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A novel approach to sexing *Lachesis stenophrys* (Serpentes: Viperidae) using radiography

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Determining the sex of captive animals has important implications for population management and social dynamics (Courts 1997; Ballou et al. 2010). This has particular importance when sex of an individual may be difficult to determine with certainty. Squamate reptiles can be particularly challenging candidates to sex as many do not exhibit sexual dimorphism (Fitch 1960). Furthermore, the paired intromittent organs of snakes (hemipenes) are invaginated within pouches when at rest and are not visible. The most widely used technique of sexing snakes is to insert a lubricated and blunt-tipped metal probe of appropriate diameter gently into the cloaca and then reverse the direction of the probe and advance it distal to the body; in male snakes of many species the inverted hemipene permits the probe to move down further than in a female snake of the same species, which lack such structures (e.g., Fitch 1960; Laszlo 1975). The sheath of the hemipenis may become filled with organic solids and this may reduce the depth that the probe can penetrate (Gnudi et al. 2009; Kane et al. 2022), leading to spurious results. Probing involves prolonged manual restraint of animals, which may induce stress in the animal (Laopichienpong et al. 2017) and cause increased risk to handlers, especially in the case of venomous or large, powerful individuals (Lock 2008; Antonio 2014). Another widely used method of sexing snakes involves applying distal to proximal pressure on the hemipene to encourage eversion of this organ (Fitch 1960). A poorly performed examination of such delicate tissues also carries a degree of risk for the snake through introduction of foreign matter or iatrogenic damage. Furthermore, hemipenes of some species such as *Psammophis* spp. (Shine et al. 2006) are relatively small which may reduce validity or complicate interpretation of results obtained. Ultrasonography has been used to identify internal primary sexual structures in reptiles (Boyd et al. 1996; Rizzi et al. 2017) with varying success and molecular markers have been demonstrably useful in the sexing of snakes (Laopichienpong et al. 2017), but molecular methods may be prohibitively expensive.

Radiography allows assessment of tissues of differing radio-opacity, including the mineralised tissues of some squamate hemipenes. The use of radiographs to determine sex is known to provide an effective and non-invasive method for sexing adult varanid lizards (Shea and Reddacliff, 1986; Card and Kluge 1995) as radiopaque structures show clearly on radiographic images. Contrast radiography, which involves inserting a radiopaque media into the cloaca to fill any cavities such as inverted hemipenes, has been used successfully on helodermatid lizards (Meza-Oviedo et al. 2020) and non-viperid adult snakes (Gnudi et al. 2009), but sex determination in snakes via traditional radiography has not been reported in the literature. This study demonstrates that *Lachesis stenophrys* (Central American Bushmaster) can be accurately sexed via conventional radiography.

Lachesis stenophrys is a pit viper native to Central America, inhabiting hilly tropical forest habitats from near sea level to over 1000 m elevation (Ripa 2002; Campbell and Lamar 2004). Human envenomation by *Lachesis* spp. is medically significant and potentially life threatening (Madrigal et al. 2012). Despite being among the longest species of viper in the world, with adults averaging over 200 cm total length (Solórzano 2004), many features of its biology are unknown. The species is reported to exhibit sexual dimorphism with males growing to a larger size than females and to have an overall darker ground colour (Ripa 2002). However, there can be substantial overlap in size, colour and pattern between different individuals of the same species and these features exist along a continuum rather than being discrete and non-overlapping (Fernandes et al. 2004). In captive snakes, a reliable method of sex determination may be to identify the presence or absence of hemipenes: these sac-like, paired reproductive organs possessed by male squamates lie in an inverted position at the base of the ventral tail when not in use and, in many species of snake, they are covered in spines or hooks to assist in anchoring the male hemipenis within the female cloaca during mating. Female snakes have paired ovaries and oviducts within the coelom but no such organs distal to the cloaca, with a few notable exceptions (Hoge et al. 1959; Mallmann-Franco and Franco 1998, 1999).

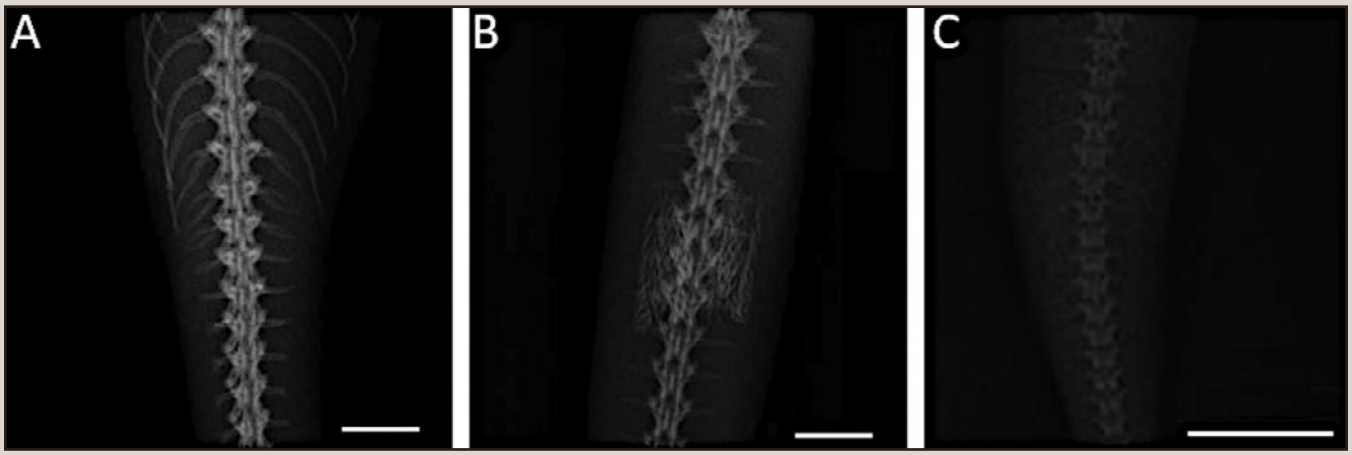

Fig. 1. Radiographs of area immediately distal to cloaca in adult female (A), adult male (B) and juvenile (C) *Lachesis stenophrys*. Note the apparently bi-lobed and paired radiopaque structures, consistent with hemipenes, visible distal to the cloaca of the adult male in Fig. 1B. These structures are approximately 40–50 mm from the cloaca. Scale bar = 10 mm.

Materials and Methods

This study probed and radiographed three adult *L. stenophrys* (>4 years old) and five juveniles (measured at 4 mo old, and then again at 7 mo old) during routine health assessment and pre-export checks between January and May 2021. Snakes were manually restrained within an unbranded transparent acrylic tube, then probed with unbranded probes between 1 and 4 mm wide at the terminal end. Probes were lubricated with KY Jelly (Reckitt, USA) prior to insertion. Digital radiographs of individuals were obtained with an Ultralight300 Collimator, model R-200V (MinXray, Inc, USA), type G01 set at 50 kVp and 1.8 mAs, processed with a Fujifilm FCR Prima 2 CR1R 391RU (Tokyo, Japan) and analysed with FCR Prima V software. Scale counts were conducted using the method described by Dowling (1951).

Results

All eight *L. stenophrys* were successfully probed and radiographed. Following transport to veterinary hospital, time for relevant restraint method (i.e., placing snake in box or restraining snake in tube) and radiographs took ca. 5 min per snake to conduct, and probing took ca. 15 min per snake. Adult snakes ranged between ca. 185.0–195.0 cm total length; juveniles measured 58.3–67.0 cm (mean: 63.4 cm) at the time of first examination and had increased to 78.4–84.6 cm (mean: 81.8 cm) at the second examination three months later. During an initial routine health check, a radiograph of one adult *L. stenophrys* identified obvious radiopaque structures ca. 50 mm distal to the cloaca. This individual was probed to a depth of 14 subcaudal scales and was confirmed to be male. Examinations of other *L. stenophrys* held at ZSL London Zoo allowed for more detailed records of sex to be made. A radiograph of another adult *L. stenophrys* did not show radiopaque features consistent with hemipene adornments near the cloaca, indicating a lack of hemipenes (Fig. 1A), and female sex was confirmed by probing to a depth of 3 subcaudal scales (11 mm); furthermore, this snake laid a viable clutch of eggs eight months prior (Kane et al. 2021). A third adult *L. stenophrys* showed radiopaque structures distal to the cloaca (Fig. 1B) and was manually probed to a depth of 8 subcaudal scales (40 mm), thus was sexed as male. When around four months of age, five young *L. stenophrys* were manually probed to a depth of 1–3 subcaudal scales (2–5 mm) and radiopaque structures were not visible (Fig. 1C). A repeat examination when the snakes were seven months old and had increased in total length by 29% yielded the same result of probes going 2–3 scales deep. These snakes were not radiographed a second time. From probing and lack of ability to evert hemipenes, it seems likely that all five juvenile snakes are female.

Discussion

The radiopaque structures visible via radiography appear to be tissue with some mineral content, as they are of a similar opacity to adjacent bone. These structures are likely from the spinose adornments on the hemipenes in adult male *L. stenophrys*. However, this study did not perform a dissection or observe an everted hemipene so other origins of radiopacity, such as mineralised hemibacula, which are present in at least some varanids (Shea and Reddacliff 1986), cannot be ruled out. Though a hemipene description is lacking for *L. stenophrys*, Wied's 1822 study, cited in Vanzolini et al. (2015), provided a line drawing of an everted hemipene of *L. muta* clearly showing spines on both terminal ends of the bi-lobed appendage and Fernandes et al. (2004) reported 81–98 spines on each lobe of the hemipenis. Our radiograph of an adult male *L. stenophrys* (Fig. 1B) would appear to be concordant with this condition. Female *L. stenophrys* appear to lack such structures and radiographs of the cloacal region show soft tissue only (Fig. 1A). Additionally, the proximal half of the tail of the adult male *L. stenophrys* was noticeably wider. It was important to ensure female *L. stenophrys* did not have hemipene-like structures given the problematic phylogenetic positioning of *Lachesis* relative to *Bothrops* (Wüster et al. 2002); intersex individuals bearing a structure similar to the hemipenis of a male snake predominate in adult *Bothrops insularis* and are known from at least three more *Bothrops* spp., with true female *B. insularis* being uncommon and infertile (Hoge et al. 1959; Mallmann-Franco and Franco 1998, 1999). Based on radiographs presented here this condition does not appear to be present in *L. stenophrys,* although adult female sample size (N = 1) is small. All five juvenile snakes were sexed as female however, as the age at which tissue mineralisation takes place in this species is unknown and was outside the scope of this study, sex determination cannot be confirmed with complete accuracy.

One main benefit to radiography was that the approximate time taken to assess each snake was shorter than having to manually restrain each snake in a tube for physical examination. Radiography required placement of each snake into an appropriate container while the radiograph was taken, whereas restraining each snake in a clear acrylic tube so that manual probing could be performed took approximately triple the time. For the snakes, this shorter duration out of the exhibit as well as not being forcibly restrained in an acrylic tube was likely associated with lower stress (Kreger and Mench 1993; Schuett et al. 2004) and a higher degree of safety for both snakes and persons involved which demonstrates the major benefit to radiographic sexing of adult *L. stenophrys*. Furthermore, probing depths in the two confirmed male snakes ranged from 14–8 subcaudal scales; a 57% difference which highlights a potential limitation of this method, though no overlap between male and female *L. muta* was noted by Boyer et al. (1989). Probing does, however, have the advantage that snakes younger than at least eight months can potentially be reliably sexed.

An advantage of standard radiography compared to contrast radiography is the lack of false positives which Gnudi et al. (2009) reported in 20% of female snakes analysed with this method. Standard radiography is likely a viable tool for confirming sex in other snake species which have ornamented hemipenes, such as *Gloydius huangi* (Wang et al. 2019) and *Gonyosoma boulengeri* (Nguyen et al. 2020) however, for species without hemipene adornments, contrast radiography may better show presence of hemipenes without natural radiopaque structures present. Such comparisons, however, were outside the scope of this study. A limitation of radiography is that if the snakes' body overlays itself then distinguishing sometimes fine structures may be difficult in a resultant image. Furthermore, radiography is not suitable for use in the field, and requires use of appropriate protective equipment for staff to limit chronic exposure to radiation.

Radiographs of a three-dimensional structure produce a two-dimensional image, which results in some structures overlapping and being difficult to distinguish (Prötzel et al. 2017). This may be a limitation to more detailed work but as this study was investigating presence/absence of such structures this constraint on detail was of negligible importance. The use of computed tomography would have permitted a more detailed analysis of hemipenes, or lack thereof, however this was outside the scope of this work and this technology is likely not reasonably accessible to most zoo veterinary facilities or individuals working with venomous snakes in a private capacity. Ultrasonography could have been used to identify internal primary sexual structures including hemipenes, testes or ovaries (Boyd et al. 1996), however the relative size of testes and follicles varies with season (Gnudi et al. 2009) and furthermore this method would have necessitated a more invasive approach than radiography while still being similarly effective in determining presence or absence of hemipenes. Moreover, ultrasonography requires expensive sophisticated equipment and requires high degree of expertise to perform accurately.

In conclusion, radiography of *L. stenophrys* is a novel and potentially reliable method of determining sex in sexually mature individuals and is shown to be a validated alternative to more invasive probing or ultrasonography, resulting in improved welfare for captive snake husbandry. Further studies investigating the applicability of radiography to determine sex, including a range of snake species at varying ages, are encouraged.

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