Acta Herpetologica 9(2): 259-263, 2014
DOI: 10.13128/Acta_Herpetol-14736

Sexing freshwater turtles: penile eversion in *Phrynops tuberosus* (Testudines: Chelidae)

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Submitted on 2014, 25th July; revised on 2014, 15th November; accepted on 2014, 17th November
Editor: Paolo Casale

Abstract. Here, we described a noninvasive method for sexing freshwater turtles by stimulating penile eversion. We immobilized the neck and limbs of animals using fingers and, after some seconds, turtles everted their penis. This method was tested in 33 male *Phrynops tuberosus*, and 28 everted the penis. The efficiency of the method was not dependent of animal size, which reinforces its applicability. Our method allows sexing turtles in the field, avoiding killing the animal or causing major injuries in order to assess the sex.

Keywords. Defensive behaviour, penis, pleurodira, sexing method.

Determining the gender of individuals is useful in most population studies. However, sexing might be difficult for many species, especially freshwater turtles, because some species lack clear morphological differences between males and females (Rueda-Almonacid et al., 2007). Examples of general noninvasive sexing methods include hormone profiles of blood and amniotic fluid (Wibbels et al., 1987; Gross et al., 1995; Xia et al., 2011), chromosome analysis in species having Genetic Sex Determination (GSD) (Ferreira Júnior, 2009), searching for mature eggs through palpation on the inguinal region of females (Rueda-Almonacid et al., 2007), sexual dichromatism in head spots (Moll et al., 1981; Bulté et al., 2013) and morphometry (Gibbons and Lovich, 1990; Valenzuela et al., 2004; Casale et al., 2005; Rueda-Almonacid et al., 2007; Readel et al., 2008). This last method is the most used in field conditions. Tail size and shape, cloaca position, claws length, and plastron morphology are examples of secondary sexual characteristics, being tail analysis applicable for all turtles (Rueda-Almonacid et al., 2007; Reed and Tucker, 2012). However, not experienced researchers might have difficulty to evaluate if a tail is large or small.

Penises of turtles are found in the cloaca's ventral surface and are inflatable copulatory organs (Zug, 1966; Carvalho et al., 2010; Cabral et al., 2011). A method to sex turtles is to insert the finger into the cloaca in order to find the penis (Rueda-Almonacid et al., 2007). However, this method is invasive and useless in small animals. Manual hemipenile eversion (copulatory structure found in Squamata) is a common method used to sex snakes, lizards, and amphisbaenians (Reed and Tucker, 2012). However, in chelonians, this method has been explored only recently (de Solla et al., 2001; Dustman, 2013; Lefevbre et al., 2013). De Solla et al. (1998) used this technique to sex individuals of *Chelydra serpentina*, but they did not explain how it was performed. Recently, Lefevbre et al. (2013) described a method to stimulate penial erection

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

and ejaculation by applying vibrations to the animal shell, while de Solla et al. (2001) and Dustman (2013) stimulated penis eversion in *Chelydra serpentina* by gently shaking the turtle horizontally and vertically, respectively.

In July 2012, we observed that when captured male turtles were much stressed (trying to bite, moving their limbs, head, and tail very much), they everted the penis during handling after a few seconds [a behaviour already observed in *Chelydra serpentina* (de Solla et al., 2001) and *Hydromedusa maximiliani* (Famelli et al., 2011), which may be considered a defensive display]. In August 2012, when we were attempting to measure a stressed male of *Phrynops tuberosus*, we held its neck and limbs against its body in order to safely and accurately measure it. When the specimen was held in this manner, the male rapidly everted the penis. These observations were the starting point for the development of the method described herein.

This study aims to describe a method for sexing freshwater turtles, based in penile eversion, which do not require any additional material and may be easily performed in field conditions.

The sexing method was tested in 33 males of *Phrynops tuberosus* (Peters, 1870) (*Phrynops geoffroanus* sensu latu, populations of Ceará referred by Rueda-Almonacid et al. (2007) as *Phrynops tuberosus*), captured at the Banabuiú River, village of Laranjeiras (05º17'S, 38º31'W, DATUM WGS84), Banabuiú, Ceará, northern Brazil, between August and November 2012. *Phrynops tuberosus* is a chelid freshwater turtle distributed in northeastern South America (Rueda-Almonacid et al., 2007). Turtles were hand-captured using snorkels, measured (Carapace Length (cm) – CL), sexed through tail size (males having a larger precloacal length than females) following Rueda-Almonacid et al. (2007) and marked following Cagle (1939).

Males had their limbs and head immobilized (Fig. 1). In some occasions, gently applying a little pressure on hind limbs and head was necessary to accelerate penile eversion in males that were calm (they did not try to bite or move their limbs, head and tail; their only action was to retract the neck and limbs when handled). The method was tested at a maximum twice in each male, the second trial being performed only if the first one had a negative result, in order to avoid excessive stress. Eversion occurred after a few seconds, which was dependent on animal stressing level (most stressed individuals completely everted the penis in 9 s, while calmer turtles took approximately 30 s to only partially evert it). All animals retracted the penis after release and had no alteration in their normal behaviour. We also observed a small cloacal wall eversion in some females (see Dustman, 2013). However, it was not employed an evaluation for females as systematic as it was performed for males. Captures were authorized by the responsible environmental institution (see license number in Acknowledgments). No animal suffered injuries or mistreatment in capture and handling processes, and no invasive method was performed.

We used a logistic regression to evaluate the relationship between the efficiency of our method and male size. A chi-square test was used to compare the number of successes and failures using the method. The sexing method reliability was assessed as a proportion of males that exposed their penises. Statistical tests were performed in R ver. 3.0.1 (R Development Core Team, 2013). Descriptive statistics are reported as mean ± standard deviation.

We found that 28 males everted the penis when we used the method reported herein. The reliability of the method was 84.85% (28/33) and the vast majority of males were correctly sexed (X^2 = 16.03, df = 1, P < 0.001). Males ranged in CL from 7.63 to 26.91 cm and averaged 20.96 ± 4.02 cm. The efficiency of the method was independent of animal size (logistic regression: z = -1.39, P = 0.16) (Fig. 2). All stressed animals everted the penis. The five individuals that did not evert were calm. Probably there was no error in sex identification, since these males had evident male characteristics, such as a large precloacal distance. If they were females, we would have observed a small cloacal wall evertion, but their tail did not show any response to the method.

The development of non-lethal techniques to sex turtles is essential in conservation and demographic studies (Rueda-Almonacid et al., 2007; Lefevbre et al., 2013). The method proposed here was able to properly sex the

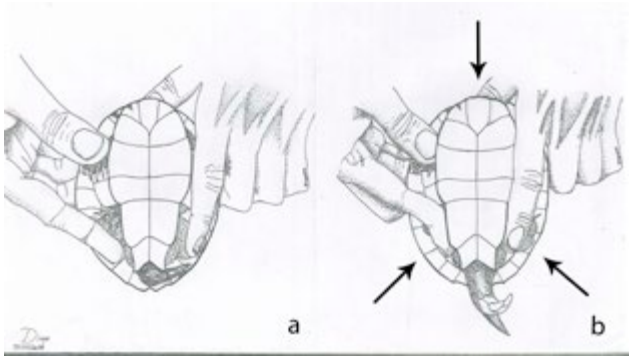

Fig. 1. Illustration of a male *Phrynops tuberosus* captured in Banabuiú River, being sexed following the method explained in this study (Rodrigues et al.). a) Male *P. tuberosus* with head and limbs hold; b) Male *P. tuberosus* everting the penis after a little pressure in the hind limbs and neck. Note the arrows indicating the pressure points. Arrows and letters were included in the original drawing using Adobe Photoshop™. Drawings: Diego de Oliveira Soares.

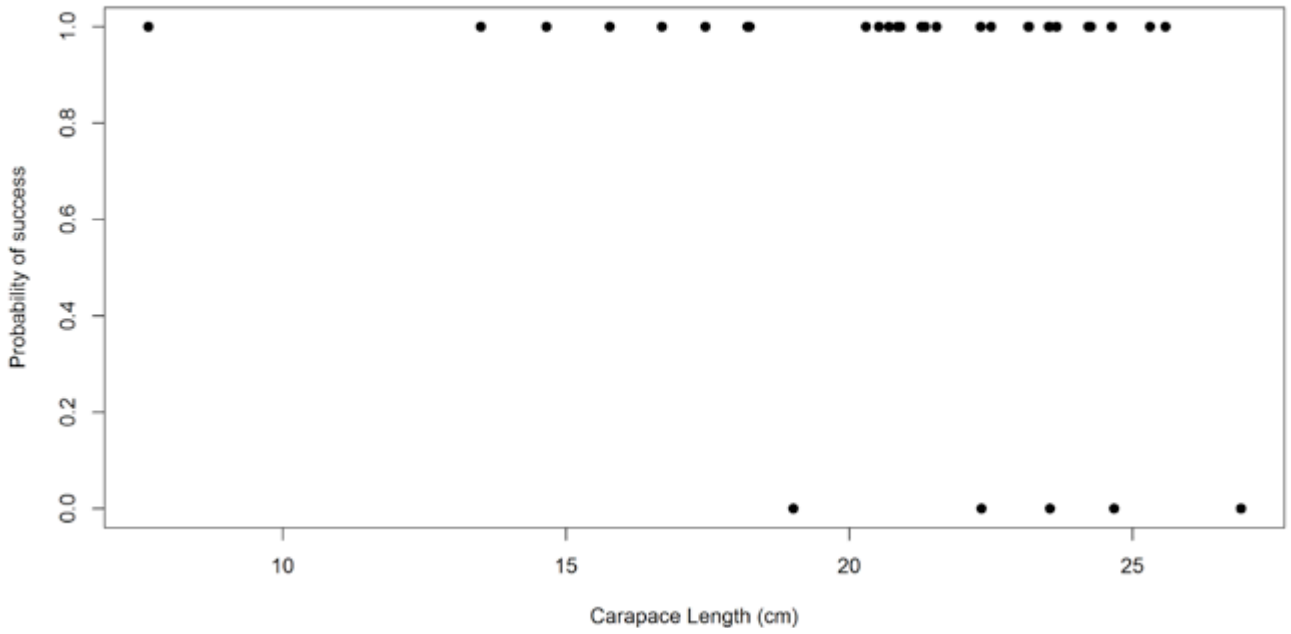

Fig. 2. Relationship between the probability of success of the method and carapace length of *Phrynops tuberosus* in centimeters.

vast majority of captured males. Our study is the first to describe a technique of penis eversion tested in Pleurodira turtles. Previous methods were successfully tested in *Chelydra serpentina* (de Solla et al., 2001; Dustman, 2013) and *Emydoidea blandingii* (Lefevbre et al., 2013). The efficiency of most methods using penile eversion was tested in only one species (including ours). However, the potential contribution of these methods to field studies cannot be underestimated due to this limitation, and future studies in other turtle populations may provide more data about their success.

Penis is an inflatable copulatory organ, which also depends on blood influx to become everted and erected (Zug, 1966; Carvalho et al., 2010; Cabral et al., 2011). Stressing conditions, such as handling, may increase heart-beating rate in freshwater turtles (Cabanac and Bernieri, 2000), which could raise blood flow, easing penile eversion. A high efficiency of a penis eversion method in stressed turtles was also found in Lefebvre et al. (2013). There were reports of other freshwater turtles everting penis after being handled (de Solla et al., 2001; Famelli et al., 2011), which reinforce the potential application of our method in other species.

Although our method was not efficient enough to sex all males, it had a high reliability (84.85%). Manual hemipenile eversion in Squamata also has some problems and is difficult to use in large bodied species, with muscular tails or in fragile species (Reed and Tucker, 2012). Penile eversion could be used in parallel to traditional methods based on tail length size, in order to have an improved result.

The lack of relationship between method efficiency and animal size is advantageous, because it reinforces its general applicability. A male with carapace length of 7.63 cm, for example, was successful sexed (see Fig. 2 for more details about the lack of relationship between efficiency and animal size). Most sexing methods of chelonians are size-dependent and could be performed only in large individuals, such as secondary sexual characteristics identification (for example, plastron concavity and claws size) and penis identification inside the cloaca (Rueda-Almonacid et al., 2007; Reed and Tucker, 2012). Unfortunately, we cannot make a prediction about the applicability of our method to immature individuals based on our data, which could be an interesting topic for future studies regarding this method.

Penis display may be considered a defensive behaviour in freshwater turtles (de Solla et al., 2001; Famelli et al., 2011). It may be an explanation for the eversion as a consequence of immobilization, because males everted the penis when they had been completely immobilized, with all their limbs and head held. Hence, this behaviour would be a final strategy to try to scary the predator (the researchers, herein) and escape.

Our method allows sexing turtles in the field, avoiding killing the animal or causing major injuries in order to assess the sex. Studies reporting sexing methods based in penile eversion are increasing, reinforcing the impor-

tance of invest in such methods, which are already very common for Squamata. Finally, this manuscript enriches the list of studies of noninvasive and reliable sexing methods for turtles, and its employment may be useful in future population studies with these animals.

ACKNOWLEDGMENTS

We thank all the friends that helped in field survey. Flávio de Barros Molina gave valuable suggestions on the first version of the manuscript and provided literature. Diogo Borges Provete and Roger Bour gave valuable suggestions in the last version of the manuscript. CNPq and Programa de Pós-Graduação em Ecologia e Recursos Naturais da Universidade Federal do Ceará provided a master fellowship to JFMR. Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) granted a measuring, marking, and capture license (Sisbio/ICMBio 32548-1).

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