



Assessment of male sexual maturity of longnose stingray *Hypanus guttatus* (Elasmobranchii: Dasyatidae) in Brazil

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Abstract: The reproductive activity of *Hypanus guttatus* was studied based on specimens caught in artisanal fisheries between August 2013 and October 2015 on the estuarine and coastal zone of Pernambuco, Brazil. A total of 73 males were sampled. All life cycle stages were present; mature individuals counted 83.73% of the sample. As the pair testes mature, it becomes lobulated-shaped; immature testes are undifferentiated and embedded in an epigonal matrix. The testicular width and mass increment observed in maturation stages are due to the male reproductive cycle phases (spermatogenesis, copula, and resting). Sexual maturity estimated (W_{50}) was 42.03 cm. Comparisons between macroscopic and microscopic gonad aspects were crucial to determining the seasonal reproductive activity. Mating is suggested to occur in November through March, and the species has a biannual cycle.

Key words: Dasyatidae, estuary, fishing, stingrays, reproductive cycle.

Evaluación de la madurez sexual em machos de la raya pico largo *Hypanus guttatus* (Elasmobranchii: Dasyatidae) en Brasil. Resumen: La actividad reproductiva de *H. guttatus* se examinó a partir de especímenes capturados en pesquerías artesanales entre agosto de 2013 y octubre de 2015 en el estuario y la zona costera de Pernambuco, Brasil. Se muestrearon un total de 73 machos. Todas las etapas del ciclo de vida presentes; los individuos maduros contabilizaron un total de 83,73% de la muestra. A medida que el par de testículos maduros, adquiere forma lobulada; Los testículos inmaduros son indiferenciados y están incrustados en una matriz epigonal. El ancho testicular y el incremento de masa observado en las etapas de maduración se deben a las fases del ciclo reproductivo masculino (espermatogénesis, cópula y reposo). La madurez sexual estimada (DM_{50}) fue de 42,03 cm. Las comparaciones entre los aspectos macroscópicos y microscópicos de las gónadas fueron cruciales para determinar la actividad reproductiva estacional. Se sugiere que el apareamiento ocurrió de noviembre a marzo, y la especie tiene un ciclo bianual.

Palavras chave: Dasyatidae, estuario, pesca, rayas, ciclo reproductivo.

Introduction

The stingray *Hypanus guttatus* Block & Schneider 1801 is a benthic species commonly

caught in shallow coastal waters and estuarine habitats (Cervigón, 1966; Thorson, 1983). There are records of this species in the southern Gulf of

Mexico, Costa Rica, Colombia, Venezuela, and Southeast of Brazil (Cervigón, 1966; Figueiredo, 1977; Costa & Chaves, 2006). *H. guttatus* is a significant component of artisanal fishing landings from Costa Rica to Venezuela (Thorson, 1983; Cordovés *et al.*, 2009; Grijalba-Bendeck *et al.*, 2012). In Brazil, the same pattern occurs with the most representative captures in North and Northeast coasts (Menni & Lessa, 1998; Meneses *et al.*, 2005; Carmona *et al.*, 2008; Lessa *et al.*, 2008; Basílio, 2011).

Despite the high contribution of *H. guttatus* to captures, the biology of this species is still poorly known, which leads the species to appear on The IUCN Red List as near-threatened (NT) (Carlson *et al.*, 2020). Besides occurrence records, age and growth, feeding habits, and connectivity aspects, the reproduction aspects remain unpublished (Gianeti *et al.*, 2018; Feitosa *et al.*, 2021). However, the available information about the reproductive biology of *H. guttatus* in its distribution area is scarce, and the existence of seasonality in the male reproductive cycle is unknown.

Pearsons & Grier (1992) recognize four testicular patterns related to Gonadosomatic Index (I_G) and mate season in shark testis. According to Maruska *et al.* (1996), I_G alone was not useful to describe mating activity in *Dasyatis sabina*, suggesting that the morphology condition of accessory organs from the male reproductive system could be a more accurate tool to understand the male reproductive cycle (Piercy *et al.*, 2004), mainly in elasmobranchs in which gonadosomatic indices are not correlated with copulatory activity (Maruska *et al.*, 1996).

This study aims to describe, for the first time, the *H. guttatus* male development establishing the first maturity size, as well as the reproduction cycle in coastal and estuarine areas of Pernambuco State, Brazil, which are crucial information for conservation assessments.

Material and Methods

All specimens of *H. guttatus* were captured between August 2013 and October 2015 by the artisanal fishing fleet, which operates to the depth of 20 m on the Pernambuco coast. The landings sites were at municipalities of Goiana, Itapissuma, Paulista, Sirinhaém e Rio Formoso (07° 33' 38" - 08° 53' 52" S, 035° 00' 09" - 5° 08' 52" W) (Fig. 1). The weather in this area has two defined seasons: the rainy season (from March to August) and the dry season (from September to February) (Cavalcanti &

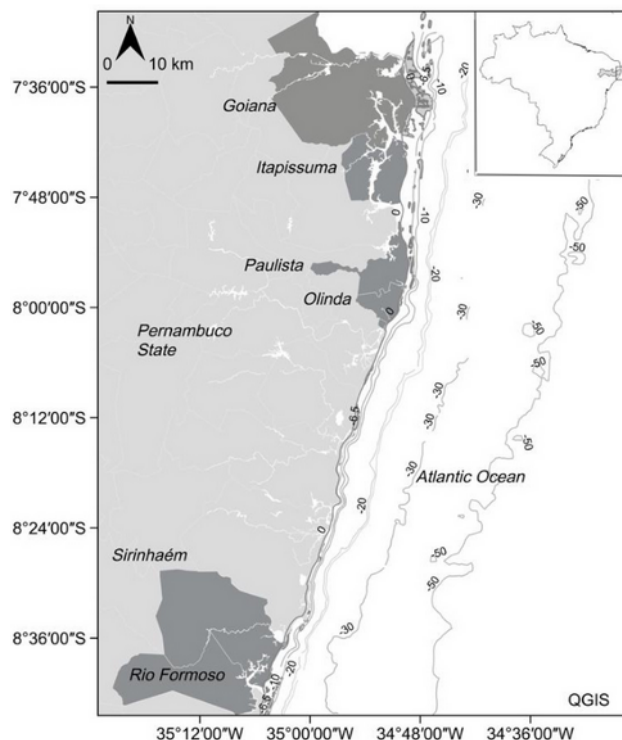


Figure 1. Location and major captures sites for males of *Hipanus guttatus* for fish examined from 2013 to 2015. Sampled regions on Pernambuco state: Goiana, Ponta de Pedras, Itapissuma, Paulista, Olinda, Sirinhaém and Rio Formoso Municipalities.

Kempf, 1970). According to monthly mean during the years 2013 -2015, Pluviometry data were analyzed using the data obtained from Agência Pernambucana de Águas e Clima (APAC).

The disc width (WD, cm), mass (M, g) were recorded for each specimen. The mass (MTes, g), length (LTes), and width (WTes) of testes were measured as well, as the internal clasper length (LC) was recorded. The values of WD, M, LTes, WTes are expressed as mean $\pm$ standard deviation.

The maturity stage description followed ICES (2013). The macroscopic maturity was modified according to the *H. guttatus* reproductive system (Table 1). The maturation stages considered in this study are immature, developing, capable to reproduce, active and regressing.

All specimens evaluated was assigned binomial maturity status (0, immature; 1, mature) (Walker, 2005) to estimate the disc width (W_{D50}), the size where 50% of males reached maturity were derived by using logistic regression analysis to describe the probability $PW_D = P_{max} [1 + EXP(-\ln(19) (W_D - W_{D50}) / (W_{D95} - W_{D50}))]$ (see Walker (2005) and Marshall *et al.* (2007)). W_D is the length of-male individuals; W_{D50} and W_{D95} are constants, and the $\ln$

Table 1. Macroscopic male sexual stages of maturity for *Hypanus guttatus* according to the scale criterion (modified ICES, 2013). W_D = disc width (cm), M_{Tes} = testes mass (g), L_{Tes} = testes length (cm), W_{Tes} = teste width (cm) and L_C = internal clasper length (cm)

* All size values are relative to the left claspers and testicle, mean and standard deviation.

Stage	Macroscopic characteristics
Immature	Claspers flexible ($L_C = 1.72 \pm 0.59$), testicles not differentiated from the epigonal organ ($M_{Tes} = 1.40 \pm 1.76$; $L_{Tes} = 3.25 \pm 1.34$; $W_{Tes} = 0.80$) vas deferens thin, seminal vesicle thin and without seminal fluid.
Developing	Claspers partially calcified but still flexible (6.87 ± 3.59), testicles differentiated from epigonal organ ($M_{Tes} = 2.76 \pm 2.08$; $L_{Tes} = 5.45 \pm 1.20$; $W_{Tes} = 1.95 \pm 0.53$), onset lobulation evident, vas deferens beginning to coil, seminal vesicles differentiated opac and without seminal fluid.
Capable to reproduce	Claspers rigids and fully calcified ($L_C = 13.16 \pm 1.86$), testicles developed ($M_{Tes} = 11.05 \pm 5.39$; $L_{Tes} = 8.01 \pm 1.55$; $W_{Tes} = 2.41 \pm 0.50$) with visible lobules, vas deferens slightly coiled, seminal vesicle with sperm.
Active	Claspers similar to previous stage ($L_C = 13.16 \pm 1.21$), testicles similar to previous stage ($M_{Tes} = 12.59 \pm 4.56$; $L_{Tes} = 8.34 \pm 1.15$; $W_{Tes} = 2.22 \pm 0.31$), vas deferens widened and highly coiled, seminal vesicle with thick and seminal fluid.
Regressing	Claspers rigid ($L_C = 12.99 \pm 1.45$), testicles shrunken and flaccid ($M_{Tes} = 9.75$; $L_{Tes} = 8.84 \pm 1.44$; $W_{Tes} = 2.34 \pm 0.21$), vas deferens empty and flaccid, seminal vesicle developed but empty.

is the natural logarithm. P_{max} is set at one because all individuals were expected to become mature (Marshall *et al.*, 2007). Maximum likelihood estimates were obtained from median values derived from 1000 sets of randomly resampled bootstrap estimates for the logistic regression analysis using SOLVE routine in Microsoft[®] Excel. The 95% confidence limits for each parameter were estimated as the 2.5 and 97.5 percentiles of the resampling analysis results. W_{D50} is the inflection point on the curve that separates immature and mature individuals.

After the macroscopic analysis, fragments of testes and epididymis from males of $\sim 2 \text{ cm}^3$ were collected. All pieces were fixed in 10% formaldehyde with seawater solution (Conrath, 2005; Rêgo *et al.*, 2015) and processed and embedded in paraplast. The tissues were cut into 5 μm sections and stained in Hematoxylin-Eosin. Male reproductive cycle periodicity was assessed using the hepato-somatic index (I_H) and gonadosomatic index (I_G), calculated by $I_H = 100 M_L M^{-1}$ and $I_G = 100 M_O M^{-1}$ (Conrath, 2005) and the microscopic analysis. Mean monthly values respective to these indices were compared using the

Kruskal-Wallis test by month in both ($\alpha = 0,05$) (Zar, 2010).

Results

A total of 73 male specimens of *H. guttatus* were caught by the fishing vessels from July to February in the study period. The W_D ranged from 15.70 to 61.50 cm. ($44.68 \pm 9.84 \text{ cm}$, $n = 73$). W_D classes' frequency indicated that the largest males were more frequent (65%) than the smallest one (Fig. 2). The W_D distribution was not normal (Shapiro-Wilk (S-W) = 0.91; $p = 0.000$), modal classes were found between 40.00 and 55.00 cm W_D . The equation that described the relationship between W_D and M was $M = 0.0168 W_D^{3,15}$ ($R^2 = 0.98$; $n = 13$).

In this study, the smallest mature male was 40.00 cm W_D , while the largest immature male was 49.90 cm W_D (Table 1). The estimated length at maturity (W_{D50}) was 42.02 (CI95% 40.91-43.23 cm) and W_{95} of 46.69 cm (CI95% 43.43-49.95 cm) (Fig. 3a). The relationship between L_C and W_D defined a sigmoid curve with an inflection point in the class 40-45 cm (Fig. 4b), which corresponded to the value of W_{D50} . The mature males' L_C ranged from 7.50 to

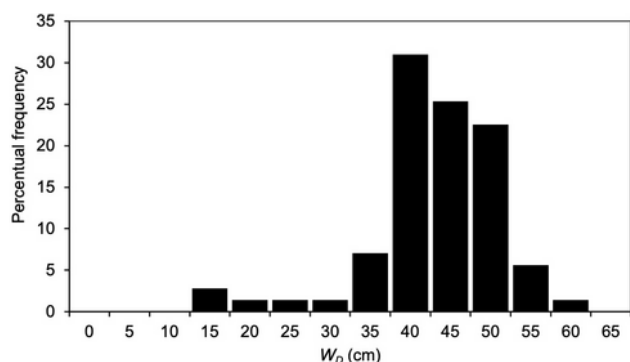


Figure 2. Size frequency of male ($n = 71$) *Hipanus guttatus* caught in Pernambuco, in intervals of five centimeters disc width (W_D) classes.

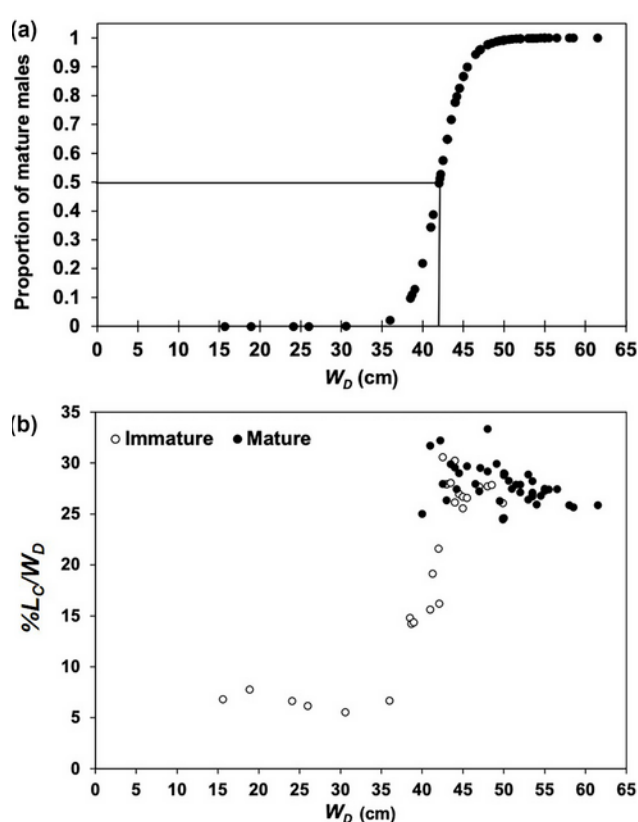


Figure 3. (a) Percentage frequency of occurrence of the mature male of *Hipanus guttatus* ($n = 71$), (b) Percentage of clasper length (L_C , cm) in relation to disk width (W_D , cm) of immatures ($n = 28$) and matures male ($n = 42$) of *Hipanus guttatus*.

16.00 cm and for immature individuals from 1.30 to 13.00 cm. W_{D50} corresponded to 68.32% of the W_D to the largest individual male in the sample.

The most frequent maturation stage in sample was capable of reproduce (29.2%), followed by developing stage (23.6%), immature (22.2%), active stage (15.3%), and regressing (9.7%) (Table 1). Both testes are functional, the length of the right and left testis, width and mass analyses demonstrated

that the left testis was longer (7.64 ± 1.87 cm, $n = 41$), widest (2.7 ± 0.50 cm, $n = 39$) and heavier (9.11 ± 7.03 g, $n = 16$) than the right testis (6.5 ± 1.62 cm, $n = 41$), (2.08 ± 0.50 cm, $n = 39$) and (7.15 ± 4.71 g, $n = 16$), respectively. However, significant difference was just observed between the average of the right and left testes length ($t = 2.93$, $n = 41$, $p = 0.004$).

As the gonad maturation progresses, there is an increase in the testis length, width, and mass (Table 1). The testis length increases significantly between the developing stage to capable to reproduce (KW-H(4:25) = 12.20; $p = 0.001$). After this point, the increment in length decreases, tends to stabilize, around the mean value of 13.15 ± 1.68 cm (mature stages). The testicular width and mass increment observed in maturation stages (capable to reproduce, active, and in regression) are due to the phases of the male's reproductive cycle (spermatogenesis, mating, and resting) (Table 1). However, significant differences were just observed in M_{Tes} (KW-H(4:25) = 11.64; $p = 0.02$). The highest values of M_{Tes} were recorded at the end of the rainy and beginning of the dry season.

Testes are associated with the epigonal organ, a yellowish lymphomyeloid tissue. Microscopic analysis indicates that the epigonal organ is more predominant in immature than mature individuals. The epigonal organ becomes laterally compressed due to the spermatocytes' development, when male stingrays becoming mature, testis surface is becoming lobulated, occupying a significant portion of the epigonal organ.

Longnose stingray testes develop from the cranial to caudal regions. They are lobulated structure and its functional unit are the spermatocysts. The spermatogenesis begins in the germinal zone in the proximal periphery of the organ, where the Sertoli cell and spermatogonia recruitment takes place to form the spermatocyst (Fig. 4a) Inside spermatocysts, spermatogenesis begins in this distal lateral surface of the testicle, and at the same time occurs a radial expansion of spermatocysts within each lobe (Fig. 4a-g). In the distal edge of the testicular lobe, the spermatozoa are released into the testicular ducts (Fig. 4h) and transported to cranial portion of epididymis through efferent ducts (fig 4h-i).

Mature males capable to reproduce and active stages presented the epididymis filled with sperm and deferent duct tightly coiled (Table I). Individuals at the beginning of the reproductive activity, observed during the dry season, had the epididymal mucosa edges compressed by the spermatic mass in

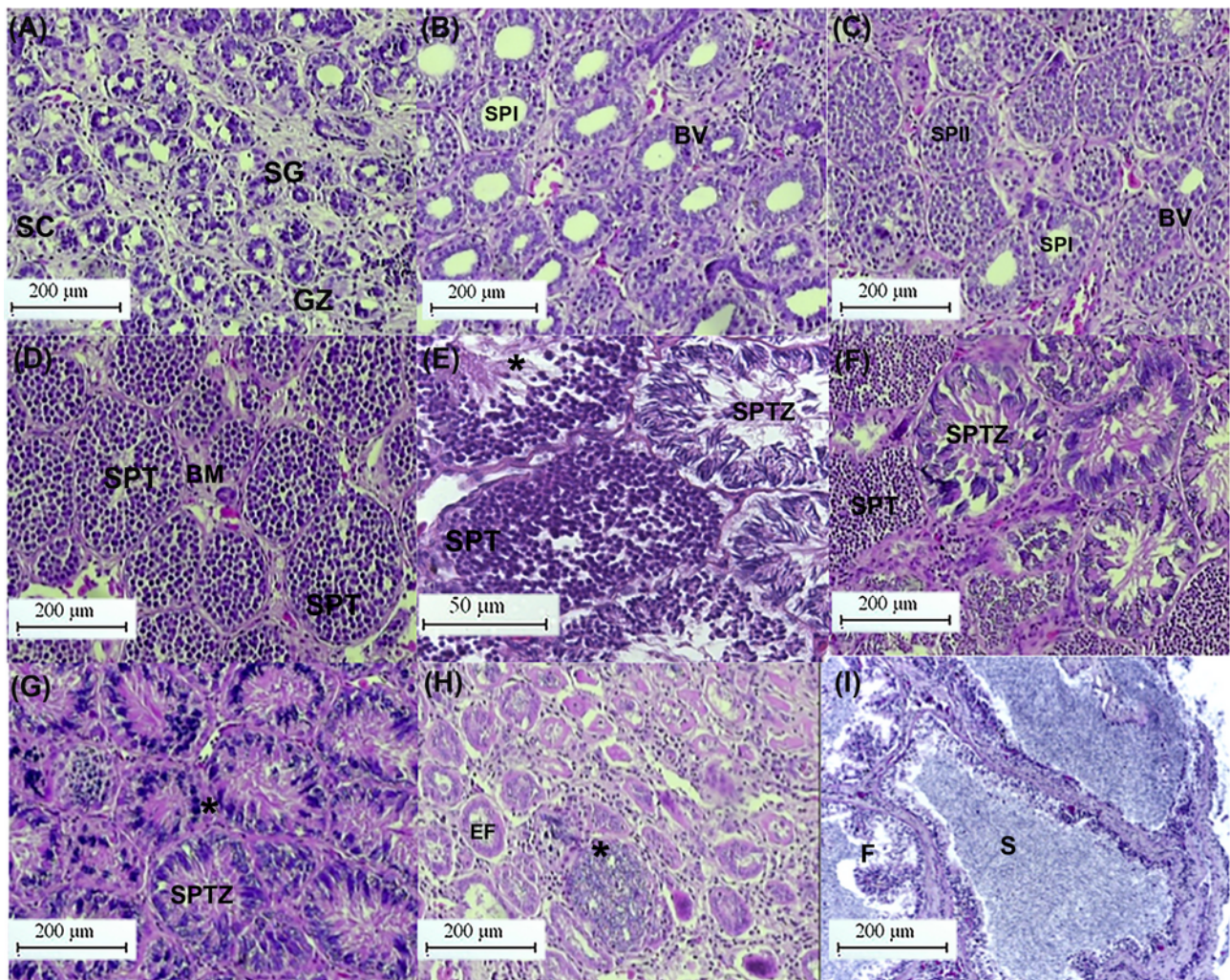


Figure 4. Photomicrograph of *Hypanus guttatus* spermatogenesis . (A) Germinal zone (GZ) where the cysts formation occur with Spermatogonia (SP) with Sertoli cell (SC) association (arrowhead). (B-G) Just after the GZ the spermatocyst observed has spermatogenic cells in the same stage arranged in concentric layers and Sertoli cells. (B) Spermatocysts with primary spermatocytes (SPI) and Sertoli cells. (C) In central area of testicle, spherical spermatocyst with secondary spermatocyte (SPII) and Sertoli cell (SC) in periphery. (D) Spermatid (SPT). (E) Spermatid in Spermiogenesis (SPTS) (40X). (F) The completed maturation process forming spermatozoa (SPTZ) associated with Sertoli (SC) cells, the spermatozoa (S) head (black arrow) faces the basal membrane and the tail to the lumen (arrowhead). (H) Efferent ducts (EF) in spermatocyst unpacked area. (I) Sperm mass (S) at cranial portion of epididymis. Staining: Hematoxilin- Eosin.

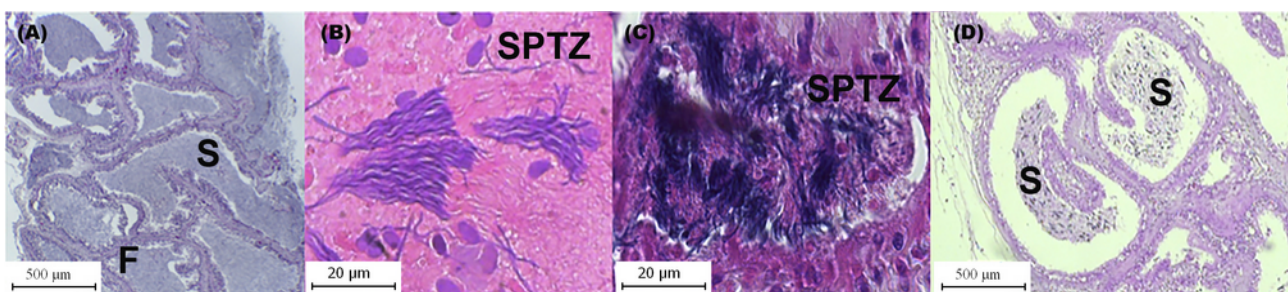


Figure 5. (A) - Epididymis of *Hypanus guttatus* (a) epididymis of an active male before the beginning of copulate season. (B) Clumps of mature spermatozoa (SPTZ) in seminal vesicle fluid during the copulate season. (C) Mature spermatozoa (SPTZ) in upper portion of female oviduct during copulate season. (D) Epididymis of regressing males. (S) sperm mass. Staining: Hematoxilin-Eosine.

the lumen (Fig. 5a). During the mating season, the spermatogenic mass in the epididymal lumen gradually declined, and the epididymis mucosa became folded (Fig. 5d). In seminal vesicle a massive presence of spermatozoa clumps at seminal fluid has occurred at the same time (Fig. 5b).

Males probably begin their reproductive activity in January, at the end of the dry season, as indicated by the number of spermatozoa in reproductive tract components and histological changes observed in epididymis structure and seminal vesicle (Fig. 5a-d). The presence of viable spermatozoa in the lower reproductive tract (seminal vesicles) (Fig. 5b) in the raining season (February to May), suggest that copulatory activities took place during this time, when is possible to find females with copulate bites and spermatozoa in upper portion of oviduct (Fig. 5c). At the end of the mating period, when the rainy season starts, males in regressing stage presented small amounts of residual spermatogenic mass, which give to the duct of the epididymis an empty aspect (Fig. 5d). The presence of males in the regressing stage occurred at the end of March (raining season) and in November (dry season). The cycle proposed to this species is biannual.

In the end of dry season (January, $IG = 0.27$; $I_H = 2.77$ - February, $IG = 0.39$; $I_H = 2.44$) and the end of the rainy season (July, $IG = 0.41$; $I_H = 3.59$ - August, $IG = 0.39$; $I_H = 3.03$). However, there is no significant differences in the mean of I_G and I_H values (I_G , $H = 10.90$; $gl = 5$; $p = 0.053$) (I_H , $H = 4.70$; $p = 0.44$) throughout the year in sampled period. These findings collectively indicate that males of *H. guttatus* are reproductively active twice a year.

Discussion

Hypanus guttatus was found throughout the year in Pernambuco estuarine and coastal areas. In the Colombian Caribe Sea, *H. guttatus* is the most abundant species captured by artisanal fisheries, where mature individuals were prevalent in the sample (Gijabalda *et al.*, 2012). They suggested that the fishing ground is a feeding and reproduction area for this species.

Mature individuals composed the major part of the capture in the present study. This bias can indicate the selectivity of the main fishing gear used by local artisanal fishing. Tagliafico *et al.* (2013), in Venezuela, attributed the highest number of mature individuals in the samples to the fishing seasonality.

The relationship between W_D and M follows a positive allometric growth $b > 3$. This 'b' value fits

the allometric range of coefficient estimated by Teixeira *et al.* (2017) for *H. guttatus* ($b = 3.01$) in the fishing ground near the study area. Similar results were found for other stingrays species such as *Gymnura micrura* ($b = 3.15$) (Yokota *et al.*, 2012); *Rhinoptera bonasus* ($b = 3.25$) (Poulakis & Grier 2014); *Himantura gerrardi* ($b = 3.03$) (Rastgoo *et al.*, 2018).

The onset of maturity determined in this study was 42.02 (CI95% 40.91-43.23 cm), which is consistent with results available in the literature for *H. guttatus* in Brazil. However, latitudinal variation in maturity size (W_{D50}) occurs in this species based on data reported by Silva *et al.* (2007) $W_{D50} = 51.0$ cm in Ceará state and Yokota and Lessa (2007) $W_{D50} = 42.00 - 46.00$ cm to Rio Grande do Norte state. Both areas are located in a northward area relative to the present study. Santander-Neto *et al.* (2016), in their work with *Urotrygon microphthalmum* in the study area, attributed the higher W_{D50} of this species in northern areas to different environmental conditions, latitudinal differences and different mortality rates in the distinct stocks. Similar observations can be addressed to the first maturity size (W_{D50}) for *H. guttatus*.

The estimated length at maturity for males corresponded to 68.32% of the largest individual size in the sample ($W_D = 61.50$ cm). This value is comparable to the percentages reported in studies with *Dasyatis sabina* (61%) (Snelson *et al.*, 1988), *D. say* (63%) (Snelson *et al.*, 1989), *D. lata* (Dale and Holland, 2012) but is smaller than the percentage reported to *D. pastinaca* (70%) (Linnaeus 1758) (Saadaoui *et al.*, 2015), *H. guttatus* (83-93%) (Yokota and Lessa, 2007), *Myliobatis californica* (70%) (Martin & Caillet, 1988) and *Urotrygon microphthalmum* (75%) (Santander-Neto *et al.*, 2016).

H. guttatus testicular lobes are visible just in mature individuals. The same occurs to *D. americana* and *M. californica* (Pratt, 1988), *Himantura signifer* (Chatchavalvanich *et al.*, 2004), *Rhinoptera bonasus* (Poulakis & Grier 2014) and *U. microphthalmum* (Santander-Neto *et al.*, 2016). Despite the fact of both testes are functional, in *Hipannus guttatus* (present study), *Rhinoptera bonasus* (Poulaskis and Grier, 2014) and *Himantura signifer* (Chatchavalvanich *et al.*, 2004), the left testis is more prominent in length than the right one.

The testis structure of *H. guttatus* is of the compound type and based on the lobate surface and spermatocyst development (Pratt, 1988). Other *Myliobatiformes* have the testis of the same pattern,

as *D. americana* and *M. californica* (Pratt, 1988) and *H. signifer* (Chatchavalvanich *et al.*, 2004).

Seasonal changes in testis structure are associated with the formation and maturation of spermatocysts, which corresponds with I_G variation and testicular structure and consequently, with all the events of the reproductive cycle (gametogenesis, mating and regressing). In *Rhinoptera bonasus*, the I_G peak coincides with the increase of seasonal sperm production (Poulanskis and Grier, 2014). For *Urobatis bucculentus*, the male breeding season coincides with the period immediately following parturition (Trinnie *et al.*, 2012). Such behavior is observed in chondrichthyan species, while fertilization occurs in the terminal zone of the oviductal gland (Hamlett *et al.*, 2005), requiring that mating precedes the ovulation (Trinnie *et al.*, 2012).

The cycle is regulated by some species and the production and storage of sperm related to environmental conditions. Mull *et al.* (2008) related these changes with the day length for round stingrays, the recrudescence of males into active breeding is correlated with a decrease in day length in the fall. In the present study, the end of the mating season is related to the beginning of the rain season, as characterized by epididymal appearance (diameter smaller, residual cells contain little or absence of sperm). These observations suggested a prolonged period of mating that begins when precipitation values decrease. Grijalba-Bendeck *et al.* (2012) observed the same behavior in males of *H. guttatus* in Colombian Caribbean waters.

Macroscopic criteria are usually employed to determine the stage of sexual maturity in elasmobranch males, but those criteria are insufficient to determine the seasonal changes that occur in genital tract components of males during the reproductive cycle. Moya *et al.* (2015), in their study with *Sympterygia bonapartii*, reported that claspers characteristics are valid and simple criteria to determine maturity, but the histology can be crucial for male reproductive study. Our histology results demonstrate the relation between the seasonal change in male genital organs during reproductive cycle of *H. guttatus* and environment conditions (rainy-dry seasons).

The I_H index did not vary significantly over the months of the year, which means that *H. guttatus* has continuous nutritional support to maintain the reproductive investment year-round. Feeding habits of *H. guttatus* individuals used in this study have demonstrated that mature individuals are caught with stomachs with full content throughout the year

(Queiroz *et al.*, 2019), this is an indication that hepatic lipidic reserves are consumed as they are stored with little variation reflecting no significant variation of the I_H index. Sherman & Gilliam (1996) noted that the lipid reserves in the liver of some fishes are influenced by dramatic changes associated with feeding, breeding, and migration. These observations suggested that *H. guttatus* presents a feeding activity throughout the cycle, and this could explain the little variation on lipid reserves during the reproductive reporting period.

Hypanus guttatus is exploited by the artisanal fishing fleet off the Pernambuco coast in all development stages (Melo *et al.* 2023). Batoids are one of the most severely threatened marine groups (Last, 2007). Little is known about the biology of the stingrays (Rosa & Furtado, 2004; Pierce, 2009; Lessa *et al.*, 2011; Tagliafico *et al.*, 2013; Dulvy *et al.*, 2014), this study provides information that can support, management and conservation measures to *H. guttatus* in Brazil.

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Ethical statement

This study was carried out under SISBIO license n. 49663-1 issued by ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade). The samples came from fishing and no live animals were handled during this research.

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