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FIELD ANESTHESIA AND GONADAL MORPHOLOGY OF IMMATURE WESTERN SANTA CRUZ TORTOISES (*CHELONOIDIS PORTERI*)

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Abstract: Evaluation of sex ratios is a critical component of chelonian captive breeding programs and may become increasingly useful to assess the demographics of free-living populations. In many reptile species, the sex of immature animals cannot be determined based on external features. Endoscopic sex identification is an accurate and safe method to identify the sex of immature individuals of some chelonian species. A number of studies describe this technique in controlled, hospital settings and report significant interspecies variations in gonad morphology; however, there are few reports describing this technique in field conditions. In the current study, the gonadal morphology of 40 immature Western Santa Cruz tortoises (*Chelonoidis porteri*) on Santa Cruz Island in Galapagos, Ecuador, was assessed. A previously described endoscopic protocol was used to perform sex identification under field conditions. Tortoises were anesthetized using an intramuscular injection of ketamine (10 mg/kg) and medetomidine (0.1 mg/kg), which provided an adequate plane of anesthesia. The medetomidine was reversed with atipamezole (0.5 mg/kg). Field conditions presented challenges such as limited control over lighting, suboptimal patient positioning, and restricted power supply for endoscopy equipment. The immature testicle in Western Santa Cruz tortoises was oval, reddish pink, and tightly adhered to the coelomic membrane ventral to the kidney. The surface of the gonads resembled other species with the notable exception that the ovaries lacked a significant number of primordial follicles. These gonadal characteristics were consistent, with only one individual identified as undetermined sex of the 40 samples. This field-based endoscopic gonadal evaluation was a safe and sensitive technique for determining the sex of free-living immature Western Santa Cruz Galapagos tortoises.

INTRODUCTION

The Western Santa Cruz tortoise (*Chelonoidis porteri*) is endemic to Santa Cruz Island in Galapagos, Ecuador.[19] The free-living population is widespread on the southern flank of Santa Cruz Island and is comprised of about 3,400 individuals, although as many as 8,000–10,000 individuals may exist based on estimated population sizes rather than census data.[5,19] Because of significant population declines, the species is listed as Critically Endangered on the International Union for Conservation of Nature (IUCN) Red List.[4] As part of a long-term captive breeding program, currently operated by the Galapagos National Park, a protected setting for egg incubation and head starting, before tortoise release into the National Park, has been operational on Santa Cruz Island since the 1970s.[3]

Captive breeding programs must ensure appropriate incubation temperatures for species that undergo temperature-dependent sex determination (TSD) to avoid producing imbalanced hatchling sex ratios.[7,10,11,14] Although TSD is common among turtles, it is not ubiquitous, and data to support the presence of TSD in Galapagos tortoises were only recently published for Espaola Giant Tortoises (*Chelonoidis hoodensis*).[25] In that study, the authors demonstrated in *C. hoodensis* that a higher proportion of males hatch at an incubation temperature of 25.5°C and more females hatch at 29.5°C.[25] Although the data were published recently, these temperature values were identified through experiments performed in the 1980s and have been used by the Galapagos captive breeding program since that time.[3,25,26]

In species in which TSD occurs, sex identification of immature animals is necessary to evaluate sex ratios and guide captive breeding programs.[7,12,13,25] In Galapagos tortoises, secondary sex characteristics that clearly distinguish males from females are not present until 15–20 years of age.[26] Recently, a study reported that female *C.*

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hoodensis hatchlings had fewer large scales on the dorsal aspect of their tails compared with males but also reported inconsistent findings depending on the methodology used for counting scales.[25] This technique has not been assessed in *C. porteri* or other Galapagos tortoise species.

Endoscopic sex identification has been described in many chelonian species as an accurate and safe method to identify the sex of immature animals based on gonadal morphology.[6,7,9–13,18,22,24,25,32] Accurate determination of sex in immature tortoises requires recognition of morphologic features that distinguish an ovary from a testis. Interspecies variations in gonad morphology have been identified, suggesting that species-specific morphologic criteria should be referenced.[8–13,32]

To the authors' knowledge, this is the first study to describe the gonadal morphology of immature Western Santa Cruz tortoises and is the first report of endoscopic sex determination in free-living, sexually immature Galapagos tortoises. Tortoises were located in three distinct nesting zones across an elevation and temperature gradient as part of a larger ecologic study of this population.[1] This paper includes descriptive data on the anesthetic and surgical procedures and the gonadal morphology of immature Western Santa Cruz tortoises. It also presents an evaluation of a field-based anesthetic protocol and discusses technical aspects of performing endoscopic sex identification of immature Galapagos tortoises under field conditions.

MATERIALS AND METHODS

The Galapagos archipelago lies approximately 1,000 km west of continental Ecuador in the Pacific Ocean. Free-living Galapagos tortoise populations are present on six islands in the archipelago.[2] Santa Cruz is inhabited by two species of Galapagos tortoises, including the Western Santa Cruz tortoise (*C. porteri*) and the Eastern Santa Cruz tortoise (*Chelonoidis donfaustoi*).[1,19] In this study, we evaluated Western Santa Cruz tortoises from the Tortoise Reserve of Santa Cruz located on the southwestern flank of the island. The site contains three distinct nesting zones along an elevation gradient. The nesting zones are separated by regions of extensive lava rock, and each contains a distinct population of juvenile and hatchling tortoises.

Immature Western Santa Cruz tortoises were located within nesting zones by either radio telemetry ($n = 15$) as part of an ongoing study or opportunistic ($n = 25$) visual searches. For each tortoise, global positioning system (GPS) coordinates were recorded at the location of capture. Individual tortoises were placed into a cloth bag for transport to a base camp where all veterinary procedures were performed.

Each tortoise received a physical examination before anesthesia and coelioscopic evaluation. Body weight was measured using a scale calibrated to the nearest gram. Tortoises were anesthetized for coelioscopy using a previously described coelioscopic technique.[6,7,11–13,18] Ketamine (Ket-A-100, 100 mg/ml; Agrovet Market S.A., Cercado de Lima 15021, Peru; 10 mg/kg IM) and medetomidine (SedaStart 1 mg/ml; Braun Vetcare S.A., 08191 Barcelona, Spain; 0.1 mg/kg IM) were administered as a single intramuscular injection in the pectoral muscles. The time of anesthetic drug administration was recorded as T_0. After injection, individuals were placed in a holding pen to be monitored for effects of the anesthetic agents. The time of first anesthetic effects (T_1) was recorded when individuals relaxed their limb and neck musculature but were responsive when touched. Recumbency time (T_2) was first recorded when individuals were completely relaxed, nonmobile, and did not retract their limb or respond when limbs were gently extended manually. At this time, an assistant held the individual in right lateral recumbency, and the left hind limb was extended caudally. A line block was administered using lidocaine (Lidomic 20 mg/ml; Microsules Laboratorios, Canelones, Uruguay; 1 mg/kg SC) in the left pre-femoral fossa approximately 5 min before creating the surgical incision. The left pre-femoral fossa was then prepared aseptically using chlorhexidine (Lirahexidina 2%; Laboratorios LIRA S.A., 170184 Quito, Ecuador). Tortoises were considered at an adequate plane of anesthesia if they remained relaxed, nonmobile, and did not retract their limbs in response to manipulation or surgical stimulation.

A 3- to 4-mm surgical incision was made in the skin using a #15 blade (Henry Schein Animal Health, Dublin, OH 43017, USA), and the coelomic cavity was entered via blunt dissection of the subcutaneous tissue and coelomic membrane using a curved mosquito hemostat. A 2.7-mm-diameter 30° rigid endoscope within a protective sheath (Karl Storz Endoscopy-America, El Segundo, CA 90245, USA) attached to an Endogo system (Envisionier Medical Technologies, Woodstock, GA 30188, USA) was inserted into the coelomic cavity to visualize the gonads. Lactated Ringer's Solution (LIFE Laboratorios Farmaceuticos, 170510 Quito, Ecuador) was used as needed to insufflate the coelomic cavity. Once

the procedure was completed, the skin incision was closed with a horizontal mattress suture using 3-0 Monocryl-plus (Henry Schein Animal Health). We recorded procedure duration (T_3) as the time from creating the surgical incision to the time of incision closure.

Medetomidine was reversed using atipamezole (5 mg/ml; Zoopharm Windsor, CO 80550, USA; 0.5 mg/kg IM). Individuals were kept in an enclosed space with both sunlight and shaded areas and were monitored during recovery. The time to recovery (T_4) was recorded when individuals became mobile and regained withdrawal reflexes in response to manual extension of hind limbs after administration of atipamezole. After complete recovery from anesthesia and at least 3 hr after reversal, individuals were returned to the capture location using GPS coordinates recorded at the time of capture. Between each surgery, the surgical instruments and endoscopy equipment were sterilized by immersion in Benz-all solution (Xttrium Laboratories, Mt. Prospect, IL 60056, USA) for 15 min and rinsed with sterile saline (0.9% sodium chloride; LIFE Laboratorios Farmaceuticos) before use.

All animal handling and procedures followed guidelines from the Galapagos National Park Service under Research Permit PC-36-17 and Institutional Animal Care and Use Committee (IACUC) protocol 2017-01 of the Saint Louis Zoo.

RESULTS

A total of 40 individuals were included in this study. Body weights ranged from 77 to 1,358 g, with a mean of 529 g. Adequate anesthetic depth was achieved in 38 individuals after administration of ketamine (10 mg/kg IM) and medetomidine (0.1 mg/kg IM). A supplemental dose of ketamine was required for one individual to obtain an appropriate anesthetic depth, resulting in a total ketamine dosage of 18.7 mg/kg. One individual received a ketamine induction dosage of 15 mg/kg. Initial anesthetic effects were observed 2–12 min after administration of the induction agents (Table 1). The average time to recumbency was 11.8 min, with a range of 4–25 min (Table 1). Procedure duration (T_3) ranged from 5 to 27 min, with a mean of 12.6 min (Table 1). Exact recovery times were recorded for 15 individuals ($n = 15$) and ranged from 1 to 10 min after administration of atipamezole (Table 1). Precise times were not recorded for 25 individuals ($n = 25$), including two tortoises ($n = 2$) with prolonged recoveries (approximately 60–90 min) characterized by an extended period of depressed mentation and lethargy after initial arousal. These individuals received subcutaneous fluid therapy (lactated Ringer's solution, 35 ml/kg) and were intubated with a 2-mm endotracheal tube (Jorgesen Labs, Loveland, CO, USA) for manual ventilation using an Ambu bag (Medline Industries Inc, Northfield, IL 60093, USA) until fully recovered within approximately 10 min of manual ventilation. All tortoises remained in a recovery corral for at least 3 hr before release, and the two individuals with prolonged recoveries were held in the corral overnight for additional observation.

Table 1. Time elapsed between anesthetic effects after administration of ketamine-medetomidine ($n = 38$), recovery after reversal with atipamezole ($n = 15$), and endoscopic sex identification procedure durations ($n = 39$) in juvenile Western Santa Cruz tortoises (*C. porteri*).

	Minimum (min)	Maximum (min)	Mean (min)
Time to initial anesthetic effects (T_1)	2	12	5.4 ± 2.25
Time to recumbency (T_2)	4	25	11.8 ± 4.17
Procedure duration (T_3)	5	27	12.6 ± 5.11
Time to recovery (T_4)	1	10	4.93

Mean values are reported with ± standard deviation.

The left gonad was visualized in all individuals ($n = 40$). Insufflation with lactated Ringer's solution was necessary to improve organ visualization in four individuals. Key features used to distinguish a gonad as a testis included its location in the cranioventral aspect of the kidney, an ovoid to cylindrical shape, and uniform pattern of surface vascularization. The testicle was located on the ventral surface of the cranial portion of the kidney. It was oval, reddish pink, and tightly adhered to the coelomic membrane ventral to the kidney. The surface of the testicle had a well-defined vascularization pattern that was uniform across its surface (Fig. 1). Key features used to identify an ovary included an elongate shape and less prominent surface vascularization. The ovary was identified near the ventral surface of the kidney, loosely attached by a suspensory ligament. It was elongate, cream colored, and extended pass the caudal portion of the kidney. The ovary lacked prominent surface vascularization, and primordial follicles were not consistently evident (Fig. 2). The oviduct was located ventral to the ovary, and it extended from a point cranial to the ovary past

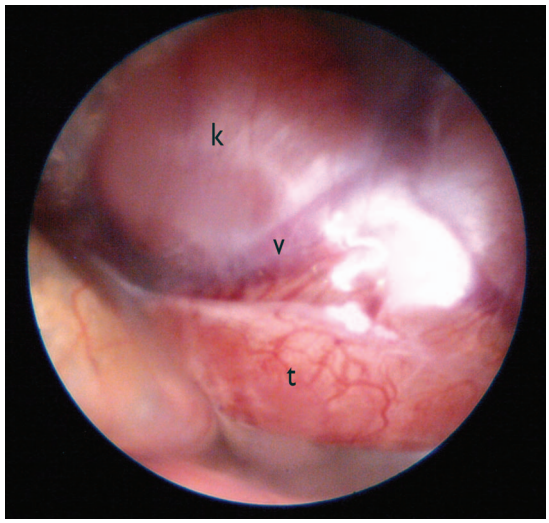

Figure 1. Male gonad of immature Western Santa Cruz tortoise (*C. porteri*). The testis (t) is tightly adhered to the coelomic membrane ventral to the kidney (k) and an adjacent vein (v). The testis has a prominent vascular supply.

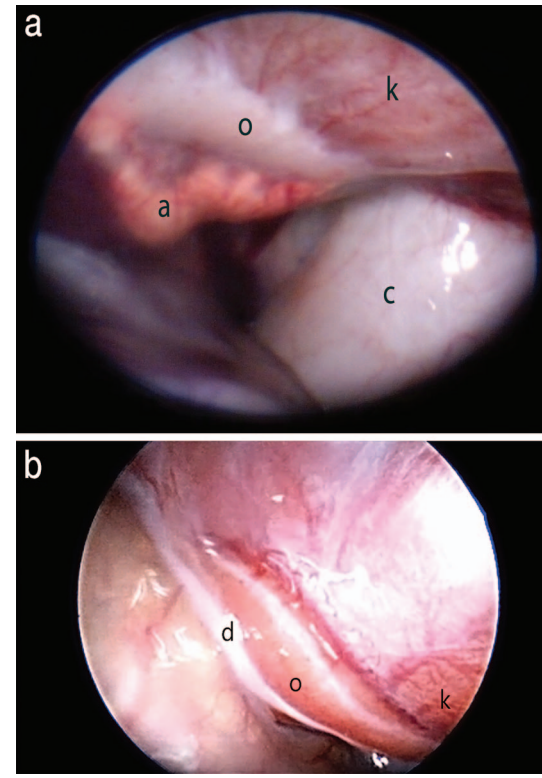

Figure 2. (a) Female gonad of immature Western Santa Cruz tortoise (*C. porteri*). The ovary (o) is located ventrocaudal to the adrenal gland (a), ventral to the kidney (k), and extends past the caudal portion of the kidney. Note the lack of visible primordial follicles in the ovary. A segment of the colon (c) is visible near the kidney and ovary. (b) Female gonad of immature Western Santa Cruz tortoise (*C. porteri*) showing oviduct (d), ovary (o), and kidney (k).

its caudal edge. All but one individual ($n = 1$) could be clearly classified as a male or female based on the above criteria for sex determination. One individual had a moderately sized white, irregular nodule with lack of surface vascularization, located ventral to the kidney. The left lung, kidney, and adrenal gland were examined in all individuals and appeared grossly normal in all but one individual with renomegaly.

DISCUSSION

Several techniques have been described to determine the sex of immature chelonians. These include cloacoscopy, cystoscopy, anti-Müllerian hormone (AMH) detection, and coelioscopy.[16,31] Cloacoscopy and cystoscopy have been successfully used to visualize gonads for sex identification in numerous species, and these techniques may be less invasive as they do not require an incision.[16] However, cloacoscopy is not a reliable method to differentiate the phallus and clitoris to determine sex because both structures appear almost identical in sexually immature chelonians.[16] Discomfort may be associated with cystoscopy and bladder insufflation, but a lighter plane of anesthesia or the use of sedation with local anesthetics could be used for this procedure, which may facilitate rapid recoveries in the field. Anesthesia times are reported to be up to approximately 20 min, usually shorter, when using cystoscopy in a hospital setting.[16] This is similar to coelioscopic procedures under hospital conditions in the experience of the authors, but in the present study, average anesthesia time was approximately 30 min in field conditions. Reported complications of sex identification via cystoscopy include difficulty identifying gonads because of distended intestinal loops, impaired visualization through the bladder wall because of inflammation secondary to infectious diseases, and the presence of yolk sacs in individuals less than 6 mo old.[16] Emesis was also reported in 16% of *Testudo hermanni* hatchlings undergoing the procedure.[29] Although cystoscopy has been successfully used in many species of Testudines, the procedure was only 10% accurate for sex identification in *Tra-*

chemys scripta, and an alarming proportion of individuals (7 of 30) had cloacal or bladder rupture after the procedure.[21] Cystoscopy may be preferable in some situations, particularly for individuals with substantial prior experience with this method. Cystoscopy may present a less-invasive approach to observe gonad morphology for sex identification, but further studies are needed to investigate the accuracy and safety of cystoscopy in additional chelonian species.

A recent publication describes a new technique for sex identification using an immunoassay designed to detect AMH, a protein important for sex differentiation, in small-volume blood samples.[31] The technique was verified by sexing individuals endoscopically or via histology, and AMH was detected in 100% of blood samples from male hatchlings and was not detected in any female blood samples from *T. scripta* and *Caretta caretta* hatchlings (1–2 days old).[31] Accuracy decreased to about 90% when differentiating the sex of 83- to 177-day-old juvenile *C. caretta*, suggesting age of hatchling/juvenile may influence when this test is most useful. AMH is thought to be highly conserved for sex differentiation across taxa, which suggests the test could be used for other species, but further studies are needed to validate this test in individuals of other species and age groups.[15,31]

Endoscopic sex identification (coelioscopy) has been described in many chelonian species that undergo TSD, with well-established safety and efficacy in most species. In addition to describing the gonad morphology in the Western Santa Cruz tortoise (*C. porteri*), this study demonstrates the feasibility of endoscopic sexing in a remote site with no access to power or constructed shelter. Nearly all studies describing endoscopic sex identification in chelonians have been performed in controlled, hospital settings. One study was performed in a primitive hospital setting in Madagascar, and these authors reported challenges related to drug availability and environmental conditions because of limited control over parameters such as ambient temperature.[22]

The medetomidine-ketamine anesthetic protocol used in the current study was a practical choice that has been used for sterilization of adult Galapagos tortoises and for endoscopic sex identification in other chelonian species.[6,7,11–13,19,23] Lidocaine was added as a local anesthetic because it has been shown to safely improve anesthetic efficacy in Chinese box turtles (*Cuora flavomarginata*).[7] This drug combination was also used for in-country anesthesia of ploughshare tortoises, and that study reported failure to anesthetize some of the smaller individuals in the study.[22] These failures were hypothesized to be associated with decreased body temperature and drug metabolism, because these individuals were unable to bask in sunlight on the day of the procedure.[22]

Opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly administered to provide postoperative analgesia after endoscopic sex identification.[6] In a field setting, the potential side effects of opioids may be more difficult to manage, and in some cases, opioid use may be limited by in-country drug restrictions.[22] An opioid was not included in this study protocol because of concern for prolonged recoveries and hypoventilation. Our intended protocol included meloxicam as a postoperative analgesic, but an abnormal appearance and viscosity was noticed when the meloxicam was opened in the field. Because of the concern that the drug might be unsafe, meloxicam was removed from our protocol. Obvious discomfort was not noted in any individuals during recovery, but the authors do advocate the use of an NSAID after endoscopic sex identification.

The tortoises in the present study had access to sunlight and shade in both pre- and postoperative periods, which allowed basking behaviors. Obvious issues related to temperature and drug metabolism were not apparent in our study, although environmental data were not recorded at the time of each procedure. The anesthetic protocol was consistently effective, but one of the smaller individuals, weighing 136 g, required an additional dose of ketamine to reach an appropriate anesthetic plane.

Tortoises were monitored postoperatively in an enclosure built with natural materials found at the field site, but the exact time of first spontaneous movement was not recorded for many tortoises because of a limited number of field personnel. The reported mean recovery times are notably shorter than recovery times in a study describing field anesthesia of gopher tortoises (*Gopherus polyphemus*), although in that study the anesthetic protocol included an opioid, which may have extended recovery time.[17] It should be noted that the mean recovery time was based on a small sample size, because accurate recovery times were only recorded for 15 individuals. Prolonged recoveries were noted in two tortoises, both of which received standard 10-mg/kg ketamine doses at induction and neither received supplemental doses of anesthetic agents. An obvious cause for prolonged recovery was not identified for either

individual, although one was the smallest tortoise in the study with a body weight of 77 g. The other weighed 177 g and had renomegaly noted during endoscopy. Underlying disease, dehydration, and body temperature are some of the factors that may have impacts on drug metabolism in these individuals, but these factors cannot be definitely linked to the prolonged recoveries. Cardiovascular and respiratory changes, particularly hypoventilation, have been reported as anesthetic complications associated with medetomidine-ketamine in gopher tortoises.[5] Hypoventilation may have been a factor in the two prolonged recoveries in this study, because both individuals recovered fully after a brief period of manual ventilation and fluid therapy. Hypoventilation may lead to respiratory acidosis and hypercapnia, which may prolong effects of some anesthetic drugs because of a decrease in plasma protein binding.[27,28] Intraoperative heart rates and respiratory rates were not measured consistently in this study, and it is possible that these prolonged recoveries may have been avoided by early identification of cardiovascular depression or hypoventilation and implementation of supportive treatment. Therefore, the authors recommend strict monitoring of at least heart and respiratory rates from induction to recovery. Heart rate could be monitored with a battery-powered doppler or endoscopically, and visual respiratory rates should be monitored routinely and recorded. Respiratory rates were monitored in this study but were not recorded consistently. Appropriate emergency drugs, such as atropine and epinephrine, should be readily available in the field, and emergency doses should be calculated before administration of anesthetics based on an estimated or exact body weight if available. Additionally, multiple sizes of endotracheal tubes should be available to permit rapid intubation and manual ventilation if hypoventilation is noted. If an individual is apneic for extended periods during recovery, administration of doxapram may be beneficial to stimulate spontaneous ventilation. In anesthetized crocodilians, doxapram has been shown to increase ventilation rates with no effect on tidal volume.[30] In that study, investigators also identified adverse drug effects including catecholamine release, arousal, increased blood pressure, tachycardia, and increased cardiac work load.[30] Overall, the partially reversible anesthetic protocol used in the present study was effective and safe for field use in juvenile Western Santa Cruz tortoises.

Field conditions presented challenges that resulted in longer procedure durations than may be expected in a controlled, hospital setting. Significant obstacles included irregular ground surfaces, lack of an examination table, and little control over environmental lighting. Inconsistent lighting and difficulty positioning patients made viewing the gonads and obtaining high-quality endoscopic images difficult. Recording video for extended periods was not feasible because of the limited battery life of the endoscopy equipment, although multiple fully charged batteries were taken to the field site. The authors recommend bringing a solar-charging unit when performing endoscopy in the field.

The morphology of the gonads varied from previous descriptions in other chelonian species. In juvenile male desert tortoises (*Gopherus agassizii*), the testicle was elongated, lobular, ribbon shaped, well vascularized, and bright yellow to yellow-orange in color with a clear membranous capsule.[24] In contrast, the immature testicle in Western Santa Cruz tortoises was oval, reddish pink, and tightly adhered to the coelomic membrane ventral to the kidney. The surface of the testicle had a well-defined vascularization pattern. The appearance of the ovary and oviduct was similar to what has been described in other species with the notable exception that the ovaries lacked a significant number of primordial follicles. This finding is in contrast to other chelonian species where primordial follicles are prominent in juvenile animals.[7,10–13,19,20,24] Gonadal morphology has been described in one other Galapagos tortoise species, the Española tortoise (*C. hoodensis*), based on both postmortem histopathologic examination and laparoscopic evaluation.[26] Histologic evaluation of ovaries from Española tortoises up to 2 years of age contained germ cells, but no developing oocytes, which may be a reason why primordial follicles were not readily visible on the ovaries during endoscopic examination.[26] The endoscopic appearance of the testis and ovary are similar between these two Galapagos tortoise species, although the endoscopic visualization of primordial follicles was not discussed in Española tortoises. One individual had a moderately sized white, irregular nodule with lack of surface vascularization, located ventral to the kidney. The lesion was not biopsied making it difficult to determine its nature; however, abnormal gonadal structures seen during endoscopic sex determination has been reported in other chelonian species.[7] Species-specific variations in gonadal endoscopic appearance should be considered when performing

endoscopic sex determination in other tortoise species.

CONCLUSIONS

In this study, endoscopy was used to identify the sex of 40 free-living immature Western Santa Cruz tortoises. The anesthetic protocol used in this study was effective, safe, and practical for a remote field setting. Field conditions (i.e., inability to control light, suboptimal patient positioning, and battery operation of equipment) made endoscopic sex identification more difficult than in a hospital setting. Therefore, an external power source should be available if performing multiple endoscopic procedures in the field over several days. The immature testicle in Western Santa Cruz tortoises was oval, reddish pink, and tightly adhered to the coelomic membrane ventral to the kidney with a well-defined surface vascularization pattern. The appearance of the ovary and oviduct was similar to what has been described in other species, except that the ovaries lacked a significant number of primordial follicles. This study highlights the importance of documenting endoscopic gonad morphology of juvenile chelonians across different species because notable differences occur.

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