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Sex Differences in the Genitalia of Hatchling *Caiman latirostris*

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Newly hatched crocodilians have a structure called a "cliteropenis" (CTP), which generally is similar in morphology between sexes (Raynaud and Pieau 1985). Neonates of *Caiman crocodilus* and several species of *Crocodylus* display discernable differences between the penis and clitoris (Hutton 1987; Lang et al. 1989; Lang and Andrews 1994; Ziegler and Olbort 2007). The cliteropenis has an asymmetrical structure with a rounded sector (head), supported from the base by an elongated structure (neck). The head has a cleft on the top, in which the tip rests when in a relaxed position, but from which it protrudes when it is erect (Kelly 2002).

The development of the sexual phenotype in crocodilians is also interesting because in all species studied the temperature at which the eggs are incubated determines the sex (Lang and Andrews 1994). Indeed, many reptiles exhibit this temperature-dependent sex determination (TSD). The TSD pattern varies among species and percentage of sexes in nest can be 100% male or 100% female according to the incubation temperature (Janzen and Paukstis 1991), making it difficult to ascertain the sex of individuals without knowing the thermal conditions they experienced during embryonic development.

The Broad-snouted Caiman (*Caiman latirostris*) is the southernmost South American crocodilian, reaching to 32.53°S in its geographic distribution (Melo 2002). This species exhibits the FMF (female – male – female) pattern of TSD (Elf 2003), where females are obtained at 30ºC and 34.5ºC and males at 33ºC (Piña et al. 2003). As in other crocodilians, sexing hatchling *C. latirostris* by cloacal examination is currently possible only when they are larger than 60 cm snout–vent length (SVL) (AL, pers. obs.). However, depending on incubation temperature, genital differentiation in hatchlings varies among species (Allsteadt and Lang 1995; Guillette et al. 1999). Previous studies have even shown differences in size and shape of the CTP in hatchling *Alligator mississippiensis* (Ziegler and Olbort 2007).

The aim of this study was to measure the genitalia of male and female *C. latirostris* at hatching to identify a method of sexing hatchlings by cloacal inspection using digital images. There is no background research on this issue for *C. latirostris*, which currently requires the sacrifice of hatchlings to ascertain sex. However, the species is a valuable natural resource in Argentina (Larriera 1998) and Brazil (Verdade 2001), where conservation and management programs have stimulated research on many aspects of its biology. We hypothesized that the CTP of hatchlings differs between males and females of *C. latirostris*. In particular, we expected to find sex-related differences in the morphometry of the CTP.

Materials and Methods.—Wild *Caiman latirostris* eggs were collected from the San Cristóbal region (30.197406°S, 61.008717°W), Santa Fe, Argentina, during the 2006 nesting season (December–January) (Larriera and Imhof 2006). Seventy-eight eggs (one week old) from three clutches (the clutches were split evenly between two temperatures) were artificially incubated at constant temperatures of 30°C or 33°C (Larriera et al. 2008) to ensure the availability of females and males, respectively (Piña et al. 2003).

At hatching, each neonate was weighed (OHAUS, CS 200, ± 0.1 g) and snout–vent length (SVL; Vernier caliper ± 0.02 mm) from the tip of snout to posterior edge of the cloaca was measured. Twenty hatchlings (10 males, 10 females) were randomly selected for examination of genital morphology. The neonates were sacrificed for removal of the CTP and examination of the gonads. Gonadal sex was identified by the shape, texture, and color of the gonads and by the presence or absence of oviducts (Guillette et al. 1995).

CTPs were removed using a scalpel and preserved in 4% formalin for two hours. Following preservation, the right and upper sides of CTPs were photographed through a stereoscopic binocular microscope (ARCANO®; 20x). A scale alongside each CTP (+ 0.001 mm) (Fig. 1) allowed calibration of the software used for measurements of digital images (Image Pro Plus Version 4.5.0.20; 1993–2001 Media Cybernetics, Inc.). The software needs to set a calibration unit for the active picture, in this case our unit was 1 mm (obtained from the digital caliper alongside the CTP).

Three dimensions were measured from digital images of preserved CTP tissue: 1) Head Width (HW), measured from the

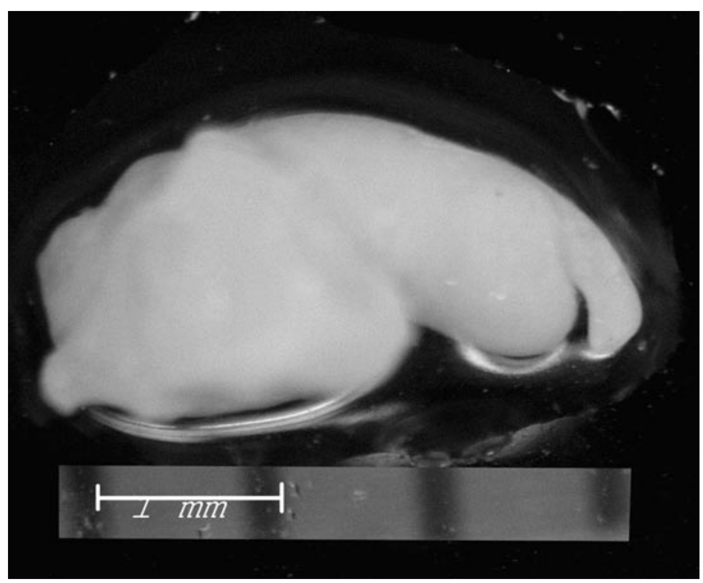

Fig. 1. Preserved CTP of hatchling *Caiman latirostris* removed from the cloaca. The scale below is from the digital caliper positioned in every picture to ensure real scale on the image in the moment of digital treatments. Scale: 1 mm

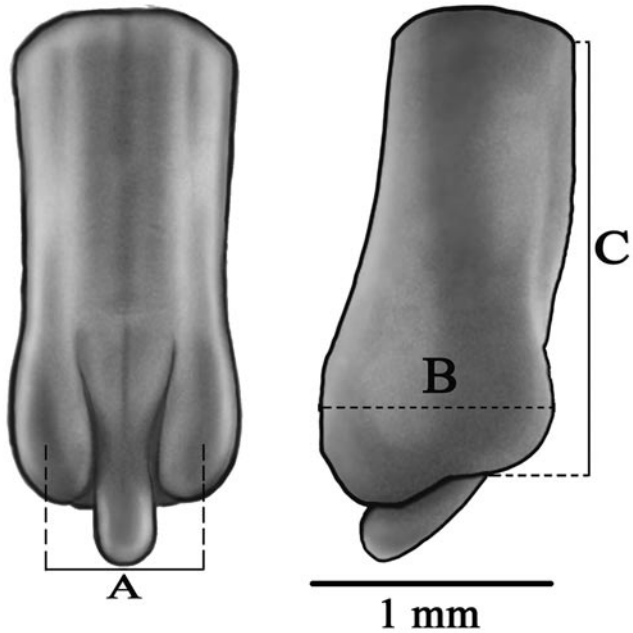

FIG. 2. Dimensions of hatchling cliteropenis *Caiman latirostris* (Left) **A** = Head Width (mm). (Right) **B** = Lateral Width (mm) and **C** = Total length (mm) of the CTP measured as the shortest distance from the base to the beginning of the tip.

upper surface (Fig. 2A); 2) Lateral Width (LW), measured as the maximum vertical width at the midsection of the head (Fig. 2B); and 3) Total Length (TL), measured from the base to the tip where it begins to separate from the head region (Fig. 2C). Also CTP volume was calculated by assuming a cylindrical structure with an ellipsoid base [$V = \pi$ (LW/2) (HW/2)TL], which slightly underestimates real volume.

InfoStat/Profesional version 1.1 was used for all statistical procedures (F.C.A 1999). All data were checked for normality and homogeneity of variances (Shapiro–Wilks and Levene test). Differences in CTP dimensions between the sexes were evaluated with t-tests (Zar 1999). A discriminant analysis with cross-validation for the variables for both sexes was subsequently run on the data set. Scatter plots were then made to create a key for future assessments of hatchling sex.

Results.—Internal sex identification: female gonads were elliptical and elongate in shape, and light beige in color with a granular texture; Müllerian ducts were clearly visible on the side of each ovary (Fig. 3). Male gonads had a similar shape, except the left testis was more elongate than the right testis (a characteristic of the sex), the color was a dark yellow, and the texture was less granular than ovaries of females; the vasa deferentia were present (Fig. 4). All females (N = 9; one of the samples was damaged in the process and we removed it from the analyses) were produced from constant incubation at 30°C and all males (N = 10) from constant 33°C incubation, consistent with the pattern of TSD in this species (Piña et al. 2003).

Cliteropenis of hatchlings: There was no significant difference in TL of the CTP between males and females ($P = 0.065$), but the CTP of males was significantly wider (t-test: LW, $P < 0.01$; HW, $P < 0.01$), and as a consequence volume was also significantly greater in males than in females ($P < 0.01$) (Table 1).

TABLE 1. Average values of variables for both sexes ± SD. (*) Significant average values ($\alpha = 0.05$) (t-test).

CTP variables	FEMALES (N = 9)	MALES (N = 10)
Lateral Width (LW*)	(1.08 ± 0.10) mm	(1.34 ± 0.07) mm
Head Width (HW*)	(0.57 ± 0.11) mm	(0.74 ± 0.13) mm
Total Length (TL)	(2.19 ± 0.48) mm	(2.73 ± 0.76) mm
Volume (*)	(1.09 ± 0.31) mm^3	(2.12 ± 0.17) mm^3

Discriminant analysis showed the presence of separate groups of males and females and the cross-validation analysis showed evidence of a strong model considering the sample size and the CTP variables (proportion hatchlings correct in classification with cross validation is 0.895 versus initial classification of 0.947). Cross-validation reduces the number of measurements, thereby reducing handling time and stress to the animals and compensates for an optimistic apparent error rate in classifications, defined as the percentage of misclassified observations (MINITAB, 2000)

A homogeneity of covariance matrix test suggested that this analysis is suitable for these data (N = 19; df = 21; $P < 0.05$), with HW the most significant variable in the group discrimination ($F = 9.03$; df = 17; $P < 0.01$). In cross classification results, all males

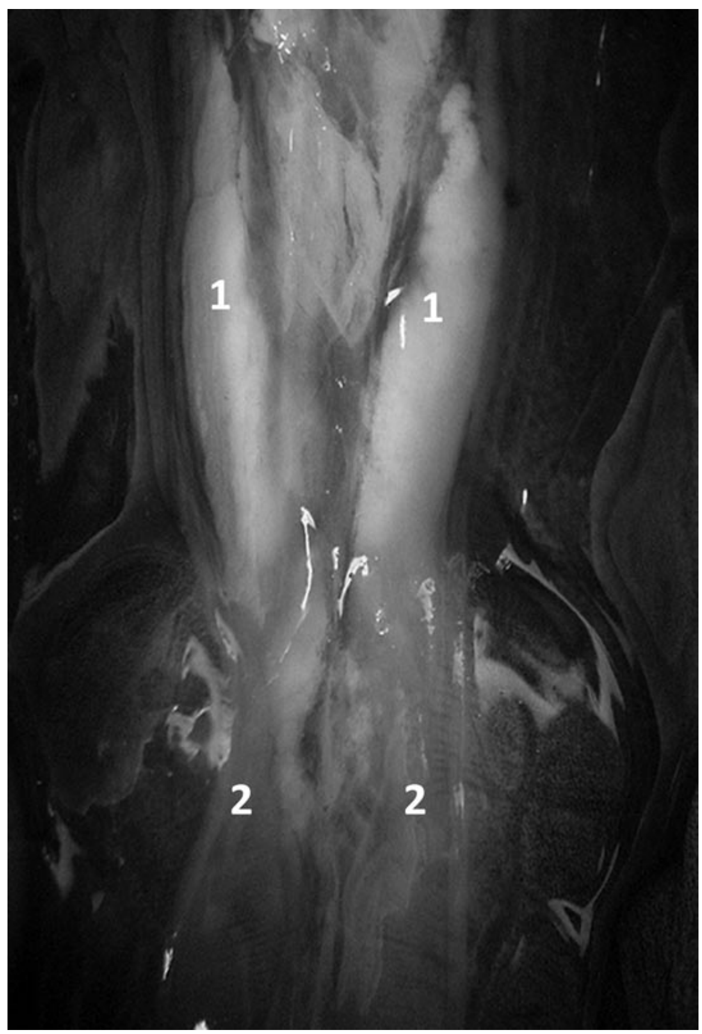

FIG. 3. Gonads of female hatchling *Caiman latirostris*. (1) ovaries, (2) Müllerian ducts.

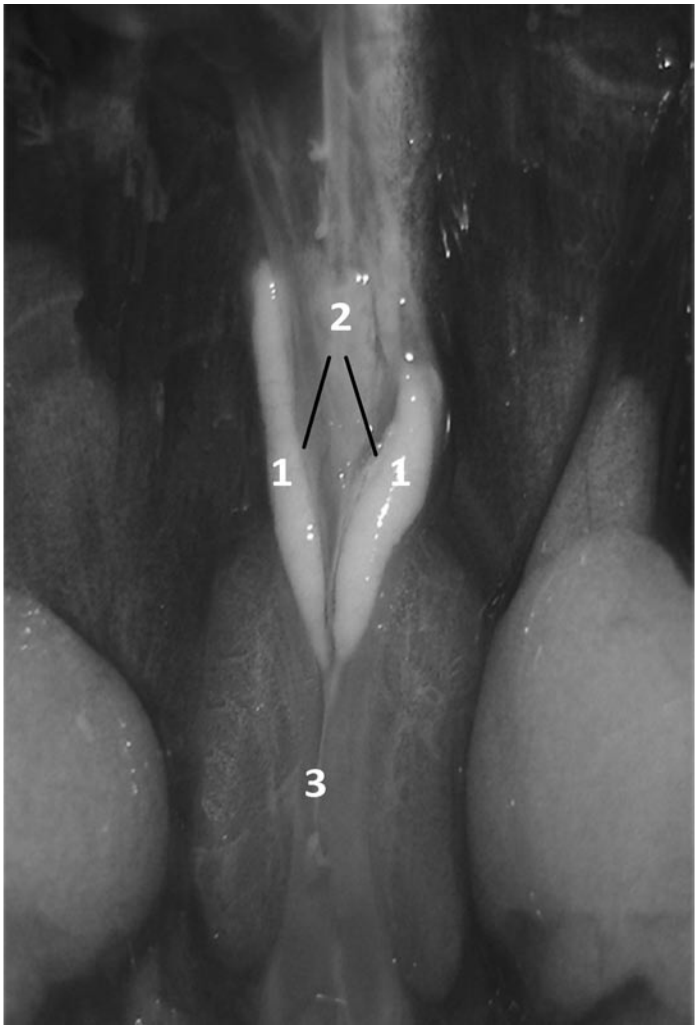

FIG. 4. Gonads of male hatchling *Caiman latirostris*. (1) testicles, (2) adrenal glands, and (3) vasa deferentia.

were classified correctly and only one female of nine was misclassified. Scatter plots of LW and HW of the CTP of hatchling *C. latirostris* revealed two groups: males formed a cluster above and to the right of the female cluster (Fig. 5).

Scatter plots of LW vs. SVL and HW vs. SVL were made to examine the distribution of these two variables in the group of hatchlings tested (Fig. 6). These results are consistent with those obtained in the discriminant analysis. We selected width limits to create a preliminary "key for sex differentiation" to classify hatchlings as males or females based on CTP measures (Table 2). Of 19 samples, we sexed nine correctly as males and seven as females in the first step of the process. One more sample was classified as a male in the second step but two remained indeterminate (no. 7 and no. 9) using the key. These latter two samples were identified as females upon subsequent inspection of the gonads. Using the size limits on the graphics, we determined sex for 85% of the samples correctly, which is similar to the error rate in the discriminant analysis (i.e., 11%).

Discussion.—Previous studies found that reptiles such as *C. latirostris* exhibit TSD (Crews et al. 1994; Lance 1997; Lang and Andrews 1994; Pieau 1999). We corroborate this finding by obtaining 100% males at 33°C and 100% females at 30°C (Piña et al. 2003) as assessed by gonadal inspection (Ferguson and Joanen

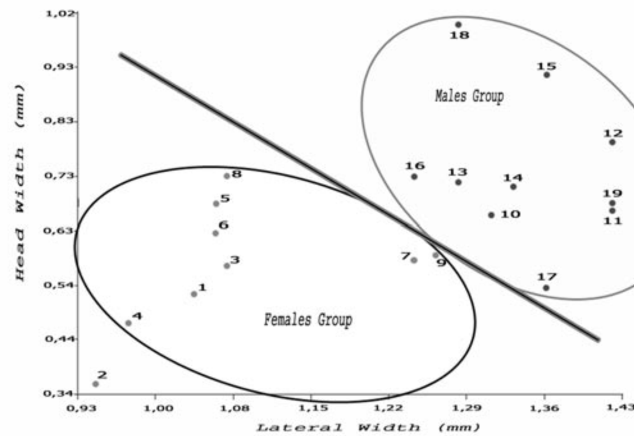

FIG. 5. Scatter plot showing the relationship between Lateral Width (mm) and Head Width (mm) of females (numbered 1–9) and males (numbered 11–19).

1983; Guillette et al. 1995).

Male CTP width and volume dimensions were significantly larger than those for females, which was somewhat surprising because the genital morphology in young crocodiles is similar. Still, prior studies found that incubation temperature affected both genital dimensions and gonadal sex determination in other crocodilians. Hatchling CTPs in *A. mississippiensis* differed in volume between the sexes and increased with increasing temperature of incubation from 30°C to 33°C (Allsteadt and Lang 1995). The CTP in hatchling *C. porosus* differed in HW and LW (Webb et al. 1984) and in size and shape in *C. niloticus* between males and

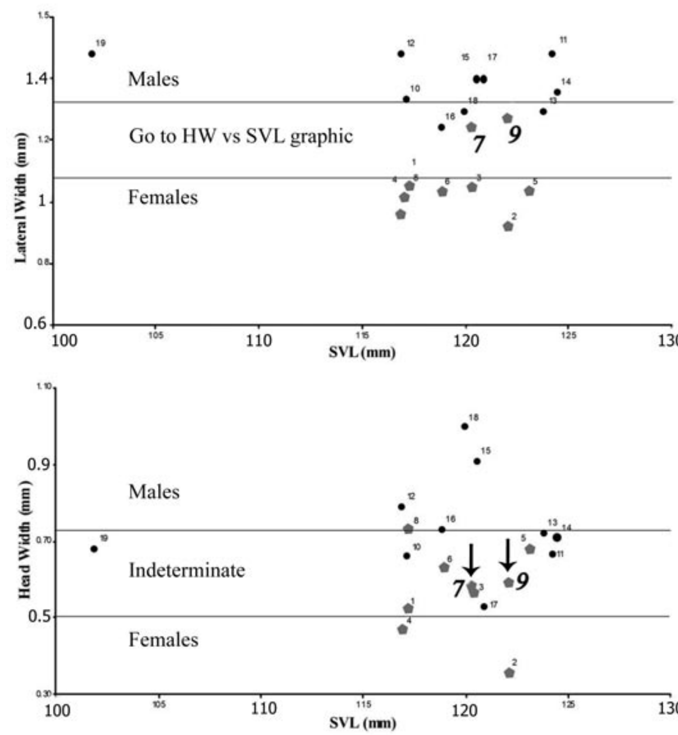

FIG. 6. Scatter plots of LW (mm) vs. SVL (mm) (*upper*) and HW (mm) vs. SVL (mm) (*lower*) values for males (black circles) and females (grey pentagons) groups. Arrows indicate hatchlings designated indeterminate by this method, but sex as females by gonadal inspection.

females (Hutton 1987).

To date the discrimination of newly hatched caiman into males and females has not been possible by observing the external genitalia. We show that such separation can be achieved from simple measures that can be recorded on the cliteropenis. The photographic measurement technique detailed herein was also tried on live hatchlings, which decreases stress on the hatchlings compared to measuring with digital calipers. This work continues in that this technique will be tried on live animals measured at hatching for which we will evaluate sex after one year of growth under controlled conditions of temperature, light, and diet.

To determine the sex in this and other species of crocodiles using a simple methodology opens the possibility of analyzing the sex ratio of offspring born in natural conditions, an unfinished agenda so far. This work is an important precedent for *C. latirostris* and similar species and lays the groundwork for a more advanced method of measurement that minimizes manipulation of individuals and measurement errors that can lead us to sex hatchlings incorrectly. Moreover, this new technique eliminates the current need to sacrifice young caimans to sex them for research work, ranching programs, and husbandry activities.

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