

Correlation between endoscopic sex determination and gonad histology in pond sliders, *Trachemys scripta* (Reptilia: Testudines: Emydidae)

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Abstract. Coelioscopy has been proven to be a valuable technique to determine the sex of juvenile chelonians. However, there is a disagreement regarding the proper way to identify testes and ovaries, which is a direct consequence of the lack of studies correlating the results of endoscopic examination with the histology of the gonad. In this blinded study we assessed two methods of sex determination in juvenile pond sliders (*Trachemys scripta*) using endoscopy: via coelioscopy, with visualization of the gonads, and via cloacoscopy, with visualization of phallus/clitoris; we then compared the results of these procedures with the histology of the gonad. The results of gonad histology correlated 100% with the results from coelioscopic examination, but only 57.77% of the results obtained by cloacoscopy were accurate. Using cloacoscopy, 83.33% of males and 38.46% of females were misdiagnosed. Sex determination of juvenile pond sliders was considered accurate when coelioscopy was used, but inaccurate when cloacoscopy with identification of phallus/clitoris was attempted.

Keywords. Reptiles, turtles, chelonians, anatomy, diagnostic imaging, endoscopy, sex determination.

Most chelonians are long-lived animals where external/secondary sexual characteristics are only evident after several years of age (Mushinsky et al., 1994). For this reason, coelioscopy has been proven to be a valuable technique to determine the sex of juvenile chelonians before they show secondary sexual characteristics (Kuchling, 2006; Hernandez-Divers et al., 2009; Kuchling and Griffiths, 2012). However, there has been a disagreement about the proper identification of testes and ovaries in juvenile chelonians using coelioscopy (Hernandez-Divers

et al., 2009; Kuchling, 2009; Innis 2012). This controversy arose from the lack of studies correlating the results of sex determination using endoscopic images with the histology of the gonad. We therefore designed a study to correlate the results of endoscopic sex determination in juvenile pond sliders (*Trachemys scripta*) via coelioscopy (with visual inspection of the gonad) and cloacoscopy (with visual inspection of the phallus/clitoris) with the result of postmortem histological examination of the gonads.

Fifteen juvenile pond sliders, weighing 93-164 g (mean = 124.5 g) and measuring 7.5-9.9 cm (mean = 8.74 cm) of straight carapace length, were captured near the city of Barcelona (NE Spain) under the pest control project (permit number 002363 from the Departament de Medi Ambient of the Generalitat de Catalunya). Animals were maintained at 22 °C and fastened for 48 hours prior to the procedures. Each animal was anaesthetised with alfaxalone (Alfaxan, Vetoquinol, Spain) at 10 mg/kg IV (subcarapacial vein) and injected with lidocaine SC (0.1 mL of a 1 mg/mL dilution) in the left prefemoral area. Coelioscopies and cloacoscopies were performed in each animal using a 2.7 mm, 30° viewing rigid endoscope with a protective sheath and a videocamera (Karl Storz Endoscopia Ibérica SA, Madrid, Spain). Briefly, for coelioscopic examination a 3-mm skin incision was performed in the centre of the prefemoral fossa and access to the coelomic cavity was performed separating the associated muscles; the endoscope was then inserted into the coelom and 10-20 mL of sterile saline (NaCl 0.9%) was used to insufflate the coelomic cavity; the endoscope was directed dorsocaudally in order to inspect the reproductive system (Divers et al., 2009). For cloacoscopic examination, the endoscope was inserted through the cloaca and directed cranially; 10-15 mL of air was used to insufflate the cloaca to visualize phallus/clitoris located in the ventral aspect of the proctodeum (Spadola and Insacco, 2009; Selleri et al., 2013).

Following the procedures, turtles were not allowed to recover and were euthanased with an overdose of intra-

venous sodium thiopental (2 mL of Tiobarbital Braun, Braun, Spain). Necropsies were performed immediately after death and the gonads were collected, fixed in 10% neutral buffered formalin, processed routinely for histology, stained with haematoxylin and eosin and assessed under light microscopy.

This was a blinded study, where the veterinarian performing coelioscopies (DP) and the veterinarian performing cloacoscopies (AM) did not know the results generated by each other; in addition, the pathologist (TC) did not know the results from coelioscopies and cloacoscopies. Furthermore, videos of the endoscopic procedures (both coelioscopies and cloacoscopies) were recorded and submitted to two independent veterinarians with experience in chelonian anatomy (JO and MG) for additional blinded evaluations. The Fleiss' kappa statistical measure was used for assessing the reliability of agreement between observers (Geertzen, 2012). Histology was considered the gold standard for gonad identification.

Results are shown in Table 1. Gonad histology revealed that 13 turtles were female and two turtles were male. Results from coelioscopies correlated 100% with the results from gonad histology, and the statistical analysis showed a perfect agreement ($\kappa = 1$) between observers. However, only 57.77% of the sexing results made via cloacoscopy were accurate, and the statistical analysis showed a poor agreement ($\kappa = -0.324$) between observers. Using cloacoscopy, males were misdiagnosed as females in 83.33% of cases and females were misdiagnosed as males in 38.46% of cases.

Table 1. Comparison of sex determination of 15 pond sliders (*Trachemys scripta*) using coelioscopy, cloacoscopy and gonad histology.

Animal	Gonad histology	Celioscopy			Cloacoscopy		
		Observer 1	Observer 2	Observer 3	Observer 1	Observer 2	Observer 3
1	♀	♀	♀	♀	♀	♀	♂
2	♀	♀	♀	♀	♂	♀	♀
3	♀	♀	♀	♀	♀	♀	♂
4	♂	♂	♂	♂	♀	♀	♂
5	♀	♀	♀	♀	♂	♀	♂
6	♀	♀	♀	♀	♂	♀	♀
7	♀	♀	♀	♀	♂	♀	♀
8	♀	♀	♀	♀	♀	♀	♂
9	♂	♂	♂	♂	♀	♀	♀
10	♀	♀	♀	♀	♀	♀	♂
11	♀	♀	♀	♀	♂	♀	♀
12	♀	♀	♀	♀	♀	♂	♂
13	♀	♀	♀	♀	♀	♂	♂
14	♀	♀	♀	♀	♀	♀	♀
15	♀	♀	♀	♀	♀	♂	♀

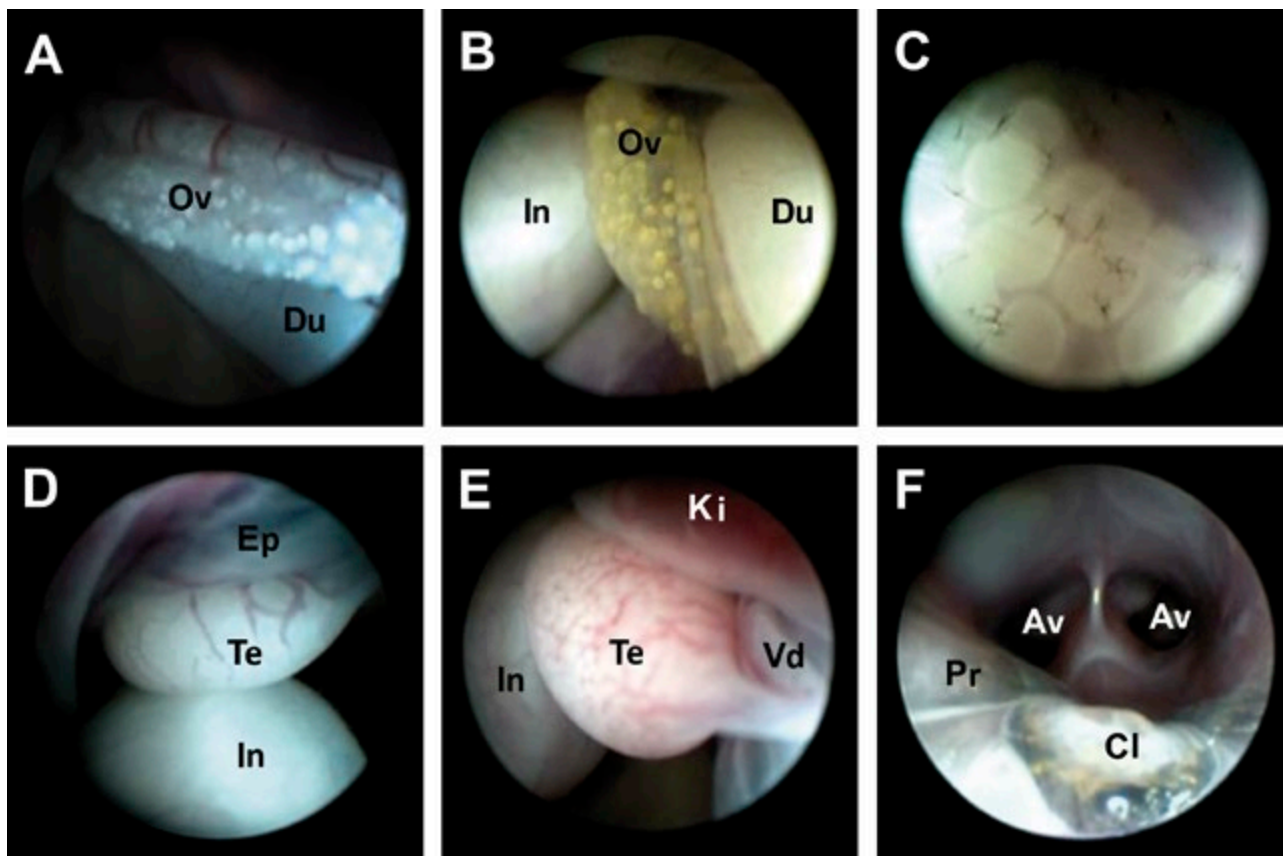


Fig. 1. Endoscopic images of ovary (A, B, C), testicle (D, E) and clitoris (F) in pond sliders (*Trachemys scripta*). Ovary (Ov), oviduct (Du), intestines (In), testicle (Te), epididymis (Ep), vas deferens (Vd), kidney (Ki), proctodeum (Pr), clitoris (Cl), and accessory vesicles (Av).

On coelioscopic examination, ovaries were long, irregular and translucent organs. They were loosely attached to the dorsal coelomic wall by long and loose ovarian ligaments on both poles of the organ (Fig. 1A). Ovarian previtellogenic follicles varied in density, size and appearance, from a few small, white to yellow round follicles embedded in the organ, to multiple prominent large yellow round follicles distributed through most of the parenchyma (Fig. 1B). The different features of the ovarian follicles were not associated with body weight or carapace length. Dark pigment and small vessels were appreciated in some ovaries (Fig. 1C). Adrenal glands were not always easily identified.

The oviduct was a white, straight and tubular structure with small blood vessels on its surface. It ran parallel to and extended further cranially and caudally than the ovary, associated with the ovarian ligaments. It was observed either dorsally or ventrally to the ovary; occasionally, it was dorsal in the cranial part of the ovary and ventral in the caudal part. The diameter of the oviduct was variable, and sometimes it had a similar diameter as

the ovary.

Testes were slightly oval, white to yellow, and had a net of blood vessels on the surface of the organ, irradiating from the area of the mesorchium (Fig. 1D). The testicle was closely attached to the coelomic wall and only tight and short ligaments were visible on both poles of the organ (Fig. 1E). A black and relatively diffuse structure (believed to be the epididymis) was closely associated with the mesenteric surface of the testicle. Melanic spots were not found on the surface of testes. Vas deferens was only seen in one animal (50% of males) as a thin and white straight tubular structure, only visible arising from the caudal aspect of the testicle.

On cloacoscopic examination, phallus and clitoris had an identical appearance; they were heart-shaped structures (with the tip of the heart pointing caudally) situated on the ventral aspect of the proctodeum. Both proctodeum and phallus/clitoris were pigmented (Fig. 1F).

Appropriate description of gonads and associated structures are provided in the present article. It should be noted that interspecific differences in gonad mor-

phology may exist; for example, images provided by Hernandez-Divers et al. (2009) showed melanic testes in Chinese box turtles (*Cuora flavomarginata*); testes with pink and orange colours have also been described in chelonians (Kuchling, 2006); testes from Aldabra tortoises (*Aldabrachelys gigantea*) in a study by Kuchling and Griffiths (2012) were much more elongated than the ones observed in the present study with pond sliders. We observed variation in density, size, colour and shape of ovarian follicles in pond turtles of similar size and body weight. It has been described that follicles become more yellow, larger and more abundant as the animal ages (Kuchling, 2006).

In our study, sex identification of juvenile pond sliders by visualization of phallus/clitoris via cloacoscopy was unreliable and therefore this method is not recommended for sex identification. However, visualization of gonads via coelioscopy was 100% accurate at determining the sex of juvenile pond sliders and, therefore, it is recommended for sexing chelonians whose secondary sexual characteristics are not evident. The small number of males included in our study should be considered a potential source of bias.

A different endoscopic technique through cloacoscopy and access to the urinary bladder or accessory urinary vesicles has been described for sex determination in adult European freshwater turtles (*Emys orbicularis*) (Spadola and Insacco, 2009) and juvenile Mediterranean tortoises (*Testudo hermanni*) (Selleri et al., 2013). This technique offers the advantage of being less invasive than coelioscopy, and further studies will need to assess efficacy and safety in juvenile pond sliders or other chelonian species.

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