Nest temperature assessment in an American crocodile (*Crocodylus acutus*) population on the central coast of Oaxaca, Mexico

René Barragán Lara [a], Jesús García Grajales [b,*], Emilio Martínez Ramírez [a]

[a] *Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional Unidad Oaxaca (CIIDIR Unidad Oaxaca), Instituto Politécnico Nacional, Calle Hornos 1003, Municipio Santa Cruz Xoxocotlán, 71230, Oaxaca, Mexico*
[b] *Instituto de Recursos, Universidad del Mar campus Puerto Escondido, Km. 2.5 Carretera Federal Puerto Escondido – Sola de Vega, Puerto Escondido, 71980, San Pedro Mixtepec, Oaxaca, Mexico*

ABSTRACT

The temperatures at which eggs of crocodilians are incubated plays an important role in embryo survival, rate of embryonic development and sex definition. The aim of this study was to assess the nest temperatures of an American crocodile (*Crocodylus acutus*) population on the central coast of Oaxaca state in Mexico. The fieldwork was carried out from February to June 2018 at Palmasola Lagoon, Oaxaca. Ten natural nests of *C. acutus* were carefully excavated to determine clutch size. When putting the eggs back in the nests, we placed a data logger in the center of the egg mass to determine the temperature parameters in the nest chamber environment, as well as the variation in temperature during the incubation period. All nests were revisited to count the number of hatched eggs (NHE) and to determine the hatching success (HS), along with the duration of the incubation period (IP). Hatching success was 89.04%. The mean clutch size in the American crocodile nests was 30.7 ± 7.83 eggs (ranging from 17 to 46 eggs), and the mean incubation period was 77.6 ± 5.89 days. The mean nest incubation temperature throughout the reproductive season was significantly different among nests. Based on the average temperature during the middle third of the incubation period, the nests should have produced both sexes, but with a higher proportion of males. This study tried to elucidate the impact of nest temperatures during the incubation period on embryo survival, as well as hatchling sex ratio in a local climate on the central coast of Oaxaca.

1. Introduction

Crocodilians face numerous threats in each stage of life, which are caused by both anthropogenic and natural factors (Brazaitis et al., 1996). Some of the anthropogenic factors affecting crocodilians include habitat destruction and poaching, which is mainly caused by the increase in human-crocodile conflicts (Thorbjarnarson et al., 2006; Mandujano, 2011), in addition to various types of pollution (Skaare et al., 1991; Bootens, 2011; Bouwman et al., 2014) that are believed to be harmful to crocodilian populations. As for natural factors, predation (Perez-Flores, 2018; Simá-Panti et al., 2020), flooding (Magnusson, 1982; Villegas et al., 2017) and the global increase in environmental temperatures (Charruau et al., 2017) threaten crocodilians. Furthermore, the temperatures at which the eggs of crocodilians are incubated plays an important role in embryo survival; rate of embryonic development; frequency of abnormalities; pigmentation and scale pattern of

hatchlings; body size and weight of the residual yolk during hatching; and thermoregulation (Deeming, 2004; Rhen and Lang, 2004; Piña et al., 2007; Parachú-Marcó et al., 2010; Charruau, 2012).

The temperature experienced by the embryo during a specific time frame during the egg incubation period was determined as the main factor driving the differentiation of its gonads (Ferguson and Joanen, 1982) and was named the temperature-dependent sex determination (TSD; Bull and Vogt 1979), of which three threshold patterns have been described in reptiles to date (Ewert et al., 1994; Valenzuela and Lance, 2004). According to González et al. (2019), the first pattern, called MF (Male-Female) or TSD Ia, establishes that low incubation temperatures lead to the predominance of males and high temperatures to females. In the second pattern, called FM (Female-Male) or TSD Ib, the inverse pattern occurs, with the prevalence of females at low temperatures and males at high ones. In the third pattern, called FMF (Female-Male-Female) or TSD II, low and high temperatures produce a predominance of

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females, and intermediate temperatures produce mostly males.

Like other crocodilian species, the American crocodile experiences the TSD in which embryo sex is established during incubation due to the effect of temperature on the sexual differentiation system during the thermo-sensitive period (TSP) (Lang and Andrews, 1994; Charruau et al., 2017). This species shows an FMF TSD pattern, in which low and high incubation temperatures during the middle third of the embryonic development produce higher proportions of females, and intermediate temperatures produce higher proportions of males (Charruau et al., 2017; González et al., 2019). This pattern also presents two transitional ranges of temperature (TRT), where both sexes are produced at different proportions, including pivotal temperatures (PT), at which 50% of each sex is produced (Charruau et al., 2017; González et al., 2019). Incubation temperatures, therefore, have an important effect on sex ratios and could affect population dynamics, generating strongly skewed sex ratios (Girondot et al., 2004). However, the temperature of wild crocodilian nests is not constant and fluctuates depending on several factors, including the metabolic heat produced by embryo development, environmental temperature, vegetation cover, solar radiation, nest material, rainfall regime and tropical cyclones (Leslie and Spotila, 2001; Thorbjarnarson et al., 2001; He et al., 2002; Ewert and Nelson, 2003; Charruau et al., 2010; Charruau, 2012).

Several studies in Mexico have focused on incubation temperatures of wild crocodilian (*Crocodylus moreletii* and *C. acutus*) nests, mainly in the Gulf of Mexico and the Yucatan Peninsula (Charruau, 2012; Escobedo-Galván et al., 2016; Charruau et al., 2017a, b; López-Luna et al., 2015; 2020). On the Pacific coast, only one study has been reported in Chiapas state (González Desales et al., 2016); thus, information about nest temperatures and their effects on embryos and post-hatching individuals in Mexican populations of *C. acutus* is still limited, principally for Pacific coast populations. One concern in particular is that current global warming could increase the incubation temperatures of natural nests, accentuating the previously existing bias in the crocodilians' sex ratio and threatening the survival of these populations already affected by other factors (Charruau et al., 2017a). Pacific populations of *C. acutus* on the coast of Oaxaca present a male-biased sex ratio with 3 males to 1 female (García-Grajales et al., 2007); therefore, it is imperative to study the level and fluctuation of nest temperatures of these populations to assess and prevent possible future problems with population dynamics.

Accordingly, the aim of this study was to perform nest temperature assessments of an American crocodile population on the central coast of Oaxaca, Mexico, in order to understand the relationship between incubation temperatures on some reproductive parameters such as incubation length, clutch size and sex ratio. This study marks the beginning of a long-term monitoring program and database collection of the nest temperatures and hatchling sex ratios of *C. acutus* on the coast of Oaxaca, so the ecological and conservational implications of the TSD on the crocodile populations – including the potential impact of global climate change – can be assessed.

2. Material and methods

2.1. Study site and species

The study took place in Palmasola Lagoon, located 15 min from the city of Puerto Escondido in the Santa Maria Colotepec municipality in Oaxaca. This lagoon extends parallel to the coast line, feeds off the fluff from the Colotepec River and maintains temporary communication with the sea in June and July (Cedillo-Leal et al., 2013). In this region, the climate is warm and sub-humid, with an annual mean temperature of 26.8 °C and annual mean precipitation of 2200 mm (García, 1973), along with a period of drought that goes from November to May (Trejo, 2010). The main type of vegetation around the Palmasola Lagoon are mangroves (*Rhizophora mangle*, *Laguncularia racemosa*, *Avicennia germinans* and *Conocarpus erectus*).

The American crocodile, *Crocodylus acutus*, has an extensive neotropical distribution range and inhabits the Atlantic and Pacific coasts (Thorbajarnarson et al., 2006). It can be found in coastal rivers, swamps, estuaries and lagoons, as well as in the open sea and island shorelines (Ernst et al., 1999). In Mexico, this species is under special protection (Diario Oficial de la Federación, 2010), classified as vulnerable by the International Union for Conservation of Nature (Ponce-Campos et al., 2012) and was recently transferred to Appendix II in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). This species is considered both a hole-nesting and mound-nesting species, the former being the most common (Greer, 1970; Campbell, 1972). Hole-nests are mainly composed of sand and leaves (Cedillo-Leal et al., 2013). The nesting season (from nest construction until hatching) in the Palmasola Lagoon begins in late February and finishes in early June. More information on the reproductive and nesting ecology of this species is available in Cedillo-Leal et al. (2013).

2.2. Field work

This study was carried out from February to June 2018. During daily walks starting in mid-February, we were able to detect the deposition beginnings in each nest, which were georeferenced (Garmin 64s, <3 m precision). We measured the distance from the center of the nest to the closest tree (DCT) and to the water's edge using a measuring tape of 50 m in length. Next, the nests were carefully excavated to determine the clutch size. Prior to their removal from the nests, the eggs were marked with pencil on the uppermost surface in order to maintain their original orientation (Charruau, 2012). Egg viability was determined by banding (Ferguson, 1985). Depth was measured from the surface of the soil to the top and bottom of the clutch (± 1 cm) using a plastic metric ruler of 60 cm in length. We placed dataloggers (UA-002-08, Onset Comp. Corp., Bourne, MA, USA) in all nests found. The remaining nests suffered from floods due to high-tide effects. In each nest, we put a datalogger in the middle of the egg chamber and programmed it to the temperature every 30 min. In addition, the direct solar radiation below canopy (DirRad) at the crocodile nests was calculated using hemispherical photographs (HP; Doody et al., 2006; Charruau, 2012). These HPs (360°) were taken with an Aukey fisheye converted lens installed on a smartphone which was placed above the soil and nest, leveled and oriented to magnetic north in accordance with Charruau (2012). The photographs were then analyzed by the Software Gap Light Analyzer v.2.0 (Cary Institute of Ecosystem Studies, Millbrook, NY).

During hatching in late May, the nests were revisited to count the number of hatched eggs (NHE) and to determine the hatching success (HS), nesting success and length of the incubation period (IP). Hatching success (HS) was defined as the proportion of embryos completely formed at the end of the incubation period and whether they were dead or alive (Charruau, 2012). In some cases, hatchlings were found perfectly formed but dead in the nest with their snout perforating the egg shell, resulting from lack of maternal assistance during hatching (Charruau et al., 2010). Only intact and fertile eggs were used to calculate proportions; broken and infertile eggs were eliminated from the analyses. The incubation period per nest was determined from the number of days between the date of egg deposition and the date of the first hatchling emergence. All nests were monitored until hatching; however, seven days after the estimated date of hatching, some nests had not experienced nest attendance or hatchling care by the mother, but we could hear the hatchlings' pre-hatching calls. Therefore, we conducted assistance activities in response to their calls and carried the hatchlings' to the water.

2.3. Statistics analyses

Linear regressions were used to test the relationship among mean daily temperature, clutch size and number of fertile eggs; between

Table 1

Characteristics of nests and nest chamber of *Crocodylus acutus* in Palmasola lagoon, Oaxaca, Mexico.

	Mean ± SD	Range	n
Nests'extern characteristics	3.28 ± 2.64	0.35–10	27
Distance from the nest to the closest tree (m)			
Distance from the nest to the water edge (m)	13.59 ± 4.78	3.7–24.1	27
Depth from the top of the nest to the first egg (cm)	25.52 ± 8.38	15–45	24
Nests' intern characteristics			
Depth of the incubation chamber (cm)	52.82 ± 9.07	35–70	24
Egg chamber maximun lenght (cm)	33.36 ± 3.73	27–42	24
Egg chamber minimun length (cm)	27.72 ± 4.36	22–35	24
Direct solar radiation below canopy %	24.78 ± 26.98	0–95	27

length of incubation period and mean clutch temperature; and between daily mean temperature and length of incubation period. The thermal fluctuations of each nest obtained during the IP were analyzed to detect the critical temperatures during the thermo-sensitive period (TSP), which was evaluated between days 25 and 45 of the incubation period, as suggested by Charruau (2012). Afterwards, this information was used to infer the sex ratio in each nest, based on Charruau's et al. (2017a) comments. An ANOVA was performed to evaluate differences among the temperatures of the TSP of each nest, and a Student *t*-test was performed to compare the incubation temperature and the temperature of the nests during the estimated TSP (González-Desales et al., 2016).

Doubtful and aberrant data caused by malfunction of dataloggers, by nest flooding or by nest predation were eliminated before analysis. Statistics analyses were carried out with XLStat software (Stat Soft Inc.), and we considered $P < 0.05$ to be significant. Descriptive statistics are presented with mean, standard deviations (SD) and range of values.

3. Results

Hatching success was 89.04%. The mean distance between the nest and the closest tree was 3.28 ± 2.64 m, and the mean distance to the water's edge was 13.59 ± 4.78 m. Nest depth varied between 35 and 70 cm; depth from the top of the nest to the first egg fluctuated between 15 and 45 cm; and the mean of direct solar radiation below canopy was 24.78 ± 26.98 SD watts per square meter (Table 1).

Although we found 27 nests in this study, only 10 of them were examined for nest temperatures in Palmasola Lagoon (Fig. 1). The mean

clutch size in the American crocodile nests was 30.7 ± 7.83 eggs (range 17–46 eggs). The mean incubation period was 77.6 ± 5.89 days. The mean nest temperature during the incubation period was significantly different among nests ($F_9 = 2969.8$, $p < 0.0001$). Likewise, mean nest temperature during the TSP (between 25 and 45 days) fluctuated between 29.13 °C and 33.46 °C, again with significant differences among nests ($F_9 = 6669$, $p < 0.0001$).

There was a positive statistical relationship between the length of incubation period and mean nest temperature ($r = 0.66$, $p < 0.05$; Fig. 2a); in addition, there was a positive statistical relationship between the number of eggs per nest and mean nest temperature ($r = 0.60$, $p < 0.60$; Fig. 2b). We found a negative relationship, however, between the mean of direct solar radiation below canopy and mean nest temperature during the incubation period ($r = -0.73$, $p < 0.05$, Fig. 2c).

Based on our results (Table 2), four nests' temperatures favored the production of males (32.15, 31.32, 31.14, 31.98 °C); four nests favored the production of both sexes (33.38, 33.46, 33.20, 33.01 °C); and two favored the production of a majority of females (30.94, 29.13 °C). Five nests presented temperatures above the mortality threshold (35 °C) during different stages of the incubation period, yet two of those nests still presented high percentages of hatching success.

4. Discussion

Both internal and external characteristics of *C. acutus* nests in Mexico have been evaluated at different locations (Casas-Andreu, 2003; Cupul-Magaña et al., 2004; Charruau et al., 2010, 2011, 2017b; Sigler, 2010; Hernández-Hurtado et al., 2011; Cedillo-Leal et al., 2013), but there has been only one study (González-Desales et al., 2016) on the Pacific slope that evaluates those characteristics and their effect on hatching success. The results of González-Desales et al. (2016) indicate that there is no relationship between hatching success and external and internal characteristics of the nest, which are similar to the results attained in our research. According to Charruau et al. (2010) and González-Desales et al. (2016), it is necessary to assess several nesting periods because variations in nest temperatures and hatching success were found after abundant rainfall; in our case, due to the swell phenomenon.

Regarding the hatching success in the 10 nests with known incubation temperatures, we found a higher success rate (89.04%) than in other studies on *C. acutus* (Ogden, 1978; Gaby et al., 1985; Rodrí-guez-Soberon et al., 2002; Charruau et al., 2010; Cedillo-Leal et al., 2013). The mean distance between the nest and the closest tree and the
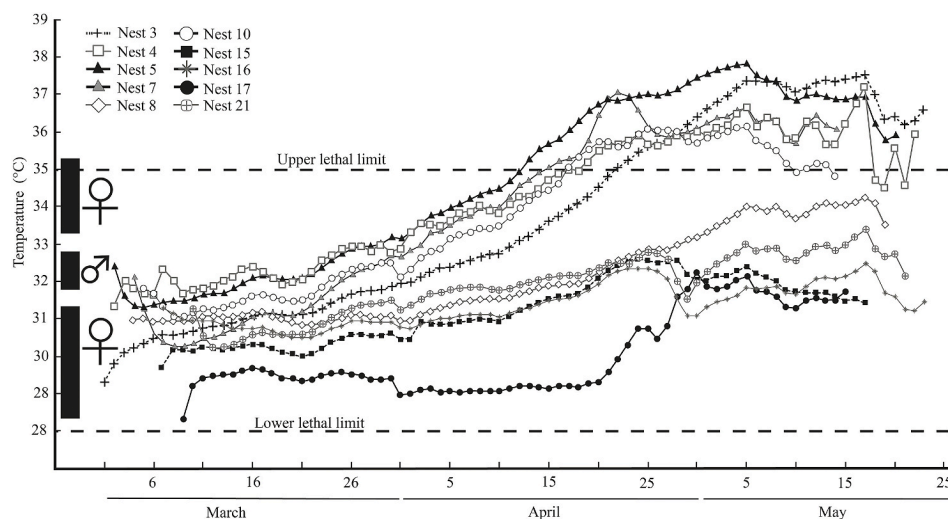

Fig. 1. Incubation temperatures of 10 nests of the American crocodile (*Crocodylus acutus*) in Palmasola lagoon, Oaxaca. Red dotted lines indicate the lethal temperature limits for the embryos suggested by Charruau *et al.* (2017).
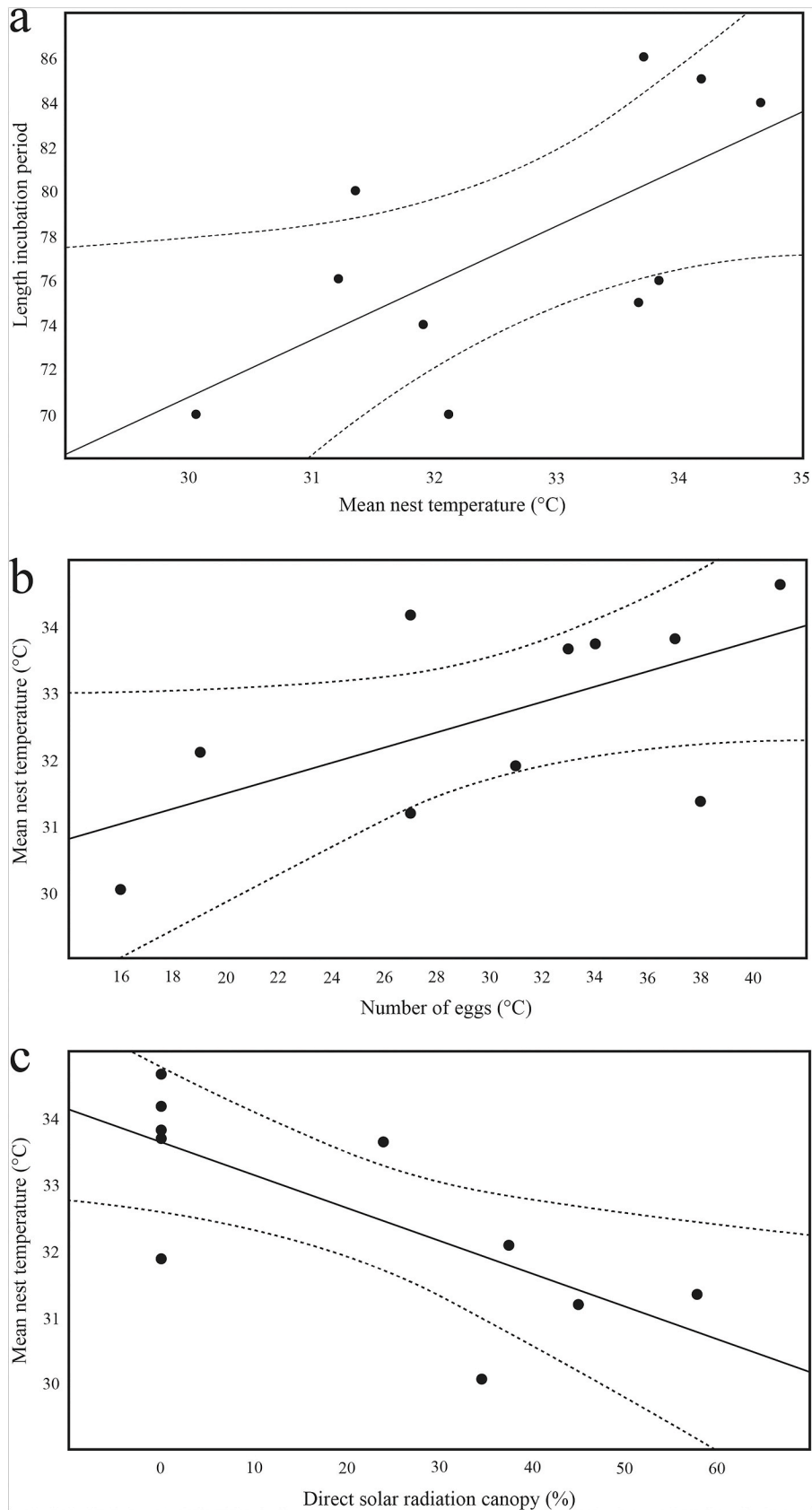
Fig. 2. Statistical relationship between mean nest temperature and a) Length incubation period, b) number of eggs and mean temperature, c) direct solar radiation under canopy, in 10 nests of the American crocodile (*Crocodylus acutus*) in Palmasola lagoon, Oaxaca, Mexico.

Table 2

Mean temperature, hatching success and other parameters evaluated in ten nests of *Crocodylus acutus* in Palmasola lagoon, Oaxaca, Mexico. * denote nests with temperatures above the mortality threshold (35 °C) during different stages of the incubation period.

Nest ID	Number of eggs	Incubation temperature (°C)	SD	Incubation temperature during TSP (°C)	SD	Length of incubation	Viability (%)	Embrionic death (%)	Hatching success (%)	Sex ratios
3	35	33.71	2.56	32.15	0.51	86	94.29	0	84.84 *	100% males
4	30	34.18	1.74	33.38	0.67	85	90	0	45 *	50% of each sex
5	46	34.66	2.25	33.46	0.73	84	89.13	8.7	26 *	50% of each sex
7	39	33.82	2.27	33.20	0.84	76	94.87	5.13	94.2	50% of each sex
8	20	32.12	1.19	31.32	0.29	70	95	0	100	100% M
10	34	33.67	1.81	33.01	0.72	75	97.06	0	28 *	50% of each sex
15	29	31.22	0.84	30.94	0.36	76	86.21	3.45	100	60% females/ 40% males
16	36	31.36	0.55	31.14	0.26	80	97.6	0	32.4 *	100% males
17	17	30.06	1.12	29.13	0.20	70	82.35	0	100	80% females/ 20% males
21	34	31.91	0.85	31.98	0.28	74	70.59	2.94	45.45	100% males

nest to the water's edge, as well as the nest depth recorded here, are similar to those reported for other American crocodile populations (Cupul-Magaña et al., 2002; Casas-Andreu, 2003; Charruau et al., 2010; Cedillo-Leal et al., 2013). Direct solar radiation is the main heat source for the nests (Charruau, 2012), but our results showed a negative correlation to mean nest temperature during the incubation period, possibly associated with the vegetation density that produces shadow on the nesting area and reduces the solar radiation heat transferred to the nest by thermal conductivity of a substrate. In Palmasola Lagoon, the nesting substrate consists of coarse sandy soil with reduced organic matter content, which provides good thermal conductivity (Cedillo-Leal et al., 2013). The lower tree cover, therefore, has a direct influence on the temperature inside nests, increasing the incubation period. This demonstrates the importance of shade to buffer solar radiation, which is the most important heat source to maintain the temperature for embryonic development (Charruau 2012; González-Desales et al., 2016). On the other hand, the IP depends on temperature; therefore, the IP decreases as the temperatures rises during incubation, and low temperatures extend IP time (Thorbjarnarson, 1989; Charruau et al., 2010). Our results, however, show a significant negative correlation (r = 0.66, $p < 0.05$) between the mean of direct solar radiation below canopy and mean nest temperature. These results were probably influenced by the lack of parental care behavior by females in five nests, specifically inside the nest, at the time of egg opening and during the transport of hatchlings to the water, which required our intervention to prevent their death.

In this study, the mean clutch size in American crocodile nests was 30.7 ± 7.83 eggs. This is slightly less than the clutch size (35.3 ± 9.95 eggs) reported in the same lagoon by Cedillo-Leal et al. (2013) and González-Desales et al. (2016) in Chiapas, but significantly less than reported by Ogden (1978). Most other American crocodile populations have a smaller clutch size (Cupul-Magaña et al., 2002; Casas-Andreu, 2003; Charruau et al., 2011), producing a mean clutch size between 16.2 and 27.9 eggs per nest. The mean clutch size is among the largest registered for this species in Mexico and in their distribution range.

Global climate change (IPCC, 2013) could have a significant impact on reptiles with TSD (Janzen, 1994; Mitchell and Janzen, 2010; Charruau et al., 2017), including crocodilians (Allsteadt and Lang, 1995; Deeming, 2004; Charruau, 2010). It has been suggested that increases in sand temperature associated with climate change will affect both crocodilian sex ratios (González et al., 2019) and hatching fitness (Allsteadt and Lang, 1995; Deeming, 2004). These changes are predicted to increase the proportion of female hatchlings, in addition to reducing hatching success and fitness, primarily at sites already experiencing biases toward female-producing temperatures (Charruau et al., 2017; González et al., 2019). On the central coast of Oaxaca, nest temperatures

may indicate the production of a range of sex ratios but with an overall female bias. This could potentially pose a problem if future temperatures increase because of global climate change. Considering that American crocodiles in Palmasola Lagoon are already producing a female-biased hatchling sex ratio and that some nest temperatures reach as high as 35 °C, the projected increases (IPCC, 2013) could result in extreme female biases and potentially impact hatching success. The reported sex ratio for the American crocodile is generally female-dominated (González et al., 2019), as we report in this study. Nevertheless, more research – including hatchlings' sexing – would be necessary to understand the influence of nest temperature on morphology and sex ratio.

Incubation temperatures (30.05–34.66 °C) are similar to those reported by González-Desales et al. (2016; 29.6–34.2 °C) and Charruau (2012; 29.8–33.1 °C) and are in the optimal range for embryonic development (Lang and Andrews, 1994). In this study, however, we recorded five nests with temperatures (>35 °C) above the mortality threshold during different stages of the incubation period with high hatching success in some nests. The success in those nests, though, was the result of the assistance activities we provided due to the absence of the female and the lack of nest attendance and hatchling care. Although embryos have been considered thermally passive, some studies have shown that they actively move toward warmer regions within the egg (Du et al., 2011; Zhao et al., 2013). Thermal conditions, therefore, may vary over small, spatial scales inside a nest, but within a single egg, the warmest part is likely to be closest to the heat source. An embryo could detect such thermal gradients and move to the warmest or coldest part of the egg, substantially affecting its own temperature (Du et al., 2011). This behavioral thermoregulation of embryos could be a response to the ability to tolerate the upper thermal limits, as we found in this study.

The effect of incubation temperatures on the life traits of ectotherms is well-known (Noble et al., 2018) – especially within TSD species – but until now, there has been a misunderstanding about how these species develop strategies to confront environmental changes and how they cope with the potential effects, such as sex ratios in clutches, that may be caused by future climate changes (López-Luna et al., 2020). Our study tried to elucidate the impact of nest temperatures during the incubation period and their subsequent effects on embryo survival and hatchling sex ratio in a local climate on the central coast of Oaxaca. Local climatic factors have an impact on incubation temperatures and, consequently, it is important to keep monitoring crocodile nests in order to determine the female adaptation strategies in response to weather variations (González-Desales et al., 2016). Finally, the conservation aspects related to sex ratio and hatching success, as well as research on metabolic heating, need to be continuously monitored for several years to understand the role of the nesting female and incubation temperatures on the sex ratio and performance of the offspring.

Credit authorship contribution statement

René Barragán Lara: Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing – original draft, Review and editing. Jesús García-Grajales: Conceptualization, Methodology, Writing – review & editing, supervision. Emilio Martínez Ramírez: Resources, Writing – review & editing, Supervision.

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