Title

Determination of maturity in male hawksbill turtle *Eretmochelys imbricata* in captivity based on tail elongation and plasma testosterone level

Running title

Determination of male maturity in hawksbill turtles

Masato Kobayashi,[1, 2*] Tomohito Shimizu,[3] Koichi Okuzawa,[4] Kiyoshi Soyano[2, 5] AND Kenzo Yoseda[6]

[1] *Yaeyama Station of the Stock Enhancement Technology Development Center, Seikai National Fisheries Research Institute, Fisheries Research Agency, Ishigaki, Okinawa 907-0451* [2]*Graduate School of Science and Technology, Nagasaki University, Nagasaki, Nagasaki 852-8521,* [3]*Chitose Field Station, National Salmon Resources Center, Fisheries Research Agency, Chitose, Hokkaido 066-0068,* [4]*National Research Institute of Aquaculture, Fisheries Research Agency, Minami-ise, Mie 516-0193,* [5]*Institute for East China Sea Research, Nagasaki University, Nagasaki, Nagasaki 851-2213,* [6]*National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Hatsukaichi, Hiroshima 739-0452, Japan*

* Corresponding author:

Tel: 81-980-88-2136

Fax: 81-980-88-2138

Email: masakoba@affrc.go.jp

Abstract

To determine the sexual maturity of fourteen male wild-caught hawksbill turtles [straight carapace length (SCL) range 63-79 cm) held in captivity, we have investigated their hormone levels in blood and morphological characteristics. Male turtles were divided into two groups: five individuals showing mating behavior (group A) and nine individuals not showing mating behavior (group B). Then, seasonal changes of plasma testosterone of both groups were studied. We also tried to determine male maturity based on tail elongation (TE) index, i.e., the ratio of tail length (TL) to SCL. Plasma testosterone level of group A gradually increased during premating, and then sharply declined to low levels between mating and postmating seasons. In contrast, that of group B remained low during the experimental periods. The distinct seasonal changes of plasma testosterone levels of group A and B correlated with their maturity. Furthermore, males were clearly divided into mature and immature based on TE values > 0.35 and < 0. 33, respectively, corresponding to results based on plasma testosterone levels. Our results indicate that TE can be used more conveniently and accurately to determine maturity of male hawksbill turtles in captivity.

Keywords hawksbill turtle・testosterone・reproductive cycle・secondary sexual characteristic・tail elongation

Introduction

The hawksbill turtle *Eretmochelys imbricata* is found throughout tropical and subtropical coral reef regions of the world [1]. In Japan, this species is seen in areas south of the Izu Peninsula on the Pacific side and Noto Peninsula on the Japan Sea side, while the northern limit of nesting occurs in the Nansei Islands [2]. Hawksbill turtles have been captured not only as a source of protein but also raw materials for ornaments, because their carapace scutes are richly colored and esthetically beautiful.

Their populations have decreased worldwide in recent years, and the hawksbill turtle has been listed as an endangered species by the International Union for Conservation of Nature (IUCN) since 1968; its status was reevaluated to critically endangered in 1996 [3]. Capture of sea turtles and their eggs is restricted to protect them in many countries around the world, and the international trade in sea turtles is prohibited entirely by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [4]. As countermeasure to the decline in populations, head-starting programs have been attempted to enhance decreasing wild populations of sea turtles, including the hawksbill turtle [5-8].

Yaeyama Station of the Stock Enhancement Technology Development Center (SETDC), Seikai National Fisheries Research Institute, Fisheries Research Agency has been studying propagation of the hawksbill turtle for stock recovery since 1999, and has started to develop captive breeding technology, especially obtaining fertilized eggs under rearing conditions [9-11].

Information on reproductive endocrinology and biology is essential to develop such captive breeding technology. Among reptiles, birds, and mammals, testosterone is

usually the major end-product of gonadal steroidogenesis in males [12]. It is known that testosterone is the major such product synthesized by turtle [13]. In sea turtles, there have been several reports indicating that testosterone plays important roles in male reproduction. In captive green *Chelonia mydas* [14] and Kemp's ridley *Lepidochelys kempii* [15] sea turtles and wild loggerhead sea turtle *Caretta caretta* [16, 17], the elevation of circulating testosterone was well correlated with spermatogenesis. Owens and Morris [18] describe that the penis and the tail of immature sea turtles elongate and precocious mating attempts occurred when testosterone was injected. Those studies strongly support that testosterone is the major sex steroid related with secondary sexual characteristics and sexual behavior in male sea turtles. Thus, testosterone is considered to be an appropriate indicator of male sexual maturity in sea turtles. It is well known that the tail of male turtles, which is a prominent secondary sexual characteristic, elongates to become longer than females [19, 20]. Limpus [20] reported that it is possible to identify mature male hawksbill turtles in the wild inhabiting the southern Great Barrier Reef using tail length based on gonadal morphogenesis observed by laparoscopic diagnosis. van Dam [21] also divided adult males from others, examining 276 hawksbill turtles in the Caribbean Sea based on tail length following the method of Limpus [20]. In hawksbill turtles, no study has been conducted to examine seasonal changes of plasma testosterone levels in captivity or the wild throughout a year. Thus, the relationships between external sexual morphological characteristics and reproductive endocrinology remain unclear.

To develop a successful breeding program for the hawksbill turtle under rearing conditions, it is important to understand whether males and females are mature or not. In the present study, to determine male maturity more conveniently and

accurately, we investigated seasonal changes of plasma testosterone levels and external sexual morphological characteristic of tail elongation in 14 male hawksbill turtles in captivity between 2007 and 2008. Finally, we identified mature males based on whether they could mate functionally with a female.

Materials and methods

Experimental turtles

A total of 14 male and 11 female hawksbill turtles were legally captured in the waters off Ishigaki Island, of which 4 males were captured in 1999, 6 in 2000, and 4 in 2001, and for females 7 in 1999, 1 in 2000, 1 in 2001, and 2 in 2002. They were transferred to tanks at SETDC immediately after being caught. The straight carapace length (SCL) of each turtle was measured at capture. Mean ± standard deviation (SD) SCL of males and females was 53.0 ± 11.1 cm and 56.0 ± 10.4 cm, respectively. The range of SCL in 14 males was 37.5-82.0 cm. One male was 82.0 cm in SCL and the other males were under 62.5 cm in SCL. The minimum SCL for adult males was 68.2 cm in wild Caribbean population [21]. Therefore, 13 individuals used in this study were estimated as immature and 1 male as sexually maturity judged by size at capture, even though the inhabited area is different from that mentioned above. The sex of captured turtles was identified by laparoscopic diagnosis in 2001, which could determine whether they had testes or ovaries. Also, they were tagged with an outer tag (Jumbo Tag; Sea Turtle Association of Japan, Osaka, Japan) and an inner microchip tag (ID-100A; Surge Miyawaki Co., Ltd., Tokyo, Japan) for individual identification.

Experimental design

We visually observed mating behavior in 110-kl tanks from March to June in 2007 and 2008. In this study, we defined an action when a male tried to mate with a female as a

mating behavior. In addition, if the female laid fertilized eggs after mating, the mating was defined as a functional. During observations, males and sexually mature females were placed in tanks at ratio of 1-7 to 1. These turtles were maintained in the same tanks during daylight, and males and females were maintained in separate tanks during the night. Thus, mating behavior was observed during daylight. When plural males tried to mate with one female, males were individually separated. Thereafter, we conducted the experiment again under the condition of one male and one female in a tank. When a female succeeded in mating, we never used the female for any other mating experiments in the season. Therefore, mating of a female was restricted to once a year. As a result, 5 of the 14 males exhibited mating behavior, and 9 males did not exhibit mating behavior at all in either 2007 or 2008. Thus, we tentatively grouped them into two groups: the former (exhibiting mating behavior) as group A and the latter as group B.

Rearing method

We rear turtles using the method described by Yoseda and Shimizu [9]. They were maintained in tanks with volume of 60-400 kl. Sand-filtered seawater was constantly provided to all tanks at a rate of 2-10 kl/h. They were exposed to ambient photoperiod, weather conditions, and seawater temperature. Anchovy *Engraulis japonicus* and squid *Illex argentinus* were fed to them 3-5 times a week, once a day. The quantity of feed was based on 1-2% of the body weight of turtle, and vitamins and calcium powder at quantity of 2.5% of feed weight of turtle were dusted onto the feed as additional nutrients.

Blood sampling and hormone analysis

Plasma testosterone analysis was conducted in 2007 and 2008. Blood sample was collected once a month. Ten milliliters of blood was obtained from the cervical sinus of each turtle using a 1.2 x 70 mm needle and nonheparinized syringe. Blood samples were immediately transferred to heparin-treated blood collection tubes and kept on ice until the samples were centrifuged. Blood samples were centrifuged for 10 min at 2500 rpm at room temperature, and the plasma was frozen and kept at - 80°C until hormone assay. The concentration of total testosterone in the plasma sample was analyzed by chemiluminescent immunoassay with ARCHITECT system (i2000; Abbott Diagnostics, Abbott Park, IL, USA). The analyses were conducted with ARCHITECT Testosterone kit (ARCHTECT Testosterone; Abbott Japan Co., Ltd., Chiba, Japan) by BML Inc. (Okinawa, Japan). The range of measurement was 0.14-15 ng/ml. The testosterone antibody used in the assay cross-reacted with 5α-dihydrotestosterone at 2.1%, 5α-androstane-3β,17β-diol at 0.2%, 11β-dihydroxytestosterone at 14.1%, and androstenedione at 0.1%.

Size measurements

Size measurements of 14 turtles were conducted monthly in 2007 and 2008, except February 2007. Their straight carapace length (SCL) and tail length (TL) were measured to the nearest 0.1 cm using Vernier caliper (MA1270BLUE; Haglöf Inc., Långsele, Sweden). SCL consisted of the distance from the nuchal scute notch to the posteriormost scute tip, and TL was measured as the distance from the posteriormost

plastron to the tail tip (Fig. 1). In this study, we used tail elongation (TE) index, defined using the formula TE = TL/SCL, to evaluate male maturity based on external sexual morphological characteristics.

Statistics

Difference in plasma testosterone level, SCL, TL, and TE between the two groups were analyzed by two-factor factorial analysis of variance (ANOVA) followed by the Tukey-Kramer multiple-comparison test at 5% significance level using the STATCEL2 program (4 steps Excel Toukei; OMS Publishing Inc., Saitama, Japan) , an add-in to the Excel software (Excel 2002; Microsoft Corp., Tokyo, Japan). Pearson's correlation coefficient test was used to examine the similarity of seasonal changes of plasma testosterone in each group between 2007 and 2008, was analyzed at 5% significance level using the STATCEL2 program.

Results

Seasonal changes in plasma testosterone level and mating behavior

Seasonal changes in plasma testosterone levels in 2007 and 2008 are shown in Fig. 2. Seasonal changes in plasma testosterone levels of group A in both years exhibited significantly high correlation by Pearson's correlation coefficient test ($F = 4.0$, $r = 0.79$, $p < 0.05$). In 2007, mean plasma testosterone levels in group A increased from January (mean ± SD, 30.7 ± 12.1 ng/ml) to May (mean ± SD, 44.6 ± 9.0 ng/ml). Then the level sharply declined and reached a minimum in August (mean ± SD, 6.8 ± 2.2 ng/ml), and then began to increase again from November. In 2008, the level increased from January (mean ± SD, 25.0 ± 8.6 ng/ml) to May (mean ± SD, 37.4 ± 14.0 ng/ml), and then gradually deceased from June to October (mean ± SD, 3.5 ± 1.3 ng/ml). The level began to increase again from November in the same manner in 2007.

In group B, seasonal changes of plasma testosterone levels in both years exhibited significantly high correlation by Pearson's correlation coefficient test ($F = 3.9$, $r = 0.78$, $p < 0.05$), as in group A. In contrast to group A, the plasma testosterone level remained low throughout the year. The levels ranged from 1.2 ± 0.6 ng/ml (mean ± SD) in April to 4.8 ± 2.3 ng/ml (mean ± SD) in August in 2007. The following year, a similar seasonal pattern of plasma testosterone levels was observed.

Mating behavior occurred in May in both 2007 and 2008. Three males in total succeeded in functional mating (Table 1); M-1 and M-2 mated in 2007, and M-2 and M-3 mated in 2008. The testosterone level of the 3 males ranged from 17.8 to 52.5 ng/ml in May. In contrast, M-4 and M-5 did not succeed in functional mating. They exhibited

mating behavior, but they were immediately removed from the female body. Their exhibited duration of mating behavior was shorter than that of the mating behavior which resulted in fertilization.

Development of morphological characteristic of SCL, TL, and TE

Mean SCL of hawksbill turtles at capture and annual means of SCL, TL, and TE of all males in 2007 and 2008 are presented in Table 2. Annual means of SCL, TL, and TE of each individual in 2007 and 2008 are plotted in Fig. 3. The mean SCL values of group A and B were not significantly different ($p > 0.05$) in either 2007 or 2008 (Table 2), and SCL overlapped between the two groups (Fig. 3). Mean TL of group A was significantly longer than that of group B in each year (Table 2, two-factor factorial ANOVA, Tukey-Kramer multiple-comparison test, $p < 0.05$), but the minimum TL in group A and the maximum TL in group B were similar (Fig. 3). TE of group A was significantly higher than that of group B (Table 2, two-factor factorial ANOVA, Tukey-Kramer multiple-comparison test, $p < 0.05$), the minimum of group A and the maximum of group B were clearly divided, the former being 0.35 and the latter being 0.33 in 2008 (Fig. 3).

Relationships of plasma testosterone level with TL and TE

The relationships of plasma testosterone level with TL and TE are shown in Figs. 4 and 5, respectively. Individuals which showed higher plasma testosterone levels had longer tails (Fig. 4) and larger TE (Fig. 5). Plasma testosterone level and TL of group A were

significantly higher than those of group B in 2007 (two-factor factorial ANOVA, Tukey-Kramer multiple-comparison test, $p < 0.05$), but a part of standard deviation of mean TL in both groups overlapped (Fig. 4). In contrast, plasma testosterone level and TE of group A were significantly higher than those of group B in 2007 (two-factor factorial ANOVA, Tukey-Kramer multiple-comparison test, $p < 0.05$), and standard deviation of mean TE in both groups did not overlap. The following year, plasma testosterone level, TL, and TE in group A were significantly higher than those of group B, in the same manner as in 2007.

Discussion

In this study, 14 male hawksbill turtles in captivity were divided into two groups: 5 turtles of group A and 9 turtles of group B based on observation whether they exhibited mating behavior or not in 2007. The maturity of each turtle, however, was not verified at that time. Consequently, we investigated plasma testosterone levels of the 14 males to determine their maturity. Plasma testosterone levels in group A began to increase from November in both 2007 and 2008, reaching a peak in April or May of 2007 and 2008, respectively, and thereafter decreasing to low levels. Mating behavior was observed in all males of group A. In contrast, plasma testosterone levels in group B remained low throughout the year in both years, and no males in group B showed mating behavior.

It is reported that testosterone levels of wild loggerhead sea turtles [16, 17] and green sea turtles [22] begin to increase during premating, and then increase significantly to peaks during the premating and mating period, thereafter decreasing to the basal level. It is known that plasma testosterone levels of Kemp's ridley sea turtles and green sea turtles in captivity increase prior to onset of mating activity [14, 15]. Also, it was reported that the testis was developed with a sharp rise of testosterone level before the mating season in captive Kemp's ridley sea turtle [15]. Jessop et al. [22] reported that testosterone level in immature males was lower than in adult males of wild green sea turtles and hawksbill turtles, and also that testosterone in nonbreeding adult male green sea turtles remained at the basal level all year around. We present herein, for the first time, annual changes of plasma testosterone levels in male hawksbill turtles, and our results for mature and immature turtles correspond with those of previous studies. These results indicate that the testosterone level in mature male turtles markedly increases

during premating, thereafter declining to a low level, while that of immature male turtles exhibits basal level throughout the year. Therefore it can be concluded that such hormonal variation of testosterone is a common phenomenon among sea turtle species.

There is some interesting knowledge about induction of endocrinology in testosterone. Owens and Morris [18] describe that precocious mating attempts occurred in immature sea turtles when they were injected with testosterone. This suggests that testosterone is one of the most import hormones to induce male maturity. Thus, testosterone level is considered as an effective indicator to determine male maturity in sea turtles. In this study, annual testosterone profiles confirmed that males of group A were mature and that males of group B were immature. However, this indicator is not appropriate for determination of male maturity during fall and winter seasons because the testosterone exhibited the basal level not only in mature but also in immature males. Therefore these sampling seasons should be avoided.

We also tried to divide the 14 males into mature and immature individuals using external morphological characteristics, namely SCL, TL, and TE. As the SCL of mature and immature males overlapped, it was impossible to judge maturity of turtles by SCL. The TL of them did not overlap, but the shortest of the mature group and the longest of the immature group were similar. Limpus [20] reported that it is possible to identify adult males based on TL in wild hawksbill turtles inhabiting the southern Great Barrier Reef. van Dam [21] also divided adult males from others among 276 hawksbill turtles in the Caribbean Sea based on TL following the description of Limpus [20]. However, it was rather difficult to determine male maturity using TL in our case. This inconsistency may be related to the origin of the hawksbill turtle, i.e., the wild or captivity, between which there is quite a difference in terms of feed, habitat

environment, etc. In contrast, TE values of mature and immature individuals were well separated; it is possible to divide mature and immature males using 0.35 of TE as a criterion. Furthermore, TE value and plasma testosterone level clearly divided males into mature and immature, being > 0.35 and 13.9 ng/ml versus < 0.33 and 4.6 ng/ml, respectively, in captive hawksbill turtles. TE of mature and immature male hawksbill turtles were clearly separated, the minimum difference between them being 0.02 in this study. van Dam [21] suggested that the tail develops rapidly in maturing male hawksbill turtles, because there was a significant difference between the range of TL of adult males and that of the others in the Caribbean Sea. Thus, the reason for the well-separated TE values of mature versus immature is provably the same as mentioned above in captivity. When tails of some male turtles in group B elongate and reach TE at 0.35, they must be sexually matured.

In conclusion, we reveal for the first time that TE can be used as a convenient and accurate indicator to determine sexual maturity of male hawksbill turtles in captivity. Furthermore, determination of male maturity based on the plasma testosterone level is not a recommended method because hormone analysis is costly and time consuming compared with determination based on TE value. Further studies are necessary to predict when male hawksbill turtles hatched in captivity mature with growth, and to examine whether TE value can be applied to other sea turtles to determine maturity.

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Figure captions

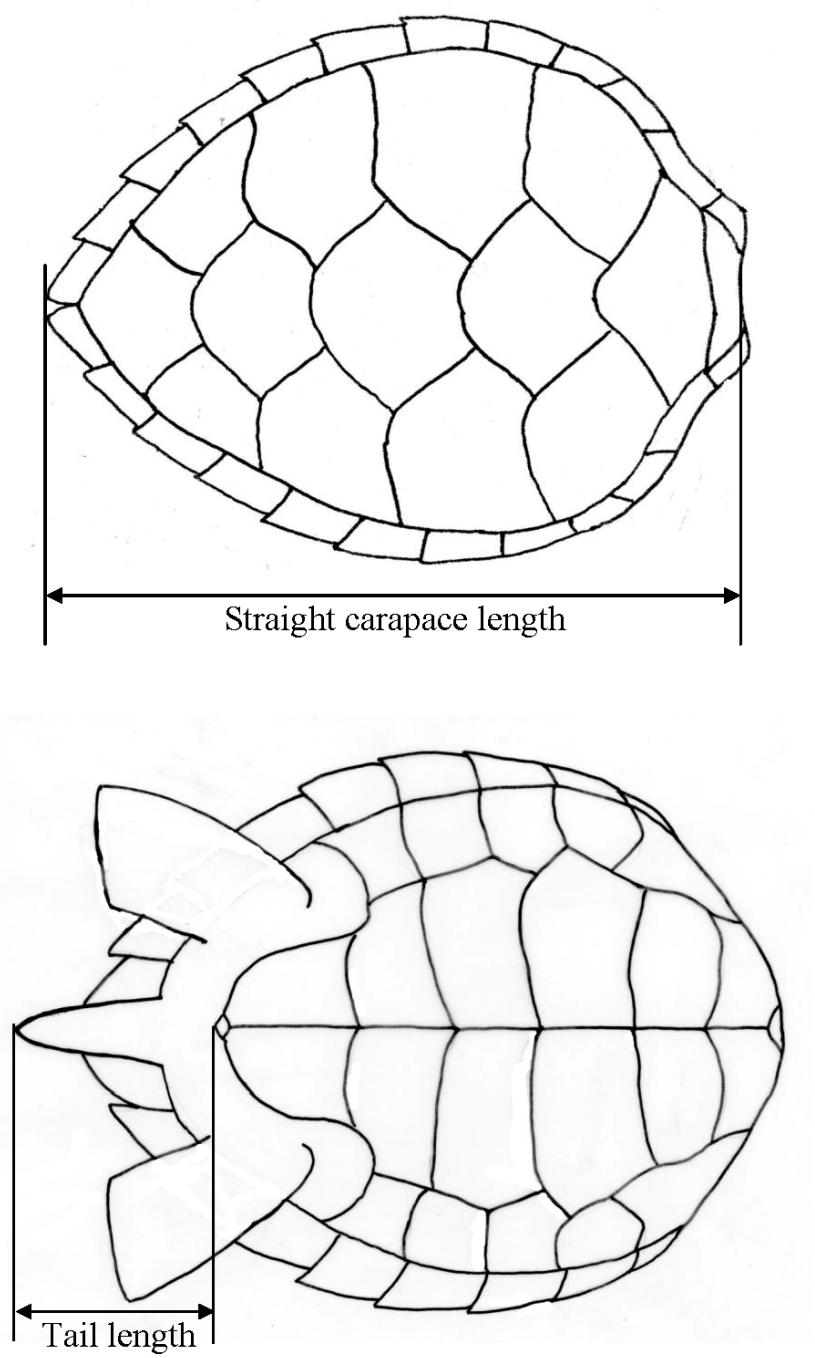

Fig. 1 Diagrams of measurement points for straight carapace length (SCL, *upper*) and tail length (TL, *lower*) used in the present study for hawksbill turtles. SCL was measured as the distance from the nuchal scute notch to posteriormost scute tip, and TL was measured from the posteriormost plastron to the tail tip.

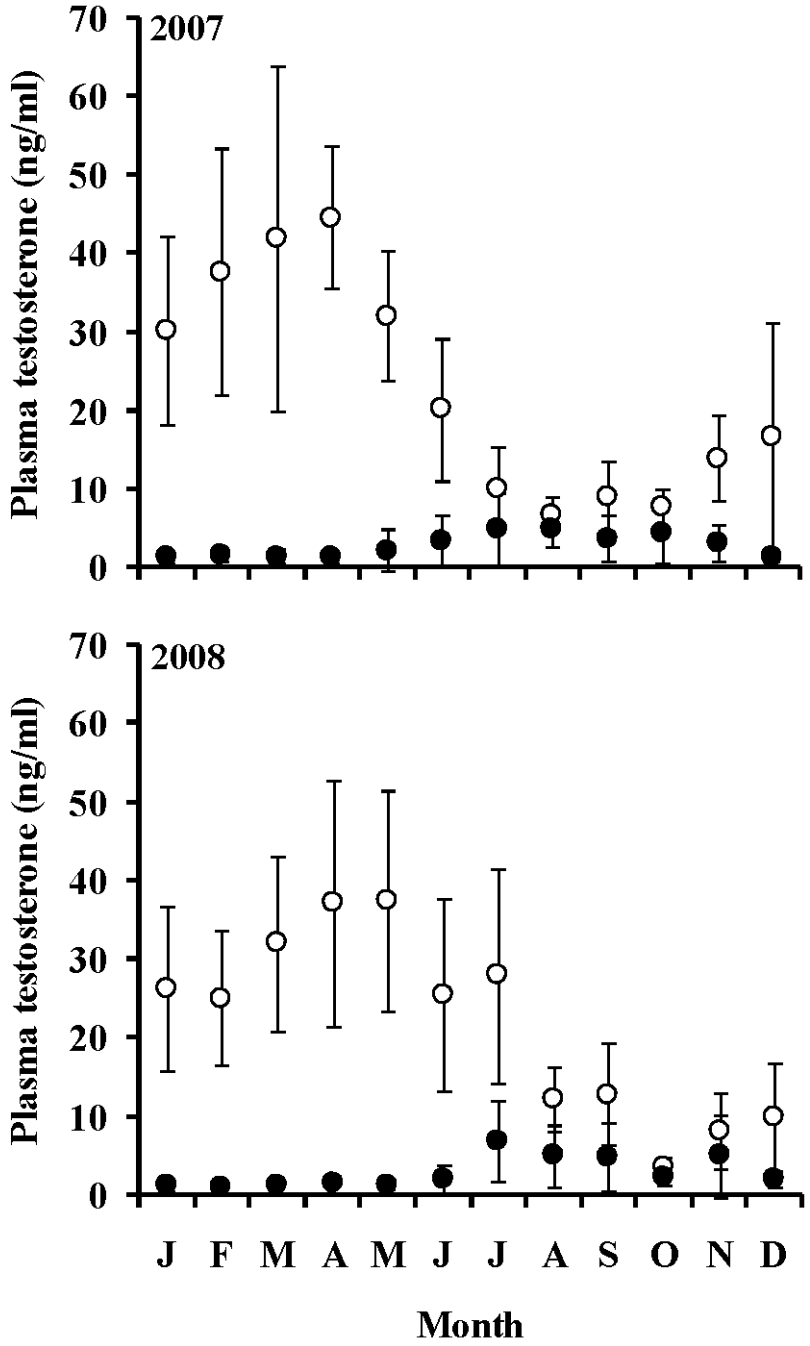

Fig. 2 Seasonal changes of plasma testosterone of group A (*open circles*) and group B (*closed circles*) in 14 hawksbill turtles between 2007 and 2008. Data are mean ± SD.

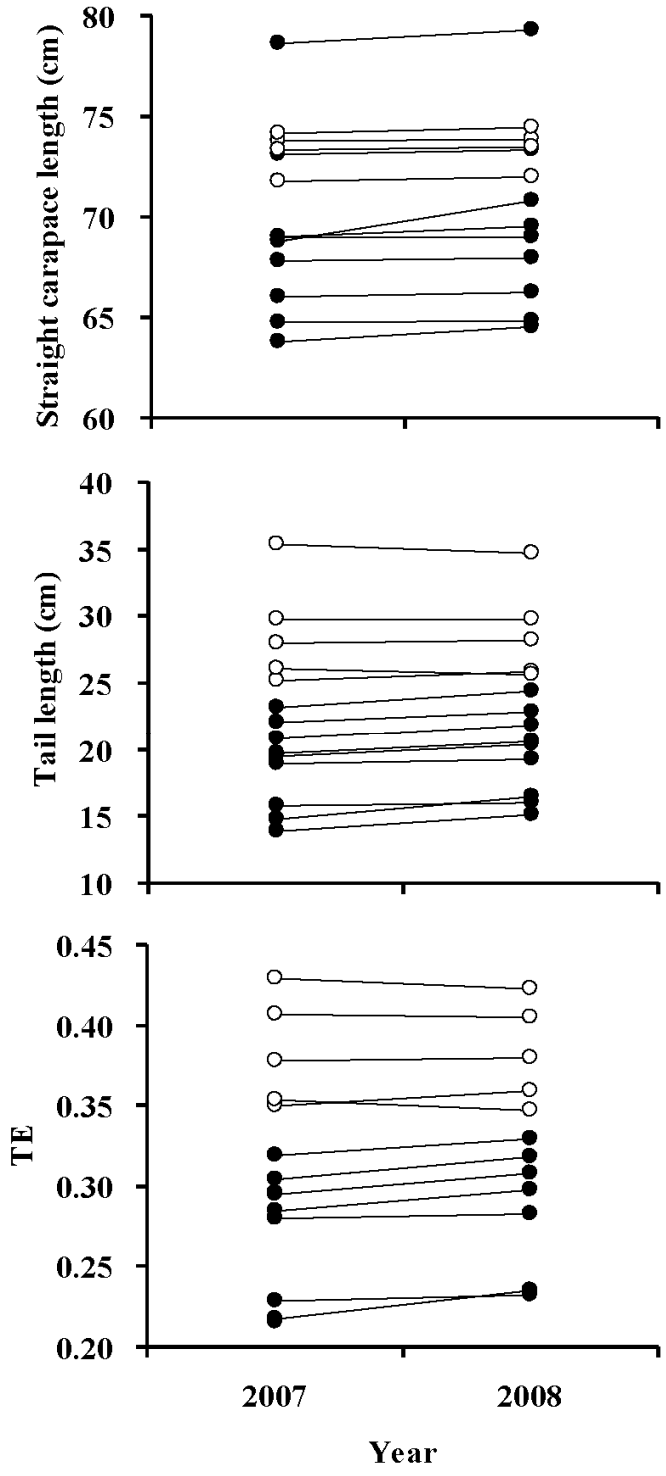

Fig. 3 Annual changes of straight carapace length (SCL, *upper*), tail length (TL, *middle*), and TE (ratio of TL to SCL, *lower*) of each individual among 14 hawksbill turtles in group A (*open circles*) and group B (*closed circles*) in 2007 and 2008.

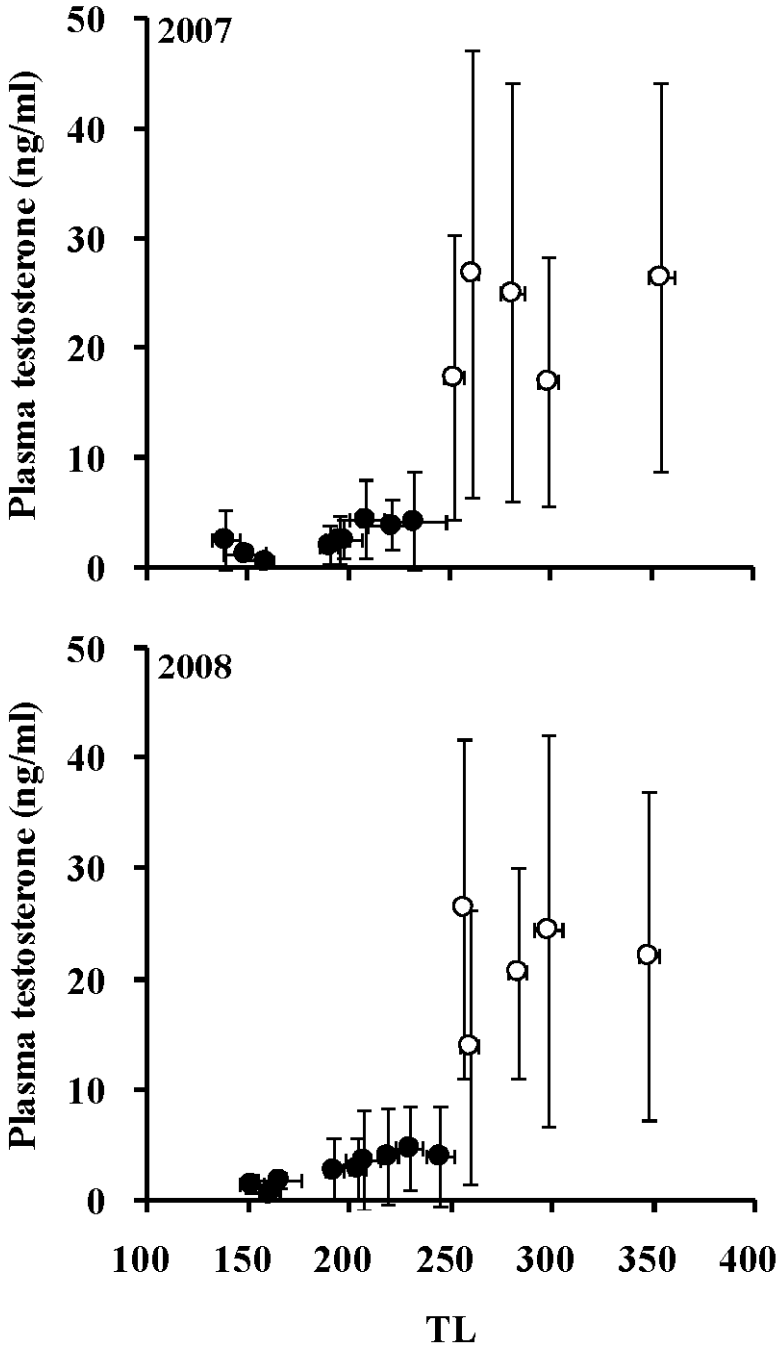

Fig. 4 Relationship between plasma testosterone level and tail length (TL) of group A (*open circles*) and group B (*closed circles*) in 14 hawksbill turtles between 2007 and 2008. Data are mean ± SD.

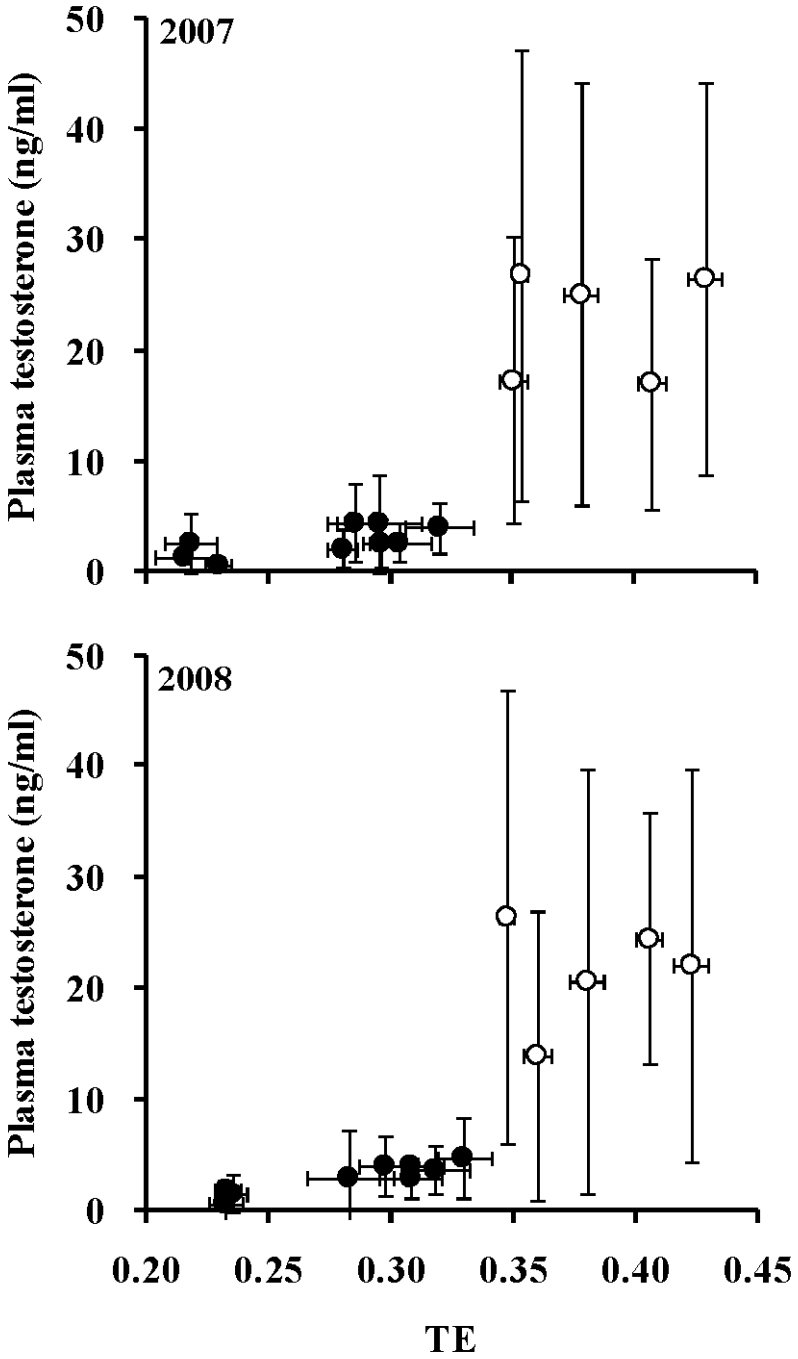

Fig. 5 Relationship between plasma testosterone level and TE (ratio of TL to SCL) of group A (*open circles*) and group B (*closed circles*) in 14 hawksbill turtles between 2007 and 2008. Data are mean ± SD.

Table 1 Results of observation of mating behavior and mating success in both groups in 2007 and 2008

	No. of turtles that displayed mating behavior		No. of turtles that succeeded in mating	
Year:	2007	2008	2007	2008
Group A	5	5	2	2
M-1	+	+	+	×
M-2	+	+	+	+
M-3	+	+	×	+
M-4	+	+	×	×
M-5	+	+	×	×
Group B	0	0	0	0
M-6	×	×	×	×
M-7	×	×	×	×
M-8	×	×	×	×
M-9	×	×	×	×
M-10	×	×	×	×
M-11	×	×	×	×
M-12	×	×	×	×
M-13	×	×	×	×
M-14	×	×	×	×

M-1 to M-14 indicate each experimental turtle. “+” and “×” symbols in the column “No. of turtles that displayed mating behavior” indicate those displaying and not displaying mating behavior, respectively. The same symbols in column “No. of turtles that succeeded in mating” indicate successful and nonsuccessful functional mating with female, respectively. In this study, functional mating means obtaining fertilized eggs after mating.

Table 2 Means SCL of male hawksbill turtles at capture, and annual means of straight carapace length, tail length, and TE of the male hawksbill turtles in 2007 and 2008

	At capture		2007		2008	
	Group A	Group B	Group A	Group B	Group A	Group B
Straight carapace length (cm)	64.2 ± 10.2	46.7 ± 5.3	75.1 ± 3.9^{ab}	69.0 ± 4.3^{ab}	75.3 ± 3.6^{cd}	69.5 ± 4.4^{cd}
Tail length (cm)	Unknown	Unknown	28.8 ± 3.6^{a}	18.8 ± 3.2^{b}	28.9 ± 3.4^{c}	19.7 ± 3.1^{d}
TE	-	-	0.38 ± 0.03^{a}	0.27 ± 0.04^{b}	0.38 ± 0.03^{c}	0.28 ± 0.04^{d}

Data are means ± SD. TE indicates the ratio of tail length to straight carapace length. Different letters represent significant difference among two groups in 2007 and 2008 (two-factor factorial ANOVA, Tukey-Kramer multiple-comparison test, $p < 0.05$, a > b, c > d).