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Additional details on temperature-dependent sex determination in *Crocodylus acutus*

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The global increase of environmental temperatures is one of the most important issues to biodiversity conservation research in this century. One important question in herpetology referring to this subject is, "What will happen to the sex ratio of reptile species with temperature-dependent sex determination (TSD)?" In these species, the sexes of embryos are determined during a specific period within incubation (i.e., the thermo-sensitive period [TSP]) by the action of temperature on the sexual differentiation system (Valenzuela & Lance 2004). Therefore, some oviparous reptiles with parental care can modify the incubation environment of their clutch and thus enhance the fitness of their offspring (Warner & Shine 2008). However, the majority of oviparous species lack this parental care behaviour, and therefore, their sole mechanism to influence offspring phenology is the choice of nesting site (Warner & Shine 2008); in this case incubation temperatures will depend mainly on environmental temperatures, extent and intensity of insolation, heat-storing/insulating properties of the substrate, and exposure to rain. The current global climate change could modify nest temperatures in such reptiles and therefore the traits of their hatchlings, leading to structural and survival problems of populations. In order to predict potential effects of nest temperature changes on hatchling characteristics and population structure, one option is to identify the effects of a large spectrum of constant incubation temperatures on hatchlings of affected reptiles.

Crocodilians form an interesting group of reptiles in which to study the TSD system and its adaptation to climatic change. The absence of sex chromosomes in all crocodilian species studied to date suggests that all species have TSD (Deeming 2004). The occurrence of TSD has been confirmed in 14 of the at least 24 currently recognized species of crocodilians (Charruau 2010). Current information suggests one TSD pattern to be universal to all species of crocodilians, the Female-Male-Female (FMF) pattern, with a prevalence of females at low (< 31°C) and high (> 33°C) incubation temperatures and a prevalence of males at intermediate temperatures (Charruau 2010, Deeming 2004). This pattern includes two transitional ranges of temperature (TRT) within which both sexes are produced at different ratios, with each TRT presenting a pivotal temperature (PT) at which both sexes are produced at equal ratios (Valenzuela & Lance 2004). Incubation temperature also determines a multitude of morphological, behavioural, and physiological traits in crocodilians (Allsteadt & Lang 1995, Deeming 2004).

Although crocodilians show well developed parental care behaviours, there is no evidence of nest microclimate regulation by adults. Thus, crocodile nest temperatures are driven mainly by environmental temperatures, insolation and rainfall (Charruau 2010, 2012, Charruau et al. 2010), and crocodilian populations could be affected by the current climatic changes through changes in nest temperature regimes. Nevertheless, crocodiles passed through several global climatic changes during their evolutionary history (Markwick 1998), which demonstrates their capacity and that of the sex determination system to adapt to such climatic processes. However, crocodilians currently face not only similar changes in temperature, but also dangers from cyclones, altered rainfall patterns, and additional threats

Table 1. Means (±SD) of incubation temperatures and incubation periods (IP), number of eggs (n), embryo survival rate (Sr), and sex ratios (SR) for each incubator. T^{tot} – temperature total; $T^{25–45}$ – temperature between days 25 and 45 of incubation; T^{mt} – temperature of the middle third of the incubation period.

	n	T^{tot} (°C)	$T^{25–45}$ (°C)	T^{mt} (°C)	IP (days)	Sr (%)	SR (male %)
2012							
Incubator 1	5	28.99±0.32	29.23±0.12	29.06±0.20	108.5±5.7	60	20
Incubator 2	6	31.22±0.45	30.97±0.12	31.01±0.13	83.0±3.3	83.3	40
Incubator 3	6	33.09±0.34	32.75±0.18	32.79±0.20	71.5±1.2	100	100
2013							
Incubator 1	5	31.74±0.14	31.67±0.09	31.67±0.10	81.2±1.6	100	100
Incubator 2	6	32.32±0.17	32.35±0.11	32.31±0.13	75.2±2.4	100	100
Incubator 3	6	33.50±0.23	33.58±0.12	33.54±0.16	71.3±0.5	100	50
Incubator 4	6	34.97±0.35	–	–	–	0	–

such as destruction, modification, and contamination of their habitats, illegal hunting, hybridisation, and impacts from invasive species.

The American crocodile (*Crocodylus acutus* Cuvier, 1807) is a coastal species classified as Vulnerable by the International Union for Conservation of Nature (Ponce-Campos et al. 2012). It faces several threats in the Mexican Caribbean including hybridisation, tropical storms and cyclones, and habitat destruction and modification (Cedeño-Vázquez et al. 2006, Charruau et al. 2010, Machkour M'rabet et al. 2009). Some populations of the Yucatan Peninsula exhibit male-biased sex ratios that could threaten the recovery of the species in this region if a warming climate accentuates this bias (Charruau et al. 2005, Charruau 2012). However, information about the effects of incubation temperature on this species is still limited. Thus, our goal in this study was to incubate eggs of genetically pure *C. acutus* from this region at a wide range of constant incubation temperatures and identify their effects on incubation periods, sex ratios of hatchlings, and the survival of embryos.

We used *C. acutus* eggs from the Punta Sur Ecological Park on Cozumel Island (Mexico). This area contains a genetically pure population (Machkour M'rabet et al. 2009), and nesting sites have previously been identified (Charruau et al. 2011). On 2 April 2012, we collected 17 fertile eggs from one nest that were estimated to have been deposited on 26 March. On 17 April 2013, we collected 23 fertile eggs from another nest that had been laid during the previous night, on 16 April. Being less than one week old, both nests had not reached the thermosensitive period yet. We marked each egg individually with a number for their identification and preserved its original orientation. We recorded the clutch size and fertility ratio for each nest. The eggs were placed in a plastic box along with substrate from the nest and then transported to the laboratory at the Instituto de Ciencias del Mar y Limnología of the Universidad Nacional Autónoma de México at Puerto Morelos, state of Quintana Roo.

The incubators (Hova Bator model 1582, G.Q.F. Manufacturing Company Inc.) were prepared before egg collection. The substrate was a 1:1 water-soaked mix of agro-lite and vermiculite. Incubators were calibrated to 29, 31, and 33°C in 2012, and to 31.5, 32.5, 33.5, and 34.5°C in 2013. The temperature of the laboratory was maintained at less than 29°C, as incubators can only warm up and not cool down. Each incubator was outfitted with a hygrometer and a thermometer to monitor humidity and temperature, respectively. We also installed a data logger (Onset HOBO® Pendant temp UA-001-08) in each incubator and in the laboratory to log temperatures every 30 minutes until the last crocodile had hatched. The eggs were distributed randomly within the incubator (Table 1).

Hatching dates were recorded and incubation periods calculated for each hatchling, corresponding to the number of days between the estimated oviposition date and the hatching date. We made necropsies of those embryos contained in unhatched eggs that had reached a development stage allowing their being sexed by directly assessing their gonads. Each live hatchling was individually marked by removing tail whorls and sexed by gently opening its cloaca with Kelly forceps to view its genitalia. Hatchlings were then maintained for one year at the Crococun Zoo (Puerto Morelos) before their release in the Punta Sur Ecological Park. They were re-sexed every month in order to verify the identified sex of each one. Hatchlings that perished were subjected to a necropsy and their sexing verified by direct assessment of their gonads.

We used linear regressions to detect correlations between mean incubation period and mean incubation temperature, between standard deviation of mean incubation period and mean incubation temperature, and between standard deviation of mean incubation temperature and temperature difference between the laboratory and incubators. Statistical analyses were performed with G-Stat 2.0.1 and results were considered significant at $p < 0.05$. To identify the temperature-dependent sex determination pattern, we used two mean incubation temperatures for each incubator: mean temperature of the middle third of the incubation period (T^{mt}) and mean temperature between days 25 and 45 of incubation ($T^{25–45}$) (Georges et al. 2004, Charruau 2012).

Although some mean incubation temperatures show a low standard deviation (e.g., 0.14°C), others show a relatively high variation (e.g., SD = 0.45°C; Table 1). Standard deviations were higher at low and high temperatures and lower at intermediate temperatures (Table 1), and they indicate a potential relationship with the difference in temperature between the incubators and the laboratory ($r^2 = 0.758$, $F_{1,5} = 15.62$, $p = 0.01$). Mean laboratory temperatures were 23.50°C in 2012 and 27.90°C in 2013, but always remained lower than the temperatures in the incubators. Relative humidity fluctuated between 80 and 90% at the beginning of the experiment. At the end of the incubation period, the humidity decreased to 70% in the incubator at 34.97°C while it remained between 78 and 90% in the other incubators. These values promote proper embryo development and do not threaten their survival or fitness (Grigg 1987). The incubation period was negatively correlated to the mean incubation temperature ($r^2 = -0.953$, $p < 0.001$; Fig. 1). The standard deviation of the mean incubation period (Tab. 1) was also negatively correlated to the mean incubation temperature ($r^2 = -0.964$, $p < 0.005$; Fig. 1). The survival rate of embryos was high (83.3–100%) at intermediate temperatures and decreased to 60 and 0.0% at the lowest and highest temperature, respectively (Table 1). The difference between T^{mt} and T^{25-45} ranges from 0 to 0.17°C and the standard deviation was low (Table 1). The TSD pattern suggested by this study is Type II (Fig. 2) with a majority of females (60 and 80%, respectively) below 31.0°C, a majority of males (100%) between 31.7 and 32.8°C, and 50% of both sexes at 33.6°C (Table 1). Estimated pivotal temperatures are 31.1 and 33.6°C (Fig. 2).

The wide range of constant temperatures used in the experiment has permitted the study of the effect of incubation temperatures on hatchling sex determination, embryo survival, and incubation periods in *C. acutus* from low (29.0°C) to high (35.0°C) temperatures. Despite always maintaining laboratory temperatures lower than incubator temperatures, it appears that differences in temperature between the laboratory and the incubators caused high fluctuations of some incubation temperatures. Differences of thermal variation around the same mean temperature can result in different sex ratios in species with TSD (Neuwald & Valenzuela 2011). However, standard deviations of mean temperatures during anticipated thermosensitive periods were very low and likely had no effects on the results. For future experiments, it would be better to maintain a laboratory temperature lower but closer to incubator temperatures to reduce standard deviation of incubation temperatures, or use incubators with more effective insulation.

Incubation periods in *C. acutus* are temperature-dependent as reported previously in this species and other crocodilians (Hutton 1987, Thorbjarnarson 1989, Piña et al. 2003); they decrease as incubation temperatures increase. Thus, embryos from colder nests are exposed to egg predators longer than embryos from warmer nests. Moreover, the standard deviation of the incubation period also decreases with the increase of incubation temperature. This signifies that the higher the incubation temperature is, the more synchronized hatching will be, which would likely mean better overall success of clutches incubated at moderately high temperatures than those incubated at lower temperatures. Indeed, hatchlings from warmer nests will hatch simultaneously or within a very narrow time window (e.g., within one day at 33.5°C in this study) compared to hatchlings from colder nests, which will hatch less synchronized over a wider time window (e.g., over 12 days at 29°C in this study). Thus, some unhatched eggs could remain in colder nests for several days after the female has started to excavate it and will be exposed to, and likely consumed by, predators or abandoned by the female in the nest (P. Charruau pers. obs., Platt et al. 2014). Also, females that need to come back to the nest repeatedly over several days to help liberate delayed baby crocodiles would
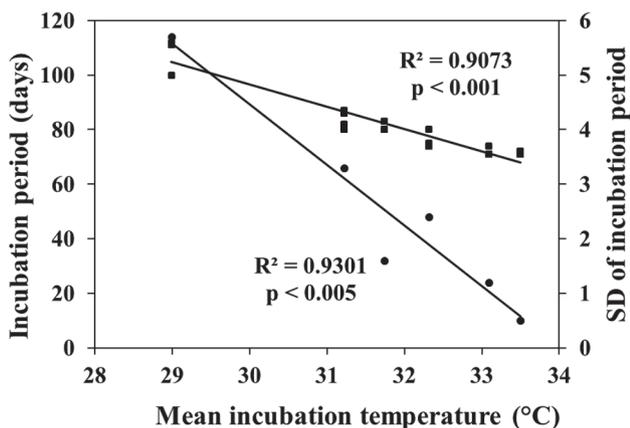

Figure 1. Linear regression between mean incubation temperature and incubation period (squares) and standard deviation of the incubation period (circles).
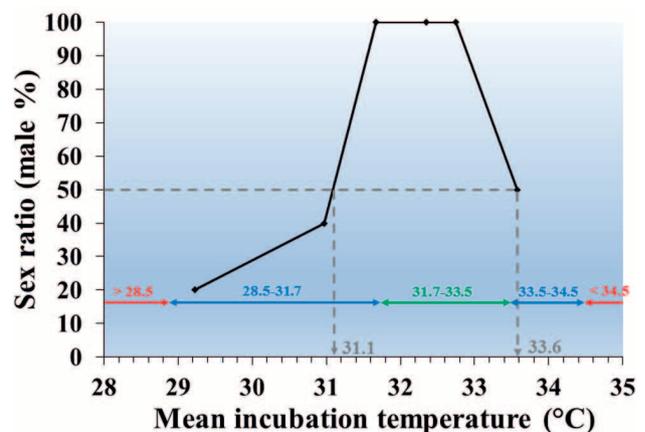

Figure 2. Temperature-dependent sex determination pattern of *Crocodylus acutus* using mean temperature between days 25 and 45 of incubation. Arrows show ranges of incubation temperatures for different embryo survival rates (green – 100%; blue – <100%; red – 0%). Dashed grey arrows indicate potential pivotal temperatures.

have to leave their recently hatched offspring unprotected in the water more often during one of their more vulnerable stages.

Low (≤ 28°C) and high (≥ 35°C) incubation temperatures generate high mortality of crocodilian embryos (Ferguson & Joanen 1982, Lang & Andrews 1994, Leslie 1997, Piña et al. 2003). This study shows the same results for *C. acutus*, with lower survival rates at extreme temperatures (i.e., 60% at 28.99°C and 0% at 34.97°C). Furthermore, Aguilar-Miguel (1994) reported a 0% survival rate of *C. acutus* embryos at an incubation temperature of 27–28°C. Thus, incubation temperatures keeping *C. acutus* embryos viable are likely to range from 28.5 to < 34.5°C, with an optimum range (i.e., 100% survival) between 31.7 and 33.5°C (Fig. 2). Moreover, embryo survival rates decrease faster at high temperatures than at low temperatures. At low temperatures, the decrease was 40% per 1.75°C while at high temperatures, the decrease was 100% over a 1.47°C interval. Thus, embryos are likely more vulnerable to increased than to decreased incubation temperatures.

Only two previous works have studied the effects of incubation temperatures on sex ratios in hatchling *C. acutus* and suggested a FMF TSD pattern for the species. One was conducted in the wild with natural variable temperatures (Charruau 2012) and one under laboratory conditions using only three constant temperatures (Aguilar-Miguel 1994). This study confirms that *C. acutus* presents a FMF TSD pattern with the production of a majority of females at low (≤ 31.0°C) and high (> 33.6°C) temperatures and the production of 100% males at intermediate temperatures (31.7–32.8°C). The pivotal temperatures estimated in this study (i.e., 31.1 + 33.6°C, respectively) differ from those estimated by Aguilar-Miguel (1994: 32.0°C) and by Charruau (2012: 31.0 + 32.5°C). One factor explaining these differences is that Aguilar-Miguel (1994) used the mean of constant temperatures throughout incubation, Charruau (2012) used the mean of fluctuating natural incubation temperatures during a certain period of the incubation, and the current study used the mean of constant incubation temperatures during the period critical for sex determination. Thus, the current study is likely to have produced more accurate results than the previous two. Moreover, sexing *C. acutus* neonates is not easy and errors are always possible. In this study, hatchlings were re-sexed monthly over one year, and no change in the sex ratio was observed after the young had turned seven months old. Another difference between the mentioned studies is the provenance of the eggs. All three studies used supposedly genetically pure *C. acutus* eggs, but from three populations with no geographic overlaps, which renders genetic differences between populations a possibility. Populations of the same species can show differences in their TRT and PT, suggesting an intraspecific genetic polymorphism of the TSD pattern and adjustments to regional climates (Chevalier et al. 1999, Ewert et al. 2004, 2005). Furthermore, the very limited sample size of this study (two nests) may not be representative of the genetic diversity of the entire population. Therefore, experiments with a larger sample size at constant incubation temperatures are necessary to elucidate the entire variation of the TSD pattern of *C. acutus* (i.e., TRT, PT, and TSP at different incubation temperatures).

This study provides important information for the prediction of changes in critical reproductive parameters (e.g., hatching success, sex ratio) of *C. acutus* as a result of changing natural incubation temperatures. Our findings suggest that an increase in incubation temperature could lead to shorter incubation periods and better hatching synchronization, which would enhance embryo and hatchling survival. However, high incubation temperatures (> 34.0°C) would also cause high mortality of embryos. Furthermore, our results on the TSD pattern of *C. acutus* can help to predict the effects of changed nest temperatures on hatchling sex ratios based on current natural incubation temperatures. Few populations of *C. acutus* show biased adult sex ratios and when they do, the bias often seems to be towards males (Escobedo-Galván et al. 2011). Recent studies have detected male-biased hatchling sex ratios in *C. acutus* populations (Charruau 2012, Murray et al. 2015), which could threaten their survival. However, Murray et al. (2015) found that some population-dynamic parameters of *C. acutus*, such as juvenile male dispersal, could permit the transition from male-biased hatchling sex ratios to equilibrate adult sex ratios. It is however still unclear what will happen to island populations where juvenile dispersal is impossible or to populations with female-biased sex ratios.

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