Turtles on the Brink in Madagascar: Proceedings of Two Workshops on the Status, Conservation, and Biology of Malagasy Tortoises and Freshwater Turtles
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Endoscopic Imaging of Gonads, Sex Ratio, and Temperature-Dependent Sex Determination in Juvenile Captive-Bred Radiated Tortoises, *Astrochelys radiata*

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Abstract. – Evaluation of offspring sex ratio is important in any large scale threatened species breeding program if temperature-dependent sex determination (TSD) is a possibility. We assessed the sex ratio of juvenile Radiated Tortoises, *Astrochelys radiata*, by direct observation of their gonads using an endoscope as part of the captive breeding program for this species at the Behler Chelonian Center (BCC). The gonads of small juvenile *A. radiata* are thin, elongate, and fixed to the dorsal part of the 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 tubular structures visible through a thin, transparent theca containing a network of fine blood vessels, but no melanocytes. The breeding program produced a female-biased sex ratio with a male to female ratio of 1:8.4 (n = 75), suggesting that *A. radiata* exhibits TSD, with cooler incubation temperatures producing males and warmer incubation temperatures producing females. Unfortunately, incubation conditions were not monitored rigorously enough to allow a precise determination of the pivotal temperature, but a preliminary estimate is that the pivotal temperature of *A. radiata* is between 28.0 and 28.9°C and the upper limit of the transitional range of temperatures (i.e., above which only females are produced) is between 28.9 and 30.0°C.

Key Words. – Reptilia, Testudines, Testudinidae, captive breeding, endoscopy, testis, ovary, sex ratio, temperature-dependent sex determination, *Astrochelys radiata*, Madagascar

The Radiated Tortoise, *Astrochelys radiata*, was once widespread and common in the spiny forest of southern and southwestern Madagascar. Today, it is gravely imperiled by human exploitation and habitat destruction (Pedrono 2008; Devaux 2010). Since this decline is ongoing, *ex-situ* assurance colonies and captive breeding operations have become increasingly important for the conservation of *A. radiata*; therefore, it is imperative that breeding programs use best practice management techniques. One area of concern is the production of skewed sex ratios, or potentially only one sex, for species that exhibit temperature-dependent sex determination (TSD). While offspring sex in most vertebrates depends on genotypic sex determination (GSD, i.e., sex is determined by maternal and paternal genes and/or sex chromosomes), temperature-dependent sex determination seems to be the most common form of sex determination in turtles, especially tortoises (family Testudinidae). As a general rule for TSD in turtles, lower incubation temperatures produce males and higher temperatures females (TSD Ia), although in some species very low temperatures can also produce females (TSD II) (Ewert et al. 2004). However, little information is currently available on the mode of sex determination in the critically endangered tortoises of Madagascar. Only a short note exists that describes the female-skewed sex ratio observed in the captive breeding project for *Astrochelys yniphora* at Ampijoroa (Kuchling and López 2000), which suggests that TSD does occur in this species.

Most tortoises have a prolonged juvenile phase and take many years or decades before they reach maturity and can be sexed externally. Traditionally TSD studies in turtles have sacrificed hatchlings to determine their sex (Ewert et al. 2004), which is prohibitive in studies regarding critically endangered species for which information on sex determination mechanisms is most urgently needed. Determining sex ratios in captive breeding projects using non-lethal sexing techniques for juveniles has become increasingly important. Endoscopy is currently the only 100% accurate non-lethal method available to sex juvenile turtles and tortoises that do not show external sexual dimorphism (Kuchling 1999, 2006; Kuchling and López 2000; Wibbels et al. 2000; Kuchling and Kitimasak 2009). This paper presents data on the endoscopic imaging of gonads and accessory ducts and on sex ratios in captive-bred juvenile *A. radiata* at the Behler Chelonian Center (BCC).

METHODS

Study Location and Breeding Group. — The BCC, which is administered by the Turtle Conservancy, is a captive breeding and management facility located in southern California, USA. The facility, certified by the Association of Zoos and Aquariums (AZA), houses some of the world's most critically endangered turtle and tortoise species. The *A. radiata* breeding program at the BCC is the largest and oldest of its kind in the United States. The program was initiated by the Wildlife Conservation Society (WCS) in the 1980s on St. Catherine's Island, Georgia, with individuals collected by Robert Baudy from Madagascar in the 1960s. When WCS discontinued their program in 2003, over 200 turtles and tortoises were transported from the island to the BCC campus. The group of *A. radiata*, which included founder animals belonging to the AZA Species Survival Plan program, continues to propagate at the BCC.

Maintenance of Breeding Stock. — The tortoises are maintained in two different breeding groups at the BCC, from which the captive-bred tortoises reported on in this study originated. The first group consists of older, wild caught specimens and is managed to meet the requirements of the SSP, with specific individuals paired to ensure healthy and genetically diverse progeny. The second group consists mainly of captive born specimens that hatched in the 1980s and are maintained in a herd. The tortoises in this group freely choose their mates. In both groups, males are separated from females for some period of time in order to maintain their interest in mating when paired again.

Tortoises have year-round access to both indoor and outdoor enclosures. They are fed a varied diet that includes natural graze cuttings, *Opuntia* cactus pads, dandelion, radicchio, endive, parsley, squash, zucchini, apples, and carrots. When outdoors, they are able to forage on natural vegetation, which includes Bermuda grass, flowering mallows, mulberry trees, *Opuntia* cacti, and autumn joy. They are also offered cuttlefish bone for calcium supplementation.

Egg Incubation. — Female *A. radiata* deposit eggs year-round. We do not have details of the incubation temperatures of eggs incubated on St. Catherine's Island prior to 2004. The eggs at the BCC used in this study were artificially incubated in modified wine coolers at the nominally constant temperature of 28.9°C up through 2007 and about 30.0°C from 2008 and on. Eggs are incubated in chunky vermiculite in a vermiculite:water ratio of 2:1 by weight. Hatchlings emerged in 90–120 days. Date of hatching was recorded for all juveniles, which were marked with small tags used for honey bees that were glued to their carapacial scutes.

Maintenance of Hatchlings and Juveniles. — Hatchlings are maintained on tables (0.9 x 1.8 m and 0.9 x 2.4 m) with 100 mm of substrate that consists of rice hulls, sand, and peat moss. The tables are planted with succulents, grasses, and other natural graze items, and a water dish is present at all times. The southern California climate allows for the tables to remain outdoors for about six to seven months (weather permitting), and in temperature and humidity controlled green houses for the rest of the year. Once the hatchlings reach an appropriate size of 100–130 mm they are transferred from the tables to larger enclosures and are fed a similar diet to that of the adults.

Endoscopy. — Sex was determined endoscopically in 75 juvenile *A. radiata* that had an average body mass of 322 g (range = 21–1333 g) during March 2010. The tortoises did not receive food for 24 to 48 hrs prior to being endoscoped. Tortoises were anesthetized by intravenous injection (carpal sinus) of ketamine hydrochloride (20–30 mg/kg body mass). Optimum anesthetic depth was achieved after about 15 minutes. Both hind legs were pulled backwards and fastened together. The left inguinal pocket and neighboring skin, shell, and leg were scrubbed with antiseptic soap and povidone-iodine. A 2.7 mm diameter rigid Storz Hopkins endoscope was inserted into the abdominal cavity through a small 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 a light source. Gonads and accessory ducts were visualized, usually behind intestinal loops, and their appearance, color, and texture 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 the endoscopy procedure the skin wound was sutured using two 4/0 vicryl stitches. The surgical procedures took between 2–10 min, depending on whether photos were taken or not. The tortoises recovered from anesthesia 1–2 hrs after the surgical procedure and were kept under observation for 24 hrs before being returned to their nursery enclosures.

RESULTS

Appearance of Gonads and Accessory Ducts. — The gonads of small juvenile *A. radiata* are 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) can 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 move and can change their position relative to the kidneys, adrenals, and lungs (which have more or less fixed positions), for example, when turtles are tilted from one side to the other during endoscopy.

Testes of small tortoises appear as small, transparent, thin, half-roundish sausage-like structures, bound to the kidneys by the mesorchium and with a net of fine blood vessels on the surface (Fig. 1A). With growth testes become thicker, but remain half-roundish sausage-like structures ventral to the kidneys, turning first pinkish-white and then yellowish (Fig. 1B) with, in close-up, tubular structures visible through a thin, transparent theca containing a network of fine blood vessels (Fig. 1C), but no melanocytes. Epididymes and vas

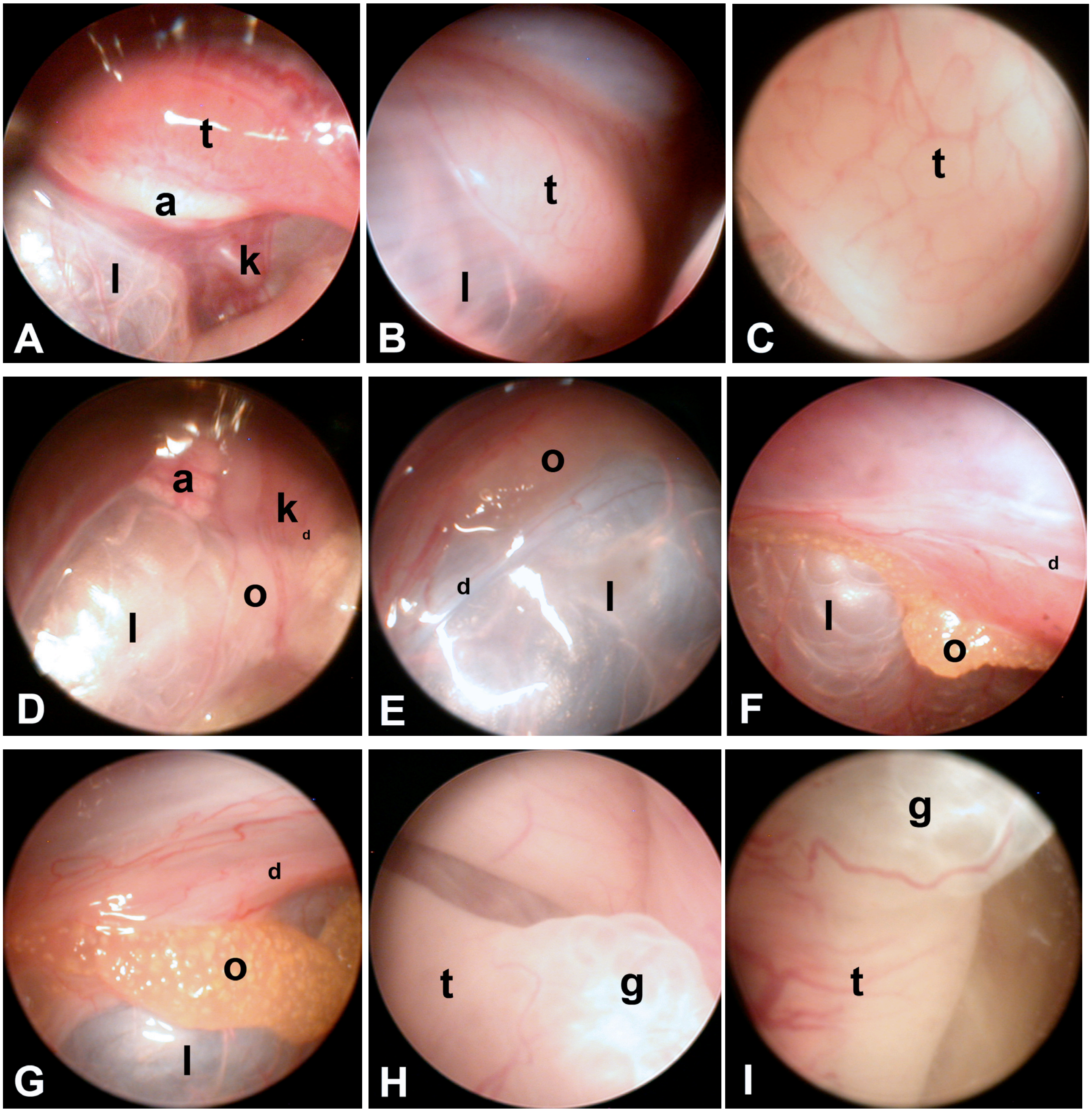

Figure 1. Endoscopic images of gonads and accessory ducts in juvenile *Astrochelys radiata*: a: adrenal; d: oviduct; g: outgrowth of testis reminiscent of ovarian tissue; k: kidney; l: lung; o: ovary; t: testis. **A:** male 3-yr-old, 212 g body mass; **B:** male 8-year-old, 1148 g body mass; **C:** close-up of testis, same male as in B; **D:** female 8-mo-old, 43 g body mass; **E:** female 2-yr-old, 128 g body mass; **F:** female 3-yr-old, 214 g body mass; **G:** female 8-yr-old, 975 g body mass. **H:** male 7-yr-old, 529 g, protruding outgrowth reminiscent of ovarian tissue on testis. **I:** close-up of protruding outgrowth on testis, same animal as in Fig. 1H.

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 to the kidneys, with some oocytes and primary follicles visible (Fig. 1D,E). With growth ovaries expand along the dorsal wall of the coelomic cavity ventrally to the lungs, increase in thickness, and masses of pre-vitellogenic follicles appear on the surface (Fig. 1F,G). 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 (Fig. 1D,E). With growth oviducts become wider and thicker and more whitish (Fig. 1F,G), but still remain straight in the size classes examined during this study.

One of the eight juvenile *A. radiata* classified as males (529 g body mass, 7-yr-old) had a normal looking right testis, but showed a small outgrowth of tissue reminiscent of ovarian cortex and stroma tissue protruding from the

Table 1. Year of hatching, sex (n = number), and body mass (SD = standard deviation) of *Astrochelys radiata* sexed by endoscopy at the Behler Chelonian Center, * = incubated at St. Catherine's Island, Georgia.

	Females				Males			
Year Hatched	**n**	**Mean body mass (g)**	**1 SD**	**Range (g)**	**n**	**Mean body mass (g)**	**1 SD**	**Range (g)**
2001*	1	1234	-	-	0	-	-	-
2002*	6	852	± 220	608-1252	2	1108	-	1067-1148
2003*	7	683	± 325	399-1333	4	681	± 197	503-997
2004*	1	428	-	-	0	-	-	-
2005	-	-	-	-	-	-	-	-
2006	6	292	± 37	223-334	0	-	-	-
2007	17	203	± 64	126-417	2	165	-	117-212
2008	12	110	± 21	81-146	0	-	-	-
2009	17	47	± 13	21-71	0	-	-	-

left testis (Fig. 1H,I; about 20% of the gonadal volume). Although some blood vessels from the theca testis extended to the surface of this outgrowth, it did not show the network of fine bloodvessels typical for the theca testis. No follicles could be seen on this outgrowth, but their appeared to be a few oocytes and primary follicles in the cortex (Fig. 1H,I) as in ovaries of hatchlings and very small juveniles (Fig 1D,E).

Sex Ratio. — Of the 75 *A. radiata*, 67 were females (89.3%) and 8 were males (10.7%), giving an overall male to female sex ratio of 1:8.4. Broken down according to the breeding location (Table 1), eggs which hatched prior and up to 2004 (n = 21) on St. Catherine's Island produced 15 females (71.4%) and 6 males (28.6%; m:f ratio = 1:2.5). Broken down according to incubation temperatures at the BCC since 2004, eggs incubated at about 28.9°C (n = 25, hatched 2006–07) produced 23 females (92%) and 2 males (8%; m:f ratio = 1:11.5), and eggs incubated at about 30°C (n = 29, hatched 2008–09) produced 29 females (100%) and no males (0%).

DISCUSSION

The good news of this study is that the breeding program produced males as well as females. The sex ratio was strongly female biased, but this is generally favored in turtle conservation programs (Seigel and Dodd 2000). The majority of juvenile *A. radiata* recovered without problems from the endoscopic procedure and continued to grow normally. Unfortunately, 3 of the 75 (4%) endoscopically sexed *A. radiata* died a few days following endoscopy. All three were from the 1-yr-age group, which was maintained in a large thermostatically controlled greenhouse and were kept together in one particular group. Two juveniles from a different group that were not endoscopically sexed, also died during this period (March 2010); these two specimens, however, were from a different age cohort (7–8 yrs), maintained in a different building ("nursery"), and were also kept together in one group. The mortality rate following the endoscopy did not differ from the overall background mortality rate of juvenile *A. radiata* that were not examined by endoscopy at the BCC at that particular time. The cause of death in both groups remains unknown and was an exceptional event; since the inception of the BCC in 2005 a total of 115 *A. radiata* have hatched and, except for those five deaths, only one other mortality occured in a 1–2 month-old juvenile. During the time when endoscopic sexing was performed (4 to 12 March 2010) southern California had unusually cold weather and the tortoises remained in the indoor winter quarters for longer than usual. No mortalities were recorded at the BCC during the same time period following endoscopic sexing of 38 juvenile *Geochelone platynota* (the majority of them in the 1-yr-age group; Kuchling et al. 2011), four juvenile *Heosemys depressa*, two juvenile *Siebenrockiella leytensis,* and the four juvenile *Chelonoidis nigra*. The mortalities in the endoscopically sexed and non-endoscopically examined juvenile tortoises were restricted to *A. radiata*. Since there have been no health issues or deaths of Radiated Tortoises in the BCC since this episode, it may be concluded that the five deaths in March 2010 were an anomaly, possibly related to the endoscopy, but not related to any systemic problem with the collection or the maintenance of the collection. In the future it would seem prudent to perform endoscopic sexing in captive breeding programs only during times of optimal environmental and husbandry conditions.

Morphology of Gonads and Accessory Ducts. — This study provides descriptions and endoscopic images of testes and ovaries of hatchling and juvenile tortoises (family Testudinidae) and shows the changes of the gonads with the growth of the juveniles. Endoscopy has been successfully used before to sex captive-raised juvenile Desert Tortoises, *Gopherus agassizii*, with a size range of 28–1250 g body mass (Rostal et al. 1994), but that paper, as well as Kuchling and López (2000) for *A. yniphora*, did not provide endoscopic images and did not describe the morphology of gonads and accessory ducts with changing age and/or body size. Other tortoise species with similar appearance of testes and ovaries in hatchlings and juveniles include *Geochelone platynota* (Kuchling et al. 2011) and Aldabra Tortoises (Kuchling and Griffiths 2012). However, the morphology of the testis in hatchling and juvenile *Gopherus agassizii* is quite different from those species and *A. radiata* with testes being bright yellow, long, flat bands (Rostal et al. 1994). Thus, there is some variability in the development of gonads in hatchling and juvenile Testudinidae. Epididymis and vas deferens in small juvenile tortoises are thin and translucent and difficult to image during endoscopy.

Figure 2. Freshly hatched *Astrochelys radiata* at the Behler Chelonian Center. Photo G. Kuchling.

So far only a few published papers have provided endoscopic images of juvenile testes and ovaries of species in other turtle families which can be used as guidance to identify gonads and sex (Podocnemididae: Kuchling 2006; Cheloniidae: Wyneken et al. 2007; Trionychidae: Kuchling and Kitimasak 2009). In hatchling and small juvenile turtles of those families and of *G. platynota* (Kuchling et al. 2011), Aldabra Tortoises (Kuchling and Griffiths 2012) and *A. radiata* (this paper), gonads and accessory ducts are of tiny size and often of transparent appearance (Fig. 1A,D,E). Testes in particular can be transparent structures, flat or half-roundish in cross section, sometimes smaller than adjacent adrenal glands (Fig. 1A) and, therefore, easily overlooked. The theca testis is always thin and translucent, never contains melanocytes, and tubular structures of different size and color (transparent, white, pinkish, or yellow) and/or a fine net of surface vasculature is visible in testis close up (e.g., Fig. 1C). However, in the Asian box turtle *Cuora flavomarginata* the juvenile testis shows melanocytes in the theca and can appear pendulous (Hernandez-Divers et al., 2009: p. 802, Fig. 2a). A close up of this structure 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: p. 802, Fig. 2b). Because of this striking differences to juvenile testes images of other turtle groups (see above) it has been suggested that Hernandez-Divers et al. (2009) may have misidentified an unrelated structure as testis (Kuchling 2009), but the identification of this structure as testis was accurate (Divers and Stahl 2009; Innis 2012) and juvenile testes of similar appearance have in the mean time also been found in *Cuora trifasciata* (Kuchling 2012).

An interesting finding in the present study was the male *A. radiata* that showed a small area of ovarian tissue on only one of the otherwise normally developed testes (Fig. 1H, I). Kuchling and López (2000) classified one out of 60 *A. yniphora* as an intersex, but in that specimen testes and ovaries appeared to develop more or less normally side by side, although the ovaries appeared to be slightly smaller than in normal females of similar body mass. Similar intersex conditions have been found in three specimens of juvenile Aldabra Tortoises (Kuchling and Griffiths 2012). However, in the case of the male *A. radiata* in this study, the ovarian tissue formed only a small outgrowth on one testis and did not show the appearance of ovaries of females of similar age and size. Since it is possible that this abnormal ovarian tissue will be suppressed with future growth and gonadal development, we did not classify this specimen as an intersex, but as a male (although with a slight abnormality on one testis).

Sex Ratio and Sex Determination. — The female-biased sex ratio of captive-bred *A. radiata* indicates that the species has temperature-dependent sex determination (TSD). At the BCC, the sex ratios of juveniles from eggs incubated at the lower temperature (hatchlings of 2006 and 2007) and at the higher temperature (hatchlings from 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. Important TSD parameters are 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); and within the TRT, the constant incubation temperature that will produce a 1:1 sex ratio, which 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 pivotal temperatures and TRTs. Therefore only crude estimate can be made, since eggs incubated at ca. 28.9°C up to 2007 produced a male to female ratio of 1:11.5, and eggs incubated at ca. 30°C since 2008 produced 100% females, the pivotal temperature of *A. radiata* is most likely below 28.9°C, with the upper limit of the TRT between 28.9–30°C. Interestingly, eggs of the Burmese Star Tortoise, *Geochelone platynota*, incubated at the BCC at the same time, at the same temperatures, and in the same incubators as those of *A. radiata,* produced a male to female ratio of 1:0.08 at ca. 28.9°C and of about 1:1.2 at ca. 30°C (Kuchling et al. 2011), indicating that this species has a higher pivotal temperature than *A. radiata*.

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 and TRT of *A. radiata*. This information is important to enable informed decisions on how to produce desired offspring sex ratios in future breeding operations. In the mean time, in order to produce a better balanced sex ratio of *A. radiata*, the incubation protocols at the BCC have been changed recently to increase the number of male hatchlings by lowering the incubation temperature to ca. 27.8°C and by moving the incubator to a climate-controlled room to avoid day-night fluctuations in temperature.

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Résumé

L'évaluation du sex-ratio de la progéniture est importante dans tout programme d'élevage d'espèces menacées à grande échelle lorsque la détermination thermodépendante du sexe (DTS) est une possibilité. Dans le cadre du programme de reproduction en captivité de l'espèce au Behler Chelonian Center (BCC), nous avons évalué le sexe ratio de tortues radiées juvéniles, *Astrochelys radiata*, par observation directe de leurs gonades à l'aide d'un endoscope. Les gonades des juvéniles de petite taille sont minces, de forme allongée, et fixées à la partie dorsale de la cavité du corps. Les ovaires apparaissent comme des enveloppes transparentes avec quelques ovocytes visibles. Quant aux testicules, ce sont des structures en forme de saucisse qui apparaissent petites, transparentes, minces, avec un réseau de fins vaisseaux sanguins à leur surface. Avec la croissance, les ovaires se développent et des masses de follicules prévitellogènes apparaissent à la surface. Les testicules sont transparents chez les petits juvéniles et, au cours de la croissance, deviennent blanc-rose puis jaunâtres, avec des structures tubulaires visibles à travers une thèque mince et transparente contenant un réseau de fins vaisseaux sanguins, mais pas de mélanocytes. Le programme de procréation en captivité a produit un sexe ratio biaisé en faveur des femelles avec un ratio mâle femelle de 1:8.4 (n = 75), suggérant que *A. radiata* présente une DTS, avec des basses températures d'incubation produisant des mâles et des températures plus chaudes produisant des femelles. Malheureusement, les conditions d'incubation n'ont pas été suivies assez rigoureusement pour permettre une détermination précise de la température de basculement, mais une estimation préliminaire la situe entre 28.0 et 28.9ºC; et la limite supérieure de la rangée de températures transitionnelle (i.e., au-dessus de laquelle seules des femelles seront produites) se situe entre 28.9 et 30.0ºC.

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