incubation can increase the proportion of male green turtle hatchlings without decreasing hatching success, providing another tool for marine turtle nesting-beach managers that are seeking to decrease the highly female-biased hatchling sex ratios currently encountered at many nesting beaches world-wide.

At nesting beaches where sand temperatures regularly are so high (i.e. >33°C) that they cause the direct mortality of embryos, regular irrigation could be used to lower sand temperatures over a prolonged period. However, in such cases, freshwater irrigation would need to be used because multiple bouts of seawater irrigation would lead to accumulation of high levels of salt in the sand. Prolonged exposure to high salt concentration would almost certainly cause an increase in embryo mortality, and therefore would not be a viable management strategy.

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