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Original article

Determination of sexual maturity of Indonesian box turtle (*Cuora amboinensis couro*) based on straight carapace length

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ABSTRACT

Predicting the turtle's reproductive status and sexual maturity is necessary for ex-situ breeding and conservation program. The histology of the seminiferous tubules of the Indonesian box turtle (*Cuora amboinensis couro*) at various ages has not been studied much. This study aims to develop equations to predict the sexual maturity of Indonesian box turtles based on straight carapace length (SCL). Six turtles with SCL sizes 10-21 cm were sacrificed and their testes were taken for morphometric measurements and histological preparations for Hematoxylin Eosin staining. Pearson’s correlation of SCL with testicular maturity parameters was analyzed, followed by Anova Regression. The results showed that there were differences in the morphometry and topographic anatomy of the Indonesian box turtle testes between SCL below 15 cm and above 15 cm. Six turtles with SCL size of 10-21 cm were sacrificed and their testes were taken for morphometric measurements and histological preparations for Hematoxylin Eosin staining. Pearson's correlation of SCL with testicular maturity parameters was analyzed, followed by Regression Anova. The results showed that there were differences in the morphometry and topographic anatomy of the Indonesian box turtle testes between SCL below 15 cm and above 15 cm.

Keywords: endangered species, Indonesian box turtle, seminiferous tubules, spermatogenic cells, testes

INTRODUCTION

The Indonesian box turtle is one of the native Indonesian turtles which is hunted to meet export and domestic needs (Qayyim, 2018). These turtles were mainly used as pets, meat and eggs for food consumption, traditional Chinese medicine, handicrafts and traditional purposes (Wang and Carey, 2014). The Indonesian box turtle was listed in appendix II (CITES, 2019) and is not protected by the Indonesian government (Nurbaya, 2018) but has been categorized as endangered in 2020 by the IUCN (Cota *et al.*, 2020). There were indications of over-exploitation of Indonesian box turtles in the long term (Widagti, 2011; Fauzi, 2022).

Turtles grew very slowly, it took four to ten years to reach sexual maturity, and the female usually laid only 2-4 eggs at a time (Tagunu *et al*., 2018). The growth and reproductive rate of the Indonesian box turtle was relatively slow compared to the invasive Red Eared Slider (*Trachemys scripta elegans*), which could lay up to 30 eggs a year (GISD, 2021). Males reach sexual maturity at a carapace length of 13 cm (Schoppe and Das, 2011) with a slightly more concave plastron, longer, thicker tail, and larger claws when mature (Barbour and Ernst, 1995).

More information is needed about turtle reproduction, especially about the peak of turtle reproduction, to maintain turtle populations through ex-situ breeding program. Studies on male reproduction of Indonesian box turtle are still rare. The gross anatomy of the reproductive organ of mature Indonesian box turtles has been studied (Ruyani *et al*., 2007). However, the histology of the seminiferous tubules of Indonesian box turtles of various ages has not been studied. The age of wild-caught turtles could be determined by the straight carapace length (SCL) as a standard measure. The SCL consisted of the distance from the notch of the nuchal scute to posterior most scute tip (Kobayashi *et al*., 2010). Therefore, this study aims to determine the sexual maturity of Indonesian box turtle based on straight carapace length(SCL).

MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (No. 2.KE.051.07.2020). Six Indonesian box turtles with different SCL sizes were purchased from the reptile market in Surabaya. Turtles were sexualy dimorphic; themale has a longer, thicker tail and concave plastron (Tiar-Saadi *et al*., 2022).

Necropsy and sample collection

The first author has been trained in an exotic animal necropsy workshop. The turtles were euthanized using the chloroform per-inhalation method, then decapitated using a sharp blade (McArthur *et al*., 2004). The testes were collected to measure diameter (cm) and weight (grams) measurement and stained histological preparations with Hematoxylin Eosin. The histological slides were evaluated under a light microscope (Nikon H600L equipped with Nikon Coolpix camera and Optilab) at 400x magnification. Tubular diameter and spermatogenic cell count were measured for five fields of view on each slide, then averaged.

Data analysis

The normality of parameter distribution was analyzed using Kolmogorov-Smirnov. Pearson correlation of SCL to testicular weight, testicular diameter, seminiferous tubules diameter, and spermatogenic cell count was analyzed using Regression Anova. All data analyses used Statistical Product and Service Solutions (SPSS) software version 23 for Windows at a 95% of confidence level.

RESULTS

Gross anatomy of the reproductive system of male Indonesian box turtles showed testes, epididymis, urinary bladder, kidneys, and hemipenis (Figure 1). Anatomical topography of the Indonesian box turtle showed testes, kidneys, and urinary bladder (Figure 2). Histological testes of Indonesian box turtle showed the seminiferous tubules and the spermatogenic cells comprised of spermatogonia, spermatocytes, and spermatids (Figure 3).

The data distribution of SCL, testicular diameter, testicular weight, tubular diameter, and spermatogenic cell count were normal ($p > 0.05$). The results showed the weight and diameter of the left and right testes (Table 1), as well as the number of spermatogenic cells and the diameter of the seminiferous tubules (Table 2) of Indonesian box turtles based on SCL size. There was a significant correlation ($p < 0.05$) of SCL with testicular weight, testicular diameter, seminiferous tubule diameter, and spermatogenic cell count (Figure 4). There was a significant ($p < 0.05$) regression equation of testicular weight (TW), testicular diameter

(TD), seminiferous tubule diameter (STD), and spermatogenic cell count (SCC) based on straight carapace length (SCL) as predictors (Table 3).

Figure 1 Gross anatomy of the male Indonesian box turtle reproductive system (17.5 cm straight carapace length, SCL); T: Testes; E: epididymis; UB: urinary bladder; K: kidney; HP: hemipenis

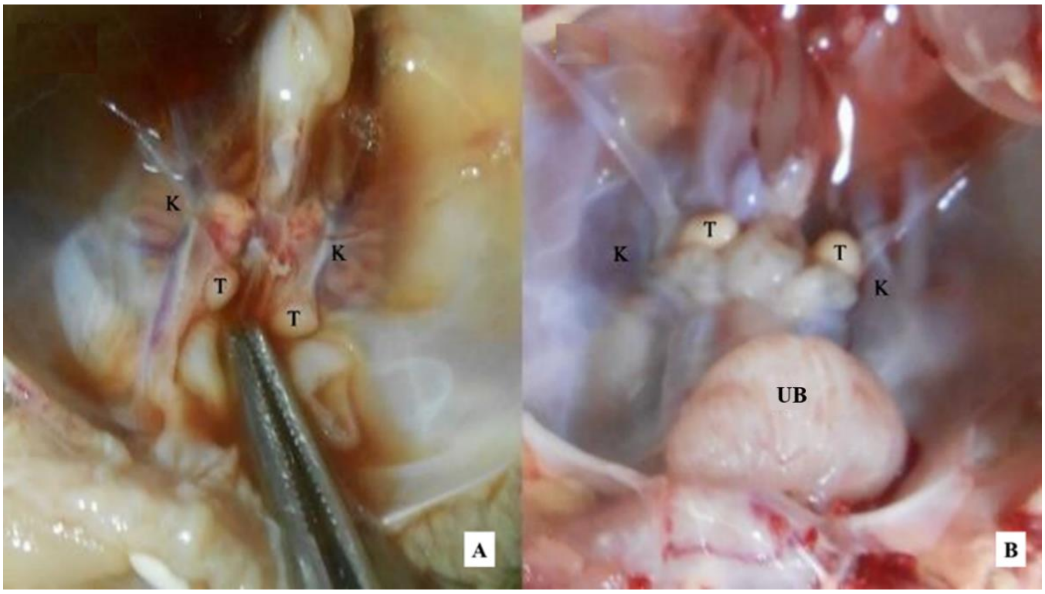

Figure 2 Anatomical topography of the Indonesian box turtle; A: 10 cm straight carapace length (SCL); B: 15 cm SCL; T: testes; K: kidney; UB: urinary bladder.

Table 1 Testicular weight and testicular diameter of Indonesian box turtle based on straight carapace length (SCL) size

SCL (cm)	testicular weight (gram)		testicular diameter(cm)	
	left	right	left	right
10	0.04	0.05	0.38	0.35
12.5	0.08	0.07	0.42	0.40
15	0.12	0.12	0.59	0.65
17.5	0.21	0.17	0.83	0.90
19	0.37	0.28	1.49	0.97
21	0.75	0.72	1.62	1.65

Table 2 Spermatogenic cell count and seminiferous tubule diameter of Indonesian box turtle based on straight carapace length (SCL) size

SCL (cm)	spermatogenic cell count		seminiferous tubules diameter (cm)	
	left	right	left	right
10	43.1	37.9	6.9	8.6
12.5	58.7	47.9	8.9	9.3
15	116.6	133.7	51.2	51.2
17.5	142.9	131.6	46.8	47.0
19	238.7	213.7	67.2	80.4
21	258.1	266.6	178.8	165.6

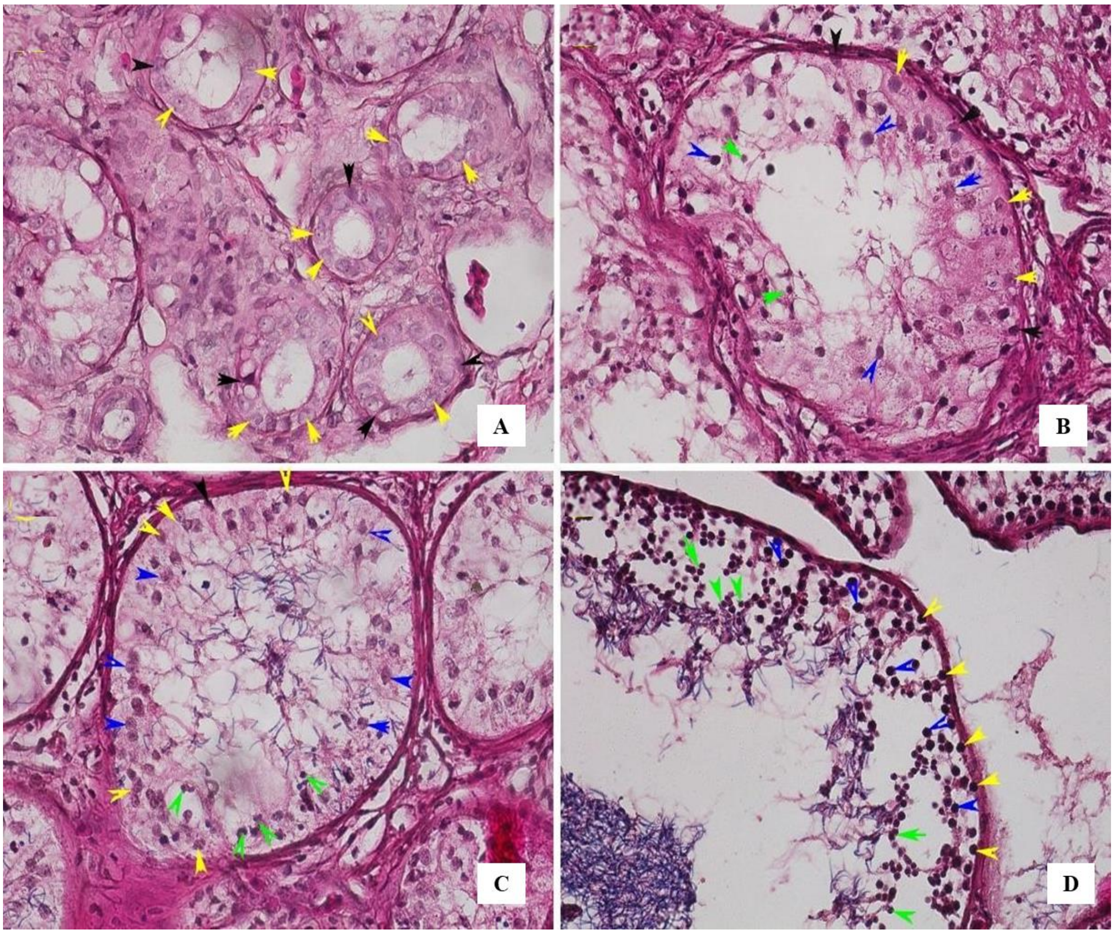

Figure 3 Spermatogenic cells in the seminiferous tubules of the Indonesian box turtles based on straight carapace length (SCL); A: 12.5 cm SCL; B: 15 cm SCL; C: 17.5 cm SCL; D: 21 cm SCL; showing spermatogonia (yellow arrows), spermatocytes (blue arrows), spermatids (green arrows), and Sertoli cells (black arrows); Hematoxylin Eosin staining preparations under a light microscope (Nikon H600L) with 400x magnification.

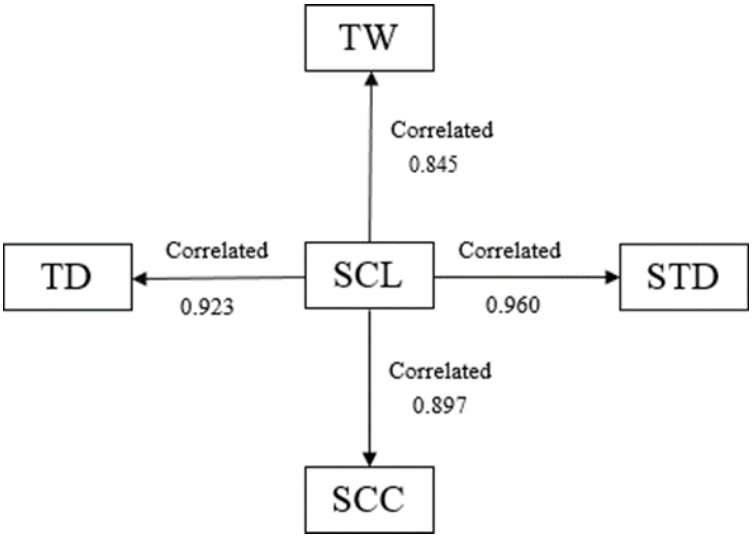

Figure 4 Pearson correlation of straight carapace length (SCL) with the testicular weight (TW), testicular diameter (TD), seminiferous tubule diameter (STD), and spermatogenic cell count (SCC); the numbers indicated the coefficient of correlation (r) ($p < 0.05$).

Table 3 Regression equations as SCL based predictors for testicular weight, testicular diameter, seminiferous tubule diameter, and spermatogenic cell count

parameters	equation	p-value	R2 (%)
TW	-0.59 + 0.05 SCL	0.001	0.714
TD	-0.96 + 115.00 SCL	0.003	0.852
STD	-189.91 + 20.89 SCL	0.000	0.922
SCC	-120.02 + 11.10 SCL	0.002	0.805

TW: testicular weight; TD: testicular diameter; STD: seminiferous tubules diameter; SCC: spermatogenic cell count; SCL: straight carapace length**.**

DISCUSSION

The testicular morphology of Indonesian box turtles with SCL sizes of 10 cm and 12.5 cm were spheroid in shape and more translucent in color. These results were in line with the results of a study on the morphological description of immature Magdalena River turtle (*Podocnemis lewyana*) (Sanchez-Ospina *et al*., 2014). However, it was different from the mature turtles that were spread on Central Sulawesi (*Cuora amboinensis*, *Leucocephalon yuwonoi,* and *Indotestudo forstenii*) which were ovoid in shaped and yellowish in color (Tagunu *et al*., 2018). The topographic anatomy study showed that testes was not always located on the anterior parts of the kidney, as theorized by Radiopoetra (1991). In this study, testes were on the posterior side of the kidney at 10 cm and 12.5 cm SCL sized turtles. Testicular diameter in this study varied between 0.35-1.65 cm and correlated with SCL sizes. This result was in line with the correlation between turtle SCL size and testicular diameter of immature green turtle (*Chelonia mydas*) (Otsuka *et al*., 2012). Variation in the size of the adult testis could be caused by the environment. SCL could distinguish adults from juvenile *Lepidochelys kempii* sea turtles (Craven *et al*., 2019). It was expected that developmental variations in the turtle would be useful for phylogenetic studies and distinguishing the reproductive status of turtle species (Olukole *et al*., 2018). For example, the testis of the African sideneck turtle (*Pelusios castaneus*) were attached to the peritoneal wall posterior to the ventrolateral kidney. The testes were yellow, smooth and ovoid in shape (Olukole *et al*., 2014). Meanwhile, the testes of *Trachemys scripta elegans* turtle were oval in shape, bright yellow in color, and were located cranially to the epididymides and caudally to the kidneys (Gradela *et al*., 2019).

The testicular histological features also differ based on the SCL size of the Indonesian box turtle. The differences were mainly in the diameter of the seminiferous tubules and the interstitial tissue between the tubules. These differences have been categorized into six stages based on the feature of interstitial tissue and the degree of widening of the seminiferous tubules in *Chelonia mydas*. Testes categorized in the first stage had indistinct seminiferous tubules with no luminal spaces. In the second stage, each seminiferous tubule had a luminal space and became distinguishable from the interstitial tissue, which contained Leydig cells with many lipid droplets. Interstitial connective tissue began to develop in the third stage, and Leydig cells with fewer lipid droplets than those in the second stage gathered closer and formed clusters. In the fourth stage, the interstitial connective tissue showed an edematous change and contained Leydig cells with fewer lipid droplets than in the third stage. In the fifth stage, widening in seminiferous tubules and reduction in interstitial connective tissue were seen. In the sixth stage, seminiferous tubules were further expanded, and interstitial connective tissue was only observed in the small spaces among the tubules (Otsuka *et al*., 2012). The seminiferous of *Trachemys scripta elegans* turtle comprised of Sertoli and spermatogenic cells, while Leydig cells in the interstitial tissue surrounding the seminiferous tubules (Gradela *et al*., 2019).

There was a correlation between the SCL of turtles and the number of Spermatogenic cell. The process of spermatogenesis differed based on the size of the SCL. The process of spermatogenesis was divided into some stages. In the first stage, the seminiferous tubule had a single layer of Sertoli cells lining the epithelium with spermatogonia interspersed. In the second stage, spermatogonia proliferated until it became several layers in the seminiferous tubule. In the third stage, several cell layers of primary spermatocytes began to appear. In the fourth stage, the seminiferous tubules have more primary than secondary spermatocytes, also spermatozoa and spermatid. In the fifth stage, the secondary spermatocytes became more abundant, with fewer primary spermatocytes. In the sixth stage, seminiferous tubules appear with primary spermatocytes, abundant secondary spermatocytes, spermatids, and a lumen full of spermatozoa (peak of spermatogenesis). In the seventh stage, there were fewer spermatids, more spermatozoa with a mixture of debris clumping in the center of the lumen and the appearance of clearing out of spermatozoa (Olukole *et al.*, 2013). Previous study reported that the SCL in the sexual maturity of male Indonesian box turtles was different compared to male Indonesian box turtles which reached sexual maturity at 13 cm (Schoppe and Das, 2011). However, in this study, based on the histology of the seminiferous tubules, male Indonesian box turtles began to mature sexually at 15 cm SCL size, marked by widening of the seminiferous tubule diameter and the development of spermatogenic cells, and spermatozoa began to appear at 17.5 cm SCL sizes. The difference in size at maturity was also reported in Loggerhead Turtles (*Caretta caretta*) between the north and south pacific (Ishihara and Kamezaki, 2011).

Spermatogenesis in turtles was influenced by many factors, and the spermatogenetic cycle was an intrinsic factor. Spermatogenesis in turtles was described as one of three patterns: prenuptial, postnuptial (dissociated pattern), and acyclic. In the prenuptial pattern, spermatogenesis immediately precedes mating. In postnuptial spermatogenesis, spring mating used spermatozoa produced in the previous summer and stored in the epididymides over winter during a sexually quiescent phase. While, in the acyclic, spermatogenesis was a continual production of spermatozoa without a defined peak or quiescence (Chaves *et al.*, 2017). Environmental factors have also been observed to impact spermatogenesis (Marn *et al.*, 2017). The spermatogenic cycle of the African sideneck turtle (*Pelusios castaneus*) was a pattern of the postnuptial spermatogenesis based on samples collected at different times (Olukole *et al.*, 2014).

SCL size of the Indonesian box turtle had a robust positive correlation (r= 80-100) to all parameters in this study. A correlation coefficient (r) of 0.80-1.0 meant a robust correlation (Sen and Srivastava, 2011; Rouaud, 2017). Our findings showed that testicular weight, testicular diameter, seminiferous tubule diameter, and spermatogenic cell count could be predicted based on the straight carapace length of the Indonesian box turtle (*Cuora amboinensis couro*). The correlation between SCL and testicular length and histology concluded that SCL was a significant predictor of the reproductive state of Kemp's Ridley Sea turtle (*Lepidochelys kempii*) (Craven *et al.*, 2019).

CONCLUSION

The size of the SCL correlated strongly with the parameters of the primary reproductive organ development of the Indonesian box turtles (*Cuora amboinensis couro*). In the mature Indonesian box turtle (*Cuora amboinensis couro*), the SCL reached 15 cm. Therefore, these findings could be used to predict the reproductive status and sexual maturity of sea turtles for ex-situ captive and conservation programs.

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