PANAMJAS
Pan-American Journal of Aquatic Sciences

Histology of the testicles and male reproductive tract of the skates *Sympterygia acuta* Garman, 1877 and *S. bonapartii* Müller & Henle, 1841 (Chondrichthyes: Rajoidei) in the Western South Atlantic Ocean

ALEXANDER BASALLO[1], ANTONIO SERGIO JR. VARELA[2] & MARIA CRISTINA ODDONE[2]

[1] *Universidade Federal do Rio Grande, Instituto de Oceanografia, Programa de Pós-Graduação em Oceanografia Biológica, Av. Itália, Km 8 s/n, Campus Carreiros, 96203-900 Rio Grande, RS, Brazil.*
[2] *Universidade Federal do Rio Grande, Instituto de Ciências Biológicas, Setor de Morfologia. Av. Itália, Km 8 s/n, Campus Carreiros, 96203-900 Rio Grande, RS, Brazil.*

*Corresponding author: alexbiology8@hotmail.com

Abstract. As in other derived vertebrates, the male reproductive tract on chondrichthyans is composed of testicles (including the epigonal gland) and reproductive ducts. The latter include efferent duct, epididymis, Leydig gland, deferent duct and seminal vesicle. The skates *S. acuta* and *S. bonapartii* are endemic to the Western South Atlantic Ocean. Specimens of these species were obtained from bottom trawl research cruises and commercial fishing trips carried out in 2011 and 2012, carried out in the area between 34° 28'S and 31° 29'S, southern Brazil, at depths between 15 and 142 m. The reproductive tracts were fixed in 10% formalin and preserved in 70% alcohol. Histological techniques for optical microscopy were performed using staining with HE, PAS and AB pH 1.0 and 2.5, with tissue sections of 6 µm. The spermatogenesis stages were described in both species. In addition, the TL50 (total length at 50% maturity) calculated in a previous work and considered here as "morphological TL50", was compared with the here estimated histological TL50, demonstrating that the latter corresponded to lower TL values. This fact indicates that the onset of spermatogenesis occurs some time the macroscopic development of the reproductive structures most frequently used to assess maturity in elasmobranches.

Keywords: Spermatogenesis, maturity, microanatomy, gonad, sexual development.

Resumo. Histologia dos testículos e trato reprodutivo masculino das raias *Sympterygia acuta* Garman, 1877 e *S. bonapartii* Müller & Henle, 1841 (Chondrichthyes: Rajoidei) no Oceano Atlântico Sul Ocidental. Assim como em outros vertebrados derivados, o aparelho reprodutor masculino em condrictes é composto por os testículos (incluindo a glândula epigonal) e dutos reprodutivos. Estes últimos incluem dutos eferentes, epidídimo, glândula de Leydig, duto deferente e vesícula seminal. As raias *S. acuta* e *S. bonapartii* são endêmicas do Oceano Atlântico Sul Ocidental. As amostras foram obtidas a partir arrasto de fundo de cruzeiros de pesquisa e viagens de pesca realizadas em 2011 e 2012, na área entre 34° 28'S e 31° 29'S, Sul do Brasil, em profundidades entre 15 e 142 m. Os tratos reprodutivos foram fixados em formol 10% e conservados em álcool 70%. Foram aplicadas técnicas histológicas para microscopia óptica, utilizando coloração com HE, PAS e AB pH 1,0 e 2,5, com cortes de tecidos de 6 um. Foram descritos os estágios histológicos da espermatogênese em ambas as espécies estudadas. Além disso, o CT50 (comprimento total 50% de maturidade), calculado em um estudo prévio e chamado aqui de "morfológico", foi comparado com o CT50 histológico resultando ser maior. Este fato provavelmente está relacionado a um atraso na atividade espermatogênica durante o desenvolvimento sexual nos machos de *S. acuta* e *S. bonapartii* em

relação ao estabelecimento das estruturas reprodutivas macroscópicas que revelam a maturidade sexual.

Palavras-chave: Espermatogênese, maturidade, microanatomia, gônadas, desenvolvimento sexual.

Introduction

The chondrichthyan fishes include sharks, rays, skates and chimaeras. These cartilaginous fishes first appeared almost 400 million years ago and are characterized by being rather diverse, especially in their reproductive modes (Compagno 1990). The skates *Sympterygia acuta* Garman 1877 and *S. bonapartii* Müller & Henle 1841 occur in the Western South Atlantic Ocean, being found in coastal waters from Brazil to Argentina (McEachran & Aschliman 2004). However, Pequeño & Lamilla (1985, 1993) record the occurrence of *S. bonapartii* in southern Chile, South Eastern Pacific. In South and Southeast Brazil, *S. acuta* and *S. bonapartii* complete their life cycles in inner-shelf waters (Vooren 1997; Vooren *et al*. 2005).

The chondrichthyan testicle in composed by a germinal zone imbibed in the epigonal gland (EG) and their functions are both the spermatogenesis and steroidogenesis (Walker 2005). The basic testicular unity in Chondrichthyan fishes is the spermatocyst (Hamlett 1999). The union of several spermatocysts constitutes a macroscopic structure, the testicular lobule (Babel 1967). Three types of testicles were described in cartilaginous fishes, based on the progression of the spermatogenesis; diametric, radial and compound, the latter being typical of batoids, i.e. rays and skates (Pratt 1988). In the compound testicle, the germinal zone (GZ) is located on the ventral testicular surface, from where lobules develop radially, migrating diametrically across the testicle (Hamlett 1999). The aim of this arrangement is the movement of the mature spermatozoa towards the efferent ducts, their final destination within the testicles (Pratt 1988, Jamieson 2005).

In male chondrichthyans, gonad is physically in contact with the genital ducts through the efferent ducts (Hoar 1969). After leaving the testicles, the mature spermatozoa produced within it are transported through the genital ducts, i.e., efferent ducts, epididymis, deferent duct and seminal vesicle (Conrath 2005, Walker 2005). The spermatozoa travel within a rich matrix of secretions produced at these sites by annex glands, e.g. the Leydig and the alkaline glands (Walker 2005, Jones & Hamlett 2006). The study of the testicle to the microscopic level allows the accurate staging the maturity of the individual, making possible to relate this with the total length of the individual or any other reproductive parameter (Maruska *et al*. 1996). Histological studies on the reproductive internal organs may help revealing aspects of the physiology and the mechanisms that regulate reproduction in Chondrichthyes. Also, the application of histological techniques to reproduction studies may lead to understand different aspects of the life history of these fishes apart from reproductive biology. Further, the analysis of the microanatomy of the reproductive tract reveals valuable insights concerning the sexual development of this animal group. Nolan *et al*. (2002), for instance, calculated the histological size at 50% maturity (TL_{50}) in males *Raja montagui* Fowler 1910, based on the gonadal histologic analysis for maturity staging. Specifically, studies of the gonads, reproductive tract and annex glands in Chondrichthyes under a histological perspective are scarce for the Western South Atlantic Ocean species, especially for rajoids. The information presented in this paper may contribute to better understand this, and other aspects of reproductive biology in skates.

The present study aims to analyze and characterize the microanatomy of the gonads and male reproductive tract of the oviparous skates *S. acuta* and *S. bonapartii* along with the spermatogenesis stages. We also provide an estimate of the histological TL_{50} for the species in question and discuss it in terms of the morphological TL_{50} calculated on a previous study by Basallo & Oddone (2014). Further, gonadosomatic indexes were calculated in both species in order to establish a correlation between the testicles growth in mass and the sexual development inferred by the histological analysis.

Material and Methods

Sample collection and biological data recorded: Male specimens of *S. acuta* and *S. bonapartii* were collected from two sources: one-day long research cruises carried out on May, June, July and August 2011, and from two commercial fishing trips that occurred from September 22nd to 30th 2011 and from January 30th to February 10th 2012. In both cases, the fishing gear used was bottom trawl. The study area

Table I.- Reproductive stages assumed for male *Sympterygia acuta* and *S. bonapartii* based on the observation and description of the reproductive organs.

Organ	Description	Stage
Testicles	Thin tissue strip with epigonal gland predominating	Immature
	Lobules differentiated, epigonal gland still extensive	Adolescent
	Lobular zone predominating	Mature
Clasper gland	Undifferentiated from surrounding tissue	Immature
	Easier to differentiate and measure, white-transparent	Adolescent
	Completely differentiated and developed, light yellow	Mature
Alar thorns	Absent	Immature
	Developing	Adolescent
	Fully developed	Mature
Clasper	With no calcification, no longer than pelvic fin	Immature
	Partly calcified, longer than pelvic fin	Adolescent
	Fully calcified, rigid	Mature
Seminal vesicle	Sperm absent	Immature
	Sperm absent	Adolescent
	Sperm absent, no mating activity	Mature
	Sperm present, mating activity	Mature

was situated between latitudes 34°28'S and 31°29'S, at depths between 15 and 142 m, on the continental shelf of southern Brazil (more details of the study area and capture sites, see Figure 1 of Basallo & Oddone 2014). The individuals collected had their total length (TL) (cm) from the snout to the extremity of the tail, the clasper length (CL) and testes weight (TW) (g) recorded. Maturity stages considered (Table I) followed Oddone *et al*. (2007) and are in agreement with Walker (2005).

Determination of the microanatomy of the testicles and ducts: For the performance of histological analysis, the complete reproductive tract of *S. acuta* and *S. bonapartii* males was removed, fixed in 10% formalin for 24 hours, and then preserved in ethanol 70%. The testicle was sectioned in the second quarter of the anterior half of the gonad (ICES 2010, 2013). Tissue samples were removed from the upper region of the epididymis (Jones & Hamlett 2006). On deferent ducts, seminal vesicle and Leydig gland coronal cuts were done. The epididymis and Leydig gland, on the other hand, were submitted to cross sections. The nomenclature of the reproductive tract was in all cases in agreement with Hamlett (1999). A description of the macroanatomy of the complete reproductive tract was also provided. Samples of gonads (including the EG) and reproductive ducts were processed for histology using a tissue Leica ASP-200 processor. Subsequently, the tissue was embedded in Paraplast Xtra (Sigma P3808) and sectioned to a thickness of 6.0 µm using an automated Rotary Microtome (Leica RM2255). The histological sections were stained with Hematoxylin and Eosin (HE), the reaction Periodic Acid Schiff (PAS), and Alcian Blue (AB) pH 1.0 and 2.5 (Carson & Hladik 2009). Images were acquired through a brightfield microscope Olympus BX 51 equipped with a high resolution camera (Olympus DP72).

Study of the sperrmatogenesis:

The classification of the histological stages of the spermatogenesis followed Maruska *et al*. (1996). The terminology for the description of spermatozoa associations follows the classification proposed by Pratt & Tanaka (1994) for elasmobranchs.

Determination of the histological total length-at-50%-maturity: The histological TL_{50} (HTL_{50}) was calculated by applying the logistic equation $PTL=1/(1-e^{(a+bTL)})$ to the proportion of mature individuals (PTL) by TL class, where a and b are equation parameters. An histologically mature male was that with had all the spermatogenesis stages detected in their testicles. The HTL_{50} value was compared with the morphological TL_{50} value (MTL_{50}) such as described by Nolan *et al*. (2002). Size at 50% maturity estimates (MTL_{50}) for male *S. acuta* and *S. bonapartii* were based on Basallo & Oddone (2014) and calculated with the exact same individuals studied in this paper.

Gonadosomatic indexes estimates: Gonadosomatic index (GSI) was calculated according to King (1995), but considering eviscerated weight instead of total weight as recommended by Peres & Vooren

(1991) for elasmobranchs, as GSI=(Wg/We)*100, where Wg=gonadal weight (g) and We=eviscerated fish weight (g). Values corresponding to GSI were expressed in terms of mean and standard deviation for each maturity stage in both species.

Results

Macro and microanatomy of the testicle of S. acuta and S. bonapartii: microanatomy of testes was similar when comparing *S. acuta* and *S. bonapartii*. Both testes in each species were morphologically and functionally symmetrical. The general shape was lobular and their position in the peritoneal cavity was dorsal. The intimate association of the testicles and the EG was indicated by the high vascularization of the latter.

In both species, the macroscopical testes analyses of the individuals classified as immature presented small dorsal testicular lobules with pale coloration. Reproductive ducts in these specimens were regionally undifferentiated, being straight and thread like. The immature gonad was mostly constituted by the EG. The ratio EG/germinal testicular tissue of the testicle decrease throughout the maturity stages by virtue of the development of the germinal testicular tissue so that in the mature gonad, the EG was limited mostly to the caudal region of the testes. In this stage, the lobular nature of the external surface of testes was visible to the naked eye.

Microscopically, the EG was composed mostly by granular cells (leucocytes) and eosinophilic granulocytes (Fig. 1a). In the middle region of the gonad tissue, ducts with simple cubic epithelium and no secretory activity were detected, corresponding to the efferent duct (ED). Positive reactivity for PAS technique was revealed in this region, surrounded by connective tissue an intratesticular secretion of glycoproteins (Fig. 1b).

Microscopically, it was also demonstrated that the ratio EG/germinal testicular tissue gradually and considerably diminished with the maturation progress (Fig. 1c). In the same way, the coloration of the EG changes according to the maturity stage, from whitish, in the immature, to reddish (highly vascularized), in the mature males. In addition, males classified as adolescents and mature had a reduced EG with respect to the germinal testicular tissue, macroscopically represented by the presence of large lobules situated on the dorsal-anterior testis zone (Fig. 1d). The mature sperm produced in the testicles is carried around in the spermatic matrix through the efferent ducts passing through the epididymis, Leydig gland, deferent duct to finally remain stored in seminal vesicle until the copula.

Stages of spermatogenesis in S. acuta and S. bonapartii: On a cross section of the testicle in the mature *S. acuta* and *S. bonapartii* male, differentiated spermatocysts were present, indicating that the spermatogenesis was in progress (Fig. 2a). Differences were observed in the stages of spermatogenesis for each maturity stage in both species studied. In *S. acuta*, stages SI and SII were recorded in the testicle of immature individuals. On the other hand, adolescents and mature males showed all stages of spermatogenesis, from SI to SVII, in their testicles. In *S. bonapartii*, the testis of immature males showed a predominance of stages SI and SII, though, some males classified as immature showed transition stages between SI and SV. In adolescent males testicular spermatocysts ranged from stage SII to stage SVII, with predominance of stages SIV and SVI. In mature individuals all stages of spermatogenesis were present, from SI to SVII. Stages SVI and SVII were predominant in the testicles of mature individuals (Fig. 2a).

The initial phase of the spermatogenesis (Stage I, SI) is characterized by the presence of dispersed germinal cells, forming a loose tissue without the delimitation of a membrane. These cells indicated that beginning spermatogenesis is irradiated diametrically. Small germinal cells with a grouping tendency were also observed. Sertoli cells were observed in association with the germinal cells. Subsequently, these initials cells arranged in spermatocysts form the spermatogonia, identified by the presence of a a large nucleus (Fig. 2b).

At SII, the spermatocysts presented internally a layer of spermatogonia resulting from the consecutive mitotic divisions of the germinal cells, with Sertolli cells migrating peripherally, arranged around a central lumen, with basement membrane delimiting the spermatocysts (Fig. 2c). At Stage III (SIII) the primary espermatocytes produced by the mitotic divisions of the spermatogonia, are visible. They have the appearance of voluminous spherical cells, with a large nuclei compared to the cells visible in previous stages, as a consequence of meiotic divisions in the primary spermatocytes, these give rise to the secondary spermatocytes. (Fig. 2d).

Stage IV is characterized by the presence of spermatids, resulting of the second meiotic division by the secondary spermatocytes. Morphologically, the spermatids have a small cell body and round nuclei. A large area of the spermatocyst is occupied

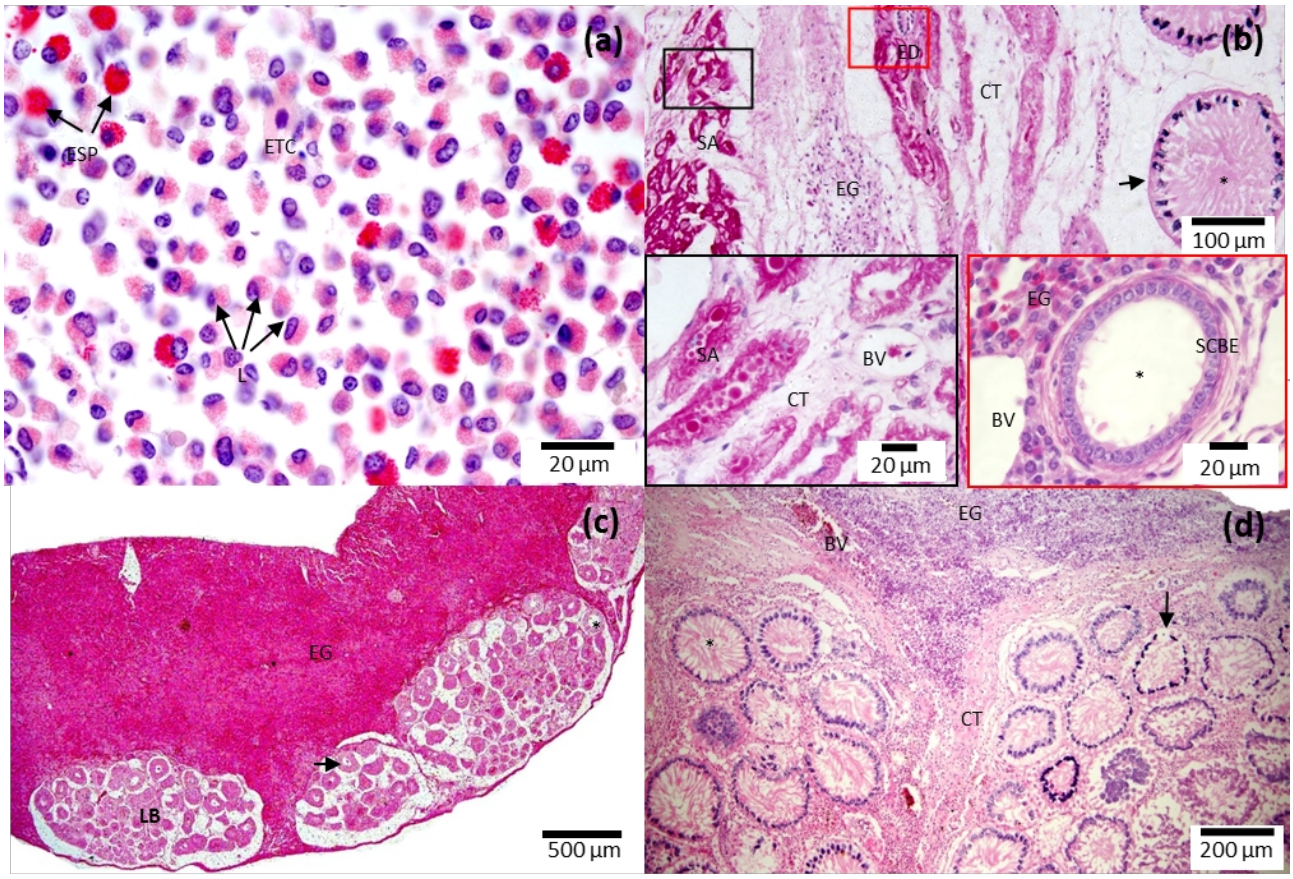

Figure 1. Cross sections of gonads of *Sympterygia acuta* (a; c) and *S. bonapartii* (b; d); (a) epigonal gland (EG) with lymphomyeloid tissue, arrows indicate eosinophils granulocytes (ESP) and lymphocytes (L) and a single erythrocyte (ETC); (b) testis and EG, the black square shows a detail of the testicle with duct secretory activity (SA), along with connective tissue (CT) and blood vessels (BV); red square shows a detail of efferent duct (ED) without secretory activity, composed of simple cubic epithelium (SCBE), arrow showing espermatocyst and the asterisk represents the lumen; (c) gonads of an immature individual with clustered spermatocysts within a lobule (LB); (d) gonad of a sexually mature individual. Figures (a-c) stained with PAS and (d) stained with HE.

by a high density of spermatids, which results in a lumen reduction in some cases (Fig. 2e). On SV, the appearance of spermatids in the previous stage marked the beginning of the spermiogenesis. During spermatids maturation, Sertoli cells migrate toward the spermatocysts periphery, allowing in subsequent stages the radial growth of spermatozoa. The immature sperm that is formed in this phase was found in a disorganized form (Fig. 2f).

On Stage VI (SVI), mature spermatozoa are visible, organized in packages, on the spermatocysts periphery, along with the Sertoli cells. The heads of the mature spermatozoa have the typical spiral shape, oriented toward the periphery of spermatocyst and forming clump type spermatozeugmata. A broad lumen within the spermatocyst communicates mature sperm toward the collecting ducts where it subsequently is communicated with the efferent vessels (Fig. 1b). Particles PAS+ secretion in nature glycoprotein were observed in this stage, near the EG ducts, where the same secretion was observed (Fig. 2g).

Finally, at SSVII, the spermatocysts undergo deformation at this stage, adopting flattened forms; with slight or no sperm. This is due to the evacuation process that indicates their degradation and the end of the spermatogenesis (Fig. 2h). The collapse of the spermatocysts cause a displacement of Sertoli cells from their initial position, becoming disperse and markedly visible.

Microanatomy of the epididymis in S. acuta and S. bonapartii: Anatomically, the epididymis in both species is a highly coiled and compressed tubule, elongated in shape, dorsoventrally flattened, attached to the dorsal wall of the abdominal cavity and externally covered by a thin membrane formed by connective tissue. However, the microanatomy of the epididymis differed in cross section between

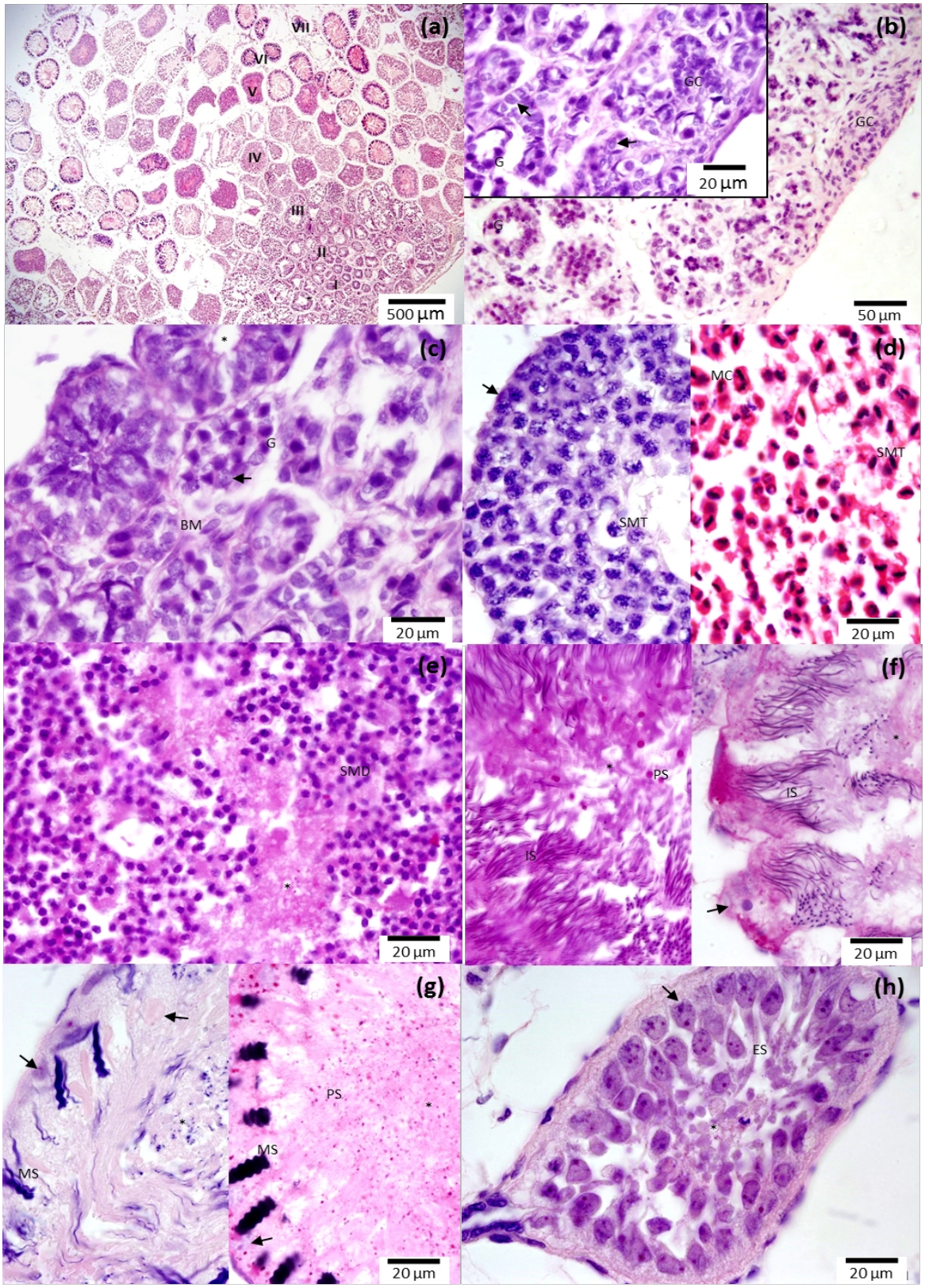

Figure 2. Spermatogenesis in *Sympterygia acuta* (a-d) and *S. bonapartii* (e-h). Testis in cross-section; (a) progression of diametric stages of spermatogenesis, stained with HE; (b) Stage I: indicating in detail germinal cells (GC), spermatogonia (G) next to Sertoli cells indicated by the arrow, stained with HE; (c) Stage II: spermatogonias with large nucleus, besides basement membrane (BM) at the peripheric spermatocysts stained with AB pH 2.5; (legend continues in next page).

Figure 2 (legend continued from previous page). (d) Stage III: formed by spermatocytes (SMT) with Sertoli of cells migrating to the periphery, mitotic cells (MC) are also observed, right micrograph stained with AB pH 1.0 and left stained with PAS; (e) Stage IV: spermatids (SMD) with small round nuclei, stained with PAS; (f) Stage V: immature sperm (IS) within the disorganized spermatocyst, showing that particle secretion (PS), stained with PAS; (g) Stage VI: mature spermatozoa (MS) packaged in the periphery, on the left micrograph the evacuation of sperm into the lumen is indicated, stained with HE, On right micrograph indicated a strong presence of particle secretion (PS) is indicated, stained with PAS; (h) Stage VII: spermatocyst in degradation with large amounts of Sertoli cells (arrow), without presence of spermatozoa, stained with AB pH 2.5.

species. Histologically, it is a duct lined by a ciliated simple columnar epithelium. This tissue bore cells with secretory activity, identifiable by a basement membrane positive with PAS; AB pH 1.0 and 2.5 (Fig. 3). In immature males, the epididymis appeared as a virtually closed duct, with a narrow lumen and with no sperm (Fig. 3a). In mature males, the epididimys was larger and vascularized with abundant amounts of seminal matrix within this cavity, forming a dense fluid with clusters of sperm in form of spermatozeugmata (Fig. 3b). Also, the glycoprotein particles PAS positive similar to those found within mature spermatocysts stage VI of the spermatogenesis in the testicles were present, integrating the seminal matrix.

Microanatomy of the Leydig gland in S. acuta and S. bonapartii: Macroscopically in *S. acuta* and *S. bonapartii*, the Leydig gland is located in the posterior region of the epididymis, in ventral view.

In a cross-section, the tissue of the Leydig gland was observed lining dorsally to the area of epididymis. This tissue was represented by a simple ciliated columnar epithelium PAS+. This epithelium showed secretory cells characterized by a light supranuclear (LS); and also cells with nucleus near the apex (Fig. 3c). The epithelium of the Leydig gland tubules in immature males showed scanty or no secretory activity, although the basement membrane showed strong reactivity, evidenced with the staining PAS and AB (Fig. 3d).

Microanatomy of the deferent duct in S. acuta and S. bonapartii: The anterior portion of the deferent duct is located posteriorly to the epididymis. This duct appears forming a coiled structure, visible to the naked eye. In mature males of both species, the deferent duct was internally lined by a columnar simple epithelium. A seminal matrix with PAS+ spermatozeugmata was detected within this duct. On its lateral sides, the deferent is inserted into the surrounding connective tissue, forming simple tubular glands highly positive with PAS, AB 1.0 and 2.5 pH. These glands were composed by a simple cuboidal epithelium with goblet cells, indicating that the secretion of this epithelium was associated with sperm inside the ducts.

Microanatomy of the seminal vesicle in S. acuta and S. bonapartii: In both species, the seminal vesicle is formed by a ciliated simple columnar epithelium, with a high amount of seminal matrix, formed by clusters of sperm or spermatozeugmata, eosinophil particles and PAS+ secretions. When observed in lateral view, the glandular tissue is similar to that found in the deferent ducts, being observed inside these tubules large amounts of secretions of sulfated and carboxylated acid mucopolysaccharides (AB pH 1.0 and 2.5 +) additionally proved whether secretion of glycoproteins and neutral substances with (PAS +).

Estimates of the histological size-at-50%-maturity in S. acuta and S. bonapartii: The HTL_{50} considering the relative proportion of spermatocysts containing mature sperm was estimated to lie in 44.7 cm (R= 0.99, n= 16) and 54.68 cm (R= 0.98 n= 79) for *S. acuta* and *S. bonapartii*, respectively (Fig. 4).

Gonadosomatic indexes of male S. acuta and S. bonapartii: The GSI values calculated for each maturity stage by species are given in Table II. Mature *S. acuta* males attained the highest IGS values (Fig. 5a-b). On the other hand, the highest values of GSI in males *S. bonapartii* corresponded to adolescent individuals (Fig. 5c-d). Clasper length values showed differences among the mean values for each stage of maturity in both species (Fig. 5b-d).

Discussion

Mature males of *S. acuta* and *S. bonapartii* showed compound testicular organization, with lobules developing from germinal zones radiating diametrically when viewed in cross section, being consistent with the classification of Pratt (1988) for elasmobranch gonads. The EG showed the typical lymphomyeloid tissue in both species, which controls functions and processes related to hemopoiesis and immune system. The EG is composed of eosinophil granulocytes cells, such as described by Hine & Wain (1987) for batoids, with lymphocytes and erythrocytes in the case of the rajoid *Raja eglanteria* (Bosc 1802) (Walsh & Luer

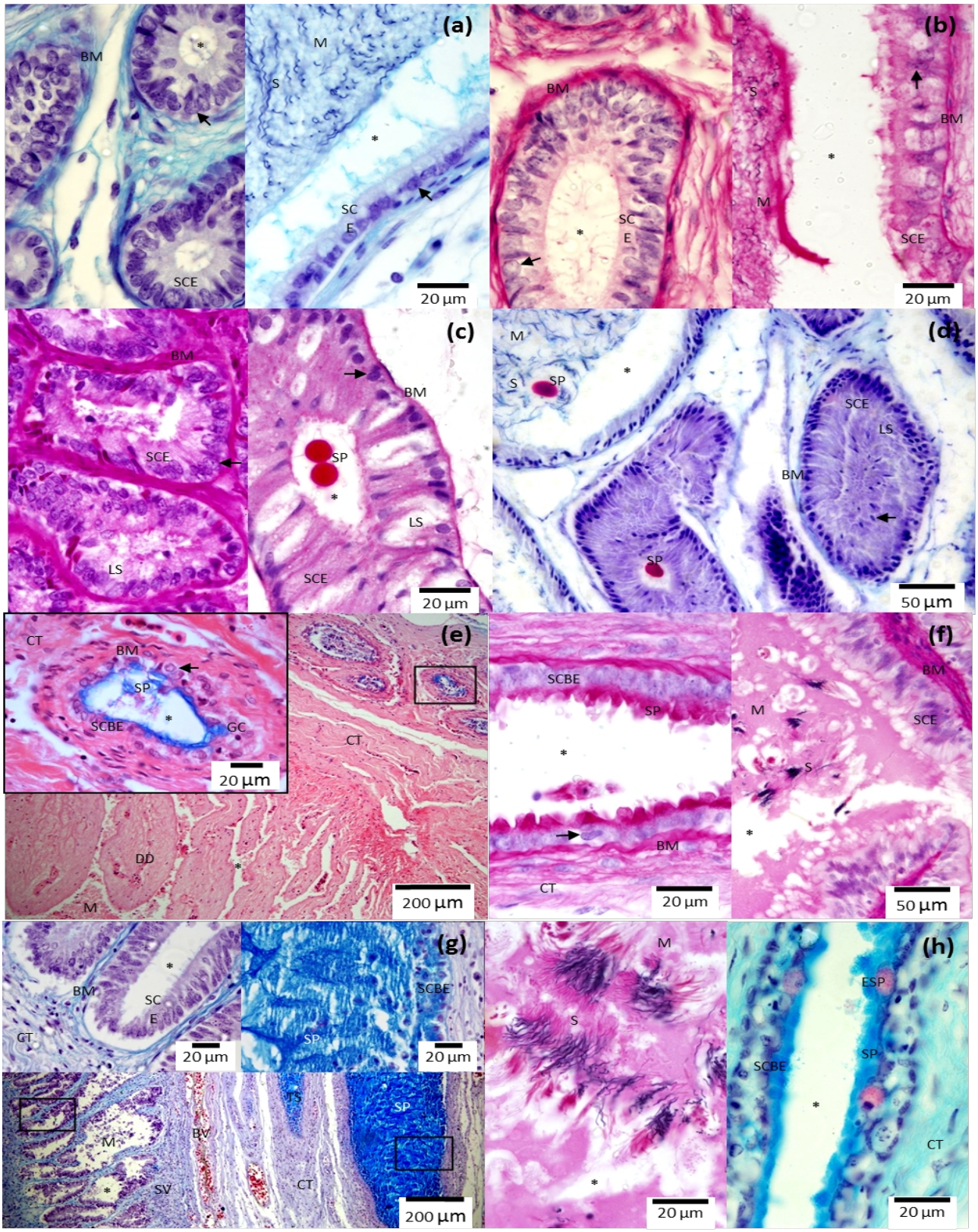

Figure 3. Male reproductive tract of *S. acuta* (a, b, c, f, h) and *S. bonapartii* (d, e, g); (a) left epididymis of an immature individual, showing a duct with simple columnar epithelium (SCE), the arrow indicates the cells nucleus, basement membrane (BM) and the small empty lumen (asterisk), on the right side, a tubule of the epididymis of a mature individual is indicated, showing a matrix (M) with spermatozeugmata (S) staining with AB pH 2.5; (legend continues in next page).

Figure 3 (legend continued from previous page). (b) presence of glycoproteins in the matrix (M) and SCE, verified with PAS staining applied in epididymis of immature and mature males, on the left and right respectively; (c) Leydig gland with simple columnar epithelium (SCE) with light supranuclear (LS), basement membrane (BM) of the epithelium together with basal nucleus (arrow), production of secretions (SP) accumulated in the lumen; stained with PAS . (d) Leydig gland contributing with secretory products (SP) in the proximity of epididymis, arrow indicates apical nuclei; staining AB pH 2.5; (e) dutus deferens (DD) with simple columnar epithelium (SCE) and matrix (M); square in black color indicates detail of glandular ducts annexes, composed of simple cubic epithelium (SCBE) and goblet cells (GC), stained with AB pH1.0 and 2.5. (f) On the left ducts annexed to DD with production of secretions (SP) PAS positive, in the right ductus deferens with SCE and a matrix with clusters of spermatozeugmata; (g) detail of the seminal vesicle (square on the upper left) composed of a simple columnar epithelium (SCE) and basement membrane (BM), inside the seminal vesicle there is a matrix with spermatozeugmata; the square on top on the right indicates large secretory tubules (TS), abundant secretory products (SP), rich in mucopolysaccharides acids, sulfated and carboxylated, evidenced by the AB pH 1.0 and 2.5 staining and (h) clusters of spermatozeugmata (micrograph on the left), stained with PAS, on the right, a secretory tubule containing granulocytes eosinophils (ESP) and secretory products (SP) AB positive. Figures (a and b) correspond to as much immature and mature individuals; figures (c-h) correspond to mature individuals.

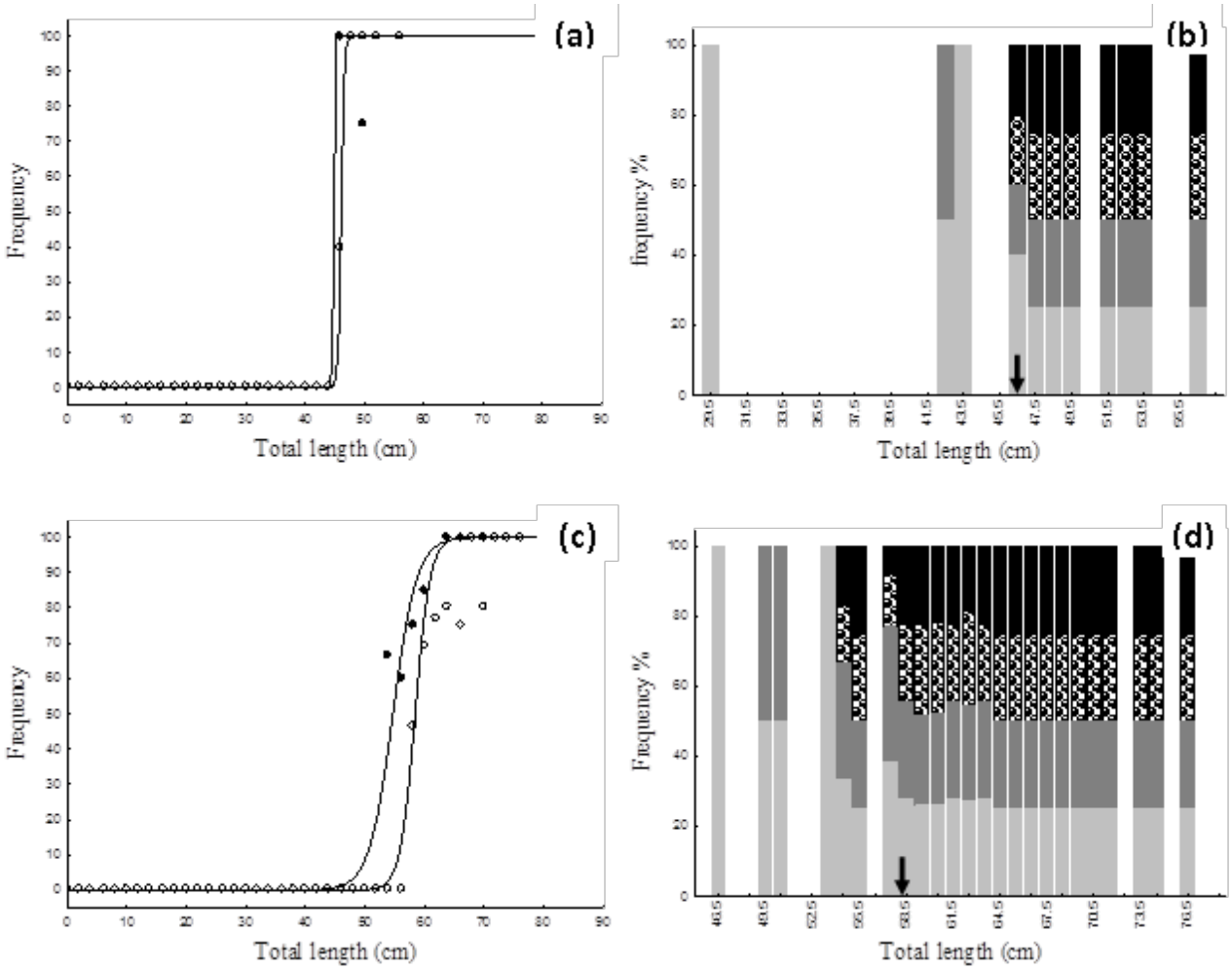

Figure 4. Logistic curves applied to the percentage of mature males by total length class for *Sympterygia acuta* (a) and *Sympterygia bonapartii* (c). Empty circle symbols represent the percentage of mature males assessed through the traditional method based on morphological characteristics of the reproductive tract and gonads by Basallo & Oddone (2014); bold circle symbols represent histological total length at % maturity as proposed by Nolan *et al*. (2002). The relative abundance of the stages of spermatogenesis detected [(spermatogonia (light gray), spermatocyt (dark gray), spermatids (black dots) and spermatozoa (black)] and the total length (cm) for *S. acuta* (b) and *S. bonapartii* (d). The arrow indicated the size corresponding to the morphological TL_{50}.

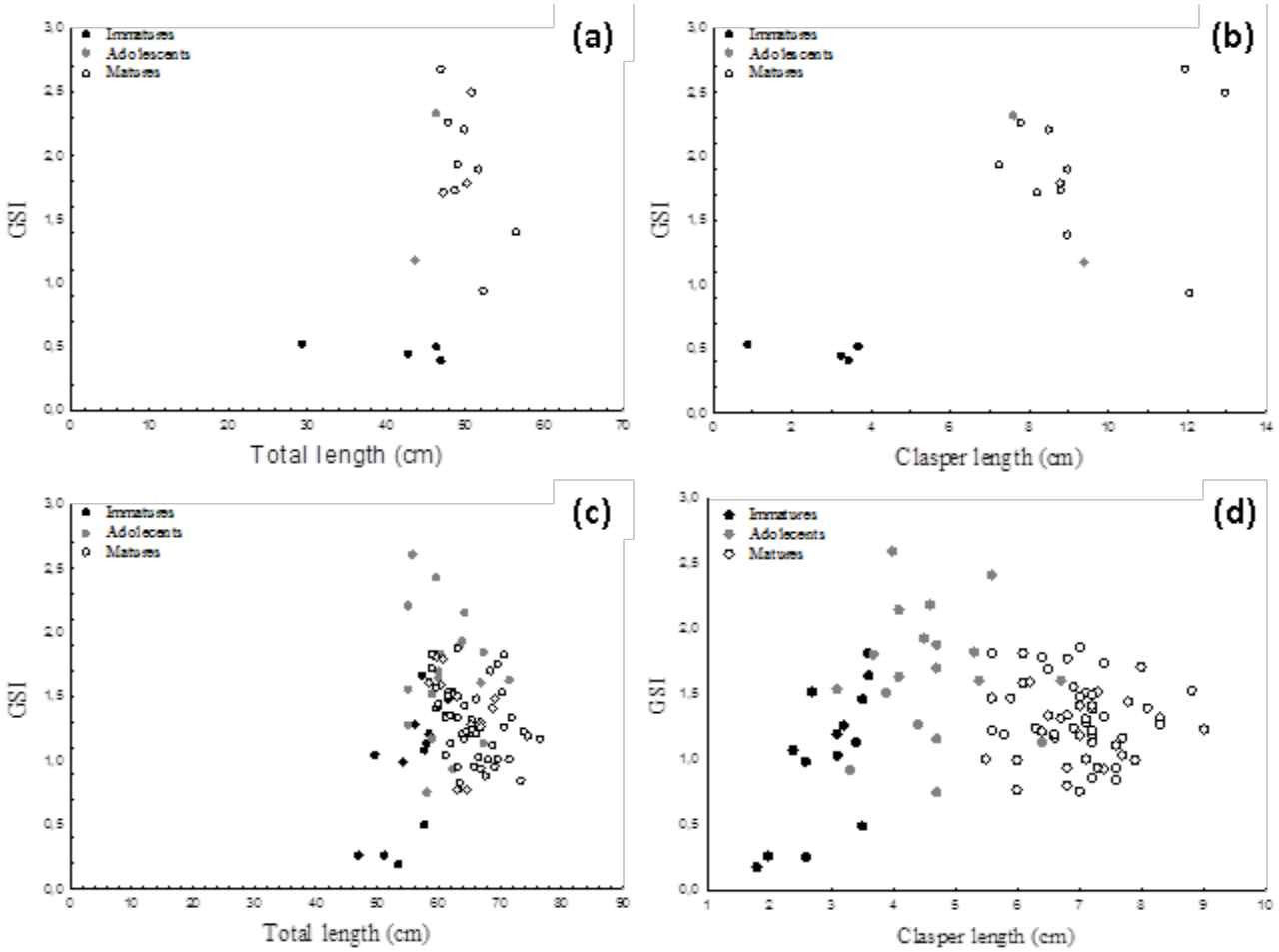

Figure 5. Relationship between gonadosomatic index (GSI) and total length (cm) and GSI and clasper length (cm) for *Sympterygia acuta* (a and b) and *S. bonapartii* (c and d).

Table II. Gonadosomatic index (GSI) calculated for male *Sympterygia acuta* and *S. bonapartii*. Mean, standard deviation (SD), sample size (n) and total length range (TL, cm) for each maturity stage (I= immature, A=adolescent, M=mature) is given.

Species	Maturity	GSI	mean	SD	n	TL
S. acuta	I	0.39-0.52	0.46	0.05	4	29.5-47.0
	A	1.17-2.31	1.74	0.81	2	43.0-46.0
	M	0.93-2.68	1.90	0.49	11	47.0-56.0
S. bonapartii	I	0.18-1.81	1.02	0.53	14	47.2-63.5
	A	0.74-2.60	1.66	0.48	19	55.0-71.6
	M	0.76-1.87	1.30	0.28	59	58.6-76.8

1998), being these cells described in other elasmobranchs by Walsh & Luer (2004).

Changes in the degree of differentiation of testicular tissue were found in the stages of spermatogenesis during the sexual development in males of *S. acuta* and *S. bonapartii*. Whereas other studies indicated changes in the phases of spermatogenesis associated with maturation stages used in *Sympterygia* spp., and *Raja clavata* (Linnaeus 1758) (Serra-Pereira *et al*. 2011). Such differentiation of spermatogenesis stages during sexual development were observed in *Leucoraja wallacei* (Hulley 1970) and *Dipturus pullopunctatus* (Smith 1964) (Walmsley-Hart *et al*. 1999), *Leucoraja ocellata* (Mitchill 1815) (Sulikowski *et al*. 2005).

Although histological differences during the sexual development were found between the epididymis of *S. acuta* and *S. bonapartii*, their micro-anatomical characteristics may be used as a reference in identifying the stage of maturity. The epididymis had seminal fluid with particles of glycoproteins (PAS +), accompanied by clusters of spermatozeugmata in both species. These secretions were also observed by Hamlett (1999) in the epididymis of *Leucoraja erinacea* (Mitchill 1825). Studies on the oviparous shark *Heterodontus portusjacksoni* (Meyer 1793) demonstrated that the amount of these secretions is related to the increase of protein concentration in the lumen of the deferens duct (Jones & Lin 1993).

The Leydig gland in mature males of *S. acuta* and *S. bonapartii* was related to the production of PAS+ secretions that form masses of material accumulated in the glandular lumen. These same secretions were detected seen in the Leydig gland of the skate *Leucoraja erinacea* (Hamlett 1999, Jones & Hamlett 2006). The nature of the secretions produced by the Leydig gland was associated with that of the particles found in the seminal fluid of the epididymis and in other parts of the male reproductive tract in deferens duct and seminal vesicle of *S. acuta* and *S. bonapartii*.

The secretory tubules observed in the deferent duct and seminal vesicle, located laterally, possibly provide elements related to the maintenance and storage of sperm in the seminal vesicle before the copula. The histochemical studies made by Jones & Hamlett (2002) described processes in glycosylation during the production of secretions along the genital tract, being important for the maturation and transport of sperm in *Leucoraja erinacea*.

The histochemical analysis performed in each duct of genital tract of *S. acuta* and *S. bonapartii* are in agreement with histological descriptions carried out in other species of skates (Hamlett 1999, Jones & Hamlett 2002, 2006, Serra-Pereira *et al*. 2011), in the oviparous shark *Heterodontus portusjacksoni* (Jones *et al*. 1984) the viviparous shark *Centroscymnus coelolepis* (Bocage & Capello 1864) (Moura *et al*. 2011) and the chimera *Callorhynchus milii* (Vincent 1823) (Hamlett *et al*. 2002). This was particularly valid in terms of the internal epithelia, type of cells found, tissue reactivity using the stains PAS and AB and presence of spermatozeugmata in the ducts.

Different methodologies are commonly used to assess the maturity in Chondrichthyes (Walker 2005). In the present study, two techniques for the assessment of maturity in elasmobranches were compared for *S. acuta* and *S. bonapartii*: the morphological and the histological TL_{50}. From this comparison, it was possible to establish differences between these two techniques for the assessment of maturity, particularly for *S. bonapartii*, where the value of histological TL_{50} was lower than the morphological. This can be explained as a result of the addition of individuals considered as adolescents from the morphological point of view, that actually had mature characteristics when histologically analyzed assessing and confirming the presence of all the stages of spermatogenesis.

Further, it was verified that some individuals that had been classified as adolescents through to morphological method and therefore with a small clasper, had on the others high IGS values. In these cases, and for both species, it was demonstrated through histology that the testicles bore mature spermatocysts corresponding to stages SV and SVI. However, in these males there was no presence of sperm in the seminal ducts. Ebert *et al*. (2008) observed the same in males of *Raja binoculata* (Girard 1854) and *R. rhina* (Jordan & Gilbert 1880), and record the clasper being developed later in relation to the spermatocysts in the gonads, a fact also documented by Sulikowski *et al*. (2005, 2006, 2007), in three species of the Gulf of Maine, *Leucoraja ocellata*, *Amblyraja radiata* (Donovan 1808) and *Malacoraja senta* (Garman 1885).

The presence of spermatozoa associations recorded within the epididymis, deferens duct and seminal vesicle of *S. acuta* and *S. bonapartii* corresponds with what Pratt & Tanaka (1994) described as spermatozeugmata. The abundance of spermatozoa in different parts of the reproductive tract in both species might be related to events previous or after the copula in mature males (Serra-Pereira *et al*. 2011). Further studies on the reproductive cycle are required for better understanding of these processes. However, it is well known that skates follow continuous reproduction throughout the year, which may or not include the presence of peaks of reproductive activity (Wourms 1977, Oddone & Velasco 2008).

Barone *et al*. (2007) showed the presence of resting gonads in terms of gametogenesis and with reduced size in mature males of *Raja asterias* Delaroch, 1809 that had fully developed claspers. A similar observation was done in *S. bonapartii*, with some individuals had mature gonads but with low weight for the maturity stage considered, what was clearly reflected by the GSI. In addition, these

individuals also had fully developed claspers (in terms of size and calcification, gonads with mature spermatocysts and no sperm in the reproductive tracts.

Posterior studies carried out for the species for which resting gonads had been reported allowed including which individual within a different reproductive stage (Serra-Pereira *et al*. 2011). Histological studies represent an accurate tool for the maturity staging in male chondrichthyan fishes. However, it is necessary to integrate these results with morphological ones, in order to get a more holistic understanding of the reproduction.

Acknowledgments

The authors are indebted to skipper Charles Da hora and crew of the fishing vessels “Da Hora C II” and “Menina Lybia”. Sample collection aboard the research vessel “Larus” was kindly enabled by Dr. Luiz Felipe Cestari Dumont (Laboratório de Crustáceos Decápodes, FURG). Technical assistants of the Laboratório de Histologia, Instituto de Ciências Biológicas (FURG) are specially thanked. This study was funded by CNPq, Brazil (Conselho Nacional de Desenvolvimento Científico e Tecnológico), postgraduate grant N° 133653/2011-0). We are indebted to an anonymous referee dramatically improved the manuscript text quality.

References

Babel, J. S. 1967. Reproduction, life history, and ecology of the round stingray, *Urolophus halleri*, Cooper. **California Fish and Game Bulletin**, 137: 1-104

Barone, M., de Ranieri, S., Fabiani, O., Pirone, A & Serena, F. 2007. Gametogenesis and maturity scale of *Raja asterias* Delaroche, 1809 (Chondrichthyes, Rajidae) from the South Lingurian Sea. **Hydrobiologia**, 580:245-254.

Basallo, A. & Oddone, M. C. 2014. Reproductive biology of the skates *Sympterygia acuta* Garman, 1877 and *S. bonapartii* Müller & Henle, 1841 (Chondrichthyes: Rajoidei) in south Brazil. **Neotropical Ichthyology**, 12(4): 771-782.

Carson, F. L., Hladik, C. 2009. **Histotechnology: A Self-Intructional Text, 3 ed, American**. Hong Kong: Society of Clinical Pathology Press, 400 p.

Conrath, C. L. 2005. Reproductive biology. Pp. 103-126. In: Musick, J. A. &Bonfil, R. (Eds.). **Management techniques for elasmobranch fisheries**. Rome: FAO Fisheries. Hoar, W. S. 1969. Reproduction. Pp. 1-72. In: Hoar, W. S. & Randall, D. J. (Eds.). **Fish Physiology Vol. 3. Reproduction and growth**. Academic Press, New York.

Compagno, J. L. 1990. Alternative life-history styles of cartilaginous fishes in time and space. **Environmental Biology of Fishes**, 28: 33-75.

Ebert, D. A., Smith, W. D. & Cailliet, G. M. 2008. Reproductive biology of two commercially exploited skates, *Raja binoculata* and *R. rhina*, in the western Gulf of Alaska. **Fisheries Research**, 94: 48-57.

Hamlett, W. C. 1999. Male reproductive system. Pp. 444-470. *In:* Hamlett W. C. (Ed.). **Sharks, skates, and rays: The biology of Elasmobranch fishes**. Baltimore, MD: The Johns Hopkins University Press.

Hamlett, W. C., Reardon, M., Clark, J. & Walker, T. I. 2002. Ultrastructure of sperm storage and male genital ducts in a male holocephalan, the elephant fish,

Callorhynchus milii. **Journal of Experimental Zoology**, 292(2): 111-128.

Hine, P. M. & Wain, J. M. 1987. Composition and ultrastructure of elasmobranch granulocytes. II. Rays (Rajiformes). **Journal of Fish Biology**, 30(5): 557-565.

Hoar, W. S. 1969. Reproduction. Pp. 1-72. *In:* Fish Physiology Vol. 3 . Reproduction and growth W. S. Hoar & D. J. Randall (Eds.) Academic Press, New York

ICES. 2010. Report of the Workshop on Sexual Maturity Staging of Elasmobranches (WKMSEL). Valetta, Malta: ICES CM 2010/ACOM: 48. 132 p.

ICES. 2013. Report of the workshop on Sexual Maturity Staging of Elasmobranchs

(WKMSEL). Lisbon, Portugal. ICES CM 2012/ACOM: 59. 68 pp.

Jamieson, B. G. M. 2005. Chondrichthyan Spermatozoa and Phylogeny. Pp. 201-236. *In:* Hamlett, W. C. (Ed). **Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, batoids and chimaeras**. Enfield (NH): Science Publishers.

Jones, R. C., Jones, N. & Djakiew, D. 1984. Luminal composition and maturation of spermatozoa in the male genital ducts of the Port Jackson shark *Heterodontus portusjacksoni*. **Journal of Experimental Zoology**, 230: 417-427.

Jones, R. C. & Lin, M. 1993. Structure and functions of the of the male Port Jackson shark

Heterodontus portusjacksoni. **Australian Journal of Zoology**, 40: 257-266.

Jones, C. J. P. & Hamlett, W. C. 2002. Glycosylation of the male genital ducts and spermatozeugmata formation in the clearnose skate *Raja eglanteria*. **Histochemistry Journal**, 34: 601-615.

Jones, C. J. P.; Hamlett, W. C. 2006. Ultrastructure of the male genital ducts of the clearnose skate *Raja eglanteria*. **Journal of Experimental Zoology,** 305(A): 1018-1029.

King, M. 1995. **Fisheries biology, assessment and management**. Fishing News Books (Ed). Osney Mead, Oxford: Blackwell Science, Ltd, 341 p.

Maruska, K. P., Cowie, E. G. & Tricas, T. C. 1996. Periodic gonadal activity and protracted mating in elasmobranch fishes. **Journal of Experimental Zoology**, 276(3): 219-232.

McEachran, J. D. &Aschliman, N. C. 2004. Phylogeny of Batoidea. Pp.79-113. *In:* Carrier, J. C., Musick, J. A. & Heithaus, M. R. (Eds.). **Biology of Sharks and Their Relatives**. Boca Raton, FL: CRC Press.

Moura, T., Serra-Pereira, B., Gordo, L.S. & Figueiredo, I. 2011. Sperm storage in males and females of the deepwater shark Portuguese dogfish with notes on oviducal gland microscopic organization. **Journal of Zoology**. 283: 210-219.

Nolan, C. P., Gallagher, M. J., Foley, D., Deane, J. & Jeal, F. 2002. Gonadal Maturity in Rajids. **NAFO SCR**., Doc. 02/131. Serial n.4753, 5p.

Oddone, M. C., Amorim, A. F., Mancini, P. L., Norbis, W. & Velasco, G. 2007. The reproductive biology and cycle of *Rioraja agassizi* (Muller and Henle, 1841) (Chondrichthyes: Rajidae) in southeastern Brazil, SW Atlantic Ocean. **Scientia Marina**, 71: 593-604.

Oddone, M. C. & Velasco, G. 2008. Observations on the reproductive seasonality of *Atlantoraja platana* (Günther, 1880), an intensively fished skate endemic to the Southwestern Atlantic Ocean. **Endangered Species Update**, 25(4): 122-129.

Pequeño, G. & Lamilla, J. 1985. Estudio sobre una colección de rayas del sur de Chile (Chondrichthyes, Rajidae). **Revista de Biología Marina y Oceanografía**, 21(2): 225-271.

Pequeño, G. & Lamilla, J. 1993. Batoídeos comunes a las costas de Chile y Argentina-Uruguay (Pisces: Chondrichthyes). **Revista de Biología Marina y Oceanografía**, 28(2): 203-217.

Peres, B. M. & Vooren, C. M. 1991. Sexual development, reproductive cycle, and fecundity of the school shark *Galeorhinus galeus* off southern Brazil. **Fish Bulletin**, 89: 655-667.

Pratt, H. L. 1988. Elasmobranch Gonad Structure: A description and Survey. **Copeia**, 3: 719-729.

Pratt, H. L. & Tanaka, S. 1994. Sperm storage in male elasmobranchs: A description and survey. **Journal of Morphology**, 219(3): 297-308.

Serra-Pereira, B., Figueiredo, I. & Gordo, L. S. 2011. Maturation of the gonads and reproductive tracts of the thornback ray *Raja clavata*, with comments on the development of a standardized reproductive terminology for oviparous elasmobranchs. **Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science**, 3(1): 160-175.

Sulikowski, J. A., Tsang, P. C. W. & Howell, W. H. 2005. Age and size at sexual maturity for the winter skate, *Leucoraja ocellata*, in the western Gulf of Maine based on morphological, histological and steroid hormone analyses. **Environmental Biology of Fishes,** 72: 429-441.

Sulikowski, J. A., Kneebone, J., Elzey, S., Howell, W. H. & Tsang, P. C. W. 2006. Using the composite parameters of reproductive morphology, histology and steroid hormones to determine age and size at sexual maturity for the thorny skate, *Amblyraja radiata*, in the western Gulf of Maine. **Journal of Fish Biology**, 69: 1449–1465.

Sulikowski, J. A., Elzey, S., Kneebone, J., Jurek, J., Howell, W. H. & Tsang, P. C. W. 2007. The reproductive cycle of the smooth skate, *Malacoraja senta*, in the Gulf of Maine. **Marine and Freshwater Research**, 58: 98-103.

Vooren, C. M. 1997. Demersal Elamobranchs. In: Environment and Biota of the Patos Lagoon Estuary. In: Seeliger, U., Odebrecht, C. & Castello, J. P. (Ed.). **Subtropical convergence environments: the coast and sea in the south western Atlantic**. Berlim: Springer-Verlag, p. 141-146.

Vooren, C. M., Klippel, S. & Galina, A. B. 2005. Os elasmobranquios das aguas costeiras da plataforma sul. Pp. 113-120. *In:* Vooren, C.

M. & Klippel. S. (Ed.). **Ações para a conservação d e tubarões e raias no sul do Brasil.** Porto Alegre: Ige.

Walker T. I. 2005. Reproduction in Fisheries Science. Pp. 81-127. *In:* Hamlett, W. C. (Ed). **Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, batoids and chimaeras**. Enfield (NH): Science Publishers.

Walmsley-Hart, S. A., Sauer, W. H. H. & Buxton, C. D. 1999. The biology of the skates *Raja wallacei* and *R. pullopunctata* (Batoidea: Rajidae) on the Agulhas Bank, South Africa. **South African Journal of Marine Science**, 21: 165-179.

Walsh, C. J. & Luer, C. A. 2004. Elasmobranch hematology: Identification of cell types and pratical applications. Pp.307-323. *In:* Smith, M. F. L., Warmolts, D., Thoney, D. & Hueter, R. **The Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays, and their Relatives**. Columbus, Ohio: Special Publication of The Biological Survey.

Walsh, C. J. & Luer, C. A. 1998. Comparative phagocytic and pinocytic activities of leukocytes from peripheral blood and lymphomyeloid tissues of the nurse shark (*Ginglymostoma cirratum* Bonaterre) and the clearnose skate (*Raja eglanteria* Bosc). **Fish and Shellfish Immunology**, 8: 197-215.

Wourms, J. P. 1977. Reproduction and development in chondrichthyan fishes. **American Zoologist**, 17: 379-410.

Received: March 2018
Accepted: August 2018
Published: September 2018