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Endoscopic Imaging of Gonads, Sex Ratio and Temperature Dependent Sex Determination in Captive Bred Juvenile Burmese Star Tortoises *Geochelone platynota*

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Abstract The possibility of temperature dependent sex determination makes it important to evaluate sex ratios in captive breeding programs of threatened tortoises. We assessed the sex ratio of juvenile Burmese Star Tortoises *Geochelone platynota* by direct observation of their gonads through an endoscope in the captive breeding program of the Behler Chelonian Center (BCC) in California. The gonads of small juvenile *G. platynota* are thin and elongate, and fixed to the dorsal part of body cavity, with ovaries appearing as transparent sheaths with some oocytes visible and testes appearing as small, transparent, thin, sausage-like structures with a net of fine blood vessels on the surface. With growth, ovaries expand and masses of pre-vitellogenic follicles appear on the surface. Testes are transparent in small juveniles and, with growth, turn pinkish-white and then yellowish, with tubuli structures visible through a thin, transparent theca containing a network of fine blood vessels. Egg incubation temperatures were not rigorously monitored, but a temperature of 28.9 °C produced a heavily male biased sex ratio whereas a temperature of 30 °C produced a balanced sex ratio. This suggests that *G. platynota* has temperature dependent sex determination.

Keywords *Geochelone platynota*, Testudinidae, incubation temperature, testis, ovary

1. Introduction

During the last decade the critically endangered Burmese Star Tortoise *Geochelone platynota*, endemic to Burma (Myanmar), was nearly extirpated in the wild due to the high prices that attract in international trade. Essentially no viable populations appear to remain in the wild and *G. platynota* is ranked as the eleventh most endangered tortoise or freshwater turtle in the world (Turtle Conservation Coalition, 2011). While protection of wild populations appears to be inadequate, several commercial and government run captive breeding operations were established for the species inside Burma. Private collectors and zoos in developed countries also started to breed the species. Once established in captivity the species appears to breed readily. However, little is known on the general biology and, in particular, the sex determination mechanism of the species. All tortoises (family Testudinidae) investigated so far show temperature dependant sex determination with males produced at low egg incubation temperatures and females at higher temperatures.

Given that the majority of the surviving *G. platynota* individuals may now reside in captivity and that captive breeding appears instrumental for the survival of the species, it is imperative to evaluate offspring sex ratios under captive conditions to ensure that both sexes are produced. Since it takes many years before external sexual characteristics can be used to assess the gender of *G. platynota* individuals, sex ratios in breeding programs

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have not yet been evaluated or published. Several methods have been trialed to sex juvenile turtles that do not show external sexual dimorphism including plasma testosterone concentration, karyotyping, H-Y antigen cytotoxicity assays, and Bkm DNA fingerprinting (Wibbels *et al*., 2000). However, endoscopy is currently the only 100% accurate non-lethal method available to sex juvenile turtles (Rostal *et al*., 1994; Kuchling, 1999, 2006; Wibbels *et al*., 2000). In particular, all other methods have to be calibrated by using known-sex individuals, which means that without knowing the sex of individual juveniles (e. g., by endoscopy) sex ratios cannot be properly evaluated by employing the other methods mentioned above.

A breeding group of *G. platynota* is maintained in the Behler Chelonian Centre (BCC), the Turtle Conservancy's captive breeding and management facility located in southern California, USA, which is certified by the Association of Zoos and Aquariums (AZA) and houses some of the world's most critically endangered turtles and tortoises. In the present paper we evaluate endoscopy to establish sex ratios in juvenile *G. platynota* during their first years of life and provide estimates of the pivotal temperature of sex determination at which an equal number of males and females are produced.

2. Material and Methods

2.1 Maintenance of breeding stock A breeding group consisting of wild caught tortoises was maintained at the BCC from which the captive bred specimens originated. These tortoises were maintained in a herd and were free to choose their mate. The males were separated from females for periods of time to keep them interested in mating. The females laid eggs year-round. Only two adult females were producing eggs up through 2008. From 2009 on there were a total of six females laying viable eggs. The average clutch size is between 4–7 eggs, but we had a large female lay 16 eggs in 2006. Typically larger clutch sizes are correlated with larger animals. The tortoises were fed a diet of natural graze cuttings, opuntia cactus pads, dandelion, radicchio, endive, parsley, squash, zucchini, apples, and carrots. Within the outdoor enclosures (to which they have access year round) was edible forage consisting mainly of bermuda grass, flowering mallows, mulberry trees, opuntia cacti, and autumn joy. They were offered cuttlefish bone for calcium supplementation.

2.2 Egg incubation Eggs were incubated in chunky vermiculite in a vermiculite water ratio of 2:1 (by weight). The eggs were kept at room temperature with day night fluctuation from about 21 to 28°C for periods of 6 to 8 weeks to provide a diapause before incubation in modified wine coolers with heating devices and thermostats at a set temperature of 28.9°C prior and up to 2007 and 30°C since 2008. The set temperatures were controlled daily with digital thermometers and the thermostats adjusted if necessary. Hatchlings emerged after 115 to 124 days. Date of hatching was available for all juveniles, which were marked with small numbers (tags for honey bees) glued to their carapace scutes.

2.3 Raising of hatchlings and juveniles Hatchlings are maintained on 0.9 m × 1.8 m and 0.9 m × 2.4 m tables with 100 mm of substrate (rice hulls, sand, peat moss). These tables are planted with succulents, grasses, and other natural graze. There is also a water dish present at all times. The southern California climate allows the tables to be outdoors during 6–7 months of the year (weather permitting). During the rest of the year the tables were in green houses with day/night temperature fluctuations between 24 °C and 30 °C. Once the hatchlings reached an appropriate size (100–130 mm), they were moved from the tables to larger enclosures. These animals' food was similar to that of the adults.

2.4 Endoscopy Sex was determined endoscopically in 38 juvenile *G. platynota* (71.2 ± 65.3 g body mass, range 26–289 g) from 8 to 11 March 2010. The tortoises did not receive food for 24 to 48 hours prior to endoscopy. Body mass and carapace length of all tortoises were recorded. Tortoises were anesthetized by intravenous injection (carpal sinus) of ketamine hydrochloride (20–30 mg/kg body mass). Optimum anaesthetic depth was achieved after about 15 minutes. Both hind legs were pulled backwards and tied together. The left inguinal pocket and neighbouring skin, shell, and leg were scrubbed with antiseptic soap and povidone-iodine. Surgical equipment was sterilized by immersion in 2.4% alkaline glutaraldehyde solution for 15 minutes and was rinsed with sterile water before use. A 3-mm-long craniocaudal skin incision was made with a No. 24 surgical scalpel blade in the lower anterior part of the inguinal pocket. A blunt forceps inserted through the incision was directed cranially and gently advanced with slow rotational movements until the coelomic aponeurosis was penetrated. A rigid Hopkins forward-oblique telescope 30°, diameter 2.7 mm, length 18 cm (7218 BA, Karl Storz, Germany) was inserted into the abdominal cavity through stab incision in the lower anterior part of the inguinal pocket. The abdominal cavity was not insufflated. A Storz cold-light fountain 482B was used as light source. The telescope was directed toward the dorsocaudal region

Table 1 Year of hatching, sex, and body mass of *G. platynota* sexed by endoscopy.

	Hatched prior to 2008[a]		Hatched in 2008[b]		Hatched in 2009[b]		
	Males	Females	Males	Females	Males	Females	Intersex
Number	9	1	3	0	11	13	1
Mean body mass	160.3 g	148 g	77.7 g	-	35.9 g	35.3 g	26 g
SD	± 76.6	-	± 2.4	-	± 6.4	± 5.2	-
Range	64−289 g	-	76−81 g	-	28−50 g	26−44 g	-

[a]: Incubation temperature 28.9 °C; [b]: Incubation temperature 30 °C

of the coelomic cavity to inspect the reproductive system. Gonads and accessory ducts were visualized, usually behind intestinal loops, and their appearance, color and texture were noted. A digital camera with macro function (Nikon Coolpix 995) was used for photo documentation. The eyepiece of the endoscope was custom-adapted to fit into the protective ring of the camera lens and photos were taken by holding the camera against the eyepiece. After completion of endoscopy the skin wound was sutured using two stitches of 4/0 vicryl. The surgical procedures took between 2 and 10 minutes, depending on whether photos were taken or not. The tortoises recovered from anaesthesia 1–2 hours after the surgical procedure and were kept under observation for 24 hours before being returned to their nursery enclosures.

3. Results

3.1 Appearance of gonads and accessory ducts The gonads of small juvenile *G. platynota* were thin, elongate, and fixed to the dorsal part of the body cavity, very close to the kidneys, adrenal glands, and lungs. The gonads and other organs (oviduct, kidney, adrenal, lung) could generally be viewed directly or sometimes through translucent peritoneal membranes such as the mesentery. Despite being attached to the dorsal coelomic wall by various membranes, gonads and reproductive tracts moved and could change their position relative to the kidneys, adrenals, and lungs (which have more or less fixed positions), for example, when turtles were tilted from one side to the other during endoscopy.

Testes of small tortoises appear as small, transparent, thin, and half-roundish structures (Figure 1 A, B), bound to the kidneys by the mesorchium and with a net of fine blood vessels on the surface (Figure 1 C). With growth testes become thicker but remain half-roundish sausage-like structures ventral of the kidneys, turning first pinkish-white and then yellowish with, in close up, tubuli structures visible through a thin, transparent theca containing a network of fine blood vessels (Figure 1 D), but containing no melanocytes. Epididymes and vas deferens in small juveniles are thin and translucent, difficult to locate and not discernible in the photographs. Ovaries are attached by a transparent peritoneum to the dorsal wall of the coelomic cavity or to a membrane that separates them from the lungs. Ovaries of small tortoises appear as transparent flat sheaths ventral of the kidneys, with some oocytes and primary follicles visible. With growth ovaries expand along the dorsal wall of the coelomic cavity ventrally to the lungs, increase in thickness, and masses of pre-vitellogenis follicles appear on the surface (Figure 1 E, F). The oviducts extend further cranially than the ovaries, and are ventral or lateral to the ovaries. They often cross ventrally over the posterior part of the ovary on the way to the cloaca. Oviducts of small females are relatively thin, transparent-whitish, straight bands (Figure 1 E, F). With growth oviducts become wider and thicker and more whitish, but still remain straight in the size classes examined during this study.

3.2 Sex ratio Of the 38 *G. platynota*, 14 were females (36.9%), 23 were males (60.5%), and one was classified as an intersex (2.6%), giving an overall male to female sex ratio of 1:0.6. Broken down according to incubation temperatures, eggs incubated at 28.9 °C (n = 10) produced 1 females (10%) and 9 males (90%; m:f ratio = 1:0.1) which is significantly different from equality ($\chi^2 = 8$, $P < 0.01$), whereas eggs incubated at 30 °C (n = 28) produced 13 females (46.5%), 14 males (50%; m:f ratio = 1:0.9) and one intersex (3.5%) which does not differ from a 1:1 sex ratio.

4. Discussion

All juvenile *G. platynota* recovered without problems from the endoscopic procedure and continued to grow normally. The breeding program produced males as well as females, with a strongly male biased sex ratio from eggs incubated prior to 2008 and an equal sex ratio

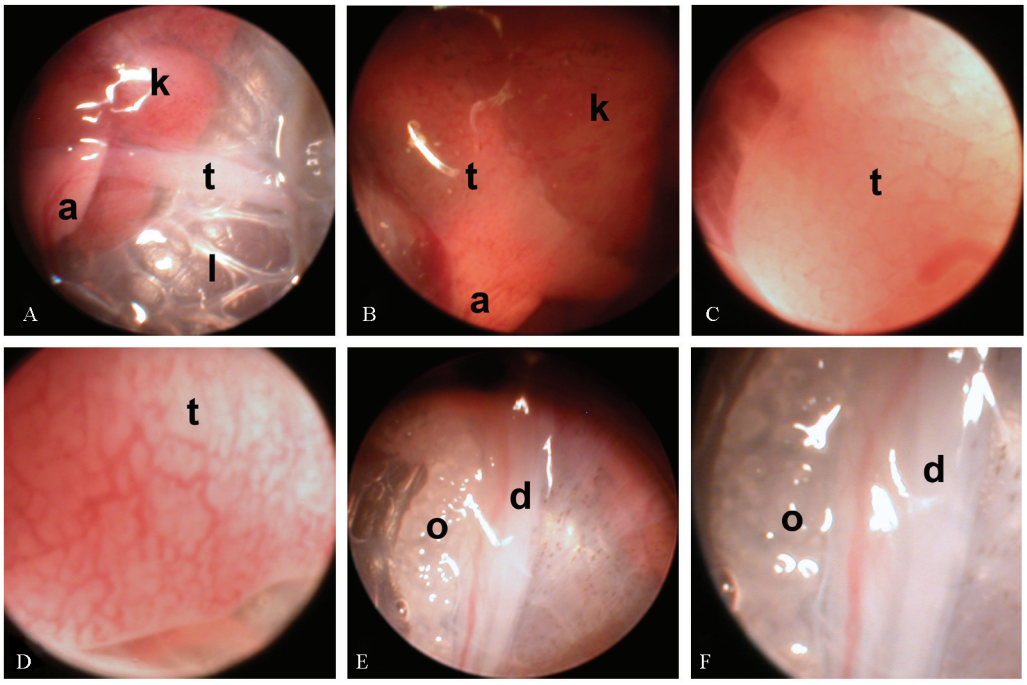

Figure 1 Endoscopic images of gonads and accessory ducts in hatchling and juvenile *G. platynota*: a: Adrenal; d: Oviduct; k: Kidney; l: Lung; o: Ovary; t: Testis. A: Male 2-year-old, 76 g body mass; B: Male 2-year-old, 81 g body mass; C: Close up of testis, 1-year-old, 37 g body mass; D: Close up of testis, 178 g body mass, age unknown (> 3-year-old); E: Female 1-year-old, 39 g body mass; F: Close up of ovary with primary follicles and of oviduct, the same animal as in Figure 1 F.

from eggs incubated since 2008. The morphology of the juvenile testes, ovaries and oviducts is comparable to that of the tortoises *Astrochelys radiata* (Kuchling *et al*., 2011a) and Aldabra tortoises (Kuchling and Griffiths, 2011), but in hatchling and juvenile *Gopherus agassizii* the morphology of the testis is quite different from those species and *G. platynota*, with testes in *G. agassizii* being bright yellow, long, flat bands (Kuchling *et al*., 2011b). Epididymis and vas deferens in small juvenile tortoises are thin and translucent and in all those species difficult to image during endoscopy.

There is some variability in the development and morphology of testes in hatchling and juvenile Testudinidae, but they share general characteristics with the testes of juvenile turtles of the families Podocnemididae (Kuchling, 2006), Cheloniidae and Trionychidae (Kuchling and Kitimasak, 2009): juvenile testes are flat or half-roundish in cross section, sometimes smaller than adjacent adrenal glands (Figure 1 A), the theca testis is always thin and translucent (Figure 1 A, B), never contains melanocytes, and tubuli structures of different size and color (transparent, white, pinkish, or yellow) and/or a fine net of surface vasculature is visible in testis close ups (Figure 1 C, D). However, a different morphological concept of a juvenile chelonian "testis" has been presented by Hernandez-Divers *et al*. (2009) for "hatchling" *Cuora flavomarginata* (age and size not indicated): a round and pendulous structure has been labeled testis (Hernandez-Divers *et al*., 2009) which in close up shows a robust, whitish external membrane with thick blood vessels and spots of melanocytes, with no tubuli structures visible through it (Hernandez-Divers *et al*., 2009). Although it has been suggested that Hernandez-Divers *et al*. (2009) may have misidentified an unrelated structure as testis (Kuchling, 2009), Sam RIVERA of Zoo Atlanta, Charles INNIS of the New England Aquarium, and Eric BAITCHMAN of Zoo New England confirmed independently that the identification of this structure as testis is accurate (Divers and Stahl, 2009).

The male biased sex ratio of captive-bred *G. platynota* indicates that the species has temperature dependent sex determination (TSD). The sex ratios of juveniles from eggs incubated at the lower temperature (2002−2007) and at the higher temperature (2008 onwards) suggest, at least inside the temperature range tested, a male-female TSD pattern in which cooler incubation temperatures produce males and warmer incubation temperatures produce females. The constant incubation temperature that will produce a 1:1 sex ratio is referred to as the pivotal temperature (Mrosovsky and Pieau, 1991).

Unfortunately the incubation conditions were not monitored rigorously enough to allow reliable estimates of the pivotal temperature. Therefore, only crude estimate can be made: since eggs incubated at 28.9 °C up to 2007 produced a male to female ratio of 1:0.1 and eggs incubated at 30 °C since 2008 produced a male to female ratio of 1:0.9, the pivotal temperature of *G. platynota* is most likely close to or just above 30 °C. Interestingly, eggs of the Radiated Tortoise *Astrochelys radiata* from Madagascar incubated at the BCC at the same time at the same temperatures in the same incubators as those of *G. platynota* produced a male to female ratio of 1:11.5 at 28.9 °C and 100% females at 30 °C (Kuchling *et al*., 2011a), indicating that this species has a much lower pivotal temperature than *G. platynota*.

With more varied incubation temperatures and more rigorous incubation temperature control and monitoring, it should be possible in the future to assess more accurately the pivotal temperature of *G. platynota* and the transitional range of temperatures (TRT), which is the range of temperatures in which sex ratios shift from 100% male (below the TRT) to 100% female (above the TRT). This information is important to enable informed decisions how to produce desired offspring sex ratios in future breeding operations.

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