The Interface of Population Dynamics, Endocrine-Disrupting Compounds and Enigmatic Disturbance: Understanding Human- Crocodile Conflict in Costa Rica

by

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Abstract

A recent survey indicates a unique male-biased sex ratio in a prized ecotourism species and aquatic sentinel taxon, the American crocodile (*Crocodylus acutus*), at Palo Verde National Park, an influential conservation area in northeastern Costa Rica. Palo Verde harbors an extensive ephemeral marsh that serves as a critical migratory-bird pathway and is home to multiple species of conservation concern in tropical dry forest habitat. Human conflict with this species heightens as the distributions of humans and crocodiles increasingly overlap in this region and media exposure of crocodile attacks becomes more widespread. Here, I test the presence of a male-biased sex ratio among crocodile age cohorts at Palo Verde and assess sex-specific immigration and emigration within these cohorts. Further, I test two hypotheses in an attempt to explain the male-biased sex ratio. First, I test the presence and effect of regional warming, as a result of global climate change, on sex determination within crocodile eggs, as well as potential compensatory nesting behaviors that may influence crocodile sex determination at the level of nest microhabitat. Second, the efficacy of a synthetic androgen used in local tilapia farming (17α-methyltestosterone, MT) to bias sexual differentiation towards males is tested. Exposure of crocodiles to this synthetic hormone is tested in natural populations of crocodiles and the role of MT as an environmental androgen is assessed in American alligators, a surrogate for the effect of this chemical on crocodiles. Demographic analysis indicates a 2:1 male-biased sex ratio in adults that results from balance migration of females and a net pattern of emigration of sub-adult males from a 4:1 male-biased sex ratio at hatching. Analysis of nest temperatures

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predicts a female bias in hatchling sex ratio based on nest thermal regimes, a pattern inconsistent with the observed male-biased sex ratio for the hatchling cohort. However, my data show a strong influence of metabolic heating on nest temperatures, a feature previously unknown for American crocodiles. Exposure of American alligator eggs to MT and female-producing temperatures results in hatchlings of both sexes including hermaphroditic individuals. Finally, sampling of the natural population of American crocodiles reveals the presence of MT in egg yolk, hatchling, juvenile and adult blood plasma in all sampling localities. These results indicate that MT is a potent environmental androgen capable of causing the male-biased sex ratio in the Tempisque River basin and other nearby drainages.

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Chapter 1

General Introduction

Abstract. Here, I introduce the organism, study site, urgency and problem that are the focus of the succeeding chapters. Relevant background on the biology of the American crocodile (*Crocodylus acutus*) and the study area of Guanacaste, Costa Rica is provided. The problem; a unique male-bias in crocodile sex ratio, and the urgency; mounting tension between local humans and crocodiles, are described. I describe three foundational hypotheses that test why the crocodile sex ratio bias exists and the study design of each. The testing of such hypothesis forms the empirical basis for my dissertation. A conceptual framework describing the association of hypotheses is included.

Key words: American crocodile, Guanacaste, temperature-dependent sex determination, methyltestosterone.

INTRODUCTION

A recent study in the Tempisque River Basin of Costa Rica documents a highly skewed (3:1), male-biased sex ratio (Bolaños-Montero 2012). This is the most heavily skewed sex ratio reported for any member of the family Crocodylidae (Thorbjarnarson 1997). Charruau (2011) also reports a male-biased sex ratio in hatchling *C. acutus* in a population in Mexico. The Costa Rican work was performed in the "humedal" in Palo Verde National Park in the Tempisque Basin, a seasonal floodplain that offers vital habitat for reproduction, feeding, and development

of American crocodiles and is a key resource in Middle America for many other vertebrates (e.g. black-bellied whistling duck).

Assuming the sex ratio assessment at this site to be accurate, two general explanations can be envisioned for biased sex ratios in crocodiles. One explanation is that this represents a fitness advantage in the local environment. However, data for sex ratios of crocodilians at other sites document that this feature varies around a mean ratio of 1:1 when including all taxa, with the Palo Verde sample being an outlier of extreme male bias within the genus *Crocodylus* (Thorbjarnarson 1997). Hence, adaptive explanations are not considered further and Fisher's (1930) 1:1 sex ratio is considered the null expectation.

Alternatively, a male-biased sex ratio could signal a disadvantageous response to environmental perturbation. Anthropogenic factors, such as regional warming and synthetic androgen use, represent factors that may bias sex ratios in American crocodiles. Because of regional warming, global climate change has been recognized as a serious threat to biodiversity in Costa Rica. This enigmatic disturbance has been attributed to local effects on specific taxa in areas within the country. Predictive models for species range shifts have been developed under projected climate change (Colwell et al 2008). As reported by Whitfield et al. (2007), the annual mean daily minimum temperature has increased about 1° C from 1982 to 2005 at La Selva Biological Station in central Costa Rica (0.04° C per year). This increase in temperature has also been negatively correlated with tree growth during that time (Clark et al. 2003). Since a 1° C temperature increase over 23 years already appears to have caused dramatic effects in La Selva's wet forest herpetofauna (Whitfield et al. 2007), one might expect even greater changes associated with the tropical dry forest. A change in climatic temperature of this magnitude could push nest temperatures in a direction that would produce male-biased clutches, rather than the

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1:1 ratio predicted by Fisher. Such a temperature-related influence has been documented by Charruau (2011) for Mexico, but not for Palo Verde National Park or other dry forests of southern Central America.

Responses to change in thermal climate have been documented in numerous vertebrate taxa. Poleward or latitudinal shifts in range serve as a biogeographic adaptation to climate change (Parmesan 2006). Colwell et al. (2008) suggest altitudinal range shifts resulting in lowland biotic attrition and mountain top extinctions in the wet tropics. In terms of reproductive behavior, Refsnider and Janzen (2012) report behavioral plasticity in nest depth, phenology, and placement in an experimental manipulation with emydid turtles. This suggests a role for plasticity in response to changing climate or other anthropogenic disturbances. Like other vertebrates, crocodilians exhibit plasticity in intra- and interspecific reproductive behaviors (Thorbjarnarson 1996). Like turtles, crocodilians have temperature-dependent sex determination (Lang and Andrews 1994) and, therefore, might alter nest placement to combat deleterious effects of climate change. Thus, it is possible that sex ratio biases might be even more extreme than observed sex ratios if behavioral plasticity in nest placement did not occur. For this reason, shifts in nesting microhabitat and clutch placement are required to test the role of nesting behaviors.

Endocrine-disrupting chemicals represent a second mechanism by which anthropogenic factors could cause male-biased sex ratios in American crocodiles. The Central American Institute for Studies in Toxic Substances (IRET) reports 12,000 tons and 404 types of insecticide, fungicide and herbicide annually imported into Costa Rica. The area under cultivation within the nation is 450,000 hectares as of 2006 and the amount of pesticide imported in kg/yr per agricultural worker has increased by 384% between 1977 and 2006. Costa Rica and Nicaragua

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currently permit the use of harmful agricultural substances and allow toxic threats to persist

(Thrupp 1988). Such chemicals threaten wildlife and humans alike. Human populations in Costa

Rica were identified as high risk for pesticide-associated cancers (Wessling 1999) and

sterilization (Thrupp 1991).

Natural aquatic habitats, both freshwater and marine, appear to bear the brunt of toxic

accumulation, with five active toxins (of the 30 most widely used) explaining more than 75% of

aquatic ecotoxicity (Humbert et al. 2006). Castillo (1997) called for study of the fate of

pesticides in tropical aquatic ecosystems, acute and chronic effects on aquatic biota, and effects

of abiotic conditions on toxicity in Central America. This review documents eighteen studies that

have been completed, most of which document organochlorine residues. Surface waters and

sediments in the Suerte River basin in Costa Rica contain six pesticides (3 fungicide, 2

nematicides, and 1 insecticide), most of which are present at concentrations that exceed chronic

risk ratios. At least one instance of toxins traveling through the protected Tortuguero National

Park region to the Caribbean Sea has been demonstrated (Castillo 2000). In addition, surface

sediments along the Pacific Coast of Costa Rica contain elevated levels of PCBs in localized

areas (Spongberg 2004) and amphibian declines in certain areas of Costa Rica have been

attributed to environmental contaminants (Lips 1998).

A more acute contaminant is associated with Tilapia aquaculture, which has increased in

Costa Rica over the past four decades, generating a major export. Considered the most important

aquaculture species of the 21st century (Gupta and Acosta 2004), Costa Rica currently sustains

the largest Tilapia farm in the Americas and is among the top 20 countries in Tilapia production

(Fitzsimmons 2000). Major farms in the country function as flow-through pond systems, often

incorporating a complex network of irrigation ditches, artificial ponds, and natural waterways

(Fitzsimmons 2000, Phelps and Popma 2000). Additionally, the three farmed species (*Oreochromis mossambicus, O. aureus* and *O. niloticus*) are characterized by uniformly large, fast-growing, and all-male populations, attained in Costa Rica by direct application of methyltestosterone, a steroid that chemically induces male monocultures (Gupta and Acosta 2004, Phelps and Popma 2000). This technique is not widely accepted because the environmental effects of methyltestosterone, are unknown (Gupta and Acosta 2004). Additionally, methyltestosterone is noted to have the potential to cause unspecified secondary effects (Phelps and Popma 2000).

The above-mentioned anthropogenic influences in the Guanacaste region present potential causes for skewed sex ratios of vertebrates with TSD. The skewed sex ratio in the Palo Verde crocodile population is hypothesized to be a result of regional climate change and androgen-based endocrine-disrupting compounds or a combination of these factors. But, compensatory mechanisms in nesting behavior may offset the anthropogenic influence on sex ratio.

STUDY SITE

The Pacific versant of Costa Rica possesses numerous rivers that drain the western slopes of the Cordillera de Guanacaste, Cordillera Central, and Cordillera de Talamanca. Such river systems begin no more than 100 km inland and discharge into the Pacific Ocean along the more than 450-mile western coast. These rivers drain urban, agricultural or unspoiled landscapes and empty into large brackish mangrove deltas. Northwest drainages collect seasonal rains from tropical dry, moist, premontane moist, and wet forest habitats before emptying into the Golfo de Nicoya at the Tempisque delta. Central and southern systems drain tropical premontane and

montane moist, wet and rain forest habitats, depositing these waters into southern portions of the Golfo de Nicoya, the Pacific Ocean, or Golfo Dulce (Kohlmann et al. 2010).

The Guanacaste region and Nicoya Peninsula are characterized by tropical dry forest, most of which, by 1950, was deforested and planted with African grasses for extensive cattle ranching. The Palo Verde region of Guanacaste is a large open wetland fed and drained by the Tempisque River. Migratory waterfowl use this wetland and it serves as a prime nesting site and nursery for crocodiles in the Tempisque Estuary. Palo Verde is likely critical habitat for the Black-bellied Whistling Duck and probably serves as the epicenter for American crocodiles in the northwest part of Costa Rica. In 1977 the Palo Verde wetland and surrounding forest was federally protected and the cattle industry was slowly removed from the park, allowing natural succession of pasturelands to forest. The 1980's saw a drastic increase in sugar cane and rice agriculture and associated construction of roads and canals. In recent years cotton, soybean, melon, and citrus agricultures have also expanded into the region. Beach tourism development, 32,000 ha of rice, and 20,000 ha of cane irrigation has contributed to a water shortage despite the intricate distribution system created by the Arenal-Tempisque Irrigation Project. In addition, 75% of wetlands in the Tempisque basin have been lost in the last 26 years (Joyce 2006). Tilapia farming has also been introduced to the Guanacaste region and the network of irrigation canals and fish farming waterways intermingle and connect to natural waterways.

STUDY ORGANISM

The American Crocodile (*Crocodylus acutus)* is the most widespread crocodilian in the New World and inhabits both Pacific and Atlantic lowland coastal slopes of Costa Rica (Manolis

and Stevenson 2010). Three of the most substantial populations in Central America are known from the Rio Tarcoles, the Tempisque Delta, and the Osa Peninsula on the Pacific versant of Costa Rica (Manolis and Stevenson 2010). Porras (2007) confirms that these populations exchange genetic information. Two Costa Rican populations, the Rio Terraba and Rio Tempisque, have been listed at the second highest "Crocodile Conservation Unit" priority level (Manolis and Stevenson 2010).

Since a wild harvest of skins was banned in the 1970's, overexploitation has been reversed and areas where crocodiles were extirpated have been repopulated via immigration (Manolis and Stevenson 2010). With increases in density, *C. acutus* populations have exploded in localized areas and have expanded inland, contributing to the local extirpation of native Spectacled Caiman (*Caiman crocodilus*) populations and creating increased contact with humans.

The American crocodile in Costa Rica serves as a sentinel species for fishes, amphibians and other reptiles and indirectly for birds and mammals, including humans. Numerous studies using American alligators (*Alligator mississippiensis*) as a crocodilian model are highlighted in Milnes and Guillette (2008) in a detailed account of role that crocodilians play in native and altered ecosystems. Demonstration of the effects of anthropogenic activity on crocodilian development and physiological function serve as evidence for the use of these creatures as ecological model species (Milne and Guillette 2008). Further, anthropogenic effects on reproduction and endocrinology of crocodilians offer insight into the potential effects of human land use on other local wildlife (Milnes and Guillette 2008).

Like all crocodilians, American crocodiles are characterized by temperature-dependent sex determination (TSD). Under this framework, temperatures within a nest during development

determine gender differentiation of embryos (Shine 1999). Lang and Andrews (1994) provide a detailed investigation of the temperature parameters involved in TSD in 6 of 12 species within the genus *Crocodylus* and 11 of the 23 extant crocodilians. The precise temperature window in which males are produced is narrow (30-33°C) and highly conserved within the family. Additionally, no temperature produces 100% males in any crocodilid taxon (Lang and Andrews 1994). Because male-producing temperatures are more difficult to create than temperatures producing females, sex ratio at hatching is female-biased, a feature that is theoretically countered by sex-specific differential mortality during juvenile stages (Lance et al. 2000, Shine 1999). Complex networks of anthropogenic waterways allow the encroachment of crocodiles on residential areas and vise versa (pers. comm. M. Sasa). Male-biased sex ratios are hypothesized to exacerbate this problem, in the short term, because males are forced to disperse as a result of territoriality (Tucker et al. 1998). Thus, anthropogenic infrastructure has contributed to human mortality from crocodiles. Spatially expanding populations of crocodiles and people have resulted in several instances of human-crocodile conflict, including fatal attacks (Ross and Larriera 2001). Current literature provides additional justification for selection of this study organism. Preliminary data from Mexico suggest that climate change may have an effect on the sex ratios of *Crocodylus acutus* and *Crocodylus moreletii* (Escobedo-Galvan 2011). Rainwater et al. 2007 identify the presence of numerous metal contaminants and organochlorine pesticides in tissues collected from *C. acutus* in Costa Rica and Belize and draw attention to the absence of literature regarding the effects of unregulated pesticide and industrial chemical use on the Neotropical fauna. Whelan (1989) foresaw the need for overfishing legislation and pollutant management in the Golfo de Nicoya to prevent lost revenue in the tourism and fishing industries.

Additionally, manipulated crocodile densities at Tarcoles have resulted in aggressive interaction among crocodiles and widespread ocular disease as a result of this conflict (Rainwater 2011).

HYPOTHESES

This study tests two main effects in determining the influences resulting in male-biased sex ratios; environmental temperatures and methyltestosterone. The influence of climate change in the Tempisque region, suggested effects on crocodilian clutch sex ratios, and the skewed sex ratio in the Tempisque basin present a system worthy of investigation. The hypothesis that global climate change is skewing the sex ratio of *C. acutus* via temperature-dependent determination of sex requires testing. If this factor alone explains the male-biased sex ratio, then nests in natural riparian habitat must produce temperatures known to yield males. Temperatures producing balanced or female-biased sex ratio falsify climate change and its effect on nest temperatures as the single factor biasing sex ratios.

The use of synthetic androgens on Tilapia fry is problematic because farming methods are locally unregulated and the effects on other vertebrate fauna are minimally tested. Methyltestosterone (MT) has been shown to effectively homogenize Tilapia populations so that all individuals are male; however, effects on other fauna are unknown, including effective concentration, entry pathway (diet, direct transport across epidermis) and physiological mechanisms of action during different life stages. The effects of MT on crocodilian sex determination will be tested on American alligator eggs, a surrogate for American crocodile eggs. Additionally, eggs from Palo Verde crocodile nests will be tested for the presence of MT in yolk, as will plasma concentrations of MT from blood samples. I predict that MT cause the

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production of male embryos at female-producing temperatures. I hypothesize that MT is present in embryos in Palo Verde nests via bioaccumulation from maternal lipids so the concentration present in *in situ* eggs can be fit to the dose-response curve developed from the experimental assay.

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Chapter 2

Cohort-Dependent Sex Ratio Biases in the American Crocodiles

(*Crocodylus acutus)* of the Tempisque Basin

Abstract. A male-biased sex ratio of 3:1 has been reported for a population of American crocodiles (*Crocodylus acutus)* in the Tempisque River Basin, Guanacaste, Costa Rica. If confirmed, this would constitute one of the largest male-biased sex ratios reported for any population of a member of the genus *Crocodylus*. Here, we examine the aforementioned *C. acutus* population and report on sex ratios of hatchling, juvenile, and adult age classes within a sample of 474 crocodiles captured in the Tempisque Basin between May 2012 and June 2014. Hatchling sex ratio is exceptionally male-biased (3.5:1), an imbalance that is maintained in juveniles but is reduced in adults (1.5:1). Mark-recapture data document that juvenile males disperse from the study site, potentially to avoid competition, a processes that reduces male bias in the adult age class. An increased role of males in human-crocodile conflict may be a result of juvenile males dispersing to human-inhabited areas.

INTRODUCTION

Sex allocation and sex ratio theory have been a topic of experimental and theoretical discussion among ecologists and evolutionary biologists for over a century (Darwin, 1871; Düsing, 1883; Fisher, 1930). Critical to the viability of sexually-reproducing organisms is an adequate proportion of both sexes, most classically modeled by Fisher (1930). This model relies on competition for mates based on the number of individuals of each sex and associated mating prospects. Parents that produce the minority sex will have increased fitness based on number of grandchildren produced and the minority sex will be selected for, thus, maintaining sex ratio equilibrium (Fisher, 1930). Numerous variables, such as sex-linked drive (Hamilton, 1967), alternative fitness influences (Charnov et al., 1981), dispersal (Bulmer, 1986), longevity (Eberhardt, 2002), operational sampling (Gibbons, 1990), and sibling interaction (Uller, 2006) have been added to the basic Fisherian model to explain unusual sex ratios observed in nature.

For organisms exhibiting environmental sex determination (ESD), abiotic cues determine patterns of steroid production, which canalizes development towards one gender or another (Valenzuela and Lance, 2004). Temperature-dependent sex determination (TSD), the most common mechanism of ESD, has obvious relevance to sex allocation theory. TSD is likely selected for when the environment produces the sex with the highest fitness in a highly variable environment (Charnov and Bull. 1977, Warner and Shine, 2008). Further, temperature is thought to lead to differential growth rates between sexes and thus, different fitness consequences (Bull and Charnov, 1989). However, sex ratios of organisms with TSD are less self-correcting than those with genotypic sex determination so that organisms with TSD may experience altered sex ratios that undermine demographic viability, a feature that may make them susceptible to anthropogenic change (Doody et al., 2006).

All extant crocodilians exhibit TSD and members of the genus *Crocodylus* produce viable offspring between 25 and 35 °C, with males generated between 31 and 33.5 °C and females produced at all other temperatures in the viable range (Lang and Andrews, 1994). With such a narrow male-specific thermal window, it has been suggested that male-bias in clutches of crocodiles is difficult to produce in nature (Thorbjarnarson, 1997; Lance et al., 2000). However, male-biased sex ratios are known in crocodilian populations (Thorbjarnarson, 1997). For example, American crocodile (Platt and Thorbjarnarson, 2000) and Morelet's crocodile populations (Rainwater et al., 1998) in Belize as well as a population of the American crocodile (*Crocodylus acutus*) in the Tempisque Basin of Guanacaste, Costa Rica (3:1 male bias; Bolaños-Monero, 2012) exhibit some of the strongest male-biases documented. Similarly, Charruau (2012) reported a male-bias in hatchling *C. acutus* in Banco Chinchorro Biosphere Reserve, Mexico. However, the study by Bolaños-Montero (2012) was based on limited sampling in the Tempisque watershed and a biased sex ratio was not found by Sanchez-Ramírez (2001) for the same area. Additionally, these previous studies were based on raw counts (observed ratios of captures animals) of individuals detected rather than accounting for segments of the population that were undetected. Therefore, the aim of this study was to use mark-recapture techniques to estimate cohort-specific population sizes of each sex independently, compare such estimates, and assess whether a male-biased sex ratio characterizes the population of *C. acutus* in the Tempisque River Basin.

METHODS

Study Area

All sampling for this study was performed in Palo Verde National Park and adjacent localities in the Guanacaste Province, in northwestern Costa Rica. Seven sampling localities in the Tempisque River basin were used; the Humedal, the Drain, the Bebedero River, Varillal Lagoon, La Bocana, Nicaragua Lagoon, and the Tower Ponds (Figure 1). All sampling localities were restricted to the areas within or immediately surrounding Palo Verde National Park and are part of the same Tempisque River Basin population. The Humedal, Varillal Lagoon, La Bocana, Nicaragua Lagoon and Tower Ponds are seasonal wetlands characterized by open expanses of *Typha*, *Thalia* and *Eichhornia* in the wet season (May to December). These areas are void of water during the dry season (December to May) when they are characterized by cracked mud with patches of vegetation. The Drain is a permanent canal that drains irrigation water from rice fields north of the National Park to the Tempisque River 10km to the southwest. This canal is influenced both by tide and agricultural discharge. The Bebedero River is a major tributary to the Tempisque River that extends to the northeast from the Tempisque River to near the town of Cañas, Guanacaste Province, Costa Rica. This river is tidally influenced towards the mouth where it meets the Tempisque River 5 km north of the Gulf of Nicoya.

Crocodile Sampling

American crocodiles were captured in the Tempisque Basin between May 2012 and June 2014. Crocodiles were captured by hand, breakaway snare pole, or top-jaw rope. Sex (by cloacal examination of the genitalia), snout to vent length (SVL), and total length (TL) were recorded followed by creation of a permanent mark via tail scute removal. Individuals were categorized into a cohort based on size: hatchling (<35cm), juvenile (35-180cm) and adult (>180cm;

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modified from Platt and Thorbjarnarson, 2000). A sub-adult size class was not included because any size cut-off between the juvenile and sub-adult cohort was deemed an artificial threshold.

Sampling of hatchlings included 13 clutches (204 hatchlings) within which all individuals were known to have been captured because we monitored the nests from which they emerged and the number of viable eggs was matched to the number of hatchlings captured (n = 6) or because we discovered aggregated individuals soon after hatching from unmonitored nests. The risk of hatchling predation and capture detection existed at unmonitored nests, but was minimized by our timing. We captured two, seven, and four clutches in 2012, 2013 and 2014, respectively. Two clutches in 2013 were captured in the Bebedero River while the rest were captured along a 10 km stretch of drainage canal along the northwest border of the national park. Because hatchlings remain aggregated at nest sites immediately after hatching and because we accounted for all individuals known to have hatched from monitored nests, we assume that sex ratios based on raw counts of hatchlings are not biased by undetected individuals emerging from unmonitored nests. A hatchling crocodilian was determined to be male if the clitero-penis possessed *all* of the following character states: bi-lobed structure, extensive vascularization, and length extending the length of the vent (Figure 2; Allsteadt & Lang, 1995).

For juveniles we used Baileys Triple Catch method (Bailey, 1951, 1952) to estimate gender-specific population size and, thus, sex ratio. This method allowed us to estimate the number of males and females by adjusting for individuals not detected during our sampling. This algorithm assumes an open population with variable gain and survival and requires three sampling events with at least 20 captures per event (Donnelly and Guyer, 1994). Each sampling event represented a complete survey of the area, with one event occurring in 2013 (January – May) and two events occurring in 2014 (January- March and April – June). Separate estimates

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for population size, loss rate, and gain rate were calculated for male and female juveniles. Because the sample of adults yielded few recaptures, unlike the juvenile cohort, we used raw counts to characterize sex ratio of this cohort.

A G-test was used to assess differences in proportion of males among cohorts and to test deviation from a 1:1 sex ratio. A runs test for non-randomness was used to assess any bias in the pattern of capture of males and females in the field.

RESULTS

A total of 474 American crocodiles were captured during our study. Analysis for raw count data provides G- test results that indicate a significant difference in the sex ratio among cohorts (G = 50.828, df = 2, p = <0.01). The hatchling cohort exhibits a sex ratio of nearly 80% male while juvenile and adult cohorts both exhibit 60% male sex ratios. Across all cohorts we recover a 2.2:1 male-biased sex ratio.

When data for juveniles were adjusted for undetected individuals we recovered a 3.4:1 male-biased sex ratio overall, an imbalance that differs from the expected 1:1 sex ratio (G = 103.05; df = 1, p = <0.001). The sex ratio differed marginally among cohorts (G = 5.447, df = 2, p = 0.07; Figure 3). The hatchling and juvenile cohorts consisted of nearly 80% males while the adult cohorts consisted of approximately 60% males.

Our juvenile sex ratio was based on population estimates associated with the second capture interval (N_1) (Table 1). These values differ markedly from the female-biased pattern indicated by juvenile raw counts. Vital demographic rates of change for juvenile females indicated a positive growth rate for this component of the population, with gains due to the

combined effects of growth of hatchlings and immigration being sufficient to counteract losses

due to the combined effects of mortality and emigration. Vital rates of change for juvenile males

indicated a negative growth rate for this component of the population, with gains due to the

combined effects of growth of hatchlings and immigration being insufficient to counteract losses

due to the combined effects of mortality and emigration. Losses were more similar for male and

female juveniles than were gains, which were reduced in males relative to females (Table 1). We

found no significant runs of either sex in our capture records ($Z = -0.447$, $p = 0.327$; Table 1);

thus, non-random sampling of sexes is rejected.

DISCUSSION

Our data document a male-biased sex ratio in the Palo Verde National Park (Tempisque

Basin) *Crocodylus acutus* population. The extent of this bias is extreme at hatching, becoming

reduced for adults. Our data for juveniles provide evidence for how such an extreme male bias is

adjusted over time. Of particular interest is our documentation that juvenile males exhibit a

negative population growth rate, likely caused by dispersal of this stage from the Tempisque

sampling localities and no immigration of juvenile males to the population. Juvenile females,

apparently, have balanced rates of immigration and emigration that maintain a positive

population growth rate for this segment of the population. Based on raw counts of individuals

captured, juvenile females were more numerous than juvenile males. This suggests either that

migration of juvenile males is extensive and immediate, that males are more adept at avoiding

capture, or that both processes are operating. Our data for adults are limited because recaptures

are too low to account for undetected animals and the important parameters of an open

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population. However, if our raw counts accurately reflect the real adult sex ratio, then increased dispersal or mortality of males beyond the juvenile stage does not appear to be occurring. If anything, immigration of juveniles to the study site to retain a male bias in detected adults may be indicated by our data.

The extent of male-bias in our hatchling cohort is unprecedented in crocodilian populations. Charruau (2012) reported male-biased sex ratios at birth for American crocodiles at a site in Mexico, but there only 66% of hatchlings were male, a percentage closer to that of adults at the Tempisque population. Because the sex of our hatchlings was determined by visual inspection of the relative size of developing genitalia, our extreme male bias might result from misidentification of sex. However, we argue that this is unlikely, based on a sample of 10 hatchlings that were identified as to sex at birth and then raised for a year at the Palo Verde Biological Station. When re-examined a year later, none of these individuals changed in status. The extreme male bias at hatching might also result from a biased sample of nests. However, hatchlings were captured within a short time following emergence from their nests during a time when nest mates remain aggregated. For monitored nests the number of hatchlings captured corresponded to the number of viable eggs known to be in the nests. We assume this to also be true of unmonitored nests. Further, the temporal and spatial disparity of the clutches analyzed accounts for climatic bias within years as well as spatial thermal variation between habitats. Therefore, the only way that our count of hatchling males and females is biased is if the pool of undetected nests produced a sex ratio differing from our detected nests. Additionally, the window of nest temperatures yielding male American crocodiles is quite narrow (Lance et al., 2000, Thorbjarnarson, 1997), making it unlikely that we selected nests within this narrow window and missed others outside this range.

The demographic patterns that we document for juvenile males and females are consistent with interpretations inferred for other crocodilians. The territoriality of adult male crocodilians has been used to suggest that juvenile males disperse from the natal population to avoid competition (Thorbjarnarson, 1989; Platt and Thorbjarnarson, 2000), features consistent with our estimates of gain and loss rates for juveniles (Figure 4).

Across the entire population of Tempisque crocodiles, we recovered a male bias that is as skewed as that reported by Bolaños-Monero (2012) and far more skewed than that reported by Sanchez-Ramírez (2001). The sampling of Bolaños-Montero (2012) and Sanchez- Ramírez (2001) may have been inadvertently susceptible to location-based biases or biases resulting from undetected individuals. Here, we present increased sampling and mark-recapture analysis to estimate animals not detected. The difference in observed versus estimated sex ratios of juveniles is extreme and renders strict observation-based counts unreliable. Dispersal of juvenile males from the core sampling areas would have likely resulted in an underestimation of males in the population based on strict count sampling.

Negative perceptions of crocodiles have reached a critical level in Costa Rica as a result of increased media coverage of crocodile/human conflicts and/or an actual statistical increase in attacks by crocodiles on humans (Valdelomar et al., 2012). A unique male bias, to the extent described by Bolaños-Montero (2012), is likely to contribute to this negative perception. The demographics discussed here indicate an expanding population, from a spatial perspective, that may lead to increased overlap with human-inhabited areas and potential for human-induced conflict such as feeding (Rainwater et al., 2011). Furthermore, if this problem persists for generations then selection for more aggressive males based on heightened reproductive competition may occur. Of utmost importance is an organized collaborative effort to assess the

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demographics of *Crocodylus acutus* along the Pacific versant of Costa Rica prior to management action.

ACKNOWLEDGMENTS

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Figure 1. Map of the study area in Guanacaste, Costa Rica. Region shown includes Palo Verde National Park and the Tempisque River Estuary. Seven sampling areas are noted: (Drain (A), Varillal Lagoon (B), Humedal (C), La Bocana (D), Nicaragua Lagoon (E), Tower ponds (F), Rio Bebedero (G)).

Figure 2. Clitero-penis (CTP) examples depicting conservative sex determination of hatchlings based on the number of lobes, vascularization, and relative length. Female exhibiting small vascularized nub (A), Female exhibiting small non-vascularized nub (B), Female exhibiting single-lobed vascularized projection (C), Male exhibiting bi-lobed vascularized projection that extends the length of the cloacal opening (D, E).

Figure 3. Cohort-specific sex ratios with juvenile cohort based on triple-catch algorithm estimates. Sample sizes do not include recaptures. G-test results show independence in male frequency among cohorts (G = 50.828, df = 2, p = <0.01).

Figure 4. Schematic depicting proposed demographics among cohorts of the two sexes, showing emigration of sub-adult (SA) males from the population.

Table 1. Population estimates, birth/immigration rates, death/emigration rates and population growth rates of males and females in the juvenile cohort based on Bailey's triple-catch algorithm (top).

Figure 1.
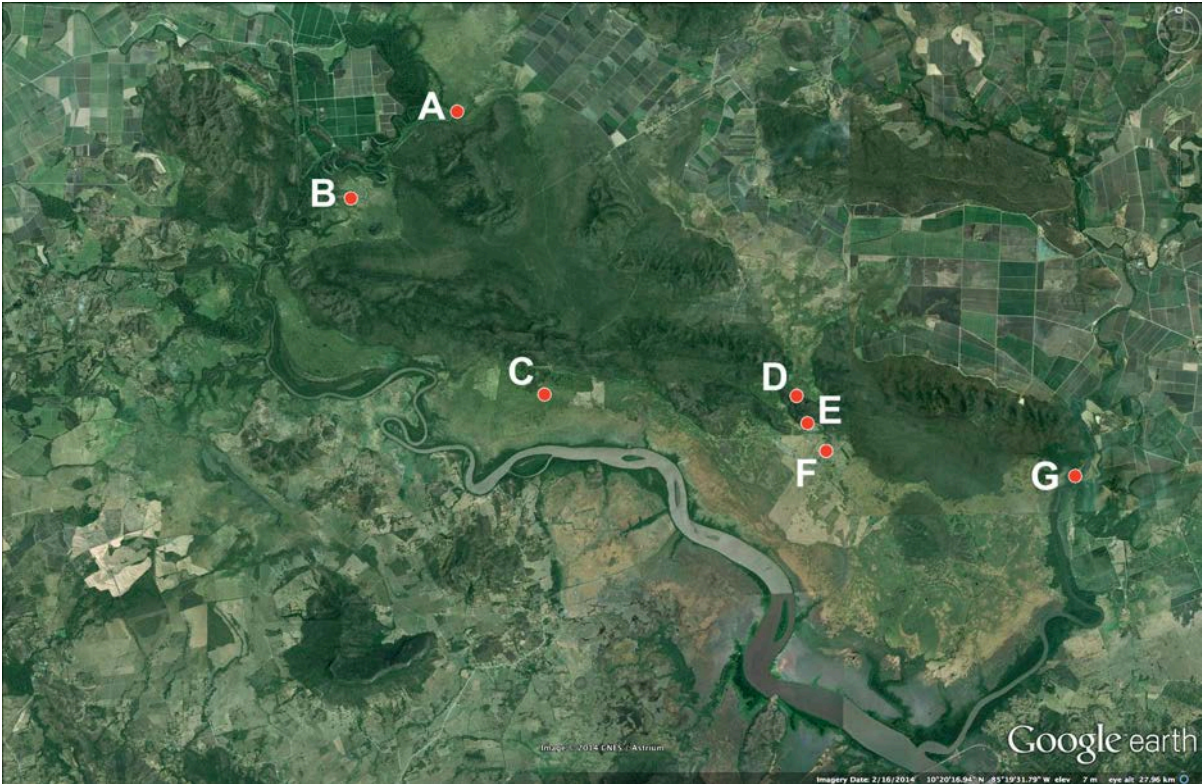
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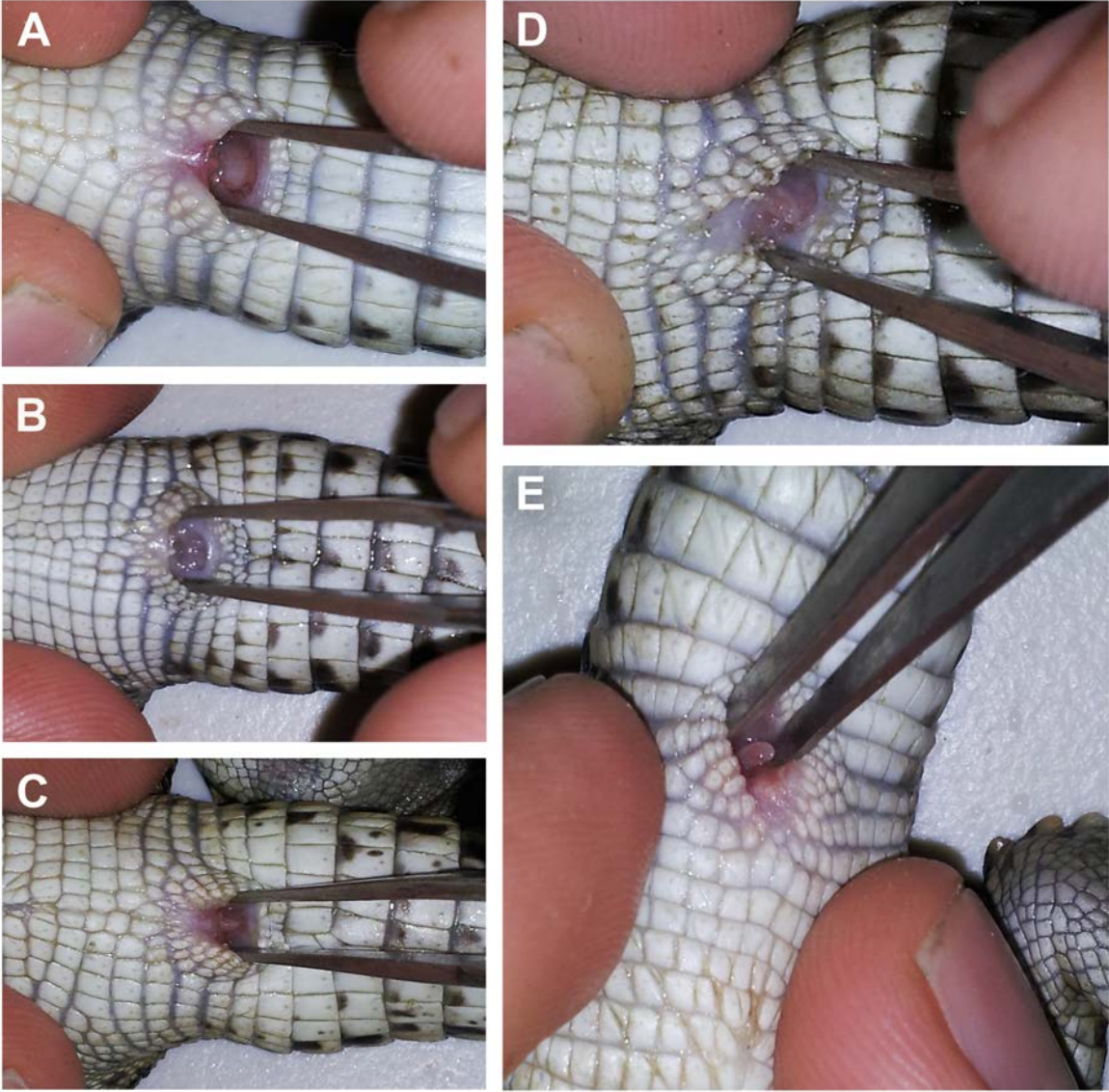
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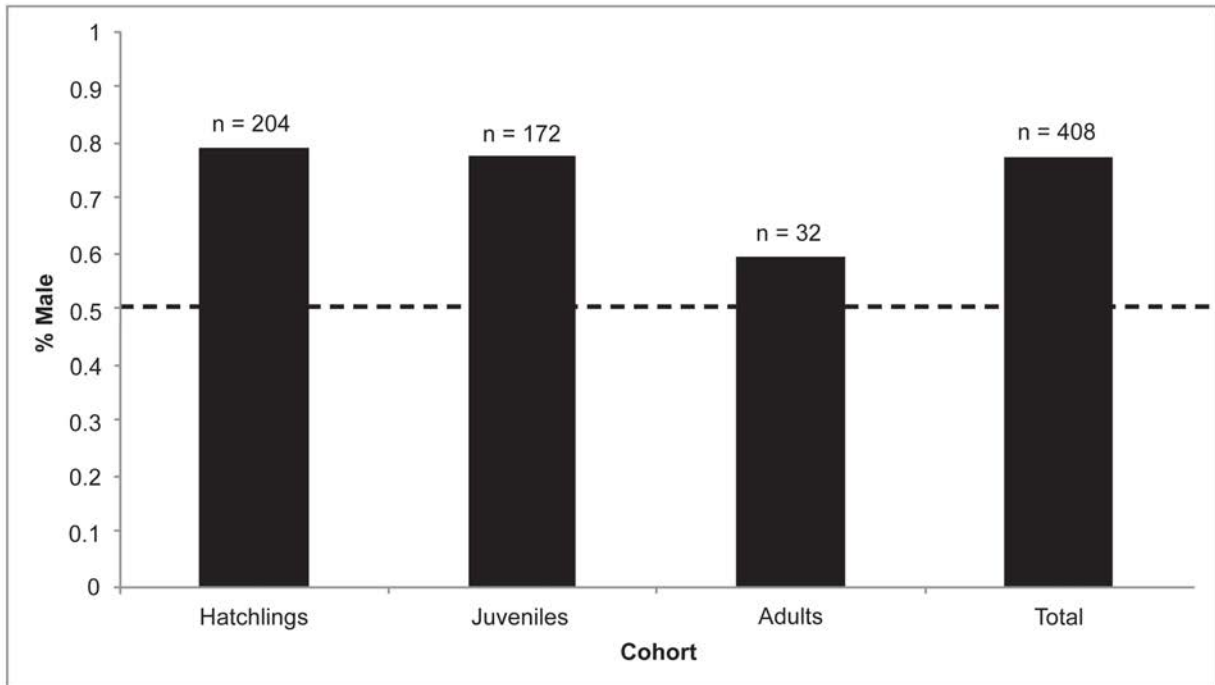
Figure 4
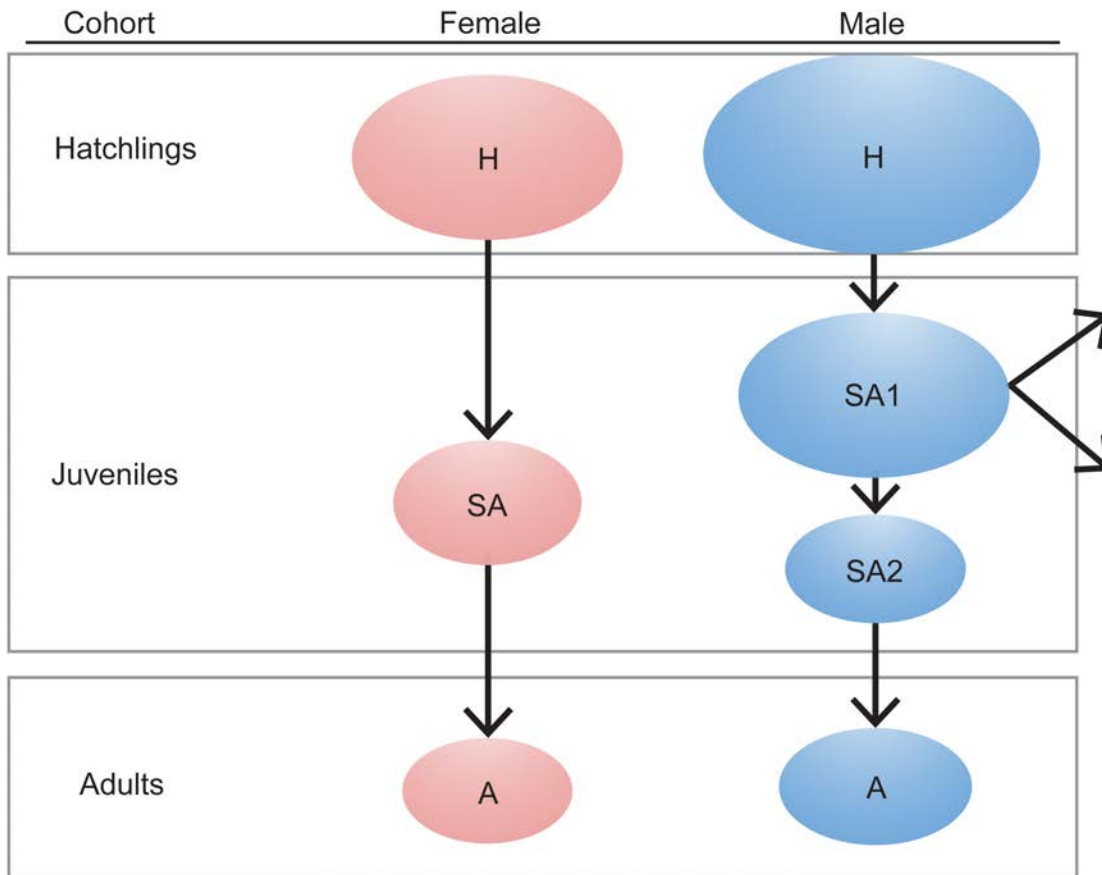
Table 1: Populations estimates, birth/immigration rates, death/emigration rates and population growth rates of males and females in the juvenile cohort based on Bailey's triple-catch algorithm (top).

Baileys Triple Catch	Males	Females
Population Size at Time 1 (N_1)	310 ± 208.2	89 ± 71.8
Raw Count	98	117
Birth/Immigration Rate (B_{12})	0.322	1.241
Death/Emigration Rate (D_{01})	0.565	0.752
Instantaneous Birth/Immigration Rate (b_{12})	-1.13	0.216
Instantaneous Death/ Emigration Rate (d_{01})	0.833	1.39
Population Growth Rate (r)	-0.586	0.073

Chapter 3

Regional Warming and the Thermal Regimes of American Crocodile Nests

in the Tempisque Basin, Costa Rica

Abstract. Spatial variation in global climate change makes population-specific responses to this enigmatic threat pertinent on a regional scale. Organisms with temperature-dependent sex determination (TSD) potentially possess a unique physiological susceptibility that threatens population viability if rapid environmental effects on sex ratios render populations non-viable. A heavily male-biased sex ratio for hatchling American crocodiles of the Tempisque Basin, Costa Rica requires assessment of how nest temperature affects sex determination at this site, how females might compensate for these effects when creating nests, and how current patterns of climate change might alter future sex ratios and survival in hatchling cohorts. We demonstrate high within-nest variation in temperature but predict a female bias at hatching based on nest temperatures quantified here. Further, our data suggest that egg size and metabolic heating associated with this factor outweighs microhabitat parameters and depth in influencing nest thermal regimes. Finally, we document regional warming in the Tempisque Basin over the last 15 years and project that further heating over the next 15 years will not yield hatchling sex ratios as male biased as those currently found at this site. Thus, we find no support for nest temperature or climate change as likely explanations for male-biased American crocodile (*Crocodylus acutus*) sex ratios in the Tempisque Basin.

Key words: temperature-dependent sex determination, climate change, sex ratio

INTRODUCTION

Research on physiological, ecological and evolutionary responses to climate change is critical for diagnosing population-level responses in relation to this enigmatic factor (Whitfield et al., 2007). Vertebrate populations are susceptible to range shifts (Colwell, 2008), physiological pressures (Huey et al. 2003) and reproductive transitions (Refsnider and Janzen, 2012) when exposed to shifts in temperature on a regional scale. Of particular concern are species with sex determination mechanisms that rely on environmental stimuli, such as organisms with temperature-dependent sex determination (TSD; Doody et al., 2006). Sex ratios are less self-correcting in vertebrates with TSD than in those species with genetic sex determination and species with TSD are also more susceptible to anthropogenic influences (Doody et al., 2006). Alteration of nesting habitat, endocrine-disrupting compounds and global climate change are all factors that can alter developmental regimes and bias the resulting sex of vertebrates exhibiting TSD. While changes in nesting phenology or location may compensate for shifts in thermal regimes on a regional scale (Refsnider and Janzen; 2012, Doody et al. 2006), these may not be sufficient to thwart demographic changes that will risk population viability (Girondot et al., 2004).

As evidenced by both experimental and field studies, climate change can negatively affect sex ratios of numerous vertebrate populations. Ospina-Álvarez and Pifferer (2008) note that climate change of a magnitude predicted by current models is capable of altering sex ratios of fish species with sex chromosomes based on a thermal override of genetic differentiation. Such a mechanism has proven common among vertebrates (Holleley et al. 2015). Among species with TSD, climate change may also prove detrimental to rare species (Hawkes et al.,

2007; Huey and Janzen, 2008; Mitchell et al., 2008) as a result of limited environmental refugia from a warming microclimate and subsequent constraints on reproductive phenology and sex ratio shifts. Although species with TSD may alter nesting phenology to compensate for climate change, such compensation may fail to match the pace of climate change (Shwanz and Janzen, 2008). Aquatic apex predators, such as crocodilians, may be particularly susceptible to sex ratio shifts that alter their interaction with humans from both positive (increased ecotourism) and negative (increased attacks on humans) perspectives. For example, Charruau (2012) reports a male-bias in hatchling American crocodiles (*Crocodylus acutus*) in Banco Chinchorro Biosphere Reserve, Mexico and relates this to recent regional temperature increases. Further, Charruau (2012) issues a warning that the sex ratio shift will depend on the magnitude of temperature increase and its effect on the female-male-female (FMF) sex determination scheme characteristic of the American crocodile, a scheme in which 31°C and 33°C are the temperatures between which males can be produced (Lang and Andrews, 1994; Charruau, 2012).

Murray et al. (2015) report an unusually high male-bias in American crocodiles of the Tempisque Basin, Guanacaste, Costa Rica. This 3.4:1 male bias is the most extensive male bias within the genus *Crocodylus* reported to date (Thorbjarnarson, 1997). The Tempisque drainage population is large, rapidly growing and has been subject to frequent conflict with humans in recent years (Valdelomar et al., 2012). Here, we describe crocodile nest temperature profiles and test for correlations between nest thermal regimes and environmental variables to assess the compensatory effect of nest location as a variable that allows regulation of hatchling sex ratios. Further, we utilize current nest temperature profiles and patterns of temperature increase over the past 15 years to predict the sex ratio of offspring in the Tempisque Basin 15 years into the future.

METHODS

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Field Methods

Between 2012 and 2014 twenty-five *Crocodylus acutus* nests were located along waterways surrounding Palo Verde National Park. These drainages included the irrigation drain on the northwest border of the park, the Tempisque River between El Viejo sugarcane farm and the Bebedero River junction, and the Bebedero River between the northeast border of the park and the Tempisque River [see Murray et al. (in press) for detailed description of the study site]. Nests were located from a boat via visual inspection of the bank for slides, cleared areas, and suitable substrate. The banks of all potential nesting waterways were searched every other day ensuring that all nests were discovered within 2 days of construction and egg deposition.

Canopy cover at each nest was quantified using the canopy-scope method (Brown et al., 2000), a more nest-specific quantification compared to the methodology of Doody et al. (2006). Laying face-up on the nest surface looking directly perpendicular to the ground, the grid is held 20 cm away from the eye, standardized by a measured string attached to the scope. The proportion of canopy foliage intersecting the line of sight between the scope and sky was recorded. Solar radiation was not recorded because all nests were situated on flat surfaces. Each nest cavity was excavated by carefully removing the loose dirt over the nest hole and nest depth, egg size (pole diameter to nearest mm) and number of eggs were recorded. Nest depth was recorded post-excavation as the distance from the soil surface to the bottom of the deepest egg. Eggs were carefully removed and their orientation marked with a pencil to avoid rotation during nest excavation. They were then replaced in the nest, along with a set of Ibutton thermal data loggers (model: DS1922L, accuracy: -40°C to +85°C, precision: ±0.5°C, Maxim Integrated.

INC.), and covered as similarly as possible to the original nest structure. The thermal data loggers were sealed in beeswax and placed in plastic eggs half filled with water and sealed with waterproof tape. To test these loggers system, Ibuttons were inserted into a small rectangular hole cut in infertile chicken eggs. This hole was covered and plastic egg and infertile chicken egg designs were paired and laid on a windowsill. The plastic egg design matched the thermal fluctuations of infertile chicken eggs in these lab trials. Four loggers were placed in each nest, one at the bottom, center, top, and on the side nearest the water. Each logger recorded temperature every 30 minutes for a minimum of two weeks and a maximum of 3.5 weeks during the 4 weeks of the middle third of incubation (days 30-60 of a 90-day incubation period; Charruau, 2012). Because the date of egg deposition was known and nest variables were quantified during the middle third of incubation, our loggers, inserted after four weeks of incubation, recorded the thermal regime during the portion of development when sex is determined (Georges et al. 2004). Loggers were recovered by re-excavating nests. Only thirteen of the 25 nests were available for data collection due to predation of the remaining nests. The eggs were covered after re-excavation so that the nests could continue to be monitored for aspects of a separate study.

In 2014, reference thermal loggers were buried one meter away from the center of each of four nests, with the reference logger placed at the same depth and same distance from water as the center of the paired nest. Nest hole substrate and canopy cover did not qualitatively differ among these four nests. Thereby, these loggers provide a control against which metabolic heat produced by a clutch might be compared. One reference logger was not recovered, so the sample size was three for this analysis.

Trends in nest thermal regimes were assessed in two ways. First, we used data from the thermal loggers to characterize the mean temperature for each location within a nest, and the variation around that mean. These summary statistics were used to describe the thermal environment within and between nests. Top, center, and bottom recordings characterized thermal profiles in the vertical axis and side temperatures were extrapolated to all sides of the nest along the horizontal axis (Figure 1). Second, we used data pooled for all loggers within a nest to explore the influence of nest parameters on nest temperature and temperature variance. For this analysis, multiple linear regression was performed in R (R Development Core Team, 2011) using mean temperature and variance as dependent variables and canopy cover, nest depth, egg number, and egg size as independent variables. We also tested these independent variables against the average logger temperature at the top position of the nest, assumed to be the most environmentally influenced location of the nest. A Welch's T test was implemented in R (R Development Core Team, 2011) to explore relationships between important nest parameters and nest temperatures.

We used data from the thermal loggers to predict the sex of offspring expected to hatch from each nest. To do this, the mean temperature for each location was converted to a constant temperature equivalent (CTE), the temperature above and below which half of embryonic development occurred (Georges et al. 1994, 2004). Data from Lang and Andrews (1994) on alligator development were used as a proxy for crocodilian developmental rates across various incubation temperatures. Developmental rates among incubation thermal regimes are not available for *Crocodylus acutus*. However, because developmental rates and mechanisms are

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highly conserved among crocodilians, and *C. acutus* has a slightly longer incubation period, larger hatchling size *and* longer time to maturity than *A. mississippiensis* (Lang and Andrews, 194; Charruau, 2012) then we assume that the rate of development is very similar and using the alligator developmental reaction norm as a proxy is justified. Lang and Andrews (1994) assessed developmental stages over time at five different incubation temperatures. We used the slope of a regression of eye orbit diameter on time as a measure of growth rate. Widths of the eye orbit at specific post-fertilization stages were taken from Ferguson's (1985) staging guide of alligator development. Growth rates (slope of orbit width regressed on elapsed time) were determined across temperatures. We then generated a histogram expressing the frequency with which each temperature was experienced during development at the four positions within a nest associated with a thermal data logger. This histogram represented the proportion of time spent at each temperature during development; when the proportion of each temperature was multiplied by the rate of development at that temperature a distribution of degree-days of development was generated. The median of a running sum of these weighted growth rates was used as the temperature above and below which half of development occurred (CTE) and, therefore, the temperature likely to be experienced when sex is determined (Georges et al. 2004). CTE for the upper and lower intermediate zones (Figure 1) were calculated as the mean of CTEs experienced by the center and top or bottom section of the nest. Mean temperatures and CTEs were used to predict the sex ratio of hatchlings expected to emerge from individual nests. Temperatures between 31°C and 33°C were deemed male producing and temperatures above and below this range were deemed female producing (Lang and Andrews, 1994; Charruau, 2012). Because crocodile nests are roughly spherical and laid very close to the soil surface, we used nest depth as the diameter of a sphere representing nest volume, calculated the volume of each region of the

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nest based on linear proportions of the radius of the nest sphere (Figure 1) and used these

calculations to determine the proportion of the overall nest volume associated with each region

of the nest. These proportions were multiplied by clutch size to estimate the proportion of eggs

experiencing the thermal regime of each region of the nest. Thus, the expected sex ratio of each

nest accounted for known within-nest thermal variation.

Predictive Modeling

Climate data were obtained from the Organization for Tropical Studies (OTS)

meteorological database. Daily maximum and minimum temperatures recorded between

December 1996 and December 2011 were downloaded for the Palo Verde Biological Station,

Guanacaste, Costa Rica. During periods of weather station maintenance, temperature data were

supplemented by the nearby weather station in Bagaces, Guanacaste, Costa Rica, approximately

20 km from the study site. Daily low temperatures were averaged by month and were analyzed

using least-squares linear regression for significant trends over time. We used the slope of this

regression to describe the rate of temperature change over the past 15 years. Further, we estimate

temperatures fifteen years into the future by simply extrapolating this slope should this trend

continue. To ensure realism in our projections we only made future predictions for the same

amount of time that we had past climate data for (15 years). We assumed that patterns of

temperature variation within current nests will be maintained in future nests with the top region

of future nests experiencing the full predicted increase in future temperatures and the bottom,

center and side locations experiencing temperature changes accounting for relationships

quantified here between surface temperatures and temperatures at deeper positions within a nest.

Thus, expected CTEs were recalculated for each nest position and for known variation in overall thermal regimes among nests. Because egg temperatures of 35°C are known to be lethal for *Crocodylus* (Lang and Andrews 1994; Lang pers. comm.), and tolerance to this lethal temperature increases with development, we assumed eggs in any nest positions reaching this temperature would die this early in development. From this, we projected nest survivorship for current and future nest thermal regimes using maximum data logger temperatures for each location and applying them to the proportion of eggs per nest region for each nest.

RESULTS

Nest Thermal Regime Profile

Thermal logger data revealed consistent trends among nest locations. Varying temperatures and amplitudes among nest locations on a diel cycle as well as influences of weather, such as rainfall, were evident. The bottom of the nest was consistently less variable than any other nest location; the top and center locations were consistently the warmest and most variable locations in the nest (Figure 2). Temperatures recorded at the top of the nest had higher variance than any other location in 11 of 12 nests while the center was the second most variable location in 10 of 13 nests. Variance in temperature among data loggers within each nest averaged 1.7 ± 0.3°C. The bottom of the nest was the least variable location in 9 nests. Average temperatures were not consistently associated with logger position. The bottom of the nest had the lowest average temperatures recorded in only 3 of the 13 nests with the top of the nest being lowest in five nests (Table 1). The center location was the warmest nest location more often than

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any other nest location, but this was true for only 5 of 13 nests. The top of the nest was warmest in 3 nests and the top and center were equally warm in one nest (Table 1)

Mean thermal logger temperatures varied as much as 1.6 °C among locations in a single nest (PVN1-13). Variation in temperature among locations within a single nest was between 0.5 and 5 °C at any specific time. Additionally, the variance at any one location within a nest was negatively correlated with the average temperature at that location (F =10.56, df = 48, p = 0.0021, R^2 = 0.18) (Figure 3).

Four common patterns in nest thermal regimes were detected (Figure 4). Pattern 'A' exhibited a hot center and top with a warm side and relatively cool bottom and was observed in 31% of nests. Pattern 'B', observed in 23% of nests, exhibited a hot top with a warm center and bottom and cool side. Patterns 'C' and 'D' were cooler in general, with pattern 'C' exhibiting a hot top with a cool center, side and bottom (38% of nests) and pattern 'D' exhibiting a cool center and bottom with a warmer top and hot side (8% of nests).

Correlates of Nest Temperature

Multiple linear regression analysis revealed overall significance between tested parameters and average nest temperature (F = 9.3; DF = 5,6; p = 0.008). Egg number (R^2 = 0.2, p = 0.03) and egg size (R^2 = 0.79, p = 0.007) were significantly correlated with average nest temperature while, canopy cover and nest depth were not significantly correlated with average nest temperature (Figure 5). No variable was significantly correlated with nest temperature variance, although canopy cover, egg size and average nest temperature explained between 40 and 60% of the variation in nest temperature variance (R^2 = 0.43, 0.59 and 0.66, respectively).

Further, mean temperature at the top of the nest was not correlated with canopy cover, nest depth, egg number or temperature variance at the top of the nest, but was correlated with egg size ($R^2 = 0.839$, p = 0.003)

Egg size and nest temperature were positively correlated (F = 16.5, df = 10, p = 0.003). More than sixty percent of average nest temperature was explained by the average size of the eggs within the clutch ($R^2 = 0.62$). Further, egg size was significantly greater in nest patterns A and B than patterns C and D (t = 2.9, df = 6.9, p = 0.02). A difference of 1.4, 1.5 and 1.7 °C was found between three nest centers and nearby isolated loggers at equal depth, with isolated control loggers being cooler in all instances (t = 15.75, df = 2, p = 0.004). All three nests used exhibited patterns A or B and had relatively large eggs.

Sex Ratio Quantification

Neither mean temperature data (Table 1) nor calculated CTEs generated a male bias in expected sex ratio based on nest thermal regimes. In fact, raw logger temperature data revealed only two nests that might have produced males because they reached the lower pivotal temperature (31°C) to produce this sex. Growth rates across temperatures, using alligator development as a proxy for crocodile growth, produced a linear relationship (y = 0.0147x – 0.2857, $R^2 = 0.91$, Figure 6). CTEs were consistently higher than mean raw logger temperatures as predicted by the increase in development above the thermal mean as opposed to below it (Georges et al. 2004). Of 52 total locations among all 13 nests, 20 exhibited male-producing CTEs, thirty-one exhibited female-producing CTEs, and one location could not be estimated due to logger failure. Because within-nest thermal variation is high and the sex of the hatchling

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cohort as a whole is of interest, reporting sex ratio predictions on the basis of individual eggs is most efficient. Of 357 eggs, we estimated 37% to have developed at male-producing CTEs, with four nests exhibiting CTEs expected to produce both sexes and nine nests exhibiting CTEs expected to produce one sex or the other (Table 2).

Predictive Modeling

Daily average minimum temperatures in Palo Verde National Park have increased significantly between 1996 and 2011($F = 9.02$, df = 14, $p = 0.009$; $R^2 = 0.392$; Figure 7). These data indicate that temperatures are increasing at a rate of 0.24 °C per year. Based on this rate, temperatures are expected to increase 3.6 °C over the next 15 years. In the future, the distribution of CTEs experienced by crocodile eggs will shift from a unimodal distribution centered well below the lower threshold for production of males, to a more uniform distribution traversing the range of temperatures producing males (Figure 8). Present CTEs do not reach the higher pivotal TSD temperature at which females are produced, but future projections indicate that the majority of eggs will produce females in the hotter female-producing thermal window or will be above lethal embryonic temperatures.

In the current environment, egg size has the strongest influence on nest temperature. Three categories of eggs characterize this variable: small eggs (71-79.1 mm in length), intermediate (79.2-83.0 mm in length) and large eggs (83.1-85 mm in length), with 28%, 34% and 38% of all eggs falling in the small, intermediate and large category, respectively. Categories were designated based on the tri-modal distribution of egg sizes observed. CTE frequency distributions at present and projected fifteen years into the future were restricted to the four nests

with the largest average egg size, four nests with the smallest average egg size and five

intermediate nests to understand the effect of egg size on sex ratio. The distribution of CTEs for

small eggs in the current environment predicts that nearly all of the eggs will produce females

because they are below the lower pivotal temperature (Figure 8A). The distribution of CTEs for

large eggs in the current environment is centered on the lower pivotal temperature, with most

eggs producing males and few eggs approaching the upper pivotal temperature (Figure 8B).

When projected 15 years into the future, the distribution of CTEs for small eggs are expected to

shift slightly toward the lower pivotal temperature with some nests expected to produce males.

The distribution of CTEs for large eggs projected 15 years into the future are expected to be

centered above the upper pivotal temperature with few eggs being cool enough to produce males,

most eggs expected to produce females, and many eggs exceeding the temperature expected to

kill developing embryos (Figure 8B). Projections based on lethal thermal maxima revealed 94%

survivorship at present, while future thermal regimes are expected to reduce survivorship to 65%

(Table 2, Figure 9).

DISCUSSION

Nest Thermal Regime Profile

The simplest model describing temperature in subterranean nests containing eggs of

ectotherms is one in which solar radiation is the only energetic input. In such a model,

temperatures at the top of the nest fluctuate most broadly, because of energetic input by day and

loss of heat to air at night. The bottom of the nest is coolest and fluctuates relatively little

because of the insulating effect of the soil and its thermal inertia against loss of heat to air. All other sites within a nest represent intermediate conditions related to nest depth. These characteristics were present in nests of Figure 4C, which was found in 38% of nests. Such nests contained eggs of small size, which appeared incapable of producing enough metabolic heat to alter the distribution of nest temperature. Thus, some crocodile nests conformed to this simple model.

Many other nests deviated from this simple model of nest temperatures because of metabolic heat associated with large eggs. Our quantified extent of metabolic heating between nest temps and isolated control loggers (1.4-1.7 °C) contrasts with the negligible influence of metabolic heating in *Paleosuchus*, a mound nesting crocodilian (Magnusson et al. 1985, 1990), but falls within the range of another hole-nesting reptile with TSD, the green turtle (*Chelonia mydas*) (0.7-2.61 °C) (Broderick et al. 2001). Nests used in our study to quantify the extent of metabolic heat all had large eggs relative to other nests, so the range of metabolic heating documented here may represent a maximum amount of heat produced by crocodile eggs early in development. Over the course of development, however, Broderick et al. (2001) indicated that metabolic heat increased over time, as embryos got larger. Since our data quantify the first and second trimester of development it is possible that our data underestimate how much heat might be generated, but not the impact of that heat on sex determination. Those nests composed of large eggs generate the warmer thermal profiles within nests (Figure 4 A&B). Increased canopy cover and egg size appear to reduce the variance in nest thermal regime, presumably by dampening nest exposure to daily maximum temperatures and elevating nest low temperatures, respectively. The negative correlation between mean nest temperature and variance corroborates the natural relationship between mean nest temperature and daily climatic variance across the

identical time period in jacky dragons (*Amphibolurus muricatus*), recovered by Warner and Shine (2011) and, as described in their experimental manipulation, can have important effects on sex ratio (see below).

Nest microhabitat parameters and their influence on nest thermal regime indicate that neither nest location nor depth is significantly influential in determining average nest temperature. Therefore, females have limited ability to influence the sex of their offspring via nest site selection. Neither canopy cover nor nest depth influence average clutch temperature, by our assessment, to a statistically significant degree. Further, neither microhabitat parameter correlated with egg size, suggesting no relationship between mother size and nest location. Instead, the only way females appear capable of altering the thermal environment of a nest is via egg size. However, it seems unlikely that female crocodilians could influence sex ratio of offspring through this mechanism since it would require adjusting egg size at the time of egg deposition or selecting nest sites based on the known size of the eggs produced. While yolk allocation has been shown to influence sex determination in a skink species (Radder et al., 2009), such a phenomenon participates in a complex interaction between temperature extremes and genotypic sex determination (GSD), a sex determination system not present in crocodilians. Further, the ability to differentially deposit eggs of various sizes among habitats with varying thermal regimes as a function of shade has been documented in emydid turtles (Roosenburg, 1996), but our results would have uncovered a relationship between egg size and canopy cover if this was occurring in our system.

Male-Biased Sex Ratios

If sex ratios at birth are correctly estimated via the method of Georges (2004) then our data eliminate current nest thermal profiles as a cause for the production of the male-bias in these specific hatchling cohorts of American Crocodiles in the Tempisque Basin documented by Murray et al. (2015). Instead, thermal data for the current environment predict a female bias within hatchlings. Our data likely overestimate the expected number of males because we projected all eggs in the nest sections with CTEs of 31-33 °C to become males. Based on experimental tests of TSD among members of the genus *Crocodylus*, *C. acutus* is highly unlikely to produce 100% males at any male-producing temperature (Lang and Andrews, 1994). Charruau (2011) reports a male-bias in nest temperatures and subsequent hatchlings that were recovered over a five-year sample in Banco Chinchorro biosphere. Data presented here contradict this finding and indicate temperatures that predict a female bias for hatchling *C. acutus* using the threshold temperatures described by Lang and Andrews (1994) and Charruau (2011).

We see three possible explanations for the discrepancy between the predicted and observed sex ratio of hatchling crocodiles reported by Murray et al. (2015). One may be that the Georges et al. (2004) CTE model is lacking in complexity. Neuwald and Valenzuela (2011) document that, if diel temperature variance in painted turtle (*Chrysemys picta*) nests is large, eggs incubated at female-producing temperatures produce mostly male offspring. Further, Warner and Shine (2011) recover similar findings in jacky dragons (*Amphibolurus muricatus*), a TSD taxon with two pivotal temperatures that, when experimentally manipulated with ±4 and ±8 degree temperature variance, produced sex ratios that were not predictable by mean incubation temperatures.

Neuwald and Valenzuela (2011) established that within-nest temperature variance of 5°C was required to observe such reversal in turtles. When temperature variance was 3°C, then the

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expected sex ratios were observed (Neuwald and Valenzuela, 2011). Crocodile nests sampled in our study do not vary by more than 3°C, so variable nest temperatures seem unlikely to explain the disparity between predicted and observed sex ratios. Further, justification for our model's accuracy and inability to predict the observed sex ratio from Murray et al (2015) rests on our knowledge of TSD parameters and necessary assumptions. The pivotal temperatures for sexual differentiation are highly conserved among genera within Crocodylia (Lang and Andrews, 1994), and such a phylogenetic constraint justifies the assumption that our pivotal temperatures from Charruau's (2012) population in Mexico are applicable to our population in Costa Rica. In addition, we can be certain that our quantification of the thermosensitive period is accurate because we know the date of oviposition, and thus, know that temperature loggers quantified nest temperature during greater than fifty percent of the trimester in which sex is determined. Weather at nests sites just before, during and just after the second trimester was also very stable, lacking precipitation and maintaining a stable diel temperature cycle. Further, we justify the assumption that the alligator is an accurate proxy for developmental reaction norms in our calculation of CTE. Because of this, we are exploring endocrine disruption as a possible explanation for altered sex ratios in crocodiles of the Tempisque Basin. Although environmental androgens are both rare and seemingly lack physiological effectiveness (Wibbels and Crews, 1995), our study area is contiguous with a large, private fish farm that uses methyl testosterone to convert all fish to males.

Predictive Modeling

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Regional warming trends in the Tempisque Basin are evident from our analysis of local weather records. The extent of increase in average daily low temperature on an annual basis is roughly 2.5°C over 16 years. This is nearly twice the thermal increase in daily minimum temperature documented at La Selva, a wet-forest site in Costa Rica, over a 22-year period (Whitfield et al. 2007). Regional warming trends in La Selva's tropical wet forest are associated with declines of terrestrial amphibians and reptiles, potentially as a result of climate-related changes in leaf litter microhabitats (Whitfield et al. 2007). Further, this very local trend corroborates the IS92 scenario model for the more general Pacific sector of Costa Rica, which predicts a 3°C increase in mean temperature for the whole region by year 2100 (IPCC, 2013) The extent to which regional warming documented for Palo Verde affects ecological and physiological traits of tropical dry forests vertebrates is unknown. However, strengthened easterly winds during July have resulted in increased precipitation on the Caribbean side of Costa Rica yielding more severe drought on the Pacific side of the country (Aguilar et al. 2005), a feature that should alter thermal environments including those of ground-nesting oviparous species.

Mortality caused by extreme nest temperatures during the late first/early second third of development, currently is low (6%) based on logger data. When extrapolated fifteen years into the future, mortality from extreme nest temperatures is expected to rise to 35% based on exposure to lethal temperatures alone. Congdon et al. (1994) suggest that nest success has little effect on population stability in extremely long-lived vertebrates, such as crocodiles. However, the rapid increase in mortality expected from climate change may require immediate monitoring. Such a decrease in survival may decrease the population's growth rate and/or may restrict

reproductive success to small mothers that lay small eggs, rather than large mothers that lay larger eggs that produce substantial amounts of metabolic heat.

Presently, large- and intermediate-sized eggs appear capable of producing males while small eggs are expected to produce females only. This yields an overall female bias at hatchling and increased fitness for large females that produce males, the limited sex. Predicted CTE distributions fifteen years in the future will reverse this trend, with small eggs achieving temperatures required to produce males and large eggs producing females from warm nests that frequently will exceed the lethal thermal maximum.

Because the laying of small eggs presents a fitness advantage in the future context of climate change, natural stress responses in reproductive allocation may rescue large mothers from a decrease in fitness. Murray et al. (2013) find that the positive allometric relationship between mother size and egg size breaks down under conditions of physiological stress, when salinity is used as a stressor for alligators. If regional warming acts as a stressor for large long-lived vertebrates with plastic egg/offspring size, then this documented breakdown in allometry may rescue size cohorts that would otherwise suffer from reduced fitness.

In summary, data presented here suggest that, while climate change is evident in the Tempisque Basin, it is not responsible for a highly male-biased hatchling sex ratio reported in Murray et al. (2015). Nor will it be caused by future thermal shifts. Further, while egg size and metabolic heating seem to be influential in sex determination, nest depth does not (in accordance with Refsnider et al, 2013), nor does canopy cover (contrary to Doody et al. 2006). Rather, egg size via maternal size may be an important factor in battling regional warming in reproductively active yet variably sized vertebrates, and the fitness of size classes may be affected by such climatic trends. Assessing, monitoring and predicting the effects of climate change on vertebrates

with TSD is of utmost importance at present and data presented here suggest that complete-nest thermal sampling is critical for sex ratio predictions. Further, reproductive output (clutch size, egg size, proportion of nest cavity filled with eggs, etc.), in addition to microhabitat parameters, should be considered as a set of important variables in future TSD—climate change studies. Lastly, and in the interest of future monitoring efforts within specific systems, the role temperature variability plays in a warming environment is of utmost importance (Neuwald and Valenzuela, 2011; Georges, 2013) when predicting sex ratios from thermal data. As Neuwald and Valenzuela (2011) and Warner and Shine (2011) describe, the relationship between temperature and developmental rate is non-linear when including all non-lethal incubation temperatures and adequate quantification of this relationship when predicting sex ratios in TSD vertebrates is critical. Further, a caveat highlighted in Warner and Shine (2011) is that the CTE model was designed for TSD taxa with one pivotal temperature and has had limited accuracy in predicting sex ratios in taxa with two, such as crocodiles, based on their findings. However, in the model described here such a decrease in growth rate at high temperatures would have predicted lower CTEs and a more female-biased sex ratio, as our nest thermal data are distributed around the lower pivotal temperature for this species (female-male-female). Therefore, we maintain that the use of a linear model was a more conservative test of our male-biased hypothesis. Limited variance within our nests is likely to account for error otherwise associated with the CTE model in TSD taxa with two pivotal temperatures. Other widely accepted models applicable to TSD vertebrates, like Mitchell et al. (2008), utilize the CTE algorithm and do not adequately account for thermal variance in sex ratio predictions, a problem likely to plague sex ratio predictions of organisms with large clutches and nest thermal variation. Our understanding

of the role of variance in TSD must be experimentally quantified for an improved algorithm to be developed.

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Figure 1. Schematic depicting a theoretical nest thermal cavity and the regions assigned to quantify thermal exposure on a per-egg basis.

Figure 2. Example thermal regime from a crocodile nest (PVN4-13) depicting temperature and amplitude variation among four different nest locations; top (red), center (blue), side (green), and bottom (purple) as well as burn-in and burn-out periods at the beginning and end of logging.

Figure 3: Scatterplot of the negative correlation between within-nest location average temperature and variance at that specific location indicating a decrease in variance at hotter nest locations. (F = 10.56, df = 48, p = 0.0021, R^2 = 0.18)

Figure 4: Thermal heat map spheres of the four most common nest thermal regimes encountered with relative within-nest temperatures depicted. Colors represent relative differences in heating within a nest but do not correspond to specific temperatures between nest types or between nests of the same type.

Figure 5: Multiple regression plot between nest temperatures and microhabitat variables hypothesized to be influential in nest thermal regime regulation. Values represent correlation coefficients between variables tested. Overall tested parameters were significantly correlated to nest temperature (F = 9.328, df = 5,6 p = 0.0085).

Figure 6: Scatterplot of *Alligator mississippiensis* optic diameter growth rate (mm/day) across incubation temperatures from Ferguson (1985). A line-best-fit provides a linear growth rate equation across temperatures utilized in calculating the constant thermal equivalent (CTE) (y = 0.0147x – 0.2857, R^2 = 0.91).

Figure 7: Average daily low temperature at Palo Verde National Park, Guanacaste, Costa from 1996 to 2012 showing a 2.5 °C increase and an increasing trend of 0.24 °C per year.

Figure 8: Frequency distribution of CTEs on a per egg basis and expected sex of small, intermediate and large eggs at present (A) and predicted CTE distributions under current climatic trends at Palo Verde in fifteen years (B). The shaded grey area represents the portion of the x-axis in which males are produced.

Figure 9: Frequency distribution of maximum temperature reached on a per egg basis using current logger data (A) and predicted thermal data in fifteen years (B). Dashed line indicates known lethal embryonic temperature early in development.

Table 1. Variance and mean temperature for four locations (top, center, side and bottom) in thirteen crocodile nests based on iButton data loggers. PVN3-13 top logger was removed by a predator and never recovered.

Nest	Location	Variance	Mean Temp (°C)
PVN3-12	Top	12.74	30.07
	Center	2.01	28.81
	Side	1.64	29.10
	Bottom	1.13	28.71
PVN4-12	Top	4.22	28.95
	Center	2.64	29.11
	Side	2.83	29.07
	Bottom	1.55	29.01
PVN1-13	Top	1.89	32.51
	Center	1.77	33.01
	Side	0.66	31.41
	Bottom	0.71	32.44
PVN2-13	Top	1.21	31.00
	Center	0.79	31.13
	Side	0.47	30.69
	Bottom	0.27	30.94
PVN3-13	Top	----	------
	Center	4.10	30.45
	Side	2.27	30.97
	Bottom	1.62	30.54
PVN4-13	Top	3.22	29.28
	Center	0.32	29.13
	Side	0.80	30.62
	Bottom	0.26	28.70
PVN5-13	Top	3.19	27.73
	Center	2.79	27.51
	Side	2.68	27.69
	Bottom	2.99	27.61
PVN6-13	Top	3.30	27.19
	Center	2.66	27.72
	Side	2.42	27.55
	Bottom	2.60	27.77

PVN7-13	Top	1.88	27.25
	Center	2.08	27.32
	Side	2.00	27.50
	Bottom	2.50	27.77
PVN2-14	Top	0.88	31.30
	Center	0.75	31.83
	Side	0.33	31.31
	Bottom	0.54	31.41
PVN3-14	Top	2.88	30.64
	Center	2.88	30.65
	Side	0.75	29.85
	Bottom	0.08	29.67
PVN4-14	Top	0.61	30.38
	Center	0.23	30.38
	Side	0.11	29.66
	Bottom	0.06	29.55
PVN5-14	Top	2.86	30.75
	Center	1.43	29.63
	Side	1.55	29.37
	Bottom	0.95	29.40

Table 2. Nest-specific within-nest and overall sex ratio predicted by CTEs at six locations quantified within each nest and proportion of eggs exposed to location-specific thermal regimes used to assign sex ratio on a per-egg basis. Additionally, maximum temperature reached at each location is included to predict survival within and among nests.

Nest	Top (1/8)	Center (1/8)	Side (1/8)	Bottom (1/8)	Upper Zone (1/4)	Lower Zone (1/4)	Clutch Size	Modeled Egg Sex Ratio (M: F)
PVN3-12 CTE	31	29	29	29	30	29	5	1:4
Max Temp	38	34	34	32	36	33		
PVN4-12 CTE	29.5	29	29	29	29.5	29	12	0:12
Max Temp	35	35	34.5	34.5	35	34.5		
PVN1-13 CTE	33	33	31.5	31.5	33	32.5	13	13:0
Max Temp	35	35	32.5	33.5	35	34.5		
PVN2-13 CTE	31	31.5	31	31	32	31.5	35	35:0
Max Temp	33	32.5	32	32	33	32		
PVN3-13 CTE	31*	31	31	31	31	31	23	23:0
Max Temp	34*	34	33.5	32.5	34	33		
PVN4-13 CTE	29.5	29	31	29	29.5	30	42	10:32
Max Temp	34	32	33.5	31.5	33	32.5		
PVN5-13 CTE	28	27.5	28	28.5	28	28.5	29	0:29
Max Temp	31	30.5	31	31	31	31		
PVN6-13 CTE	27.5	28	28	28	28	28	17	0:17
Max Temp	30.5	31	30.5	31	31	31		
PVN7-13 CTE	27.5	27.5	28	28	27.5	28	37	0:37
Max Temp	31	30	30	31	30.5	30.5		
PVN2-14 CTE	31.5	31.5	31.5	32	31.5	32	22	22:0
Max Temp	35	34.5	35	34.5	35	35		
PVN3-14 CTE	31	31	30	29.5	31	30	36	15:21
Max Temp	34.5	34.5	31.5	32	34.5	32		
PVN4-14 CTE	30.5	30.5	29.5	29.5	30.5	29.5	42	0:42

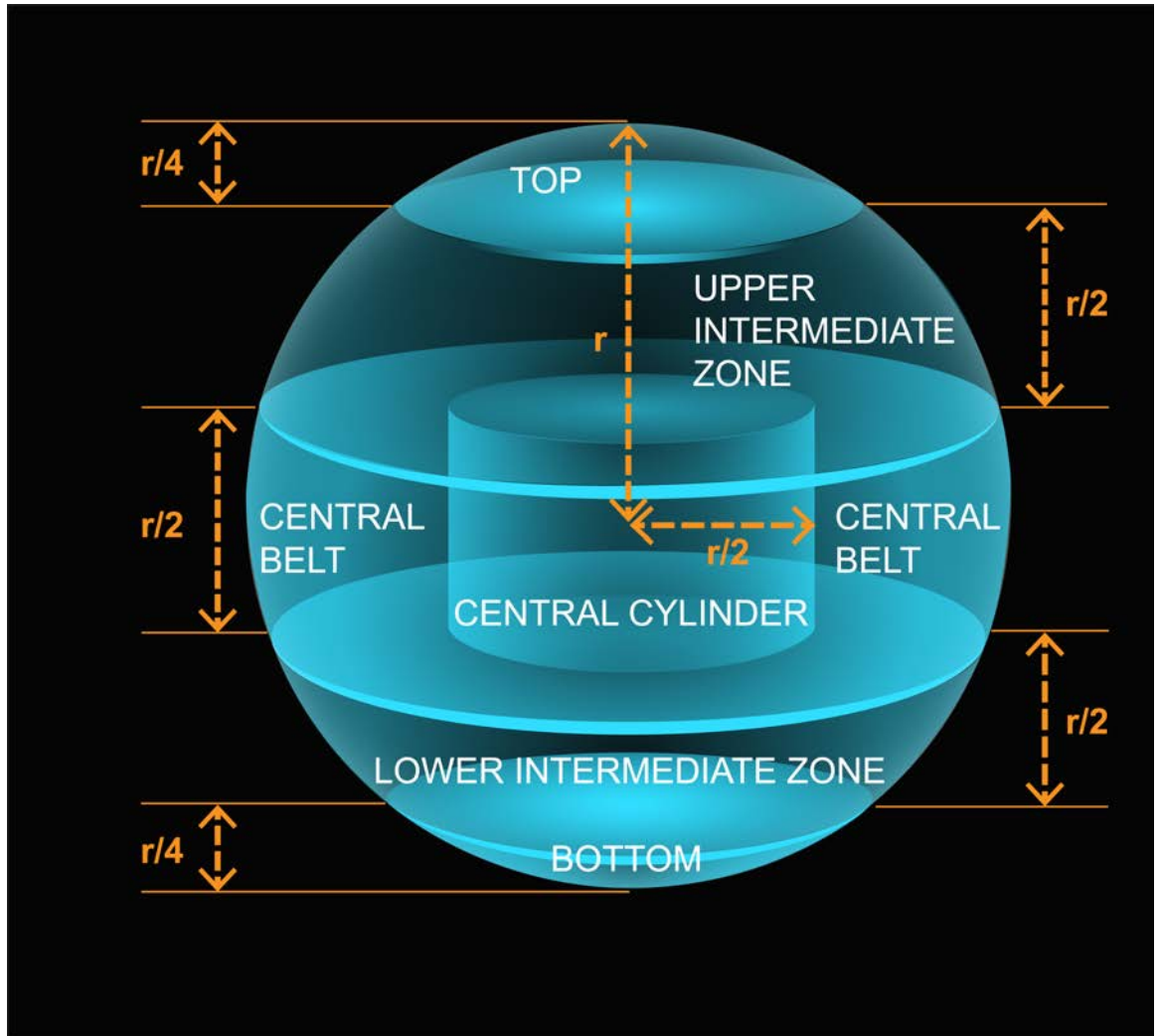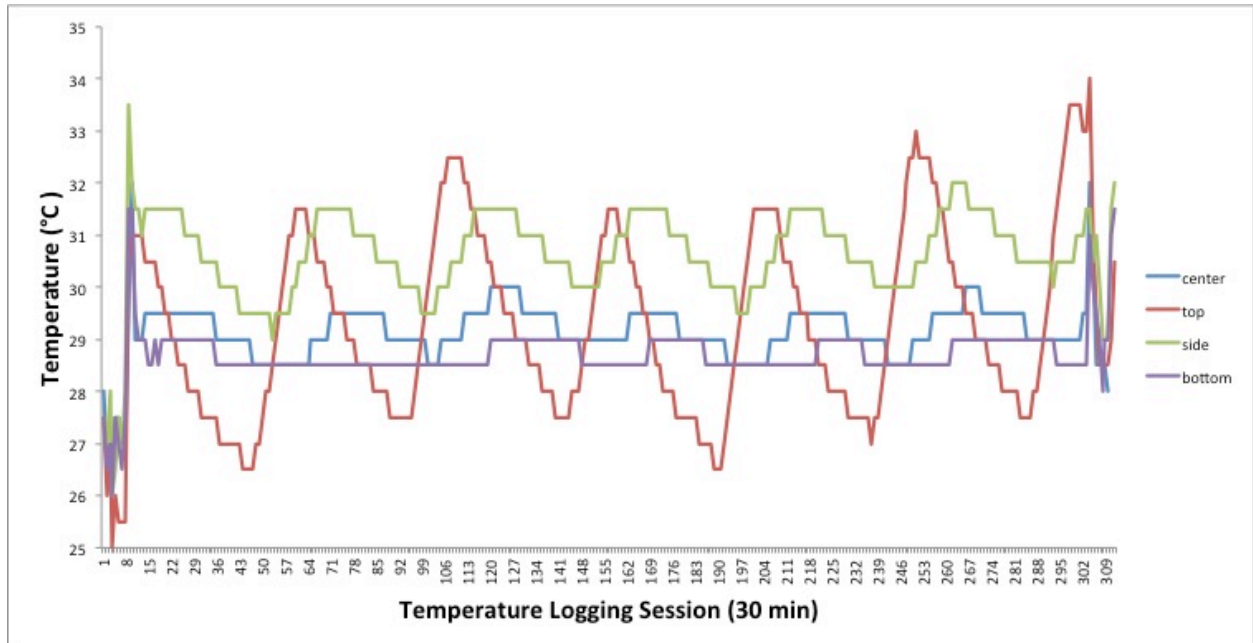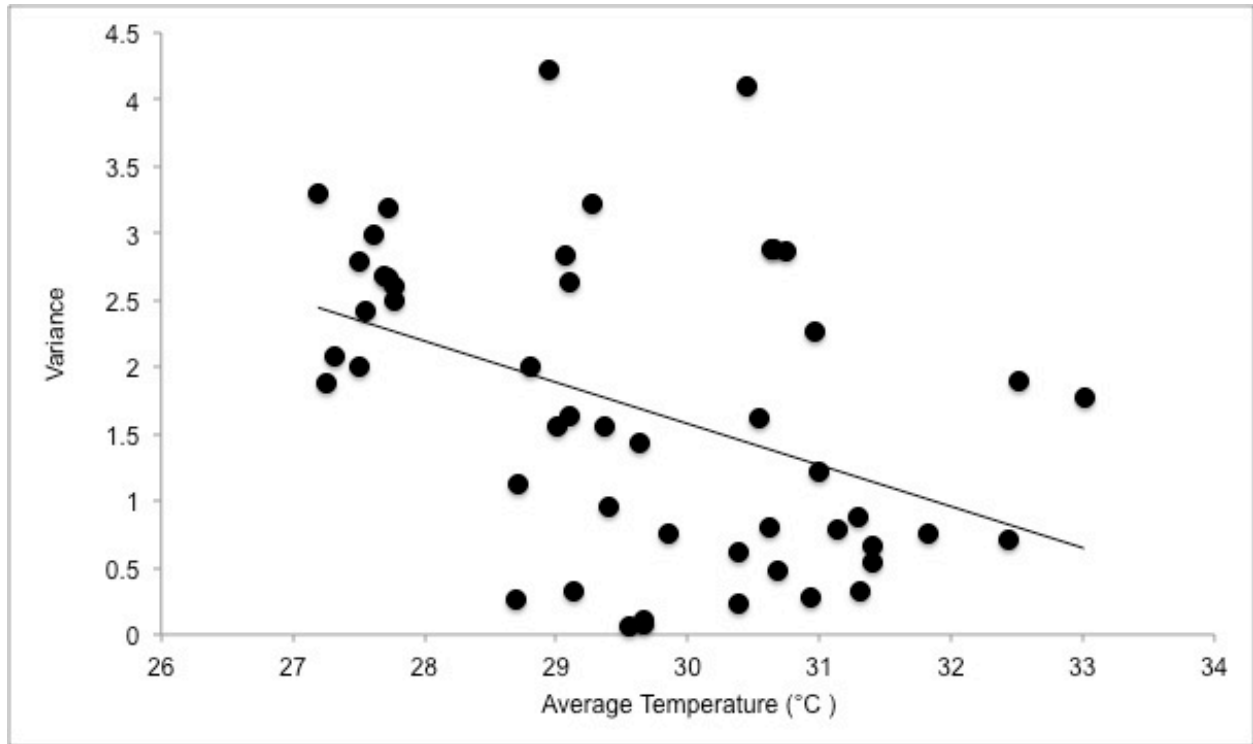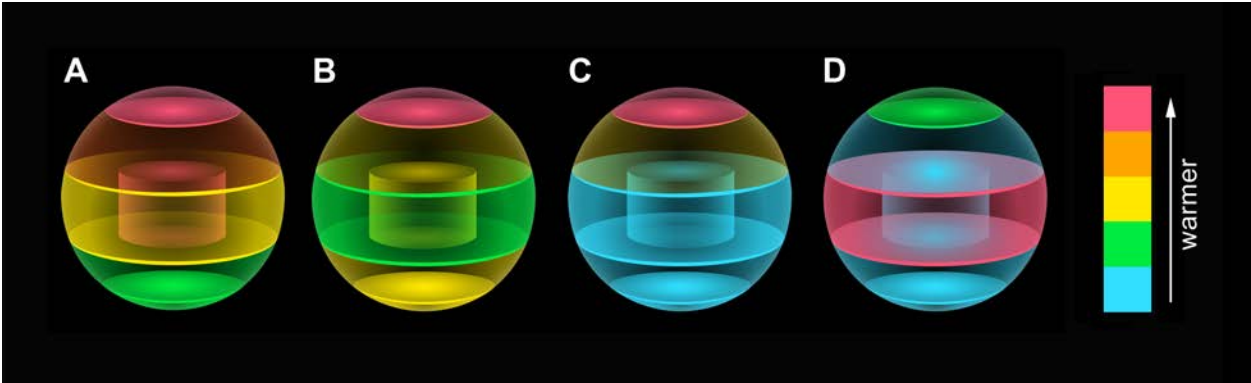|							
Max Temp	31.5	31	30	30	31.5	30		
PVN5-14 CTE	31	30	29.5	30	30.5	30	46	9:37
Max Temp	34	31.5	32	30.5	32.5	31.5		Total: 128:231 (1:1.8)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

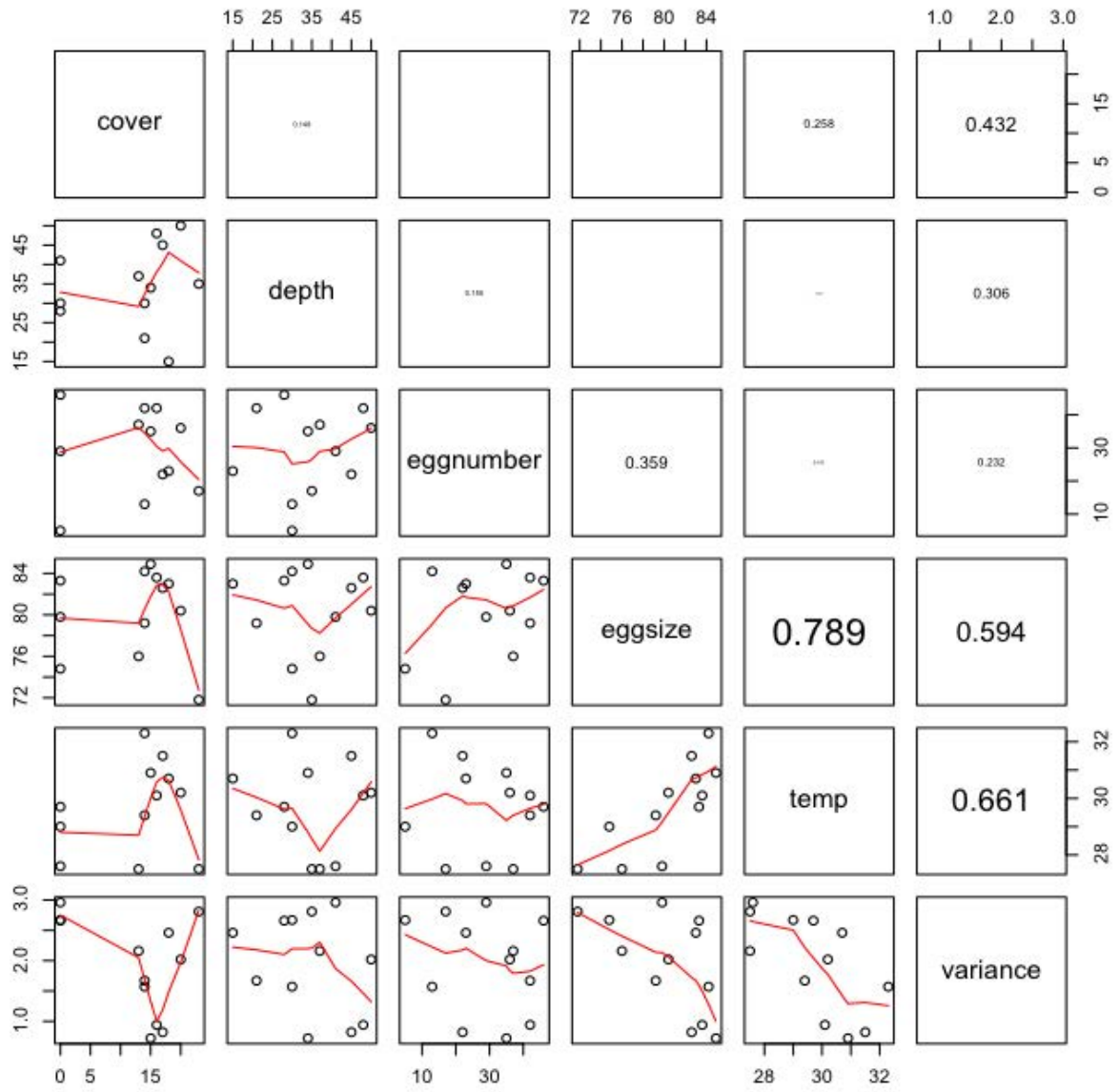Figure 6
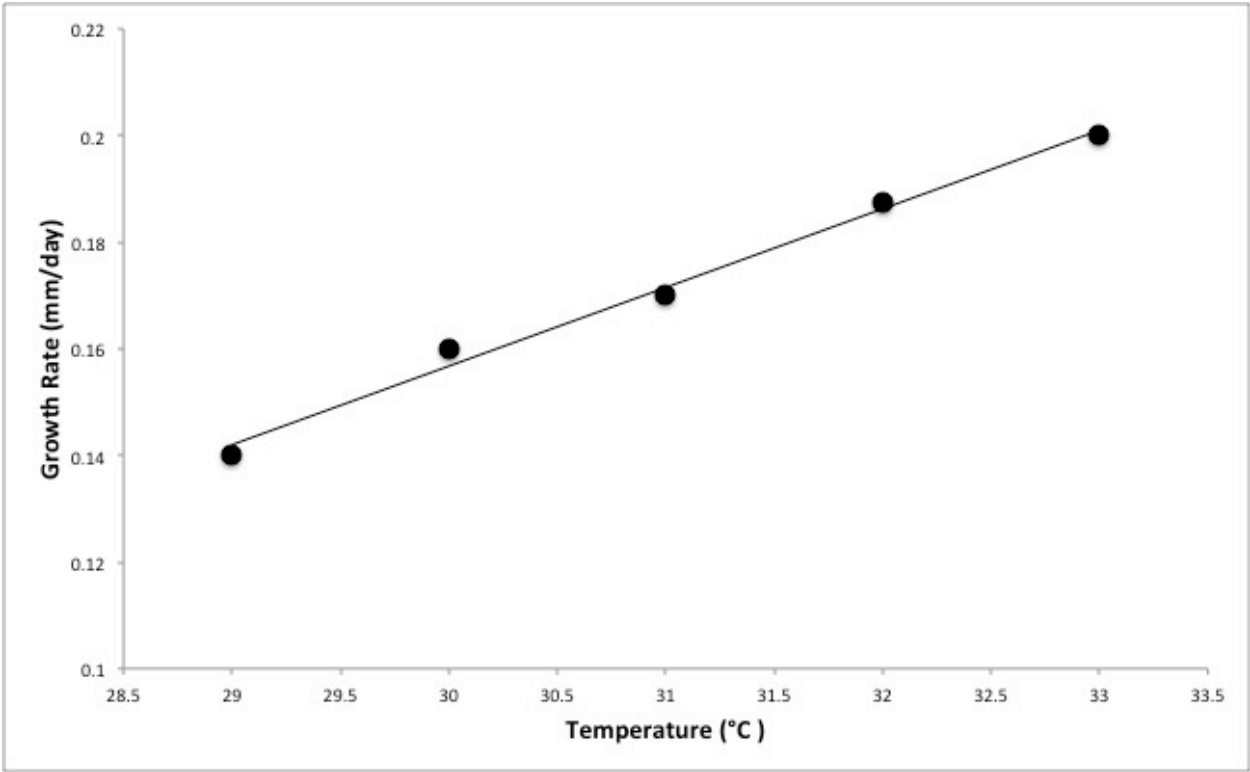
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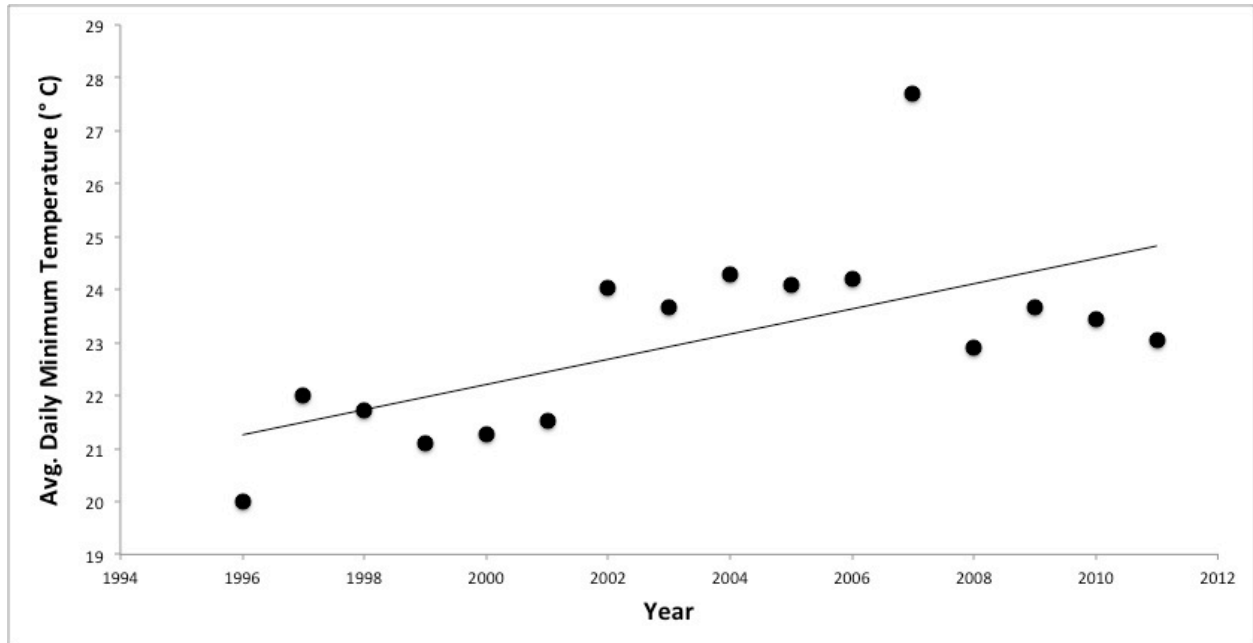
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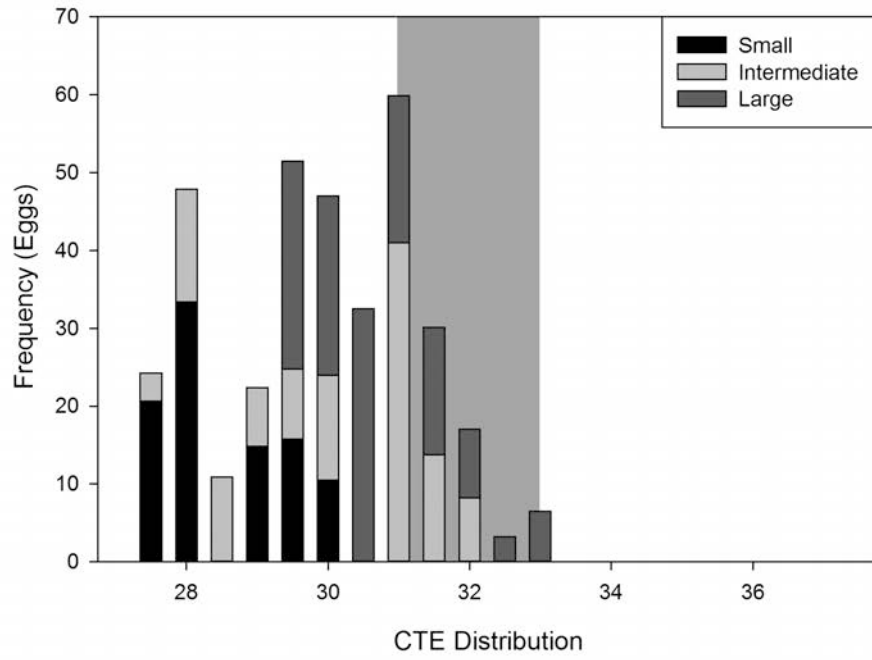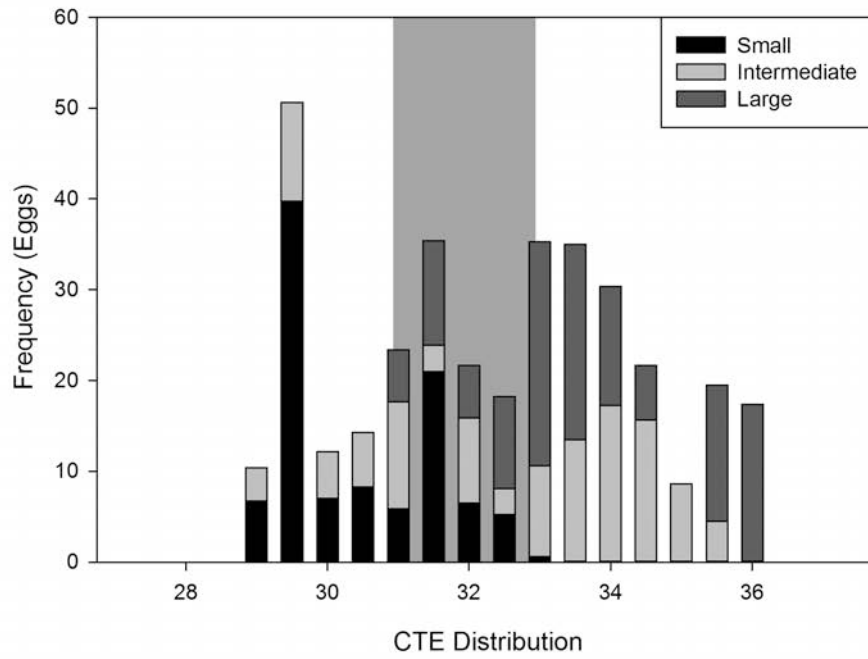
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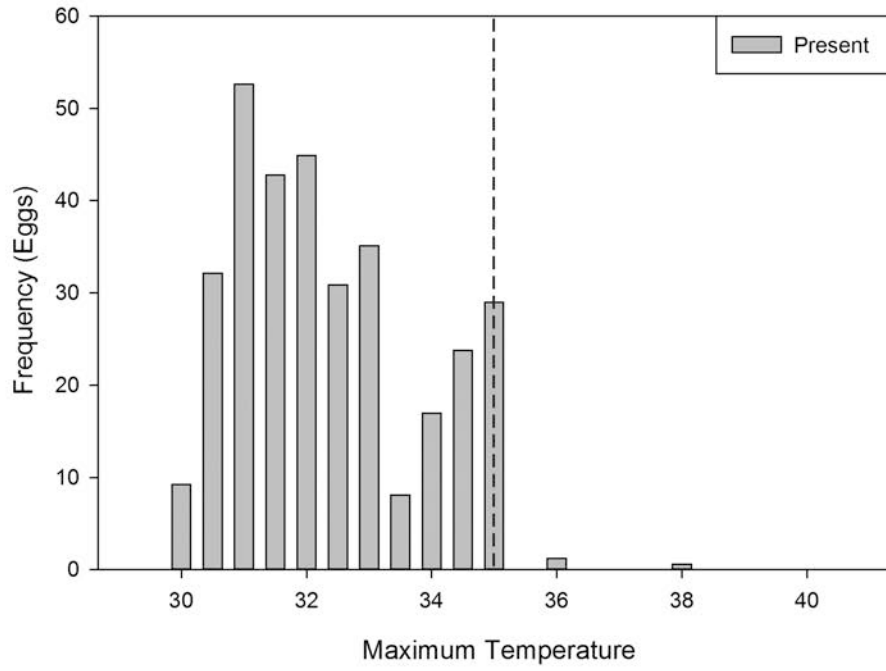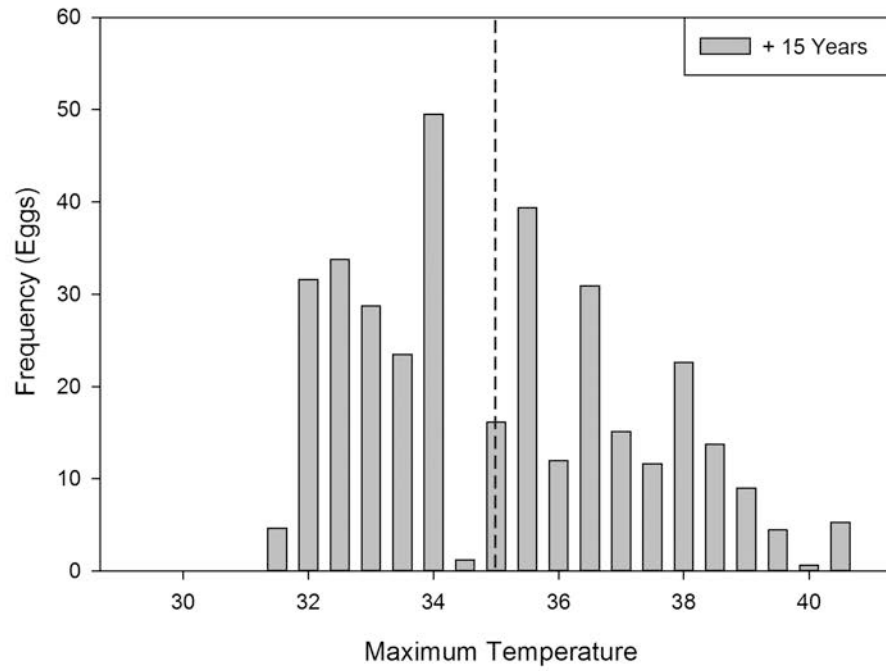
Chapter 4

Methyltestosterone Alters Sex Determination

in the American alligator (*Alligator mississippiensis*)

Abstract. Effects of xenobiotics can be organizational, permanently affecting anatomy during embryonic development, and/or activational, manipulating transitory actions during adulthood. The organizational influence of endocrine-disrupting contaminants (EDC's) produces a wide variety of reproductive abnormalities among vertebrates that exhibit temperature-dependent sex determination (TSD). Typically, such influences result in subsequent activational malfunction, some of which are beneficial in aquaculture. For example, 17-αmethyltestosterone (MT), a synthetic androgen, is utilized in tilapia and other fish farming to bias sex ratio towards males because they are more profitable. A heavily male-biased hatchling sex ratio is reported from a crocodile population near one such tilapia operation in Guanacaste, Costa Rica. In this study we test the effects of MT on sexual differentiation in American alligators, which we assume is a reasonable surrogate for all crocodilians. We find that MT is capable of producing male embryos at temperatures known to produce females and demonstrate a dose-dependent gradient of masculinization. Embryonic exposure to MT results in hermaphroditic primary sex organs, delayed renal development and masculinization of the clitero-penis (CTP).

Key Words: methyltestosterone, *Alligator mississippiensis*, sex determination, androgen.

INTRODUCTION

Recent theory maintains that most vertebrates exist somewhere along a continuum between strict genetic control of sex determination and strict environmental control (Sarre et al. 2004) and that placement along this continuum exists as a function of environmental influence on fitness (Charnov and Bull, 1977). Sex determination mechanisms either maintain a default primary sex and subsequent secondary sex characters or steer development towards the alternate primary sex and secondary characters. Genotypic sex determination (GSD) is a preset mechanism of primary and secondary sexual structure differentiation based on the presence and subsequent expression of a single gene, or lack of that gene, resulting in production of a default sex (gene absent) or the alternate sex (gene present). In mammals, for example, the SRY (or SOX9) gene steers development away from a default female phenotype via promotion of a male reproductive tract and simultaneous suppression of a female tract (Kovacs and Ojeda, 2011). While GSD allows for a heritable sex, it can be influenced by gonadal sex disorders (Kovacs and Ojeda, 2011).

Environmental sex determination (ESD) is susceptible to many more external influences (Wibbels et al. 1994). Temperature dependent sex determination (TSD), a form of ESD, relies on specific thermal regimes to dictate the expression of aromatase and/or reductase, thus governing the sex steroid regime of the developing embryo and subsequent sex characteristics (Wibbels et al. 1994). Thermal thresholds or 'critical' temperatures, that serve as a thermal pivot between development of one sex or the other, are species-specific and vary widely among vertebrates with TSD (Janzen and Paukstis; 1991). TSD mechanisms also vary among taxa and three main mechanisms exist; male production at low temperatures and female at high, female production at

low temperatures and male at high, and female at low, male at intermediate, and female at high temperatures (Valenzuela, 2004). A remarkable series of sex steroid and temperature manipulations have elucidated some constants among TSD mechanisms; 1) A critical period exists at which an embryo's sex determination is sensitive to both temperature and sex steroids; 2) feminization and masculinization are mediated by steroid-specific receptors; 3) aromatase and reductase inhibitors can manipulate sex regardless of temperature, however with unequal potency; 4) response to estrogens can produce female embryos at male-producing temperatures, but androgens can only masculinize embryos at threshold temperatures; and 5) mixed sex ratio clutches are produced at threshold temperatures rather than hermaphrodites (Wibbels et al., 1991; Wibbels et al, 1994; Wibbels and Crews, 1994; Crews et al, 1994; Wibbels and Crews, 1995; Crews; 1996).

Our understanding of how hormone sources, storage, and internal feedback mechanisms affect sexual differentiation mechanisms has greatly improved as a result of research in temperature-dependent steroid expression and utilization (Kamel and Kubajak, 1987; Janzen et al., 1998; Paitz and Bowden, 2010; Pfannkuche et al., 2011; Paitz et al., 2011). Unfortunately, so has our understanding of anthropogenic influences on such processes (Guillette Jr. et al. 1995). Numerous industrial and agricultural compounds, when introduced to natural systems, have endocrine-disrupting affects. Endocrine-disrupting compounds (EDCs) have negative activational effects on endocrine systems, but more daunting is the organizational role they play as hormone mimics during sexual differentiation and embryonic organization (Guillette et al. 1995). Because sexual differentiation mechanisms vary among reptiles, these taxa have become model indicators of EDC exposure. Among reptiles, contaminants that mimic estrogen are common (e.g. PCBs, Dioxin, Furans, DDE), while environmental androgens are far more rare,

presumably as a result of the aromatizable nature of testosterone and the dominance of the estrogen pathway associated with TSD (Wibbels and Crews, 1995).

Among reptiles the most notable sentinel taxa for EDCs are crocodilians (Milnes and Guillette, 2008). Their popularity as 'model' organisms emerged because of the Lake Apopka superfund site, where dicofol, DDT and subsequent metabolites were discovered as contaminants in 1980 (Guillette et al, 1994). Such contaminants were deemed potent environmental estrogens after female alligators displayed unnaturally high 17β -estradiol plasma concentrations, polyovular follicles, polynuclear oocytes (Guillette Jr, et al., 1994), and reduced gonadal-adrenal mesonephros (GAM) aromatase activity (Crain et al., 1997). Males exhibited decreased plasma testosterone, reduced phallus size (Guillette Jr. et al., 1996) and poorly organized testes (Guillette Jr., 1994). This case study exemplified the utility of crocodilians in understanding the activational and organizational effects of EDCs.

In Guanacaste, Costa Rica, three large tilapia farms utilize 17-αmethyltestosterone (MT) to produce all-male offspring that grow faster and reach larger maximum size than females. Preliminary data on MT persistence in water and soil was noted during initial testing of this fish farming practice, however, lipid persistence of the compound and its effects on vertebrates other than fish are unknown (Phelps and Popma 2000; Gupta and Acosta 2004). The nearby Tempisque Basin harbors a rapidly expanding population of American crocodiles (*Crocodylus acutus*) that exhibits a male-biased sex ratio (Bolaños-Montero 2012; Murray et al. 2015). Hatchling sex ratios from this population do not match the ratios predicted by clutch thermal regimes and this sex ratio bias differs among clutches, with some clutches being male-biased and others not (Murray et al. in review). Here, we test the potential for MT to produce male crocodilian embryos at female-producing temperatures and histologically analyze

organizational effects of urogenital development from MT exposure during the experimental

assay.

METHODS

For this experiment, 108 American alligator (*Alligator mississippiensis*) eggs were

collected from five clutches in June 2013 and 76 eggs from three clutches in June 2014. All eggs

were collected at J D. Murphree Wildlife Management Area, Port Arthur, TX, within five days

of deposition, as assessed by daily nest monitoring and the width and length of banding (Masser,

1993). Eggs were transported to an Auburn University live animal facility and incubated at 28°C,

a female-producing temperature (Lang and Andrews, 1994). In 2013, eggs were misted with

water daily in an incubator (Fisher Scientific, Isotemp model 655D) to maintain humidity.

However, 50 eggs failed to complete development, likely because of dehydration. Therefore,

eggs in 2014 were maintained at approximately 100% humidity using a vermiculite substrate and

steam heating. Each year, four eggs were opened periodically to stage the embryos as described

by Ferguson (1985). Prior to the temperature-sensitive period each year (stage 20, when sex

determination occurs; Lang and Andrews, 1994), eggs were randomly assigned to one of five

treatments using a random number generator. Eggs were randomly dispersed among plastic bins

in the incubator with 10-14 eggs per treatment per year. Two treatment groups served as

controls. One control received no treatment while the other received 5μl of 95% ethanol (ETOH)

to control for effects of the vehicle used to deliver MT to all treatment groups. Treatment groups

received 4 mcg/ml, 40 mcg/ml, or 400 mcg/ml of 17α- MT in 95% ETOH. These treatments

exposed eggs to between 10 and 1000 times the natural amount of testosterone in alligator egg

yolk (Conley et al. 1997), a range of doses standard for ecotoxicological dose-response assays

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with sex steroid hormones or related endocrine-disrupting compounds (Wibbels and Crews 1995; Crain et al. 1997). Treatments were applied topically as 5 μl of solution deposited on the surface of an egg at stage 21, a technique that is used to transport compounds inside reptilian eggshells (Crews et al. 1991, Paitz et al. 2012). Using this method, Crews et al. (1991) found that at least 90% of applied compound was incorporated into the embryo. Additional eggs were incubated separately at male-producing temperatures (32°C) to serve as control males for primary and secondary sex organ comparison.

Upon hatching, chorioallantoic fluid (CAF) and yolk were collected and frozen to quantify the concentration of 17α- MT that reached the embryo. Steroid hormones were extracted from egg yolk using a 3:2 volume solution of ethyl acetate and hexane, respectively. Samples were dried under vacuum at 25°C and dissolved in 100 μL assay buffer supplemented with 10 μL of DMSO to encourage dissolution. 17α-methyltestosterone concentrations were quantified using a sandwich ELISA kit (MaxSignal® methyltestosterone kit, Bioo Scientific, Austin, TX). Cross-reactivity with testosterone was 0.3% and samples were not analyzed in duplicate as all other wells were occupied for another study. Optical density was determined using a Benchmark Plus microtiter plate spectrophotometer (Bio-Rad, Hercules, CA) at 450 nm. Hatchlings were individually marked via caudal scute removal, and snout-to-vent length and total length were recorded. The sex of each hatchling was determined by cloacal examination with an MDS 105 x 2.7mm endoscope. A hatchling was determined to be male if the clitero-penis (CTP) possessed *all* of the following character states: bi-lobed structure, extensive vascularization, and length equal to or greater than length of the vent (Fig. 2; Allsteadt & Lang, 1995).

A subset of twenty hatchings was euthanized and preserved for detailed CTP examination using a Leica M165C stereoscope with Leica DFC425 camera attachment. These sacrificed individuals were examined histologically for internal verification of sex and description of testicular and ovular development among treatments. The remaining surviving hatchlings (n = 16) were housed at Auburn University and McNeese State University so that sex of each individual could be verified at five months of growth.

Whole animals were fixed in 10% neutral buffered formalin and preserved in 70% EtOH. The developing urogenital tissues were excised under a stereomicroscope and placed in 70% EtOH for further manipulation. Tissues were washed with deionized water, dehydrated in a series of increasing concentrations of EtOH, and placed in paraffin wax overnight to allow the wax to fully infiltrate the tissues. Tissues were then placed in embedding molds with paraffin wax and allowed to cure for 24 hours. The urogenital tracts were then serial sectioned sagittally on an American Optical rotary microtome, and sections were then placed on albuminized slides and stained with Ehrlich's Hematoxylin and Eosin.

Slides were viewed at various magnifications using an Olympus microscope to identify structures of the developing urogenital system and to determine if gonads were of the male or female category for each of the above-mentioned treatments. Representative slides from each treatment along with any abnormalities were photographed using an attached digital camera and images were compiled into composite micrographs using Adobe Photoshop CS5.

 G tests for independence were performed on the proportion of male hatchlings among treatments, the proportion of male hatchlings among clutches, the proportion of survivors among treatments, and the proportion of survivors among clutches. A two-sample T test was used to test for difference in size between control or treatment individuals. Welch's two- sample T-tests were

used to determine difference in CTP length and width (Figure 1) between treated individuals and control individuals because sample sizes were low among individual experimental groups.

RESULTS

Of 126 experimental or control eggs, only 36 survived long enough to be categorized as to sex. Survival did not differ among experimental groups but did differ among clutches ($G = 28.3$, df = 7, p = 0.0002). Alligator eggs incubated at a female-producing temperature and treated with 17α- MT produced a significantly higher proportion of male hatchlings than control groups ($G = 20.2$, df = 4, p = 0.0005; Figure 2). There was no difference in proportion of males among clutches. CTP lengths, but not widths, were significantly larger in treatment (pooled across treatment groups) versus the pooled control groups (t = 2.65, df = 10.72, p = 0.02, Figure 3). Sixteen surviving treatment eggs were housed for sex confirmation five months later. All but one of these was confirmed to have the same sex as morphologically determined at hatching. The only hatchling whose sex diagnosis differed was deemed a female at hatching and a male five months later.

Stereoscope examination of twenty individuals sacrificed at hatching revealed novel CTP characters for differentiating males from females as well as treatment effects on CTP development. Control females exhibited flatter, non-vascularized structures that extended from the dorsal cloacal surface in a low-lying "T" shape (Figure 6 A, B). Control male CTPs exhibited a basal bi-lobed structure with a large projection characterized by a mid-sagittal groove, presumably for sperm delivery. Four to four hundred mcg/ml MT treatment concentrations present a gradient from minimal to extreme vascularization, bi-lobed shape and projection length

(Figure 4). Such characters are more pronounced within the 4 mcg/ml treatment than in control females and nearly mirror control males for individuals in the 400 mcg/ml treatment group.

Alligators sacrificed for histological examination had gonads that had completely separated from the mesonephros and had begun differentiation. In controls incubated at female-producing temperatures, the ovaries extended along the medial border of the developing mesonephros but were distinctly separated from the mesonephros by a band of loose connective tissue. Along the posterior border of the mesonephros separation between the ovary from the mesonephros was more distinct (Fig. 5A). The ovaries had begun differentiation with a layer of germ cells at the periphery of the gonad near the coelomic cavity (Fig 5A). Large lacunae were evident near the medullary region of the ovary and were surrounded by a low epithelium (Fig 5B). Germ cells of increased size were concentrated in various regions of the medullary region as well (Fig 5C).

Controls incubated at male-producing temperatures had testes that were separated from the mesonephros by a layer of loose connective tissue (Fig 6D). Germ cells were concentrated along the peripheral border of the gonad and seminiferous tubules were evident (Fig 6E). The seminiferous tubules contained spermatogonia with large nuclei giving the seminiferous tubules a dark-staining appearance. Development of the testicular ducts had initiated but differentiation and regionalization had not occurred at this stage of development (Fig 6F).

Individuals treated with 4 mcg/ml of 17α- MT had a mesonephros with well-developed nephritic tubules (Fig. 6A). The gonad was separated from the mesonephros by a thin band of connective tissues (Fig. 6B). The gonad exhibited large lacunae similar to those found in control females (Fig, 6B). However, at the peripheral border of the gonad seminiferous tubules were evident (Fig. 6C). No seminiferous tubule development was found on the interior of the gonad.

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At 40 mcg/ml of 17α- MT individuals developed a mesonephros that was underdeveloped and the nephritic cords were unorganized with a lack of an epithelium (Fig. 6D). The gonad was separated from the mesonephros by a thin band of connective tissue and displayed large lacunae (Fig. 6E) indicative of female ovarian development. Scattered throughout the gonad large germ cells with enlarged nuclei were found developed in seminiferous tubules (Fig 6F). Although no quantitative analyses were performed the number of seminiferous tubules present appeared to be greater than that found in the 4 mcg/ml treatment and seminiferous tubule development was not isolated to the periphery of the gonad. Individuals in the 400 mcg/ml treatment had a mesonephros that was well developed and the nephritic tubules were highly organized with well-developed epithelia (Fig. 6G). A thin band of connective tissue separated the gonad (Fig 6H, Go) from the developing mesonephros (Fig 6H, Mn). The gonad had lacunae that appeared smaller in size and contained abundant seminiferous tubules (Fig. 6H&I).

Salvaged yolk contained 17α- MT in all treatment samples, although concentrations did not differ among treatment groups (F = 1.61, DF = 3, p = 0.35). Yolk was not obtained from control eggs for comparison. All concentrations among treatment groups were on the order of ng/mL compared to the initial mcg/mL dosage. Small sample size and previously quantified steroid conjugation mechanisms further prohibited construction of a dose-response curve relating initial dose to yolk concentration at hatching.

DISCUSSION

Results presented here indicate that MT has a masculinizing effect on crocodilian embryos during sexual differentiation and produces male hatchling alligators at female-producing

temperatures. This response is observable in both the gonad and secondary sex structure (CTP). Additionally, the effect of MT on developing embryos is dose-dependent. MT does not affect survivorship of exposed individuals and, regardless of exposure concentrations, is barely detectable in yolk late in development. Further, methodology used here to determine the sex of hatchlings was conservative in the sense that individuals deemed male were unlikely to be female, but some deemed female could have been male. This method proved to be accurate when assessing male versus non-males at hatching (15 out of 16) including the potential diagnosis of hermaphroditic individuals if some, but not all, male character criteria are met. However, hatchlings used to determine the accuracy (male versus non-male) of our methodology after five months were not histologically analyzed for hermaphroditic gonads.

Stereoscopic and histological examination reveals a gradient of characters among treatment individuals from feminine to masculine. As exposure concentrations increase, so does vascularization, lobature and length of the CTP. Control female CTP morphology appears to be a non-vascularized laterally folded tissue that is slightly thickened medially. Control male CTP morphology appears to be twice as long as control female CTP and is highly vascularized with a low-lying second lobe posterior to the lengthy primary structure. The primary structure is characterized by a medial, anterior groove that may serve to assist sperm transport. The gradient of secondary sex characters noted among MT treatment groups appears to mirror the relationship between MT exposure and urogenital differentiation. Gonads of control hatchlings exhibit differentiated gonads with associated tissues and precursors to gamete production. Low MT exposure at female-producing temperatures results in slight masculinization of the ovary or underdevelopment of both kidney and gonad. This underdevelopment may be a result of a competitive interaction between steroid reception by tissues and a subsequent delay in

development. Exposure to higher concentrations promotes a qualitatively higher ratio of seminiferous tubules to follicles with more gonadal masculinization at higher MT concentrations. Although the sexing of hatchlings by CTP morphology was conservative for this study, the gradient of CTP masculinization appears to be correlated with gonadal masculinization at a fine scale. In future studies, determination of sex based upon CTP may prove to be more accurate in determining male versus non-male hatchling than previously thought (See Zielger and Olbort, 2007 and Otaño et al., 2010 for review).

The ability of MT to bias structural differentiation towards male morphology indicates that it is not aromatizable and is, thereby, a potent androgen. Its effective use in fish farming indicates affinity as an androgen at the cellular level via steroid 'swamping,' or forcing masculinization by changing relative concentrations of sex steroids from estrogen rich to androgen rich while the concentrations of estrogens stays the same (Phelps and Popma, 2000). Results presented here indicate a potent organizational role for MT as a sex steroid in vertebrates with temperature-dependent sex determination.

The minimal detection of MT among experimental yolks suggests one of two possibilities. First, a limited amount of MT may have reached the yolk of each treatment egg. Given that dose-dependence was not observed within yolks, but was observed in other aspects of this study, limited crossing of MT into the egg seems unlikely. More likely is a scenario in which we detected only a portion of MT in yolk samples. Paitz et al., (2012) discovered the conjugation of maternally-supplied steroid hormones within the egg of a TSD reptile and further noted accumulation of such hormones in the albumin. In our analysis, conjugation of experimentally supplied MT may make it invisible to our assay and higher detected concentrations may be

evident in albumin as opposed to yolk. Because we were unable to sample albumin, this possibility awaits further testing.

Results presented here contradict two observations previously regarded as constants in the mechanics of TSD. The efficiency of androgens at producing males has previously been restricted to threshold temperatures. In addition, our data contradict the expected result of mixed-sex clutches. Instead, the production of hermaphrodites was observed in this study. Instead of these patterns, we demonstrate the experimental production of male alligators at a strong female-producing temperature, as well as production of dose-dependent hermaphroditic individuals. This finding is likely a result of the non-aromatizable nature of MT as opposed to natural androgens used in prior studies. Crews (1996), in a review of TSD mechanisms, notes the production of male-biased clutches in turtles as a result of exogenous application of the non-aromatizable dihydrotestosterone. The male-biased sex ratio documented in the Tempisque Basin, Costa Rica appears unrelated to nest thermal regimes (Murray et al, in revision). However, MT utilized by local tilapia farms is capable of acting as a potent androgen yielding male-biased sex ratios at hatching. If this represents the mechanism by which the population of crocodiles of the Tempisque Basin became male biased, then we predict wild crocodiles from this site will demonstrate exposure to MT and evidence of hermaphroditism among females.

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Figure 1. Schematic illustrating cliteropenis (CTP) width and length measurements during stereoscopic examination in both A) control females and B) masculinized CTP at 40 mcg/ml.

Figure 2. Sex ratios among control, ETOH, 4 mcg/ml, 40 mcg/ml and 400 mcg/ml experimental groups. Percent male based on morphological sexing at hatching was significantly different among groups (G = 20.2, df = 4, p = 0.0005).

Figure 3. Clitero-penis (CTP) lengths significantly larger in treatment groups than control individuals (t = 2.65, df = 10.72, p = 0.02).

Figure 4. Stereoscope images of experimental alligator clitero-penises at hatching including control females (A, B), 4 mcg/ml treatment group (C), 40 mcg/ml treatment group (D), 400 mcg/ml treatment group (E) and natural male hatchling incubated at male producing temperatures (F).

Figure 5. Histological analysis of the urogenital tracts of control females and control males. A;B;C: Urogenital system of control females depicting the development of the meseneprhos and the ovary. D;E;F: Urogenital system of control males depicting the development of the mesonephros and the testis. Connective tissue (Ct), Glomerulus (Gl), Lacuna (La), Mesonephros (Mn), Seminiferous tubule (St), Testis (T), Testicular duct (Td).

Figure 6. Histological analysis of the urogenital tracts of treatment individuals. A;B;C. Urogenital system of individuals treated with 4 mcg/ml showing seminiferous tubule development at the periphery of the developing gonad. D;E;F: Urogenital system of individuals treated with 40 mcg/ml showing seminiferous tubule development and enlarged germ cells. G;H;I: Urogenital system of individuals treated with 400 mcg/ml showing well developed mesonephros and increased amounts of seminiferous tubule development. Connective tissue (Ct), Gonad (Go), Lacuna (La), Mesonephros (Mn), Seminiferous tubule (St)
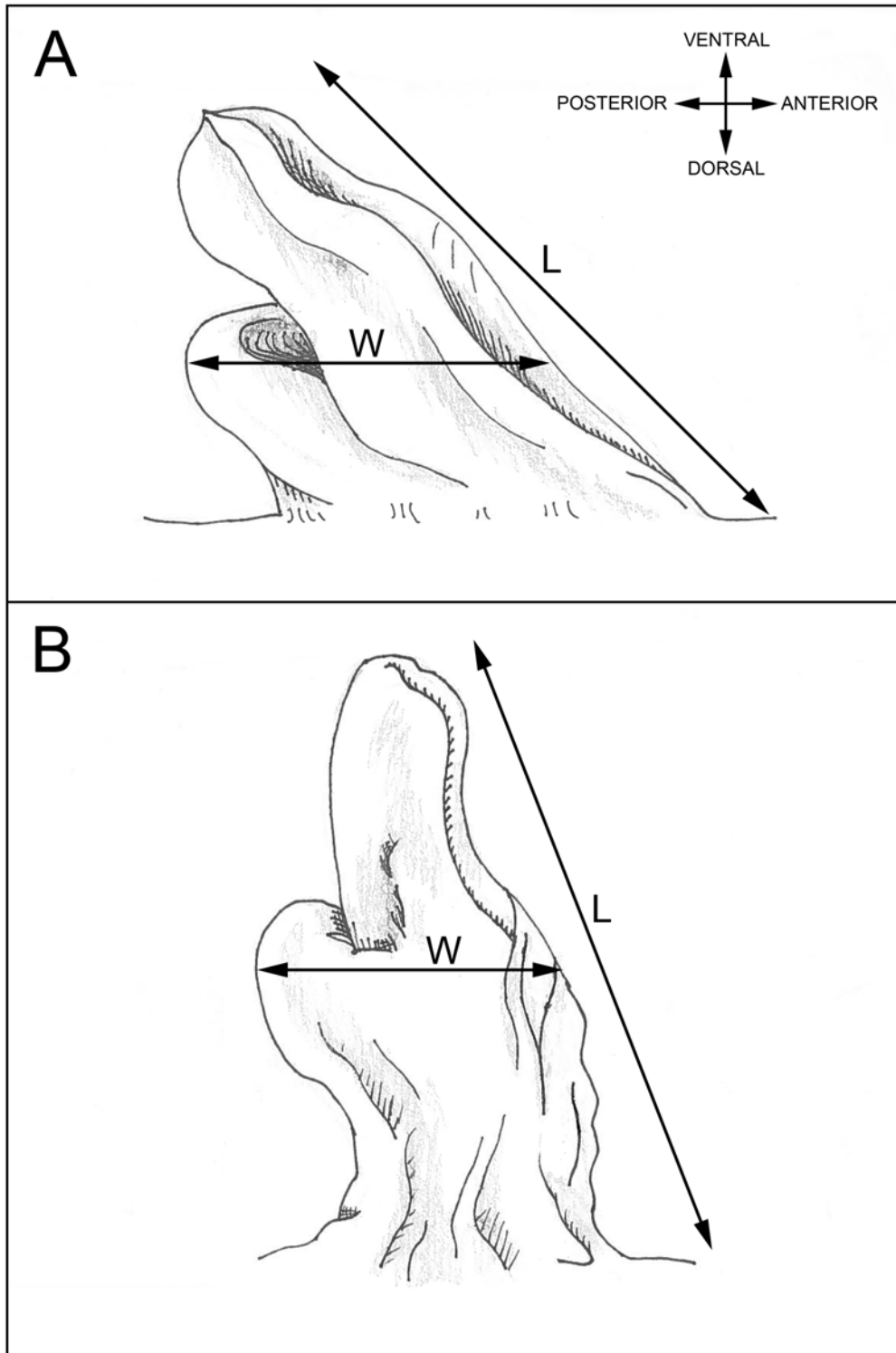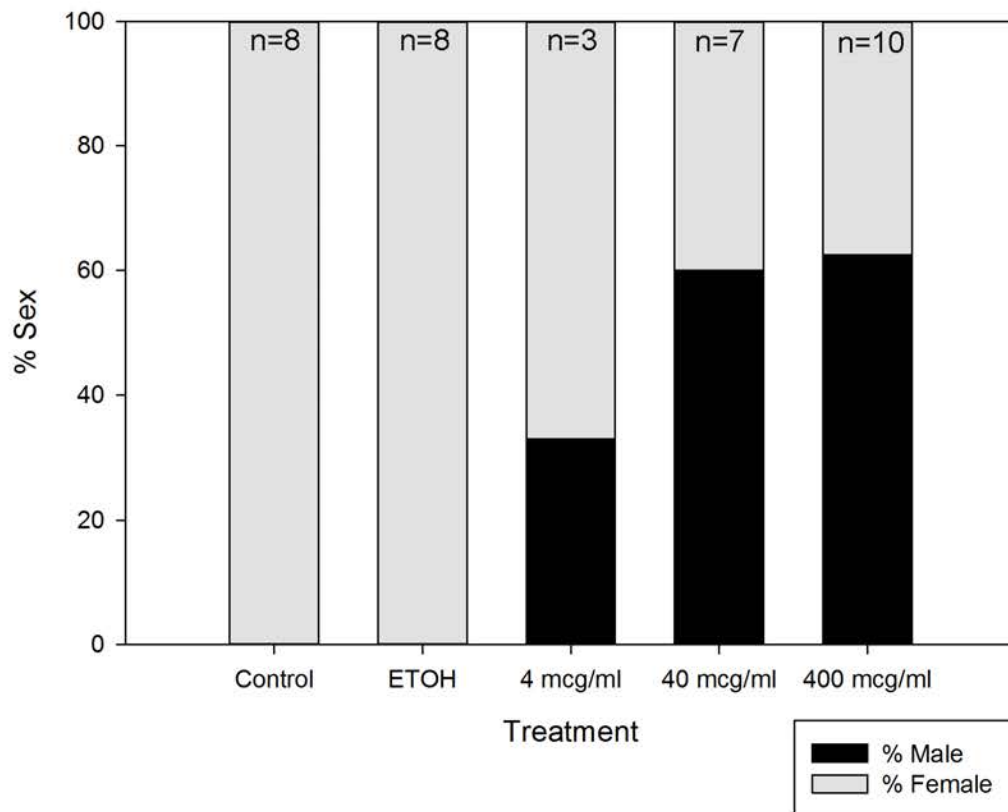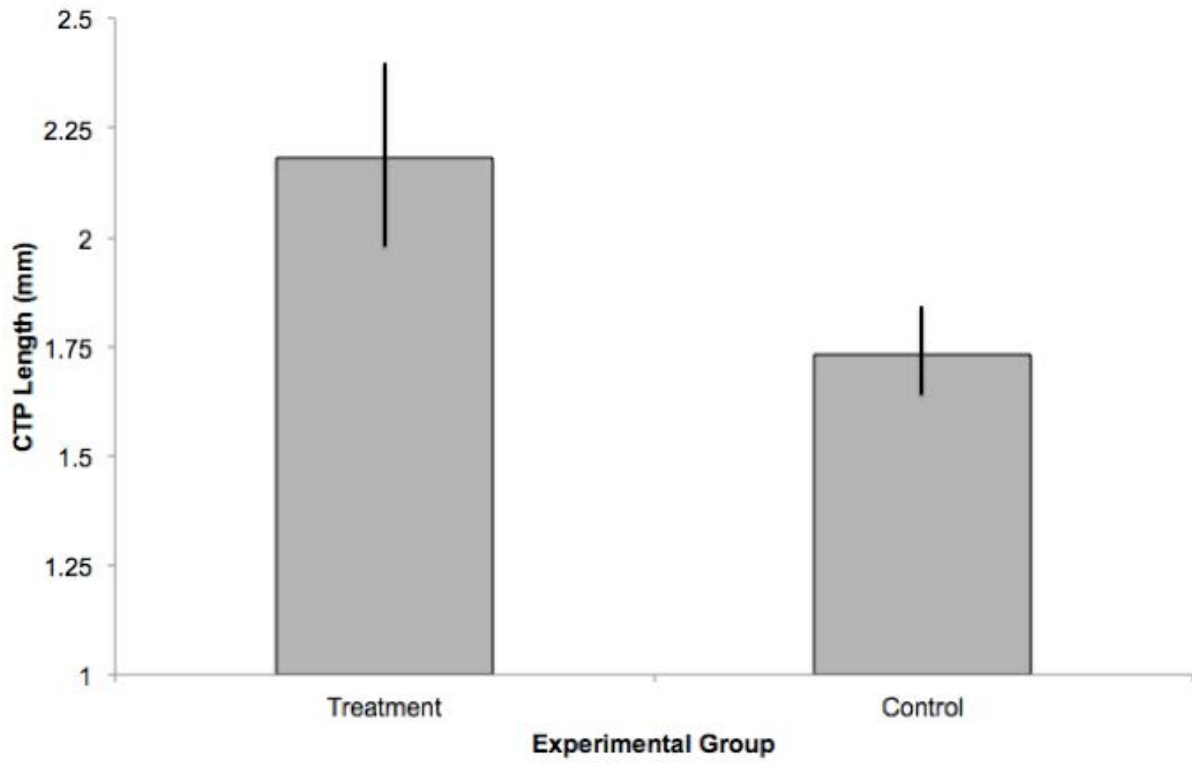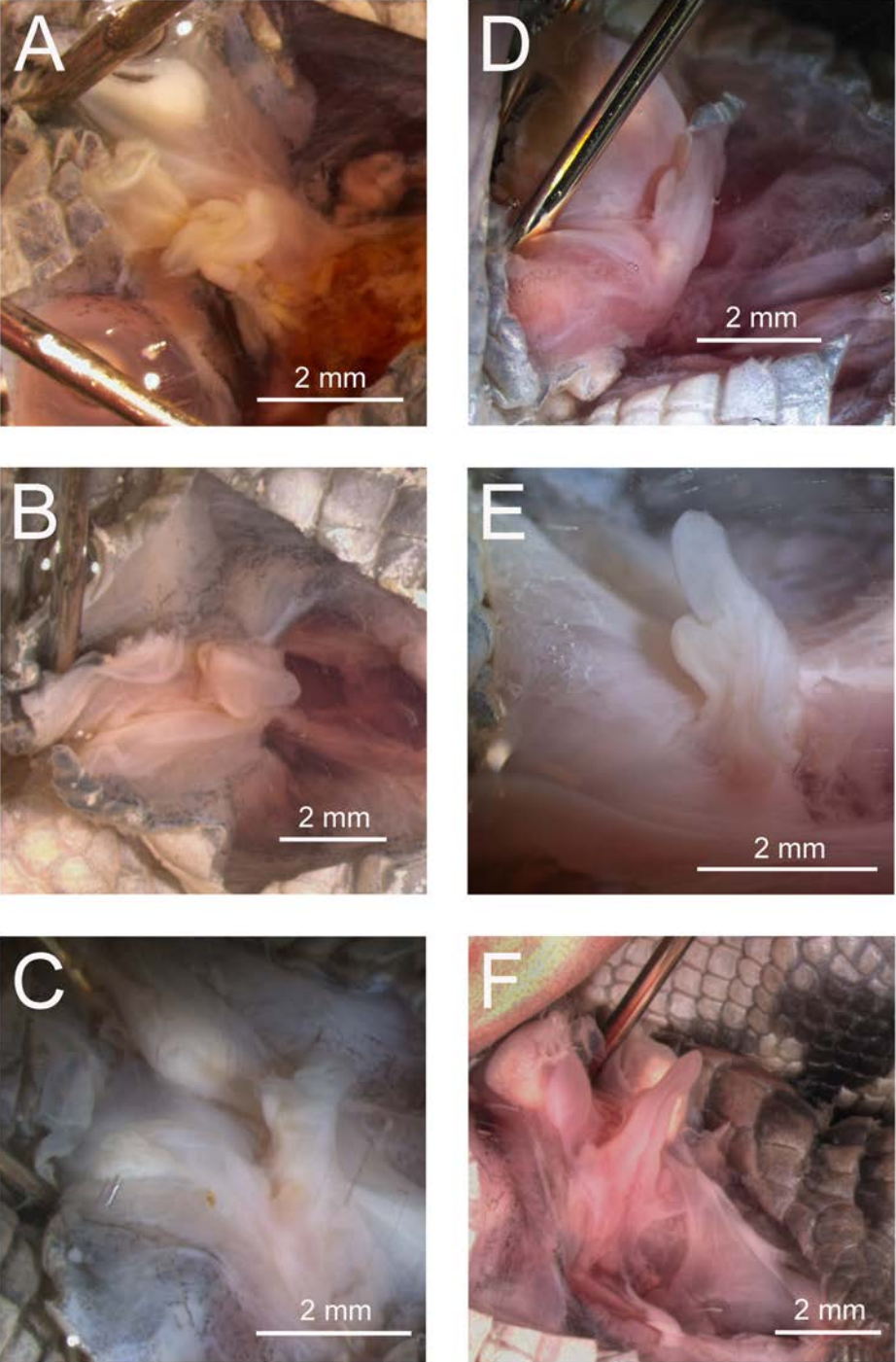
Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Control Female | Control Male

A Ct Ov * Mn

B Ov Ct Mn La

C La Gl

D T Ct Mn
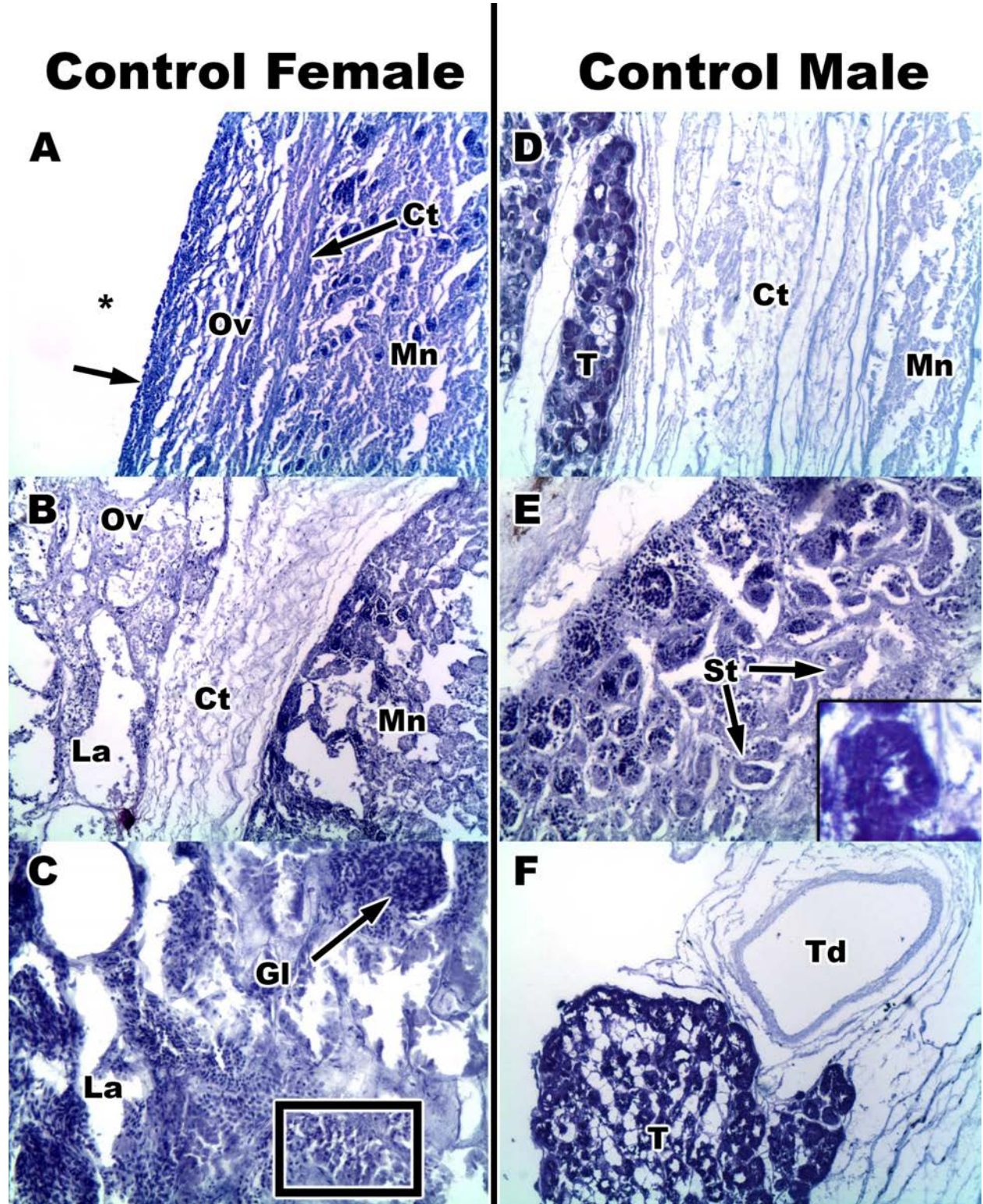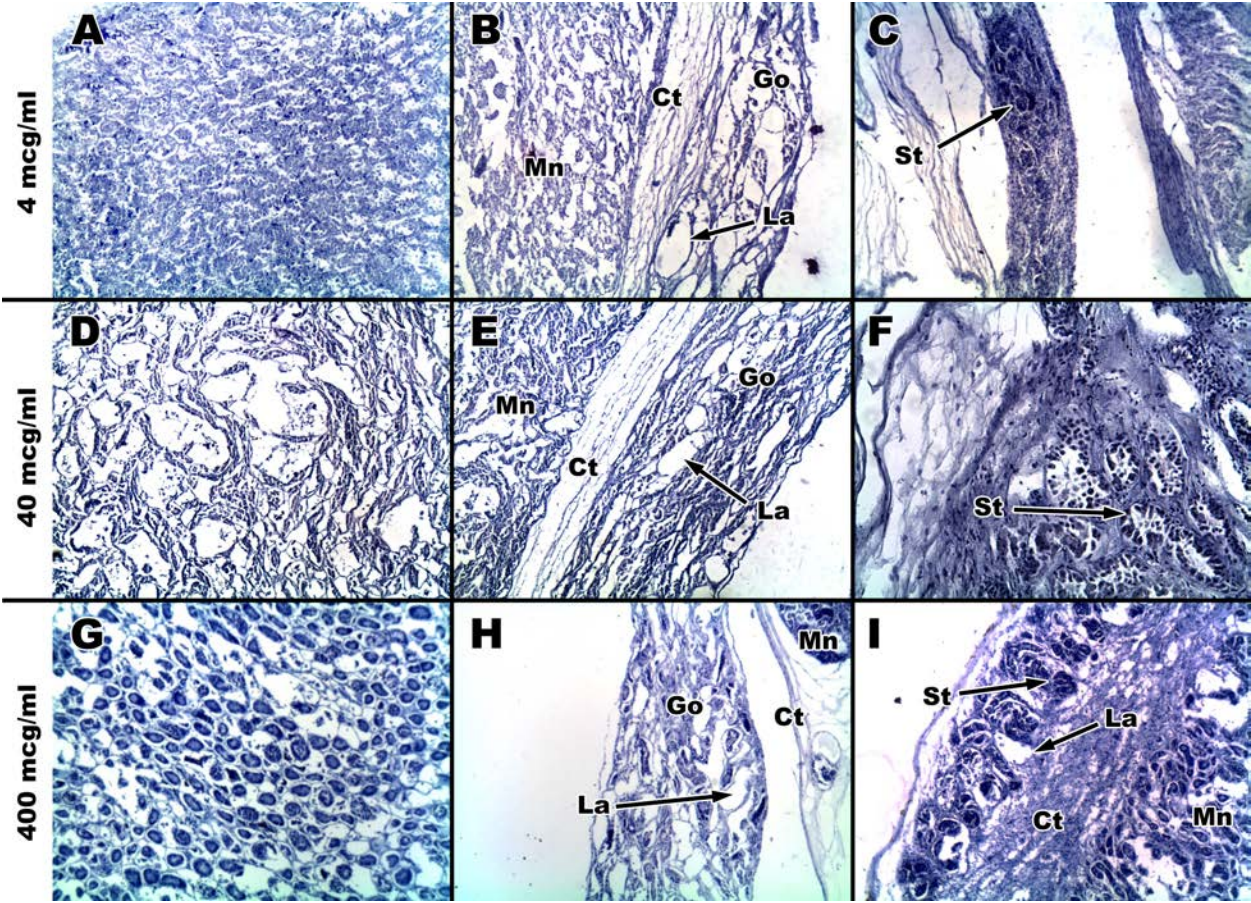
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Figure 6.

Chapter 5

Identification of a Synthetic Sex Steroid as an Environmental Androgen

Abstract. Endocrine-disrupting contaminants (EDC's) are well known to alter sexual differentiation among vertebrates via estrogenic effects during development, particularly in organisms characterized by temperature-dependent sex determination. However, substances producing androgenic effects typically lack potency when tested in laboratory settings and are virtually unstudied in field settings. Here, we assay levels of a synthetic androgen, 17α-methyltestosterone (MT), in a heavily male-biased population of American crocodiles in the Tempisque River Basin of Costa Rica. Nearby tilapia farms utilize MT to bias tilapia differentiation towards the more profitable male sex and we test the hypothesis that this chemical is an EDC in developing crocodilian embryos. The presence of MT was documented in all field-collected samples of egg yolk and in plasma samples of all age classes in the population of crocodiles. A mechanism for the bio-transport of MT and its subsequent effects is proposed.

INTRODUCTION

Pollutants, referred to as endocrine-disrupting contaminants (EDC's), have been shown to alter endocrine function in all vertebrate classes (Hayes et al. 2002), affecting many organ systems and associated processes within each system. The magnitude of this anthropogenic epidemic has spurred pollutant-related operational terminology, such as bio-indicators (Hyne et al., 2009) and sentinel species (Milnes and Guillette Jr., 2008). Commonly, EDC's mimic

estrogens or aromatizable androgens, or act as androgen antagonists, thus biasing secondary sexual characteristics towards a female morphology and associated physiology (Guillette Jr. et al. 1995). Environmental estrogens can result in hermaphroditic males (follicular growth within seminiferous tubules) or 'super' females (rapid and poorly regulated follicular development; Guillette Jr. et al., 1994). To date, the phenomenon of biasing sexual differentiation using androgens is known from lab settings only around pivotal temperatures, or the temperatures at which sex differentiations changes between male and female (Crews et al. 1994), but environmental androgens are virtually unknown from field settings, probably as a result of the aromatizable nature of testosterone and its structural mimics (Wibbels and Crews 1995). Parks et al. (2001) identified paper-mill effluent as having androgenic substances associated with the masculinization of female mosquitofish, providing the only known environmental androgen.

17α-methyltestosterone (MT) is a synthetic androgen used widely in commercial fish farming (Phelps and Popma 2000). It is commonly applied to fish fry to divert sexual differentiation away from female development and towards male, the more profitable sex in most cases. The half-life of MT in water and soil is short (days), suggesting that MT is unlikely to be an EDC. However, the persistence of this compound in a hydrophobic, steroid-friendly medium, such as lipids, is unknown (Phelps and Popma 2000; Gupta and Acosta 2004, Murray et al. in prep). Use of this synthetic androgen is a regular practice in tilapia farming, perhaps the most prominent New World fish industry, where it is applied liberally via feed (Gupta and Acosta 2004). Containment regulations of the compound, either by water or animal exchange with the surrounding environment, vary widely by region and detailed studies of MT fate in the environment are not available (Phelps and Popma 2000).

Bolaños-Montero (2012) and Murray et al. (2015) described a uniquely male-biased sex ratio of American crocodiles (*Crocodylus acutus*) in the Tempisque drainage of Costa Rica. Thermal data from nests within this same population demonstrate that the male-bias is *not* a function of temperature effects on hatchling sex determination (Murray et al. in review). Additionally, exposure of crocodilian eggs to MT generates male hatchlings when incubated at a temperature that should produce only female offspring. The goal of this study is to determine if MT is present in eggs as well as free-ranging hatchling, juvenile and adult crocodiles within the Tempisque Basin, Costa Rica. In addition, a mechanism for its physiological action in the Tempisque system is proposed.

METHODS

Between January 2013 and September 2015 we hand captured American crocodiles at Palo Verde National Park and surrounding areas. Juveniles and adults were captured in four locations within the Park and two outside of it. El Humedal, La Bocana, Nicaragua Lagoon and Varialle Lagoon are all seasonally flooded wetlands within the Park. Varialle Lagoon is in very close proximity to the Tempisque River while the other wetlands are all relatively equidistant and farther from the permanent Tempisque and Bebedero waterways. We also surveyed waterway banks of a drainage canal along the northwestern border of Palo Verde National Park, as well as the Bebedero River near its junction with the Tempisque River, in order to find fresh nests. These banks were then surveyed frequently enough to assure that hatchlings were captured within days of hatching. Other locations sampled included the Tarcoles River south of Guanacaste and a nuisance crocodile captured at a large, commercial tilapia farm near Cañas. For

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each individual sampled we extracted 0.5 to 2.5 cc of whole blood (18-27 gauge needles, depending on the size of the individual) from the spinal vein. Whole blood was kept on ice for no more than 2 hours before being centrifuged so that plasma supernatant could be removed and frozen (-20°C).

During the 2015 nesting season six eggs were collected from five nests late in the first trimester or early in the second trimester of incubation. Eggs were obtained from the waterway banks of a drainage canal along the northwestern border of Palo Verde National Park. Canal banks were monitored multiple times per week as part of another study to ensure accuracy of deposition and hatching date to within two days. Eggs were frozen at -20°C. Upon capture, sex was determined for individuals *via* inspection of the secondary sex organ. Individuals were diagnosed as male is the clitero-penis exhibited all of the following characteristics: bi-lobed structure, extensive vascularization, and length extending the length of the vent (Murray et al. 2015).

Steroid hormones were extracted from egg yolk and plasma using a 3:2 solution (volume: volume) of ethyl acetate and hexane, respectively. Samples were dried under vacuum at 25°C and dissolved in 100 µL assay buffer supplemented with 10 µL of dimethyl sulfoxide (DMSO) to encourage dissolution. We quantified 17α-methyltestosterone using a commercially available sandwich ELISA kit (MaxSignal® methyltestosterone kit, Bioo Scientific, Austin, TX). Cross-reactivity with testosterone was listed as 0.3% and samples were not analyzed in duplicate. Optical density was determined using a Benchmark Plus microtiter plate spectrophotometer (Bio-Rad, Hercules, CA) at 450 nm.

A T-test was performed to assess difference in means between yolk MT concentrations of alligator eggs given doses of MT known to cause sex reversal from female to male (Murray et

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al., in prep) and wild yolk concentrations collected for crocodiles sampled during this study. Concentrations of MT in blood plasma and eggs were compared among crocodile cohorts using one-way ANOVA. Tukey's HSD post-hoc test was used to elucidate differences among cohorts. Among localities, all Palo Verde field sites were pooled and adult plasma MT concentrations were compared to Tarcoles and one sample collected at a tilapia farm via T-Tests. These tests determined whether exposure is spatially acute to Guanacaste or widespread. Within cohorts, T-tests were used to assess statistical differences in MT concentrations between morphological sexes. All statistical analyses were performed in program R (R Development Core Team, 2011).

RESULTS

MT concentrations in yolk sampled from eggs in natural nests did not differ from concentrations known from yolk of alligator eggs dosed with MT concentrations known to cause masculinization (Figure 2; Murray et al. in prep.). MT was detected in every egg yolk sample analyzed.

Hatchlings, juveniles and adults differed significantly in plasma MT concentrations (F = 13.7, DF = 68, p < .0001; Figure 3). Hatchlings exhibited higher plasma MT concentrations (23.06± 18.0 ng/mL) than juveniles and adults, who did not differ from each other (7.09± 7.78 and 5.2± 2.38 ng/mL, respectively). Individuals were identified to sex via inspection of the secondary sex organ and MT concentrations did not differ between males and females within any cohort. Every plasma sample contained a measureable level of MT and MT concentrations of hatchlings were much greater than those in yolk of eggs producing those hatchlings (mean = 0.04± 0.19 ng/g).

Concentrations of adult plasma MT did not differ significantly between pooled Palo Verde samples (4.16± 0.17 ng/mL) and samples of adults from Tarcoles (4.85 ± 0.3 ng/mL). Adults from both localities exhibited lower MT plasma concentrations than one individual captured at the tilapia farms (11.31 ng/mL), the potential source of MT.

DISCUSSION

Analysis of crocodile egg yolk and blood plasma from samples collected in Tempisque Basin and surrounding localities indicated that animals experience heavy MT exposure and retain MT as they grow. The presence of MT in blood plasma of juveniles and adults suggests that exposure to this synthetic hormone is chronic. If exposure to MT were acute within individuals, a steroid of this nature would be filtered by the liver and stored (Guillette Jr. et al., 1995), making acute past exposure of MT undetectable in plasma. Further, degradation of MT in the environment is rapid (Gupta and Acosta 2004) potentially lending support for a bio-transporter.

Our observation that yolk MT concentrations do not differ between wild eggs and those experimentally dosed with MT suggests that Palo Verde crocodile eggs contain MT at levels known to produce male offspring despite incubation at female-producing temperatures (Murray et al. in prep). In fact, of 357 eggs monitored by Murray et al. (nest temp paper) at Palo Verde, 225 were estimated to produce females and 132 were estimated to produce males, based on temperature alone. Data from Murray et al. (in prep) suggest that 60% of the females would convert to males in the presence of MT. Thus, 75% of eggs incubated at field temperatures known from Palo Verde would produce males in the presence of known levels of MT in the yolk.

This value approaches the 80% male bias for hatchling crocodiles recorded at Palo Verde (Murray et al. 2015).

Concentrations of plasma MT for adults at Palo Verde do not differ from levels measured at Tarcoles. Initially, we thought that crocodiles at Palo Verde were affected by a single large tilapia farm located just outside the Park boundary. Therefore, the Tarcoles population was sampled to serve as an uncontaminated control site. However, our results instead demonstrate either that MT is widespread in Costa Rica or that Palo Verde crocodiles are dispersing widely. The Tarcoles River drains San Jose, the major industrial and residential center of Costa Rica. It is known to be a highly polluted river, accumulating and concentrating waste from many sources (Rainwater et al., 2011) and may very well be accumulating MT input from other smaller or unknown fish farming operations. Further, restaurants and tourists surround the site and fish waste deposited in the river may be frequent despite not being directly downstream from a tilapia farm.

Among cohorts, eggs collected after one-third of incubation exhibit concentrations of MT that are barely detectable, while hatchlings exhibit extremely high concentrations of MT in their blood plasma without sufficient time for exposure after hatching. Juveniles exhibit slightly higher MT concentrations than adults (although not statistically different). This may be indicative of less frequent exposure by adults and/or more rapid utilization or storage of this exogenous androgen in reproductively mature individuals. Based on these results we propose a likely mechanism for chronic crocodile exposure to MT in the Tempisque Basin. Initially, tilapia bio-accumulate MT in adipose tissue, a common storehouse for cholesterol-based exogenous steroids (Guillette Jr. et al., 1995), from the supplied feed. Since the escape of tilapia from farms to surrounding natural waterways is observable (Personal observations by authors CMM, ME,

MSM) and crocodiles readily move in and out of tilapia farm ponds, tilapia has become an available food source for crocodiles in the system (Personal observations by authors CMM, ME, MSM). Crocodiles, like the tilapia, sequester the MT acquired via the consumption of tilapia, and likely also use adipose tissue for storage after plasma circulation. Some processes during folliculogensis likely mobilize MT stored in adipose tissue during the preparation and deposition of yolk, allowing deposit of MT in eggs (Paitz and Bowden, 2010). Such processes have been suggested (Guillette Jr et al. 1995) and the detectability of MT in hatchlings and documented masculinizing effects (Murray et al., in prep) implies that MT is not aromatizable. Supplied doses are known to bias sexual differentiation in crocodilians (Murray et al. in prep) and act as an environmental androgen within the eggs in the Tempisque basin, resulting in the male-biased sex ratio recovered by Murray et al. (2015).

Of interest is the low concentration of MT detected in egg yolk and high concentration detected in hatchling plasma before the hatchlings were large enough to consume tilapia. The persistence of MT in water and soil is on the order of hours or days (Phelps and Popma, 2000), so environmental exposure without a bio-transporter is unlikely. Because the concentrations of MT recovered in wild eggs in this study is comparable to the concentrations of experimentally-dosed crocodilian eggs from Murray et al. (In prep), it is likely that much MT persists within developing eggs but is unrecognizable in our analysis. Paitz and Bowden (2011) and Paitz et al. (2012) posit that maternally-derived steroid hormones (in their case, estradiol) are conjugated to a water-soluble sulphates in painted turtles, a TSD reptile. Such conjugates are transported from yolk to embryo and utilized later in development, while the intended embryonic response to the modified steroid is the same as if it lacked conjugation. When experimentally treated, conjugation of sex steroid hormones can occur within hours (Paitz and Bowden, 2015).

Such a process would make MT undetectable to our analyses but result in high hatchling concentrations when (and if) the steroid is biologically reactivated.

Results presented here, in combination with previous experimental studies, suggest that 17α-methyltestosterone (MT) acts as a non-aromatizable environmental androgen on crocodiles in the Tempisque and surrounding basins in Costa Rica and is responsible for a male-biased sex ratio. Interestingly, because the short environmental half-life of MT limits exposure to bio-transport and accumulation, and MT concentrations are highly detectable in juveniles and adults as well as newborn hatchlings, MT likely follows a rare pathway for maternally supplied exogenous sex steroids or their mimics in developing embryos. Further hypothesis testing regarding the physiological mechanism of maternal supply, and bio-reactivation during late development, is needed. Specifically, analysis of MT concentration among yolking follicles within female crocodiles is critical to our proposed mechanism. Additionally, study of the travel and conjugation of MT within eggs among yolk, albumin, and embryo is needed. Environmentally, it is necessary to quantify MT in tilapia and other potential crocodile food sources to isolate the mechanism of bioaccumulation. Lastly, the sex ratios and endocrine profiles of other vertebrates in the area require quantification to fully understand the breadth and mechanism of effects of this environmental androgen. If the proposed mechanism is isolated to diet, then predatory fish and piscivorus birds may likely be affected, either in sex ratio and/or endocrine profiles. However, if MT is widely present in the food chain then one may expect a male-bias in the TSD mud turtle population or other aquatic reptiles and amphibians. Testing of such hypotheses is critical in falsifying the proposed model.

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Figure 1. A. Map of general sampling localities in Costa Rica including the Palo Verde National Park region (Area 1) and Tarcoles River (Area 2).

B. Map of Area 1 including egg sampling locality (A), hatchling sampling localities (A, B), Varialle Lagoon (C) Humedal (D), La Bocana (E) and Nicaragua Lagoon (F). Specific tilapia farm localities are not included to protect privacy of the organizations.

Figure 2. Bar graph comparing yolk MT concentrations between experimental eggs from Murray et al. (in prep) and wild eggs collected for this study. MT concentrations did not differ indicating the potential for biasing sexual differentiation towards males in wild eggs in Palo Verde.

Figure 3. Bar graph showing variation in plasma MT concentrations among crocodile age cohorts with associated standard error (F = 8.924, DF = 4, p = 0.0000066). Coding above bars indicate post-hoc Tukey's HD statistical difference and sample size, respectively.
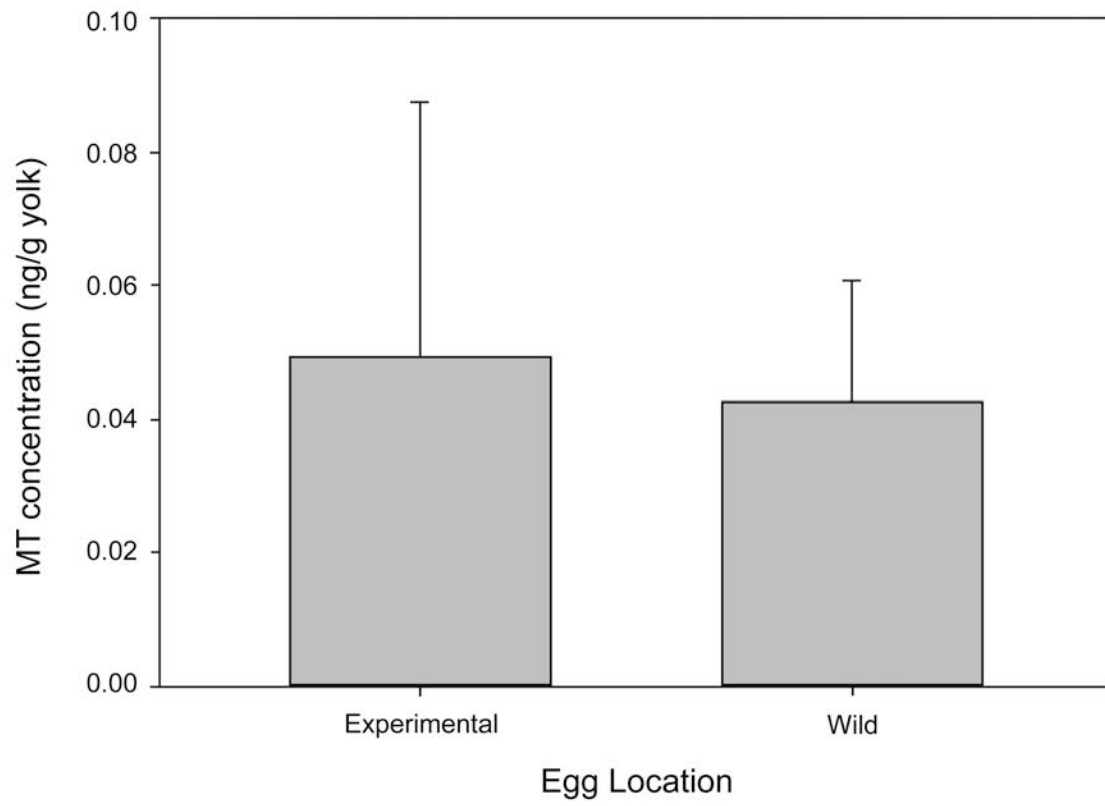
Figure 1

Figure 2

Figure 3