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ONTOGENY OF GONAD AND GENITAL MORPHOLOGY IN JUVENILE ALLIGATOR SNAPPING TURTLES (*MACROCHELYS TEMMINCKII*)

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***Abstract.*—Juvenile turtles often lack sexually dimorphic morphological features, and as a result in many studies sex is often simply not determined. There are several alternatives for ascertaining sex, but they tend to be error-prone, expensive, time consuming, or require invasive surgery. We compared the age-specific efficacy of laparoscopic evaluation of gonads to cloacoscopic evaluation of genitalia to non-invasively determine sex of juvenile Alligator Snapping Turtles. These techniques were, in turn compared to sex determination by pre-cloaca tail length, an approach frequently used for assessing sex of many turtle species. Laparoscopy was reliable for identifying sex of juveniles as young as eight months and as small as 35 mm midline plastron length, cloacoscopy was reliable for animals that were at least eight years old and at least 165 mm midline plastron length. Tail morphology began to diverge between males and females at approximately 150 mm midline plastron length, but divergence was not complete except among sexually mature animals that were > 295 mm midline plastron length. Thus, laparoscopy likely remains the only reliable technique for sexing small juveniles, but cloacoscopy presents a viable non-invasive alternative for larger juvenile size classes.**

Key Words.—cloacoscopy; endoscopy; genitalia; gonad; laparoscopy; *Macrochelys temminckii*; reptile; sex

INTRODUCTION

Correctly identifying the sex of turtles is important in many contexts, including ecological and life history studies where precise demographic data are required. Additionally, correctly identifying the sex of juvenile turtles is critical to successful reintroduction efforts for threatened and endangered species. Whereas with many species an even primary sex ratio can be assumed (Fisher 1930), most turtles exhibit temperature-dependent sex determination, a trait that increases the likelihood of producing skewed ratios (Vogt and Bull 1984; Freedberg and Wade 2001; Girondot et al. 2004).

Many turtle species can be easily sexed as adults based on external secondary sex characteristics. Depending on the species under consideration, such characteristics may include dimorphism in color, plastron concavity, foreclaw length, and body size. Perhaps one of the most readily distinguishable characteristics, however, is differences in tail morphology. With few exceptions, male chelonians exhibit longer, thicker tails than females, or have more distally-located cloacae (Ernst and Barbour 1989). The reason for this distinguishing and generally reliable characteristic is straightforward: the difference reflects males' need for space in the cloaca to house a penis that, among adults, is considerably larger than the homologous clitoris of females (Seshadri 1956).

In contrast to adults, juveniles of most turtle species are not easily sexed based on external morphological characteristics. As a result, several alternative approaches to sexing young turtles have been employed with varying degrees of success. Historically, many studies have relied on dissecting sacrificed hatchlings in order to visualize gonads and distinguish ovaries from testes (Mrosovsky and Yntema 1980; Schwarzkopf and Brooks 1987; Spotila et al. 1987; Ewert and Nelson 1991; Wibbels et al. 1991). However, several non-lethal approaches have been developed. For instance, a landmark-based geometric morphometric method was used to detect subtle anatomical dimorphisms in hatchling *Chrysemys picta* and *Podocnemis expansa*, with accuracy rates of 98% and 90%, respectively (Valenzuela et al. 2004). Investigators have also used plasma testosterone concentrations to differentiate male from female *Gopherus agassizii* with 98% accuracy (Rostal et al. 1994), and in species for which circulating testosterone concentrations don't differ predictably between juvenile males and females, dosing subjects with follicle stimulating hormone prior to collecting blood will cause a spike in male, but not female, testosterone levels (Owens et al. 1978).

In recent decades, however, laparoscopic surgery has been the most commonly used non-lethal method for sexing juvenile turtles. This method is superior to others in that it can be 100% accurate (Wibbels et al. 1989; Rostal et al. 1994; Ligon et al. 2009). However, it also comes with several drawbacks: it is an invasive surgical procedure and can cause complications that can damage or kill turtles; it requires expensive equipment that may

not be widely available; and it is a veterinary procedure that requires surgical training and access to controlled anesthesia drugs to which many investigators may not have access.

Cloacoscopy is a minimally invasive technique that has been proposed as an alternative to laparoscopy for sexing adult turtles of species that exhibit reduced sexual dimorphism (Coppoolse and Zwart 1985; Spadola and Insacco 2009). Although male and female genitalia are generally indistinguishable in very young turtles (D. Ligon, pers. obs.), we hypothesized that the genitalia may differentiate in older juveniles and sub-adults, thus providing a useful means of discriminating sex of individuals before other external sexually dimorphic characters are evident.

Conservation considerations.—Patterns of temperature-dependent sex determination are well documented for some turtle species (Ewert and Nelson 1991; Ewert et al. 1994; Ewert et al. 2004), and therefore it is often easy in head-start programs to obtain desired sex ratios by simply incubating eggs at two different temperatures: one that is known to disproportionately produce females and another that will produce males. This approach is not always applicable, however, because patterns are not well-studied for many species that are of critical conservation concern.

Alligator Snapping Turtles are a species of growing conservation concern, and head-start efforts are underway in Oklahoma and Louisiana. Propagation of this species is problematic in that no constant incubation temperature produces an all-male cohort (Ewert et al. 1994; Ligon and Lovern 2009). Additionally, embryo survival, as well as hatchling size and morphology, is affected by incubation temperature, so care must be taken to select temperatures that ensure that high quality hatchlings are produced (Ligon and Lovern 2009). Because of these constraints, the head-start effort in Oklahoma has incubated eggs at an intermediate temperature that is known to produce a female-biased mixed sex ratio, and proportions of males and females that are released has either been assumed to approximate desired ratios, or on occasion been determined via surgical observation of gonads when small numbers of subjects were involved (Riedle et al. 2008a). Our objective in this study was to determine the minimum ages at which this species can be accurately sexed via laparoscopy and by cloacoscopy, both to enhance ongoing head-start and reintroduction efforts for the species, and to establish the efficacy of cloacoscopic sex identification for turtles generally.

MATERIALS AND METHODS

Animal acquisition.—We used Alligator Snapping Turtles from two different sources. Tishomingo Nation Fish Hatchery supplied hatchling and juvenile turtles that were produced as part of a propagation/reintroduction project. These animals' precise ages were known at the time of surgery. The adult turtles that we used were also obtained from Tishomingo National Fish Hatchery, but originated from a group of adult animals that were confiscated from an Arkansas turtle farm in 2007. These animals are members of the brood stock maintained by the hatchery. Their origins are unknown, but genetic analyses confirm that they originated from Mississippi River drainage wild stock (unpubl. data).

In addition to examining genital and gonadal characteristics, we assessed the size and age at which turtles could be sexed based on tail morphology. Pre-cloacal tail length, measured from the posterior edge of the plastron to the center of the cloacal orifice, was regressed against midline plastron length to determine the size at which this character began to diverge in males and females. We used midline plastron length instead of midline carapace length in our analyses for two reasons. First, carapace length is measured differently among studies, including curved midline carapace length (common in sea turtle studies; e.g., Bjorndal et al. 2000) and maximum carapace length (Riedle et al. 2008b). Second, one subject in our study exhibited significant kyphosis that made midline carapace length an inaccurate measure of body size. Its plastron, in contrast, was well-formed.

Surgical procedure.—Each turtle was anesthetized using a 10 mg/kg ketamine (Ketaset, Fort Dodge Laboratories; Fort Dodge, Iowa, USA) and 0.1 mg/kg medetomadine (Diamondback Drugs; Scottsdale, Arizona, USA) injected intramuscularly (IM). This drug combination has a proven record for this and other chelonian species (Ligon and Lovern 2009; Ligon et al. 2009; Carpenter and Marion 2013). The anesthetized turtle was positioned with its head and right front limb directed downward. We extended the left rear leg caudally and scrubbed the surgical site with chlorhexidine (Chlorhexidine Gluconate Vetone, MWI Veterinary Supply; Boise, Idaho, USA). We then made a stab incision with a #11 surgical blade in the left prefemoral fossa cranial to the leg and directed towards the opposite front leg and slightly dorsal to avoid iatrogenic puncture of bowel or lung tissue. The stab incision punctured the skin and coelomic membrane. A 2.7-mm 30° viewing scope (Karl Storz Endoscopy–America, Inc.; Culver City, California, USA) was inserted through the incision into the coelomic cavity and pivoted dorso-cranially to visualize the gonads. Physiological saline was injected into the cavity through a sleeve on the scope to suspend the organs and improve visibility. We captured both video and still digital

Table 1. Morphometrics of Alligator Snapping Turtles (*Macrochelys temminckii*) within each of the nine size classes examined. Values are mean ± 1 SD.

Age Class (mo)	n	Mass (g)	Midline Carapace (mm)	Midline Plastron Length (mm)	Pre-cloaca Tail Length (mm)
2	8	28 ± 1	44 ± 1	35 ± 2	11 ± 3
8	5	35 ± 5	49 ± 12	39 ± 3	12 ± 2
16	7	169 ± 37	84 ± 6	69 ± 6	23 ± 2
32	5	331 ± 47	108 ± 5	89 ± 7	27 ± 3
44	4	524 ± 111	123 ± 10	102 ± 7	32 ± 3
60	2	790 ± 155	147 ± 13	120 ± 5	38 ± 9
88	2	1,696 ± 10	163 ± 30	152 ± 2	44 ± 11
96	2	1,890 ± 67	174 ± 29	163 ± 29	52 ± 9
Adult	2	25,650 ± 15,203	463 ± 60	362 ± 60	155 ± 89

images for future identification. After examination, we closed incisions in both the skin and coelomic membrane with one simple mattress suture of 3–0 polydioxanone (PDS, Ethicon, Inc.; San Angelo, Texas, USA) and sealed the skin with topical placement of surgical glue (VETbond Tissue Adhesive, 3M; St. Paul, Minnesota, USA). Atipamezole (Antisedan, Pfizer Animal Health Group; New York, New York, USA) was administered at 0.1–0.2 mg/kg IM following surgery to reverse medetomidine. This dose is at the low end of the recommended dose range (Carpenter and Marion 2013) but has proven highly effective for Alligator Snapping Turtles and other chelonia on which we have conducted laparoscopic surgery (Ligon et al. 2009; Ligon et al. 2012). Each subject was maintained in shallow water (approx. 1 cm) for 24 h or more to prevent drowning during recovery from anesthesia.

Cloacoscopy.—We performed cloacoscopy of turtles after atipamezole reversal but before complete recovery from anesthesia. Turtles were placed in sternal recumbancy with their back legs and tail extending off the edge of the table. We gently cleaned the cloacal orifice with chlorhexidine to remove loose skin and debris. We then flushed the proctodeum with tap water to remove feces and debris. A speculum was lubricated with sterile lubricating jelly (First Priority, Inc.; Elgin, Illinois, USA) and inserted into the proctodeal segment of the cloaca. We performed a final proctodeal rinse with 1–2 mL of 2% lidocaine (Lidocaine injectable, Sparhawk Laboratories; Lenexa, Kansas, USA) diluted in 15–20 mL of physiological saline (0.9% sodium chloride, Abbot Laboratories; Chicago, Illinois, USA) to relax the cloaca. Depending on the size of the turtle, we used specula ranging 4–9 mm diameter. An endoscope was inserted, using the speculum as a sleeve to expand the cloaca and proctodeum to better visualize the genetalia. Video and still digital images were captured for future examination.

Gonad and genital identification.—We ascertained and recorded gonadal sex of each subject at the time of laparoscopic examination. Cloacoscopies were not conducted in a blind fashion, as they were conducted on the same day and on the same animals that laparoscopies were performed.

Digital images and video files were sorted by turtle. We stored images of gonads separately from those of genitalia, and no files contained information that observers could correlate with specific turtles. These grouped files were then examined and categorized by three observers who were not present during surgical procedures. Identification of gonads from this exercise were compared to those made by the authors at the time of surgery. The procedure was not repeated for images of genitals because the authors' general inability to differentiate penises from clitori at the time of examination precluded meaningful comparisons. Sample video footage of juvenile alligator snapping turtle genitals and gonads are archived in an on-line appendix (available at: http://www.herpconbio.org/Volume_9/Issue_1/Ligon_et al_2014-appendix).

Several indivdual turtles were identified as males based on presence of testicles, but also exhibited tubular structures that appeared to be oviducts (see Results). One such animal was sacrificed, a necropsy was performed, and tissue samples were submitted to the Oklahoma Animal Disease and Diagnostics Laboratory (Oklahoma State University, Stillwater) to confirm our preliminary identification of oviductal tissue.

RESULTS

We conducted laparoscopic surgery on 47 animals representing nine age classes: 2, 8, 16, 32, 44, 60, 84, and 96 mo, and adults of indeterminate age (Table 1). Cloacoscopic examinations were conducted on a subset of subjects ≥ 16 months of age as younger age classes

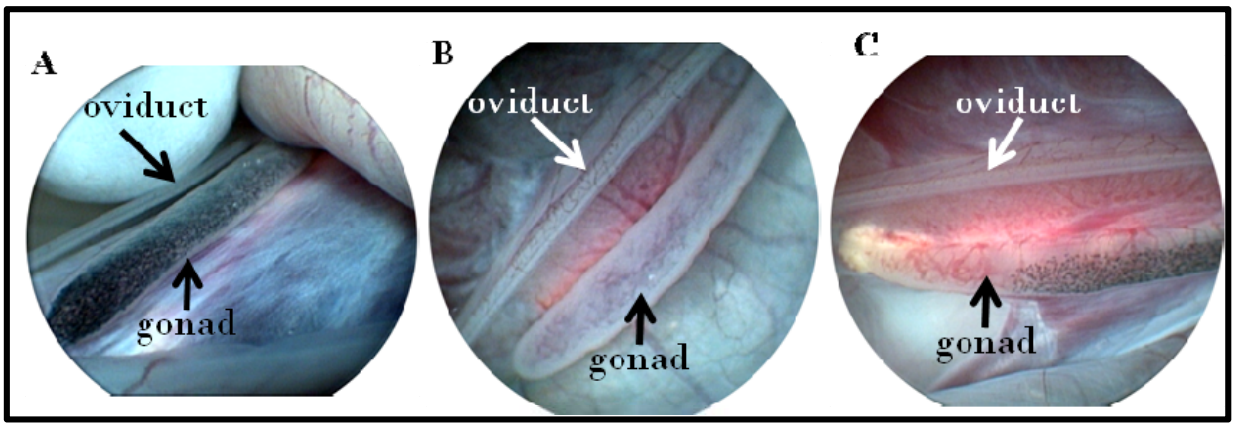

FIGURE 1. Pigmentation of gonadal tissue varied within and among 2-mo-old and 8-mo-old Alligator Snapping Turtles (*Macrochelys temminckii*). Shown are the A) right and B) left gonads of a single 2-mo-old. C) A partially pigmented left gonad of another 2-mo-old individual. All animals from this age class that were examined appeared to have oviducts, but it is unlikely that this structure is a reliable means of determining sex at this young age, as several older males exhibited tubular structures that were indistinguishable from oviducts.

were deemed too small for the procedure. The three blind observers' scoring of gonads was in 100% agreement with one another and with the initial observations made at the time of surgery, including the scoring of 2-mo-old turtles as uniformly unsexable based on gonad morphology.

Melanocytes were common on the gonads of 2-mo-old and 8-mo-old Alligator Snapping Turtles, as well as in some older males. Such pigmentation was not uniform, even within individuals, and ovaries and testes were indistinguishable in 2-mo-old animals (Fig. 1). However, gonads could be differentiated at eight mo and beyond by the presence of primordial follicles on the ovaries that increased in number and density with age (Table 2, Fig. 2). By 16 mo, follicles on ovaries had proliferated to the point that the melanistic tissue was mostly obscured. Testes had the appearance of being more vascularized than ovaries at all stages at which sexes were differentiable. Interestingly, we observed ductal structures that were visually indistinguishable from oviducts in some but not all males, and the oldest male that retained these female secondary sex organs was 88 mo old (Fig. 2). Histological examination of these structures confirmed their tubular nature but was unable to confirm whether or not the tissue was indeed homologous to female oviducts. We do not know whether oviducts regressed completely beyond this age or whether a larger sample size would have revealed these female secondary reproductive organs in 96-mo-old and adult males.

We could not differentiate clitorises and penises from one another in individuals up to 88 mo. At 96 mo,

TABLE 2. Ages and sizes of Alligator Snapping Turtles (*Macrochelys temminckii*) for which images of gonads and genitals are presented in Figs. 2–4. The two adult turtles were not captive hatched and therefore are of unknown age. Two-mo-old individuals were omitted because sex could not be ascertained.

Sex	Age (months)	Mass (g)	Midline Carapace (mm)	Plastron Length (mm)	Pre-cloaca Tail Length (mm)
Female	8	31.8	47.3	38.3	10.7
Male	8	28.0	43.0	35.6	9.2
Female	16	149.3	79.0	64.7	19.7
Male	16	210.6	90.2	74.8	24.6
Female	32	277.5	103.2	82.5	25.9
Male	32	350.0	111.2	88.3	29.8
Female	44	639.2	131.6	111.5	33.2
Male	44	496.5	126.3	102.0	34.8
Female	60	680.2	137.3	117.0	31.1
Male	60	899.8	155.9	123.5	44.3
Female	88	1,703.0	142.1	150.1	36.2
Male	88	1,689.0	184.5	153.3	51.8
Female	96	1,937.0	154.3*	162.0	45.8
Male	96	1,843.0	194.6	164.6	58.1
Female	Adult	14,900.0	393.0	319.5	92.0
Male	Adult	36,400.0	533.0	404.0	217.2

*Indicates an animal that exhibited significant kyphosis, resulting in an atypically short straight midline carapace length.

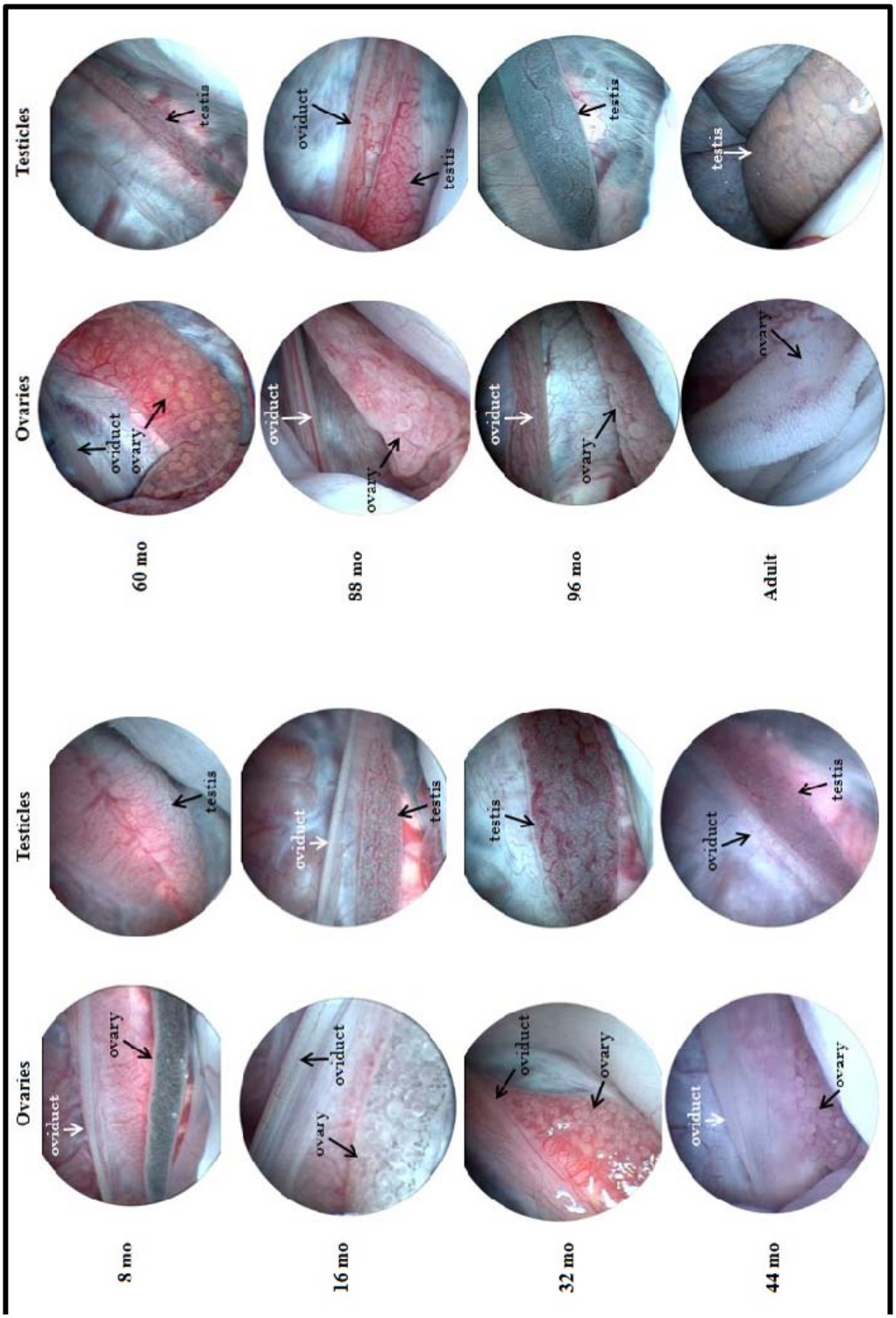

FIGURE 2. Comparison of juvenile Alligator Snapping Turtle (*Macrochelys temminckii*) gonads at eight ages. Images are not similarly scaled.

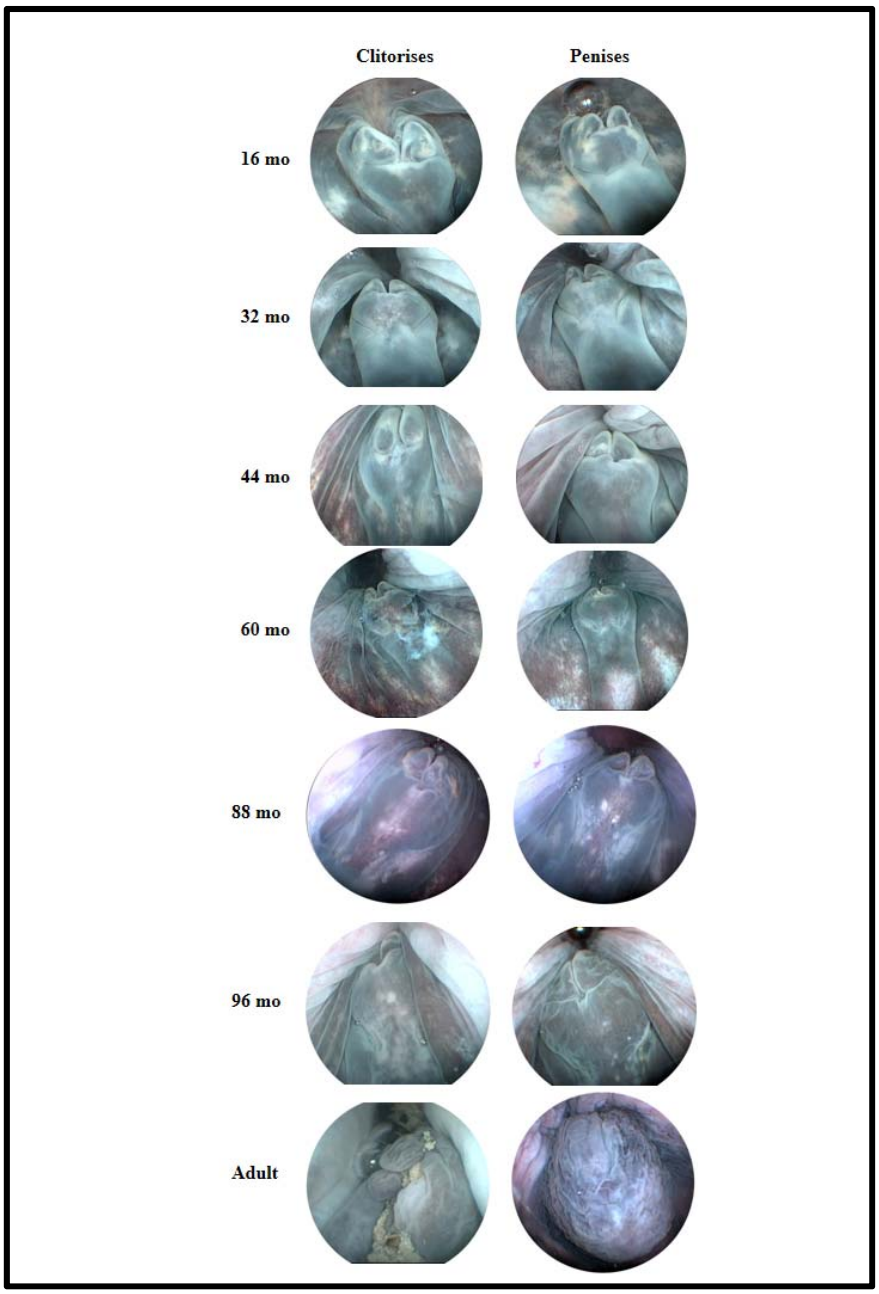

FIGURE 3. Comparison of juvenile Alligator Snapping Turtle (*Macrochelys temminckii*) genitalia at seven ages. Images are not similarly scaled.

however, penises were noticeably larger and darker in color, whereas clitorises remained proportionally similar in size to those observed at earlier stages (Fig. 3).

Additionally, penises in older animals appeared more wrinkled when flaccid than did clitorises, and during examination sometimes became turgid (Fig. 4). Importantly, these differences in genital morphology preceded unambiguous divergence of male from female pre-cloaca tail length (Fig. 5).

DISCUSSION

Unsurprisingly, Alligator Snapping Turtles could be sexed via laparoscopy at a much earlier age than was possible by viewing the genitalia via cloacoscopy. Although greater resolution in the age at which laparoscopy becomes useful could be obtained by examining animals between the two-mo and eight-mo age classes used in this study, we suspect that eight mo

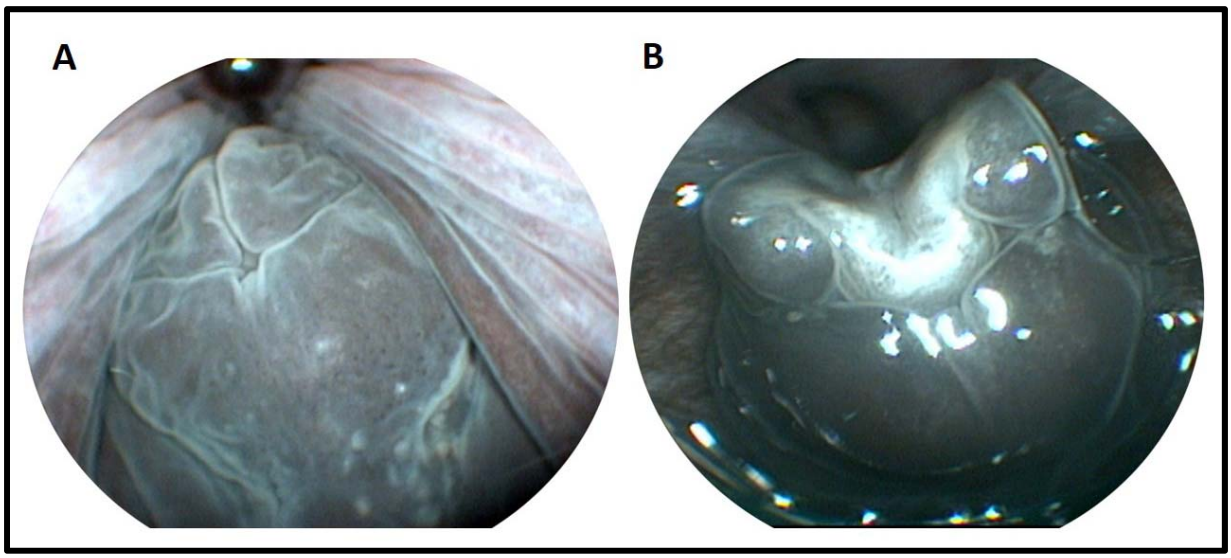

FIGURE 4. After 88 mo, Alligator Snapping Turtle (*Macrochelys temminckii*) penises appeared larger and more wrinkled than clitorises and began exhibiting erectile behavior. Images depict the penis of a 96-mo-old animal in A) flaccid and B) turgid states.

very nearly defines the lower limit at which sex can be reliably determined without employing histological methods because ovarian follicles were so sparsely distributed on the ovaries at this stage. This presents limitations for studies in which true secondary sex ratios are desired or in cases when hatchlings cannot be reared in captivity for months. Additionally, such delayed gonadal differentiation is not necessarily typical of turtles, as studies have reported success surgically sexing very young individuals (*Chelydra serpentina* at 3.3 mo, Janzen 1992; *Chrysemys picta* at hatching, Janzen 1994; *Cuora flavomarginata* at hatching, Hernandez-Divers et al. 2009).

In a study in which Alligator Snapping Turtles were sexed by inserting a lubricated finger into the cloaca to check for the presence of a penis, sex could not be reliably determined for animals < 334–340 mm maximum carapace length and < 242–258 mm midline plastron length (Riedle 2001). Furthermore, pre-cloaca tail length did not accurately indicate sex because, unlike in the present study, male and female pre-cloaca tail length did not clearly diverge (Riedle 2001). In another study, Alligator Snapping Turtles < 400 mm midline carapace length were considered juveniles and sex was undetermined (Jensen and Birkhead 2003). We found that cloacoscopy could not substitute for laparoscopy for sexing Alligator Snapping Turtles until animals were roughly eight years old and/or 195 mm straight midline carapace length (165 mm midline plastron length). However, this age and size class is still markedly younger and smaller than can be sexed based on external morphology or digital probing, and cloacoscopy therefore represents a useful relatively non-invasive technique for a range of juvenile age classes for which only surgical methods of sexing have previously been practical. This approach could have significant impacts in ecological studies. Based on the size distribution of juveniles reported, Riedle (2001) might have been able to determine the sex of as many as 53 additional animals using cloacoscopy. Jensen and Birkhead (2003) did not report morphological data for individual turtles, so it is unknown for how many of the

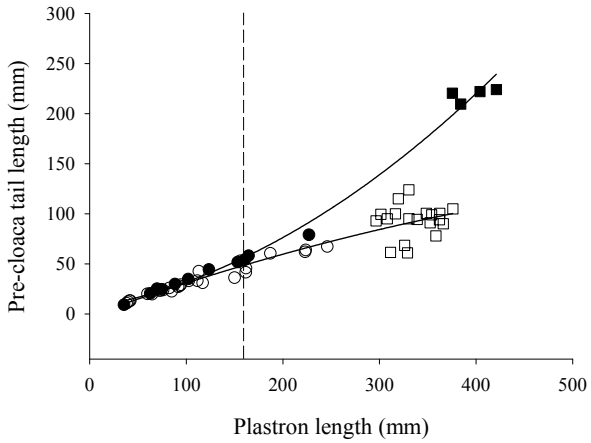

FIGURE 5. Pre-cloacal tail length relative to midline plastron length of Alligator Snapping Turtles (*Macrochelys temminckii*). Males and females did not begin to differentiate until reaching approximately 150 mm plastron length. Among the captive-reared turtles used in this study, this size threshold was achieved at approximately eight y. Dashed line indicates the minimum size at which sex could be determined via cloacoscopy. Symbols: filled = males; open = females; circles = juveniles; squares = adults.

11 juveniles in the study sex might have been determined. Finally, in a diet study, 39 juvenile Alligator Snapping Turtles were trapped that ranged 151–257 mm straight midline carapace length, all smaller than the minimum size at which this species can be reliably sexed based on external morphology. However, 28 of the animals (72%) were within the size range for which cloacoscopy may have been useful (East et al. 2013). In retrospect, knowing the sex of these juveniles could have addressed a potentially important source of variation in diet among individuals. Without these data, no option was available but to lump males and females into a 'sex unknown' category.

The extent of interindividual variation in gonadal pigmentation was interesting, but its significance unclear. Importantly, in early age classes where gonads were least differentiated, pigmentation neither aided nor hindered our ability to differentiate testes from ovaries. Among females, the pigmented tissue appeared to compose a basement tissue over which ovarian follicles formed. This was evident in several young females (e.g., 16-mo age class) that were examined at a stage when the follicles were readily identifiable but still somewhat transparent, revealing the underlying pigmented layer.

Identification of gonads from still images was at times challenging because not all turtles generated consistently high quality images. On occasion, for instance, intestines blocked the visual field of the endoscope. However, video footage was consistently useful because even brief glimpses of the gonadal tissue were easily captured.

Several studies have used the presence of secondary sex organs (namely oviducts) to aid in assigning sex to young turtles that lacked highly differentiated gonads (Janzen 1994; Rostal et al. 1994; Wyneken et al. 2007). Therefore, it is interesting that we identified several individuals with clearly formed testes paired with oviducts that were indistinguishable from those in females of comparable age. It was unclear whether these structures have a tendency to regress with age, but at least one individual retained these structures to at least 88 mo and 184.5 mm midline carapace length (153.3 mm midline plastron length). Oviducts were also retained in 56% of young male Desert Tortoises (*Gopherus agassizii*) examined, but the structures appear less robust and less vascularized than in females (Rostal et al. 1994). No comparable difference between sexes was apparent in our study. Further investigations will be necessary to determine how ubiquitous this phenomenon is, but we caution against relying on the presence or appearance of oviducts alone for assigning sex in Alligator Snapping Turtles.

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