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# Endoscopy gender determination and reproductive hormone profiles of Painted Terrapins (*Batagur borneoensis*) subjected to *ex situ* incubation

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**ABSTRACT.** Chelonian exhibit temperature dependent sex determination, and *ex situ* incubation of eggs in conservation hatcheries may render a gender bias. The gender of juvenile Painted terrapins (*Batagur borneoensis*) produced at a conservation hatchery in Malaysia was determined by endoscopy of the gonads. Circulating reproductive hormones (testosterone, progesterone and estradiol) were profiled for 31 juveniles and nine captive-reared non-breeding adult terrapins. Endoscopy revealed a gender bias of 96.8% (30/31) females. Testosterone levels in the juvenile females (2.49 ± 1.29) were significantly lower than that of the adult females (12.20 ± 4.29), and lower than values in the juvenile male (9.36) and adult males (27.60, 35.62). The progesterone levels in the juvenile females (107.12 ± 68.68) were significantly higher than that of the adult females (51.13 ± 24.67), but lower than values in the juvenile male (33.27) and adult males (3.43, 8.51). Estrogen levels were significantly lower in the juvenile females (1.57 ± 1.35) compared to the adult females (77.46 ± 53.45). Negative correlations were observed between levels of progesterone and testosterone, and progesterone and estrogen. A positive correlation was noted between estrogen and testosterone. The present study constitutes the first attempt to determine the gender and reproductive hormone profiles of juvenile Painted terrapins produced by *ex situ* incubation, and captive non-breeding adults. Endoscopy of the gonads is a useful techniques for gender determination among juvenile turtles, while the use of testosterone as a gender biomarker warrants further investigation.

**KEY WORDS:** *Batagur borneoensis*, endoscopy, gender determination, reproductive hormone

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The Painted terrapin (*Batagur borneoensis*) is a hard-shelled chelonian species endemic to Southern Thailand, Peninsular Malaysia, Borneo and Sumatra [1, 2]. Although these terrapins are spread across Southeast Asia, their actual distribution is limited to only a number of large river systems in the region. In Peninsular Malaysia, major populations have been reported to inhabit the Setiu and Paka Rivers in Terengganu, the Linggi River in Melaka/Negeri Sembilan [17]. There has been a dramatic reduction in the population of this terrapin species causing it to be listed as critically endangered on the IUCN Red List of Threatened Species [1], and under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II [4]. Wild populations of this chelonian species are rapidly declining due to the international trade of live terrapins for pets, food and medicine, the local consumption of eggs and meat, habitat loss and improper coastal and estuarine development [16, 22].

Painted terrapins are riverine turtles which are known to be able to tolerate higher amounts of salinity, allowing them to inhabit estuarine areas [2, 6]. The adults are mainly herbivorous, feeding on leaves and fruits of mangrove vegetation [2, 7]. Adults have

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marked sexual dimorphism. The female is generally larger with an olive colored head, while the head of the non-breeding male is charcoal grey, which turns white with a red dorsal streak during the breeding season [2, 18]. The hatchlings and juveniles are monomorphic, which makes them difficult to be sexed by appearance alone. Breeding is seasonal, and females will swim out to the estuary and into the sea to lay their eggs on the sandy beaches [2]. As the nesting sites for the terrapins are the sandy beaches, the eggs have higher risk of being poached or succumb to predation. Thus, to increase the chances of survival, hatcheries have been set up at the major nesting sites. In Malaysia, these hatcheries can be found in Pahang, Terengganu and Malacca, which were set up by the State Fisheries Department. The terrapin eggs are collected and re-buried in a safer and enclosed environment (hatchery) for *ex situ* incubation, away from predators and poachers. However, human manipulation of the eggs leads to changes in the incubation temperature, which influences the gender of the hatchlings as chelonians exhibit temperature dependent sex determination [15, 23]. *Ex situ* incubation in hatcheries may therefore lead to a bias in the gender ratio of these terrapins.

As part of the Painted terrapin conservation program, it vital to determine the sex ratio of the terrapins hatchlings that are produced by these conservation hatcheries. Previous gender determination studies in other chelonian species had noted biased sex ratios when these eggs are artificially incubated [9, 13]. To date there has not been any attempt to study the gender ratios of hatchling Painted terrapins either in the wild or those produced *via* artificial incubation. In addition, there are no published data on the appearance of the gonads of this terrapin species. Endoscopy could be a minimally invasive way to determine the sex of the hatchling and juveniles of this species, which will be useful for the hatcheries to manage their incubation temperatures and gender ratios. The present study was therefore undertaken to provide the much needed information on the gender ratios of Painted terrapins produced by *ex situ* incubation at conservation hatcheries in Peninsular Malaysia. It also provides baseline data on the reproductive hormone profiles of these terrapins which could also be an alternative method of minimally invasive gender determination. It is envisaged that the data obtained will assist in the protection and conservation of this endangered terrapin species and assist in the management of conservation hatcheries in the region.

## MATERIALS AND METHODS

### *Animals*

Thirty one, two year old Painted terrapins were randomly selected from pooled clutches that were incubated in *ex situ* nests on the beach at a turtle conservation hatchery on the east coast of Peninsular Malaysia. Upon completion of the study, the terrapins were released back into the river where the adult females originated. Prior to endoscopy, morphometry of all juveniles were recorded, including measurements of curved carapace length (CCL), curved carapace width (CCW), straight carapace length (SCL), straight carapace width (SCW), plastron length (PL), plastron width (PW), pectoral to abdominal scutes length (Pc-Ab), and width of the anal scutes (WAS) (Table 1). Morphometry was done using a vernier caliper and a flexible measuring tape. Weights of the terrapins were recorded using a digital weighing scale.

### *Endoscopy and histology of the gonads*

All surgical equipment were autoclaved and endoscopic equipment were soaked in glutaraldehyde solution for 15 min and then rinsed with autoclaved distilled water prior to the endoscopy procedure. The terrapins were fasted for 24 hr before being anaesthetized with Ketamine (Ketamil®, Troy Laboratories, Glendenning, New South Wales, Australia) 50 mg/kg, intramuscularly (IM). The terrapins were then intubated and Intermittent Positive Pressure Ventilation (IPPV) was performed at four breaths per min. The hind limbs were retracted to expose the left prefemoral fossa and the site was routinely prepared for surgery. A stab skin incision was made at the center of prefemoral fossa and a small straight mosquito forceps was inserted and advanced cranially until the coelomic aponeurosis was penetrated. A sheathed rigid 30° viewing telescope with a diameter of 2.7 mm (Karl Storz, Tuttlingen, Germany) was inserted into the coelomic cavity and 30 to 40 m*l* of air was used for insufflation. Gonads were identified as ovaries or testes at the dorsocaudal region of the coelom. After gender was determined, the ceolomic aponeurosis, muscle layer and skin was sutured. Meloxicam (Metacam®, Boehringer Ingelheim, Ingelheim am Rhein, Germany) (0.2 mg/kg) was administered for three days and enrofloxacin (Baytril®, Bayer, Leverkusen, Germany) (5 mg/kg) was administered over five days. Recovery was uneventful and sutures were removed after three to four weeks. Four female juvenile terrapins that had died previously from hydrocoelom of uncertain aetiology, were subjected to postmortem examination and the gonads were harvested for

**Table 1.** Morphometry of juvenile *Batagur borneoensis* from Peninsular Malaysia

| Parameter (cm) | Mean ± SD | Min | Max |
|---|---|---|---|
| Curved carapace length | 17.6 ± 0.9 | 15.7 | 19.9 |
| Curved carapace width | 17.4 ± 0.9 | 15.5 | 19.2 |
| Straight carapace length | 15.8 ± 1.0 | 14.0 | 18.2 |
| Straight carapace width | 14.2 ± 1.0 | 12.1 | 16.0 |
| Plastron length | 15.7 ± 1.1 | 13.8 | 17.8 |
| Plastron width | 16.5 ± 1.0 | 14.8 | 18.2 |
| Pectoral to abdominal scute length | 7.4 ± 0.5 | 6.4 | 8.1 |
| Width of the anal scutes | 2.8 ± 0.2 | 2.5 | 3.3 |
| Weight (g) | 721.9 ± 123.7 | 481 | 1,017 |

histological evaluation. Ethical guidelines follow those prescribed by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (Animal Utilization Protocol Approval No. R088/2014).

*Reproductive hormone profiling*

Plasma levels of the reproductive hormones (testosterone, progesterone and estradiol) were profiled for the juvenile terrapins, seven adult female and two adult male captive-reared terrapins during the non-breeding season. Blood was drawn from the jugular vein in the juveniles and the brachial vein in the adults. There was no obvious contamination with lymphatic fluid in the blood samples. Reproductive hormone assays were done for the adult terrapins as a comparison to the values obtained from the juveniles and to act as a baseline for the species. Commercial ELISA kits (Cayman Chemical, Ann Arbor, MI, USA) were used for hormone assays according to the manufacturer's protocols. The testosterone ELISA kit has a range from 3.9–500 *p*g/m*l* with a sensitivity of 6 *p*g/m*l*, and cross reactivity of 140% to 19-Nortestosterone and 100% to testosterone. The progesterone ELISA kit has a range from 7.8–1,000 *p*g/m*l* with a sensitivity of 10 *p*g/m*l*, and cross reactivity of 100% to progesterone. The estradiol ELISA kit has a range from 6.6 to 4,000 *p*g/m*l* with a sensitivity of 15 *p*g/m*l*, and cross reactivity of 100% to estradiol. Plasma was extracted using diethyl ether and evaporated using a water bath at 30–31°C under a lamina flow hood. Standards were prepared by serial dilution with the supplied buffer; two-fold dilution factor for testosterone and progesterone, and 2.5-fold dilution factor for estradiol. Plate preparation included two blanks (B), two non-specific binding wells (NSB), two maximum binding wells (BO), eight standards in duplicates and 24 samples that were run in triplicates. The B wells were used to determine the background absorbance caused by the Ellman's reagent and was subtracted from the absorbance readings of all the other wells. The NSB was a measurement of low binding of the tracer to the well and BO was the maximum amount of tracer that could bind to the antibody. For all the plates, BO wells were between 0.3–1.0AU. The Ellman's reagent that was added to the wells causes a colorimetric enzymatic reaction that was quantified using an ELISA reader at wavelengths of 415 nm or 420 nm.

*Statistical analysis*

Relationship between the circulating values of the reproductive hormones (estrogen, progesterone and testosterone) were explored using the Spearman's rank-order correlation. Significant differences between the hormone among the juvenile and adult female terrapins was tested using an independent *t*-test after log transformation of the data.

## RESULTS

*Endoscopy and histological evaluation*

Endoscopic examination revealed that 96.8% of the juvenile terrapins were females (30 out of 31), with only one recognized as a male. The gonads of both sexes were clearly visualized and were situated between the kidneys, intestine and lungs. The testes were whitish, sausage shaped with round edges (Fig. 1), and possessed a network of fine blood vessels on its surface. The epididymis and vas deferens were not clearly visible upon endoscopy. The ovaries were shiny, yellowish in colour with small round follicles on its surface (Fig. 1). The oviduct runs adjacent to the ovary and it appeared as a white transparent sheath with a parallel blood vessel. Histological examination of the ovaries revealed clearly demarcated follicles of varying sizes, consisting of an oocyte surrounded by single layer of cuboidal cells (Fig. 2). This confirmed the endoscopy identity of the organ.

*Reproductive hormone profiling*

Profiles of the circulating reproductive hormones (testosterone, progesterone and estrogen) in the juvenile and captive-reared non-breeding adult terrapins are presented in Table 2. Values for the juvenile male were only obtained from one individual. The juvenile females showed testosterone values that were significantly ($P<0.01$) lower than that of the adult females. The mean values of testosterone in the juvenile females (2.49 ± 1.29 *p*g/m*l*) was approximately five times lower than that observed for the adult females (12.20 ± 4.29 *p*g/m*l*). In addition, the highest testosterone value (4.83 *p*g/m*l*) obtained from the juvenile females was almost half that observed in the juvenile male (9.36 *p*g/m*l*), and six times lower than that recorded for the lowest value observed in the adult males (27.60 *p*g/m*l*). Similarly, the two adult males had much higher plasma testosterone values compared to the range observed in the adult females. The level of this hormone was more than two-fold greater in the adult males compared to the mean value obtained for their female conspecifics.

The plasma progesterone values in the juvenile females were significantly ($P=0.033$) higher compared to that observed in the adult females. The mean values of progesterone in the juvenile females (107.12 ± 68.68 *p*g/m*l*) was approximately two fold higher than that observed for the adult females (51.13 ± 24.67 *p*g/m*l*). The progesterone value obtained from the juvenile male was within the range observed for that of the juvenile females. However, the higher plasma progesterone value observed in the adult males (8.51 *p*g/m*l*) was approximately three times lower than the minimum value obtained in the former, and close to half that detected in the adult females (15.22 *p*g/m*l*).

Levels of circulating estrogen were significantly ($P<0.01$) lower in the juvenile females compared to the adult females, where the mean values in the latter (77.46 ± 53.45 *p*g/m*l*) were close to 50 times higher than that in the latter (1.57 ± 1.35 *p*g/m*l*). The range values of this hormone did not overlap between the juvenile and adult females, whereby the highest value in the juveniles (4.89 *p*g/m*l*) was almost half that of the lowest value detected in adult females (9.57 *p*g/m*l*). Although there was an overlap in the ranges of estrogen between the adult females and males, the former exhibited a much higher mean value (77.46 ± 53.45 *p*g/m*l*) compared to the highest level detected in the males (9.96 *p*g/m*l*). Estrogen was not detectable in the juvenile male terrapin.

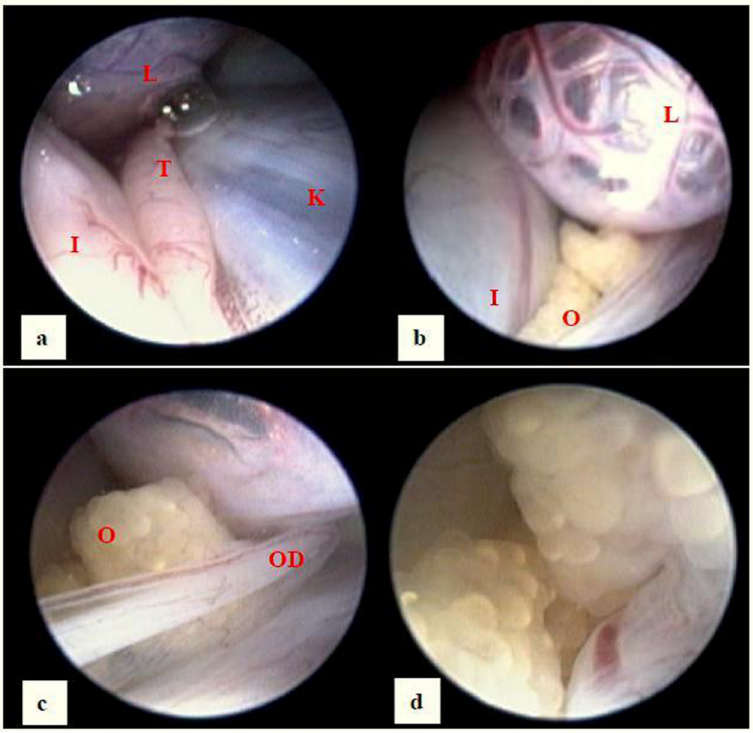


**Fig. 1.** Endoscopic appearance of male (a) and female (b, c) gonads, with a close up view of ovaries (d) in juvenile *Batagur borneoensis* from Peninsular Malaysia. Kidney (K), testis (T), lung (L), intestine (I), ovary (O) and oviduct (OD).

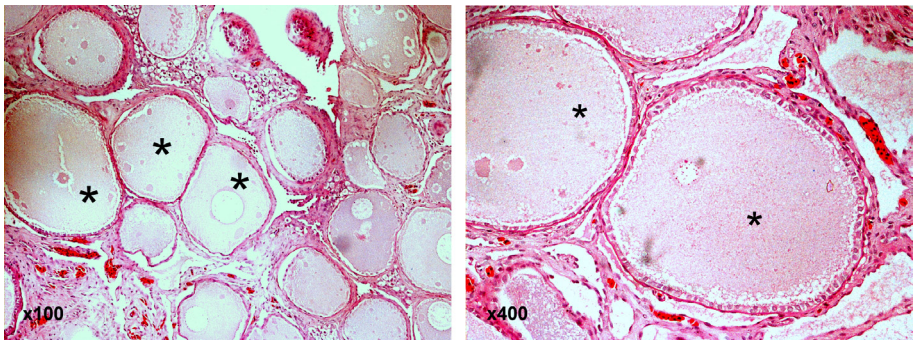


**Fig. 2.** Histology section of ovaries in juvenile *Batagur borneoensis* from Peninsular Malaysia. Asterisks indicate the follicles.

**Table 2.** Reproductive hormone values in juvenile and adult *Batagur borneoensis* from Peninsular Malaysia

| | Testosterone | Progesterone | Estrogen |
|---|---|---|---|
| Females | | | |
| Juveniles | | | |
| N | 22 | 29 | 12 |
| Mean ± SD | 2.49 ± 1.29* | 107.12 ± 68.68* | 1.57 ± 1.35* |
| Range | (0.53–4.83) | (25.21–252.34) | (0.22–4.89) |
| Adults | | | |
| N | 7 | 7 | 7 |
| Mean ± SD | 12.20 ± 4.29* | 51.13 ± 24.67* | 77.46 ± 53.45* |
| Range | (6.34–17.79) | (15.22–81.29) | (9.57–158.33) |
| Males | | | |
| Juvenile (N=1) | 9.36 | 33.27 | ND |
| Adults (N=2) | 27.60, 35.62 | 8.51, 3.43 | 5.44, 9.96 |

Sample size (N) includes only individuals that had detectable plasma levels (*p*g/m*l*) of the respective hormone as determined by the ELISA assay. An asterisk (*) denotes significant differences ($P<0.01$) in values between the juvenile and adult females. ND, Not detectable by the ELISA assay.

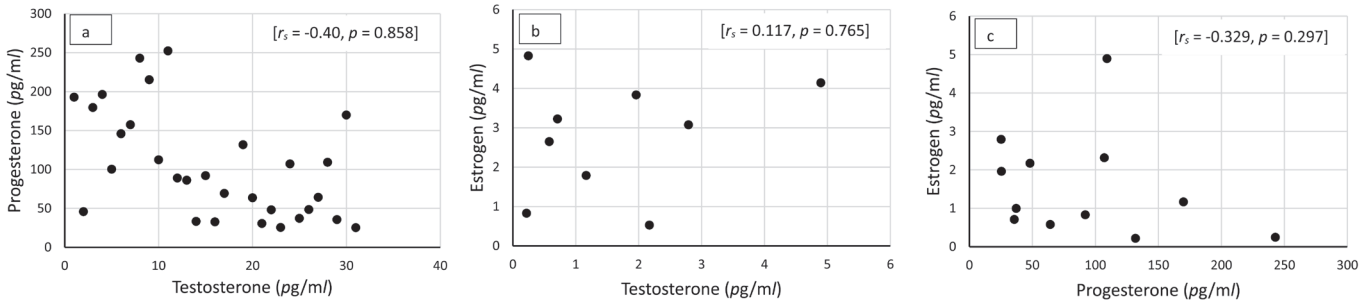


**Fig. 3.** Spearman's rank-order correlation between the various reproductive hormones in hatchling *Batagur borneoensis* from Peninsular Malaysia. a) progesterone and testosterone; b) estrogen and testosterone; c) estrogen and progesterone.

The Spearman's rank-order correlation showed a negative correlation between progesterone and testosterone [$r_s$ (21)=–0.40, $P$=0.858] (Fig. 3a), a positive correlation between estrogen and testosterone [$r_s$ (7)=0.117, $P$=0.765] (Fig. 3b), and a negative correlation between estrogen and progesterone [$r_s$ (10)=–0.329, $P$=0.297] (Fig. 3c). However, none of the correlations were statistically significant.

## DISCUSSION

The present study constitutes the first attempt to determine the gender of Painted terrapins produced by *ex situ* incubation at conservation hatcheries in Malaysia. It is also novel in reporting the reproductive hormone profiles among juveniles and captive non-breeding adults of these endangered terrapins. Determining the gender of artificially incubated chelonians is vital for the success of conservation programs that employ *ex situ* incubation techniques. Unfortunately, manipulation of these eggs and removal from its natural nests may render alterations in the incubation temperature, which is a major determinant of the gender of the hatchlings. A captive breeding program of Chinese box turtles, *Cuora flavomarginata* showed a female biased sex ratio (6.9% male, 93.1% female, n=58) [9]. In another conservation breeding program for Burmese star tortoises (*Geochelone platynota*), a male biased sex ratio (n=38, 60.5% male, 36.9% female) was noted when the eggs were incubated at 28.9°C [13]. Conversely, an equal sex ratio (50% male, 46.5% female, n=28) was produced when the eggs when incubated at a higher (30°C) temperature [13]. The turtle conservation hatchery examined in this study had a highly female biased sex ratio. While incubation temperatures were not collected for the nests, based on previous reports, it is reasonable to suggest that the incubation temperatures of the nests at this hatchery were too high resulting in a female gender bias among the hatchlings. Generally for chelonians, lower temperature yields more males and higher temperature yields more females. However, masculinizing and feminizing temperatures differ between species. For example, in the European pond turtle, the masculinizing temperature is 25°C, while the feminizing temperature is 35°C [5]. In captive bred Burmese Star tortoises, a temperature of 28.9°C yielded a male biased sex ratio and a 30°C temperature produced more balanced genders [13]. Therefore, future studies should focus on monitoring the incubation temperatures at conservation hatcheries, followed by gender determination of the hatchlings, in order to produce a balanced gender ratio.

Over the years, endoscopy has been used by many researchers to study reproduction in chelonians. It has been used to identify the sex of the hatchlings of various species of chelonians including Desert tortoises (*Gopherus agassizii*) [21], Madagascar big-headed turtle (*Erymnochelys madagascariensis*) [10], Chinese box turtles (*Cuora flavomarginata*) [9], Asiatic soft-shell turtles (*Amyda cartilaginea*) [12], Burmese star tortoises (*Geochelone platynota*) [13], Aldabra Giant tortoises (*Aldabrachelys gigantean*) [11], and Radiated tortoises (*Astrochelys radiat*) [14]. Endoscopy is said to be absolutely accurate in determining the sex of turtles [10, 12, 21]. In the endoscopic view, the gonads in juvenile turtles appear as thin and elongate structures located near the kidneys [12, 13]. Ovaries have a transparent sheath with small whitish-yellowish follicles while the testes are sausage shaped with smooth surface and fine vasculature on it [9, 12, 13]. Younger female hatchlings/juveniles have whitish follicles while older females have yellowish follicles [10]. As for the male, the colour of the testes will change from pinkish white to yellowish with age [13]. There was no appreciable variation in the appearance of the gonads of the Painted terrapins compared to that previously reported for other chelonian species. However, there were subtle variation in terms of coloration of the ovary follicles and testes, which may be attributed to age.

Hormone assays have also been proven useful in determining the gender of juveniles or hatchlings of monomorphic chelonians. Many studies in sea turtles have used testosterone levels as a gender indicator, whereby higher testosterone levels have been demonstrated in juvenile male Green turtles (*Chelonia mydas*) [20], Kemp's Ridley turtles (*Lepidochelys kempii*) [8], and Loggerhead turtles (*Caretta caretta*) [3]. However, testosterone levels are known to differ between species [19], and as such, it is imperative that species-specific baseline levels should be determined. The oestradiol assay used in this study had a range of 6.6 to 4,000 *p*g/m*l*, and was therefore not sensitive to detect low levels of estrogen found in the juvenile terrapins. In addition the testosterone assay employed had a range of 3.9 to 500 *p*g/m*l*, and proved insensitive to detect the hormone in the juvenile females. A study that was conducted to evaluate different sexing methods in chelonians found that plasma testosterone was 98% accurate to determine the gender in juvenile and immature Desert tortoises, (*Gopherus agassizii*) [21]. However, these juveniles have much

higher testosterone levels compared to the Painted terrapins; females less than 200 *p*g/m*l*; males above 200 *p*g/m*l* to 9.48 *n*g/m*l*. This demonstrates the variation in the testosterone ranges among juveniles of different chelonian species.

The present study reports for the first time the endoscopy examination of the gonads, and the circulating levels of reproductive hormones in the critically endangered Painted terrapin. Endoscopy is an efficient method for sexing juvenile chelonians and can be used as a routine tool for gender determination for Painted terrapins in conservation hatcheries or breeding programs. While the data on the testosterone values obtained from a single juvenile terrapin may indicate that circulating testosterone may be employed in gender determination, it would be necessary to obtain values from more male juvenile terrapins before any conclusive inferences could be made on the use of this biomarker as a tool for chelonian sexing. As such, this reproductive hormone may serve as a useful biomarker in determining the gender of hatchlings produced by artificial incubation in conservation hatcheries. Future studies should focus on obtaining incubation nests temperature data in order to determine the cut-off value for producing male and female hatchlings. In addition, a larger sample size for the males would be necessary to obtain reference range values for circulating testosterone levels for both sexes in these terrapins.

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