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Source: South American Journal of Herpetology, 16(1) : 50-55

Published By: Brazilian Society of Herpetology

URL: https://doi.org/10.2994/SAJH-D-18-00075.1

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South American Journal of Herpetology, 16, 2020, 50–55

Sexual Identification of *Caiman latirostris* Hatchlings by Cloacal Inspection

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Abstract. Sexual identification of crocodilians is important in population studies and provides useful information for conservation and management plans and monitoring populations over time. It is possible to distinguish between male and female *Caiman latirostris* by cloacal palpation or eversion of the penis in individuals larger than 75 cm total length, but smaller animals possess a barely differentiable cliteropenis. In hatchlings, sex determination methods involve surgical examination, necropsy, or analysis of cranial dimorphism, which cannot be applied in the field. We classified hatchlings of *C. latirostris* by observing the color and shape of their genitals. The penis is a milky white organ with a rounded shape at the tip and a purple hue at the end, whereas the clitoris is shorter, whitish, and has a pointed end. The procedure was tested on hatchlings from three nests at the "Proyecto Yacaré" study area (Santa Fe province); half of the eggs of each nest were incubated at a constant temperature of 31°C (producing females) and the other half at 33°C (producing males). To observe the sexual organs by cloacal inspection, we used a modified instrument whose function during palpation is like that of a finger applied in large animals to evert the penis or clitoris. In the first days after hatching we correctly scored the sex of 80% of the individuals. The number of correct identifications was slightly lower for males than for females. This technique might be a useful tool for field studies, as it allows the sex of small caimans to be estimated in situ.

Keywords. Broad Snouted Caiman; Cliteropenis; Cloacal sexing; Genital morphology; Sex identification; Sex ratio.

Resumen. La identificación del sexo de los cocodrílidos es de importancia en estudios sobre ecología de poblaciones, ya que este dato permite elaborar o proponer planes de conservación y manejo, así como también, el seguimiento de estas poblaciones en el tiempo. En la naturaleza, es posible diferenciar machos y hembras de *Caiman latirostris,* mediante la palpación cloacal o eversión del pene en animales de tamaño mayor a 75 cm de largo total. Mientras que en animales pequeños se observa un cliteropene difícilmente diferenciable. En el caso de los neonatos, los métodos de determinación del sexo implican exámenes quirúrgicos, necropsias o análisis de dimorfismo craneal, los cuales no son aplicables a las condiciones de muestreo en campo. En este trabajo, clasificamos ejemplares recién nacidos de *C. latirostris,* observando el color y forma de sus genitales. Pudimos caracterizar al pene como un órgano de color blanco lechoso con forma redondeada en la punta y una tonalidad púrpura en el extremo; mientras que el clítoris es más corto, blanquecino y con el extremo puntiagudo. El procedimiento se testeó en crías de tres nidos, provenientes de sitios de trabajo de "Proyecto Yacaré" (Provincia de Santa Fe); la mitad de los huevos de cada uno fueron incubados a una temperatura constante de 31°C (productora de hembras) y la otra mitad a 33°C (productora de machos). Para observar los órganos sexuales mediante inspección cloacal, utilizamos un instrumento que modificamos y cuya función es similar a la de un dedo, como frecuentemente se utiliza en animales grandes para realizar la eversión del pene o clítoris. De acuerdo con nuestras observaciones, en los primeros días de vida asignamos correctamente el sexo del 80% de los individuos. La cantidad de aciertos fue ligeramente menor para los machos que para las hembras. Esta técnica sería una herramienta de utilidad para los estudios de campo, ya que posibilita la identificación del sexo de animales pequeños in situ.

INTRODUCTION

All species of crocodilians undergo temperature-dependent sex determination (Lang and Andrews, 1994), with the temperatures that produce males or females varying according to the species (Janzen and Paukstis, 1991; Valenzuela and Lance, 2004; Piña et al., 2015). In nature, nest temperature cannot be used to deduce hatchling gender because researchers are unaware of the temperatures at which the embryos developed, and these temperatures vary due to the effect of environmental conditions or nesting habitat (Campos, 1993; Lang and Andrews, 1994; Simoncini et al., 2014).

Many crocodile species, including *Crocodylus acutus* (Cuvier, 1807), *Crocodylus moreletii* (Duméril and Bibron, 1851), and *Crocodylus porosus* Schneider, 1801, show sexual size dimorphism when mature, and it is possible to identify males when they exceed the maximum size of females; however, males cannot be distinguished from females of the same size (Platt et al., 2009; Platt et al.,

How to cite this article: Ciocan H., Leiva P.M.L., Simoncini M.S. 2020. Sexual identification of *Caiman latirostris* hatchlings by cloacal inspection. *South American Journal of Herpetology* 16: 50–55. http://doi.org/10.2994/SAJH-D-18-00075.1

Submitted: 06 November 2018
Accepted: 11 September 2019
Available Online: 31 August 2020

Handling Editor: Luciano Martins Verdade
http://doi.org/10.2994/SAJH-D-18-00075.1

2011; Barrios-Quiroz et al., 2012; Fukuda et al., 2013). Similarly, individuals can be sexed by cloacal palpation or eversion of the penis once they reach a total length greater than approximately 60 cm (Chabreck, 1963; Webb et al., 1984) or 75 cm (Honegger, 1978; Combrink et al., 2012). Studies on *Alligator mississippiensis* (Daudin, 1802) and *Caiman latirostris* (Daudin, 1802) report that hatchlings possess an undifferentiable cliteropenis (Joanen and McNease, 1978; Ziegler and Olbort, 2007; Nuñez-Otaño et al., 2010); in contrast, in other species of crocodilians the hatchling penis and the clitoris can be differentiated, as for example in *Caiman crocodilus* (Linnaeus, 1758) and several species of *Crocodylus porosus* and *Crocodylus johnstoni* Krefft, 1873 (Webb et al., 1984), *Crocodylus niloticus* Laurenti, 1768 (Hutton, 1987), and *Crocodylus palustris* (Lesson, 1831) (Lang et al., 1989; Lang and Andrews, 1994).

The penis of crocodilians is normally hidden within the cloaca (Ziegler and Olbort, 2007; Johnston et al., 2014), and its size depends on the species and the size of the animal (Moore and Kelly, 2015). It is usually cylindrical, somewhat laterally compressed, and reinforced by connective tissue (Ziegler and Olbort, 2007; Moore et al., 2012; Kelly, 2013). Protrusion during copulation is produced by muscular pressure and vascular dilatation (Ziegler and Olbort, 2007; Moore et al., 2012), and the penis has a dorsal groove that functions as a spermatic channel (Cabrera and García, 2007; Moore and Kelly, 2015); this groove is also found in the clitoris of females, but it lacks any known function (Ziegler and Olbort, 2007). At the end of the genital organ, a loop-like structure is observed, called the tip, on to which the spermatic groove extends, and a softer structure is demarcated from the tip by a small groove. For mating, the penis protrudes forward (cephalad) from the cloaca and is bent towards the venter (Ziegler and Olbort, 2007; Johnston et al., 2014).

The cloacal palpation method used to evaluate the sex of crocodilians usually consists of introducing a finger or speculum in the cloaca and everting the penis (Chabreck, 1967; Brazaitis, 1969). However, this method can only be used in animals larger than about 75 cm in total length (Honegger, 1978; Ziegler and Olbort, 2007; Combrink et al., 2012), whose minimum cloacal size allows the insertion of a finger. In the case of smaller specimens, a speculum can be used to observe differences in external genital morphology, although it is difficult to apply (Hutton, 1984; Allsteadt and Lang, 1995; Leslie, 1997; Combrink et al., 2012). Another method proposed by Whitaker et al. (1980) consists of everting the penis by compressing the cloaca laterally and flexing the tail towards the ventral part. Differentiation of genital structures occurs at very small sizes in some species and at sizes of at least 1 m in others (e.g., *Gavialis gangeticus* [Gmelin, 1789], Lal and Basu, 1982). Dissection of *Alligator mississippiensis* and *Caiman latirostris* hatchlings have revealed that the penis is long and has a rounded shape and reddish coloration, while the clitoris is short and pale white (Allsteadt and Lang, 1995; Nuñez-Otaño et al., 2010; Gredler et al., 2015).

In the case of neonates, palpation is not possible due to their small size and undifferentiated penis and clitoris (Ziegler and Olbort, 2007; Nuñez-Otaño et al., 2010). Therefore, for the evaluation of sex in neonates, invasive methods such as surgical examination of the gonads (Magnusson et al., 1990) or necropsy are applied (Nuñez-Otaño et al., 2010; Murray et al., 2016). Cranial dimorphism can also be analyzed, but it has an efficacy of approximately 70% and is not applicable in the field (Piña et al., 2007a), or one must for the animals to grow, which requires a lot of time (Piña et al., 2007b).

The ability to identify the sex of crocodilian hatchlings and small juveniles in the field would provide an important tool to obtaining data without transporting or collecting the animals or impacting the study population. This is important because sex identification is fundamental to understand population dynamics and ecological/biological processes, and knowing the proportions of males and females is essential for management plans, either for conservation or harvest (Ziegler and Olbort, 2007; Platt et al., 2009; Fukuda et al., 2013). Consequently, the objective of this study is to validate a technique that allows sex identification of *Caiman latirostris* hatchlings through observation with the naked eye. We also test if the results are affected by the size of the hatchlings and the nest of origin.

MATERIALS AND METHODS

We collected 100 eggs of *Caiman latirostris* from three nests collected in the localities of Cacique Ariacaiquín, Espín, and Intiyaco, which are areas of the "Proyecto Yacaré," a sustainable use and conservation program (Leiva et al., 2019). Once the nests were collected and transferred to the laboratory, we determined the embryonic development time of the nest according to the criteria of Iungman et al. (2008). This was done by observing the opaque band (no greater than 37% of egg surface) and the morphological characteristics of the embryo. We only used nests at a developmental stage less than or equal to 5 d (stage 5). All caimans of the three nests hatched on 7–8 March 2016. We randomly separated the eggs of each nest into two equal groups (50 each) and incubated them at 31°C (which produces a 100% female development) and 33°C (to produce 100% male development; Piña et al., 2007b; Parachú-Marcó et al., 2017). After birth, we identified the animals individually by scute clipping (Richardson et al., 2002).

We carried out four measurement events in which we considered different morphometric parameters: total length (TL), snout–vent length (SVL), and weight (W).

The first measurement and observation were carried out at the month of birth; thereafter, measurements and observations were made every 2 months until the hatchlings reached 7 months of age. We considered ranges of size of the animals to observe the sex according to the TL; these size intervals in total length (cm) were: 22–27, > 27–33, > 33–36+. Hatchlings that remained at the same size interval at the next measurement period were re-examined and their sex re-evaluated. However, the second measurement and sex identity were not included in the analysis. If the second sex identity was the same as the first, we classified it as successful; if not, we classified it as failure, regardless of which identification was confirmed by necropsy (see below).

For data collection, we obtained hatchlings from unidentified plastic trays and measured and weighed each individual. Later, the same experienced observer identified the sex—not knowing the mark, nest of origin, or incubation temperature—throughout the experiment. To observe the sexual organs by cloacal inspection, we used an instrument that we modified and whose function during palpation is like a finger applied in large animals to perform the eversion of the penis or clitoris. This instrument consists of a curved forceps (London College Tweezer) covered with latex to increase adhesion and prevent injury (Fig. 1). However, it can also be built with other materials, and we have also constructed versions made of wood and aluminum covered with latex. At the end of the study, we corroborated the sex of the animals via direct observation of their gonads by necropsy (Nuñez-Otaño et al., 2010; Murray et al., 2016) by randomly selecting 10% of each group to confirm that the hatchlings from eggs incubated at 31°C were females and those incubated at 33°C were males.

We analyzed the data using the Chi-square test, considering successes and failures in sex identification for each TL interval; this analysis was performed for each sex separately and with all individuals pooled. We considered no differences in the identification between size intervals studied if $P > 0.05$. For each growth interval, the reliability of the method was also calculated as successes/total individuals. Further, we evaluated if there was an effect of the nest of origin on the effectiveness of the identification in the animals whose origin was known.

RESULTS

Among the 100 incubated eggs, hatching success was 76%, including 41 females and 35 males (i.e., 54% F; 46% M). The observer identified males by the presence of a longer sexual organ with a broad, rounded tip and milky whitish coloration with a purple hue on the tip (Fig. 2). In contrast, females had a shorter clitoris with a fine tip termination and whitish coloration (Fig. 3).

We did not observe a size effect in the correct assignment of the sex of individuals in general ($X^2 = 0.74$, df = 2; $P = 0.6896$) or for males ($X^2 = 0.97$, df = 2; $P = 0.6146$) or females ($X^2 = 1.09$, df = 2, $P = 0.5785$). The Chi-square was calculated to observe if there was influence of the origin nest in the identification of sex, and we did not find differences between the different nests ($X^2 = 0.43$, df = 2; $P = 0.8049$).

DISCUSSION

According to Leslie (1997), the use of the cloacal test to identify sex is not reliable in *Crocodylus niloticus* from the Lake St. Lucia region during the first year of development. At approximately 14 months, the morphological differences between the male and female genitalia, mainly the length and shape of the cliteropenis, were more evident and enabled external sex identification. However, that study did not mention the size of those animals at 14 months. In contrast, Hutton (1984) was able to identify the sex of small 4-week-old *C. niloticus* with 80–85% effectiveness, which increased to 95% in animals larger than 40 cm TL. In our study, we achieved a success rate of 80.5% (71.4% for males and 87.5% for females) for individuals that were 4 weeks old and 22–27 cm TL—approximately half the size reported by Hutton (1984). Although we did not observe statistically significant differences in reliability according to the increase in size of the sexed individuals, the reliability was 88.9% (75.0% in males and 92.8% in females) for individuals of 33–36+cm TL. It is worth mentioning that the caimans in this study reached the last interval of TL at approximately 7 months under captive conditions (held at a controlled temperature of 32°C and fed three times per week).

For individuals of *Crocodylus porosus* and *Crocodylus johnstoni* of 10–14 cm SVL (~20–30 cm TL), the penis and clitoris can be distinguished with 95% accuracy using forceps (Webb et al., 1984). As reported by Webb et al. (1984), there is a bias in sex identification, confusing the long clitoris of females with penises, although it is rare for males to be classified as females. In contrast, in our study errors occurred when a small penis was mistaken for a clitoris, resulting in males being misclassified as females (Tables 1, 2). According to Moore et al. (2018), there are morphological differences between the sex organs of crocodiles and alligators. These differences could be observed in hatchlings, whereby it would be easier to identify sex in crocodiles, whose genital structures are more prominent than those of alligators. However, we were able to obtain a high percentage of successes in *Caiman latirostris,* although we did not test it in other alligatorids; therefore, we recommend that the tests should be extended to other species. According to Joanen and McNease (1978), *Alligator mississippiensis* individuals less

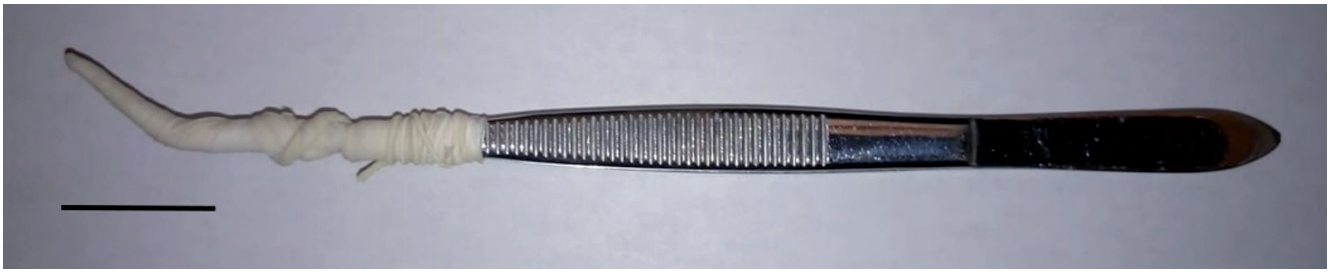

Figure 1. Curved forceps, closed at the end and lined with latex, used like a finger to evertthe cliteropenis. Total length: 165 mm. Width from the rubber tip to elbow: 2–4 mm. Scale bar = 2 cm.

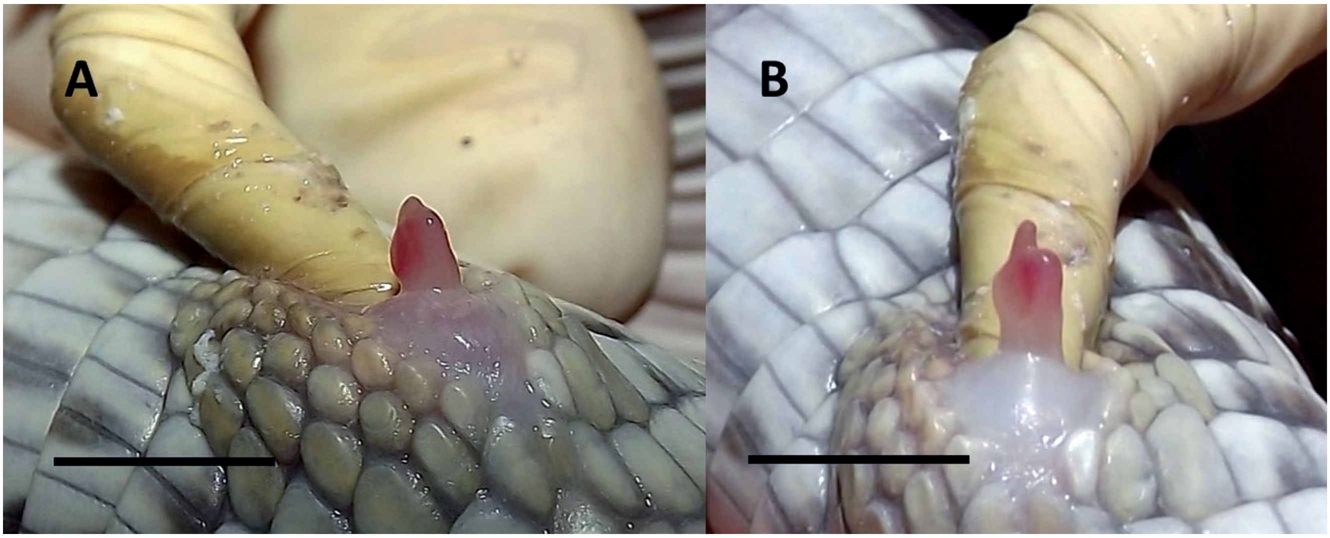

Figure 2. Penis with the tip retracted **(A)** and penis with the tip deployed **(B).** The milky white coloration with purple color/tint hue at the end is observed. The instrument used to make eversion is observed behind. Scale bar = 5 mm.

Figure 3. Clitoris. The whitish coloration and the sharpest termination can be observed. Behind it is the instrument used to evert the clitoris. Scale bar = 5 mm.

than 30 cm SVL (~60 cm TL) possess and undifferentiated cliteropenis; nevertheless, Allsteadt and Lang (1995) were able to classify the sex of hatchlings and juveniles of *A. mississippiensis* with the naked eye with a high percentage of effectiveness. Other studies have found morphological differences in alligatorids, but through necropsies or histological observations (Nuñez-Otaño et al., 2010; Kelly, 2013; Gredler et al., 2015).

Previous studies have claimed that sex could be identified in most crocodilian species through direct observation of individuals greater than 60 cm (Chabreck, 1963; Webb et al., 1984) or 75 cm TL (Honegger, 1978; Combrink et al., 2012). In the particular case of *Caiman latirostris,* to date sex identification has been recorded only in animals between 60–120 cm TL (Piña et al., 2007b; Nuñez-Otaño et al., 2010). In this species, although the penis is wider and has a greater volume than the clitoris, this was only observed after euthanasia of the hatchlings and analysis of photographs taken using a stereoscopic binocular microscope (Nuñez-Otaño et al., 2010). Through this method of observation, Nuñez-Otaño et al. (2010) achieved approximately 85% accuracy for hatchlings, which is similar to the results obtained in our study by direct observation of live animals.

We did not find statistically significant differences in the number of successes with respect to the size of the animals in the intervals evaluated. Since the nest effect is reflected in hatchlings size, it was expected that it would not influence the identification of hatchling sex (Garnett and Murray, 1986; Hutton, 1987; Schulte and Chabreck, 1990; Ciocan et al., 2018). Regardless, we do not dismiss the possibility that there could be differences in the sexual organ development associated with the nest of origin. However, reliability seems to increase as the size of the animal increases, reaching almost 90% for both sexes (Table 3).

Table 1. Male assessment, differentiated by individual size intervals (total length). *n* = No. individuals sexed in the corresponding size range.

Males				
Total length (cm)	***n***	**Successes**	**Failures**	**Reliability**
22–27	56	40	16	0.714
27–33	36	29	7	0.806
33–36+	4	3	1	0.75
Mean ± SD				0.757 ± 0.046

Table 2. Female assessment, differentiated by individual size intervals (total length). *n* = No. individuals sexed in the corresponding size range.

Females				
Total length (cm)	***n***	**Successes**	**Failures**	**Reliability**
22–27	72	63	9	0.875
27–33	40	33	7	0.825
33–36+	14	13	1	0.928
Mean ± SD				0.876 ± 0.052

Table 3. Assessment of both sexes, differentiated by individual size intervals (total length). *n* = No. individuals sexed in the corresponding size range.

Males and females				
Total length (cm)	***n***	**Successes**	**Failures**	**Reliability**
22–27	128	103	25	0.804
27–33	76	62	14	0.815
33–36+	18	16	2	0.889
Mean ± SD				0.836 ± 0.046

Previous studies affirm that the size of the cliteropenis of neonates of *Alligator mississippiensis* increases according to incubation temperature among animals of the same and different sexes (Allsteadt and Lang, 1995; Gredler et al., 2015). In the present study, we worked with animals from eggs incubated at temperatures producing only males (33°C) or only females (31°C), but it is possible that *Caiman latirostris* individuals incubated at temperatures producing both males and females (e.g., 32°C; Parachú-Marco et al., 2017), external sexual morphology might be more difficult to distinguish. Given that incubation temperatures in nature vary due to environmental conditions (Simoncini et al., 2014), the results of our method of sex identification could also vary.

We can conclude that it is possible to observe and distinguish between the male and genital structure of *Caiman latirostris* at an early age and small size using the cloacal palpation method and this new tool to perform the eversion. The method is simple, inexpensive, and easy to use in field conditions, without requiring the complex logistics required to study cranial morphometry or resorting to highly invasive practices such as surgical examination or necropsy. We also found that accuracy increased as the size of the hatchlings increased. For all individuals the accuracy was 84%, being slightly higher for females (88%) than males (76%). The percentage of failures might vary according to the experience of the observer, who should understand the method and be able to clearly distinguish between male and female genitalia. Through this study, we considerably reduced the size in which a hatchling can be classified as male or female with a high effectiveness, which allows to expand the database of sexual proportion in small animals in the field.

ACKNOWLEDGMENTS

We would like to thank CICyTTP-CONICET for support. We are grateful to all members of Proyecto Yacaré, Dr. Carlos Piña for his advice, Msc. Ricardo Ojeda and Lic. Leonardo Scarpa for their help, and Dr. Perran Ross for help and advice. Special thanks to CSG-IUCN members for their contribution.

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