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SPECIAL SECTION: FISHERIES REPRODUCTIVE BIOLOGY

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

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Abstract

There is a need for a unified terminology to describe reproductive phase assignment across fish taxa, regardless of the reproductive strategy involved. Reproductive terminology already adopted for teleosts has been applied to oviparous elasmobranchs of both sexes. A historical review of the terminologies used by previous authors and how these correspond to the new terminology is presented. Five reproductive phases are considered: immature, developing, spawning capable (which includes an actively spawning subphase), regressing, and regenerating. By using an oviparous elasmobranch, the thornback ray *Raja clavata*, as an example, the different phases are described based on both macroscopic and microscopic features of the reproductive tract, including the ovaries, oviducal glands, and uterus in females and the testes, claspers, and sperm ducts in males. The regressing phase was observed in females, but the regenerating phase was not; neither of these two phases was observed in males. Records from other species suggest that all five reproductive phases can be found in oviparous elasmobranchs, depending on the reproductive strategy of the species.

Knowledge of elasmobranch reproductive cycles is still scarce. Therefore, details on reproductive cycles for most elasmobranch species and standardized reproductive terminology are not yet available. New standardized terminology for teleost reproduction has only recently been proposed (Brown-Peterson et al. 2011, this special section), despite increased knowledge of reproduction for a significant number of teleost species. For oviparous teleost species, the reproductive cycle is divided into five reproductive phases: immature, developing, spawning capable (which includes an actively spawning subphase), regressing

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TABLE 1. Comparison among the reproductive phase terminologies adopted for studies of oviparous elasmobranchs. The terminology from Brown-Peterson et al. (2011) is highlighted in bold as the one proposed for standardizing the terminology across all oviparous elasmobranchs.

Authors	Number of phases	Maturity scale terminology phase (or subphase)					
Brown-Peterson et al. 2011	**5**	**Immature**	**Developing**	**Spawning capable**	**Actively spawning (subphase)**	**Regressing**	**Regenerating**
Frisk and Miller 2009	4	Immature	Adolescent	Onset mature	Functionally mature		
Barone et al. 2007	6	Immature	Virgin/maturing	Mature	Extruding	Resting	
Coelho and Erzini 2006; Ruocco et al. 2006	3	Immature	Maturing	Mature	Mature		
Ebert 2005[a]; Ebert et al. 2006	3	Juvenile	Adolescent	Mature	Mature		
Ivory et al. 2004	4	Juvenile	Maturing (adolescent)	Mature (adult)	Running/laying (adult)	Resting (adult)	
Templeman 1987; Stehmann 2002[b]; Sulikowski et al. 2005	6	Immature (juvenile)	Maturing (adolescent)	Mature (adult)	Active/ advanced/ extruding		
Walmsley-Hart et al. 1999[c]	3	Immature (juvenile)	Immature (subadult)	Mature (adult)	Mature (adult)		
Stehmann 1987[d]	2	Immature	Immature	Mature	Mature		
Richards et al. 1963; Kyne et al. 2008	3	Immature	Adolescent	Mature	Mature		

[a]Followed by Ebert et al. (2008a, 2008b).
[b]Followed by Costa et al. (2005) and Moura et al. (2007).
[c]Followed by Colonello et al. (2007) and Quiroz et al. (2009).
[d]Followed by Demirhan et al. (2005) and Whittamore and McCarthy (2005).

(ending of the spawning season), and regenerating (preparation for the next season). Given the high number of current classifications, the standardization of reproductive terminology across different fish taxa is of great importance for allowing comparisons among different studies (Brown-Peterson et al. 2011).

For elasmobranchs, there are several reproductive terminologies. One proposed by Stehmann (2002) for oviparous species has been adopted by some authors (e.g., Costa et al. 2005; Moura et al. 2007). It divides the reproductive cycle into three ovarian phases (immature, maturing, and mature) and three uterine phases (active, advanced, and extruding). Stehmann's (2002) terminology is one of the most complete terminologies since it differentiates spawning females from mature females, whereas the remaining terminologies have generally considered only three phases: immature, adolescent, and mature (Table 1; e.g., Richards et al. 1963; Walmsley-Hart et al. 1999; Ebert 2005; Coelho and Erzini 2006; Ruocco et al. 2006; Kyne et al. 2008). Most studies on reproductive development of oviparous elasmobranchs have relied only on macroscopic features (e.g., Richards et al. 1963; du Buit 1976; Ebert 2005; Oddone and Vooren 2005; Ruocco et al. 2006; Ebert et al. 2008a, 2008b), yet some good descriptive works on gonadal histology are also available (e.g., Stanley 1966; Hamlett et al. 1998, 2005; Andreuccetti et al. 1999; Lutton et al. 2005). However, these works generally only apply to adult specimens in the spawning capable phase. Barone et al. (2007) are the only authors that have applied histology to improve the description of the reproductive phases in elasmobranchs. However, those authors noted that their work was not complete since the phases described were based only on gonadal development.

All elasmobranchs have internal fertilization, so all species require specialized behavioral, morphological, and physiological mechanisms to ensure the success of fertilization (Hamlett and Koob 1999). Skates are oviparous, producing eggs enclosed in hard egg capsules, which are released into the water 0.5–2.0 d after the beginning of egg encapsulation (Holden et al. 1971; Ellis and Shackley 1995). After extrusion of offspring, parental care is absent. The skate reproductive tract has adapted to oviparity by allowing egg encapsulation and sperm storage in the oviducal gland (Hamlett et al. 1998) as well as egg capsule sclerotization via quinone tanning in the uterus (Koob and Cox 1990). In general, the complex life cycle of oviparous elasmobranchs is translated into an extended reproductive cycle. In addition to

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the batoid family Rajidae, this type of reproductive strategy is also shared by most carcharhiniform cat sharks (Scyliorhinidae), bullhead sharks (Heterodontiformes), and orectolobiform carpet sharks (Parascylliidae, Hemiscylliidae, and Stegostomatidae; Compagno et al. 2005; Musick and Ellis 2005). It is important to note that the scyliorhinid cat sharks *Halaelurus* spp. and some orectolobiform sharks share a different type of oviparity, the multiple or retained oviparity, which differs in the retention of multiple eggs in the oviduct for most part of development and the consequent extrusion of eggs containing well-advanced embryos (Compagno 1990; Dulvy and Reynolds 1997). The long reproductive cycle of oviparous species is associated with high energy requirements, which is related to greater ovarian follicle size prior to ovulation in females. For most species, mature size is reached at least 1 year after hatching and may take up to 7 years, as is the case for the thornback ray *Raja clavata* (Serra-Pereira et al. 2008).

In skates, the embryo develops inside the egg capsule while using yolk reserves for nourishment. The gestation period is species specific. The thornback ray is the most abundant species in northeast Atlantic landings (Dulvy et al. 2000). Spawning occurs between February and September in British waters (Holden et al. 1971; Holden 1975). Incubation time is estimated to be 5 months (Ellis and Shackley 1995). Compared with the majority of teleosts, the thornback ray has a number of *K*-strategist characteristics, including late maturation occurring at around 80% of the maximum size (Walker 1999; Whittamore and McCarthy 2005), slow growth (growth coefficient $k = 0.117$ per year; Serra-Pereira et al. 2008), large maximum sizes (adult females and males can reach 1,070 and 1,016 mm total length, respectively; Holden 1972; Nottage and Perkins 1983), and low fecundity (the maximum estimate for the thornback ray is an average of 140–150 eggs·female^{-1}·year^{-1}; Holden et al. 1971; Holden 1975).

The main objectives of this study were to (1) describe the reproductive tract development and gametogenesis in rajid species, particularly focusing on the thornback ray; and (2) adapt the recent reproductive terminology for teleosts (Brown-Peterson et al. 2011) to oviparous elasmobranchs (excluding retained oviparity) by using the thornback ray as an example in an effort to unify the reproductive terms used among all fish studies. The objectives were accomplished through macroscopic and microscopic analyses of female and male reproductive structures, following the work developed by Barone et al. (2007). The standardized terminology will not be extended to retained oviparity because little is known about the development in such species, and therefore different reproductive adaptations may occur.

METHODS

Sampling.—Thornback ray samples were collected between 2004 and 2008 from (1) landings of Portuguese commercial artisanal fleets (at Matosinhos and Peniche) under the scope of the National Data Collection Program (Plano Nacional de Amostragem Biológica, European Union Data Collection Regulation) and (2) Instituto de Investigação das Pescas e do Mar (IPIMAR) bottom-trawl research surveys carried out along the Portuguese continental shelf.

The reproductive organs of females (ovaries, oviducal glands, and uteri) and males (testes and sperm ducts, both epididymis and vas deferens) were extracted and preserved in a 10% solution of buffered formaldehyde.

Histological procedures.—Sections of reproductive organs were extracted and processed by use of an automated tissue processor (Model TP1020; Leica, Nussloch, Germany) according to the standard protocol (Bancroft and Gamble 2002). Samples were embedded in paraffin wax blocks with a standard heated paraffin embedding system (Model EG 1140H; Leica). The paraffin blocks were then sectioned to a thickness of 3–5 μm by use of a sliding microtome (Model SM 2000 R; Leica) or a rotary microtome (Model RM2125RT; Leica). The following staining techniques were used to analyze the histological structure of the oviducal gland: (1) hematoxylin and eosin, (2) toluidine blue, (3) periodic acid–Schiff (PAS), and (4) combined PAS and alcian blue (AB). Histological protocols followed Bancroft and Gamble (2002).

Histological slides were observed with a stereo microscope (Model SZX9; Olympus, Center Valley, Pennsylvania) and an optic microscope (Axioplan 2; Carl Zeiss, Oberkochen, Germany). Images were obtained with the imaging software programs TNPC version 4.1 and AxioVision version 4.1, respectively.

The different reproductive phases for females and males of the oviparous thornback ray based on macroscopic and microscopic features of the reproductive system during maturation were described. In total, 183 samples of thornback rays were observed. The current reproductive phases described by Stehmann (2002) were adapted to accommodate the terminology recently proposed by Brown-Peterson et al. (2011).

RESULTS AND DISCUSSION

Comparison of Terminologies Used for Oviparous Elasmobranchs

The terminology commonly used to describe different reproductive phases in oviparous elasmobranchs is variable among authors (Table 1).

The term "immature" is used by most authors to designate specimens with small gonads and an undeveloped reproductive tract (e.g., Richards et al. 1963; Walmsley-Hart et al. 1999; Stehmann 2002; Coelho and Erzini 2006; Ruocco et al. 2006; Barone et al. 2007; Kyne et al. 2008; Frisk and Miller 2009). In immature females, ovaries do not have visible follicles, the uterus is undeveloped, and oviducal glands are absent; in immature males, claspers are shorter than the pelvic fins, the testes are small and do not have visible lobes, and sperm ducts are undeveloped. The term "juvenile" is also used to designate

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immature specimens (Ivory et al. 2004; Ebert 2005; Ebert et al. 2006). Other authors (e.g., Stehmann 1987; Templeman 1987; Sulikowski et al. 2005) have even used the term "immature" to classify all specimens prior to maturation (i.e., both immature and developing individuals).

The developing phase (Brown-Peterson et al. 2011) is used to designate specimens in prespawning condition. In developing females, ovaries have small follicles and the uterus and oviducal glands are developing; in developing males, claspers are larger than pelvic fins, testes have visible lobes, and sperm ducts are developing. The developing phase is also termed "adolescent" (Richards et al. 1963; Ebert 2005; Ebert et al. 2006; Kyne et al. 2008; Frisk and Miller 2009), "maturing" (Stehmann 2002; Ivory et al. 2004; Coelho and Erzini 2006; Ruocco et al. 2006; Barone et al. 2007), "immature-subadult" (Walmsley-Hart et al. 1999), and "virgin" for males (Barone et al. 2007). Barone et al. (2007) subdivided developing males into "virgin" and "maturing," the former describing males with soft claspers and developing testes and sperm ducts and the latter only characterized by hardened claspers.

The spawning capable phase (Brown-Peterson et al. 2011) is used to designate adult specimens in reproductive condition. This phase is most often termed "mature" (e.g., Richards et al. 1963; Stehmann 1987; Templeman 1987; Walmsley-Hart et al. 1999; Ebert 2005; Sulikowski et al. 2005; Coelho and Erzini 2006; Ebert et al. 2006; Ruocco et al. 2006; Kyne et al. 2008). In oviparous elasmobranchs, the spawning capable phase refers to specimens that are capable of reproducing: females have ovaries full of large vitellogenic follicles and well-developed uterus and oviducal glands, and males exhibit hard and enlarged claspers, enlarged testes full of developed lobes, and developed sperm ducts. Terms that have been used instead of spawning capable include "onset mature" (Frisk and Miller 2009) and "mature" (Stehmann 2002; Ivory et al. 2004; Barone et al. 2007). In oviparous elasmobranchs, the actively spawning subphase within the spawning capable phase is used to describe females with egg capsules inside the uterus and males with reddish, swollen clasper glans and sperm flowing in the sperm ducts and seminal vesicle. The actively spawning subphase has previously been called "functionally mature" for both sexes (Frisk and Miller 2009); "active" (Stehmann 2002) or "running" for males (Ivory et al. 2004); and "extruding" (Barone et al. 2007), "laying/resting" (Ivory et al. 2004), or "active/advanced/extruding" for females, depending on the stage of development of the egg capsule (Stehmann 2002).

The regressing phase, also termed "resting" (Barone et al. 2007), is used to identify mature adults that have ceased spawning. Oviparous elasmobranch females in this phase have ovaries containing follicles with different sizes, postovulatory follicles, and small oviducal glands, whereas males have large and hard claspers and undeveloped testes. This term was only applied in the starry ray *Raja asterias* (Barone et al. 2007). Ivory et al. (2004) used the term "resting" to identify adult female spotted dogfish *Scyliorhinus canicula* in spawning condition without further description of an actual "resting" phase. The regenerating phase has never been applied to oviparous elasmobranchs.

Female Thornback Rays

Female thornback rays possess two ovaries, each located on the distal surface of the epigonal organ and containing developing follicles distributed on its surface (Figure 1). Macroscopically, the ovary is not distinguishable from the epigonal organ since the latter is a thin layer surrounding the gonadal tissue. This ovary is classified as an external ovary according to Pratt (1988). Ovaries possess follicles in all stages of development but no dominant stage (Figure 1), a sign of asynchronous follicle development; thus, the thornback ray is most likely a batch spawner (Murua and Saborido-Rey 2003). As in all other skates, each oviduct opens into an oviducal gland (Figure 1). The uterus is composed of a pair of anterior ducts connected anteriorly to the oviducal gland and converging in a unique posterior duct, which opens to the exterior through the cloaca (Figure 1). The main structure of the reproductive tract, including the position of the epigonal organ, is shared by all rajid species as well as by oviparous sharks, such as the spotted dogfish (Stehmann 2002).

The immature phase is clearly identified in female thornback rays. Macroscopically, it is not possible to identify follicles in the ovary, and the oviducal glands cannot be distinguished from the uterus, which is very thin (Figure 1a). However, microscopic analysis shows that the epigonal organ dominates most of the gonad. The epigonal organ, an autonomous lymphomyeloid tissue, is highly vascularized and contains different types of blood cells; leukocytes are the most abundant, mainly consisting of granulocytes and lymphocytes (Figure 2a). Ovarian follicles (i.e., oocyte and associated membranes, surrounded by the epigonal organ) are located under the germinal epithelium and under the tunica albuginea, a thin layer of connective tissue to which the follicles seem to be connected, especially during early development (Figure 2b). No oogonia are present. In skates as well as in all other elasmobranchs, oogenesis occurs early in life and oogonia are only observed during embryonic development (Prisco et al. 2002, 2007; McMillan 2007). In immature females, the first two stages of ovarian follicles observed are primordial and primary follicles. Ovarian follicle structure changes during development. The primordial follicles (<0.3 mm) consist of a primary oocyte surrounded by a single layer of flattened follicle cells (squamous cells; Figure 2c). Primordial follicles are transformed into primary follicles (diameter between 0.3 and 1.0 mm), in which the oocyte increases in size and the follicular epithelium thickens into a columnar epithelium containing two types of cells: small cells and large or intermediate cells (Figure 2d). The primary follicle stage is intermediate between primordial and previtellogenic follicles. No analogy could be made with teleosts since their follicles transform from primary to cortical alveolar oocytes, which differentiate just prior to vitellogenesis and therefore are not present in immature females. In summary, in oviparous elasmobranchs, immature females seem to have, in fact, some gamete development occurring in the

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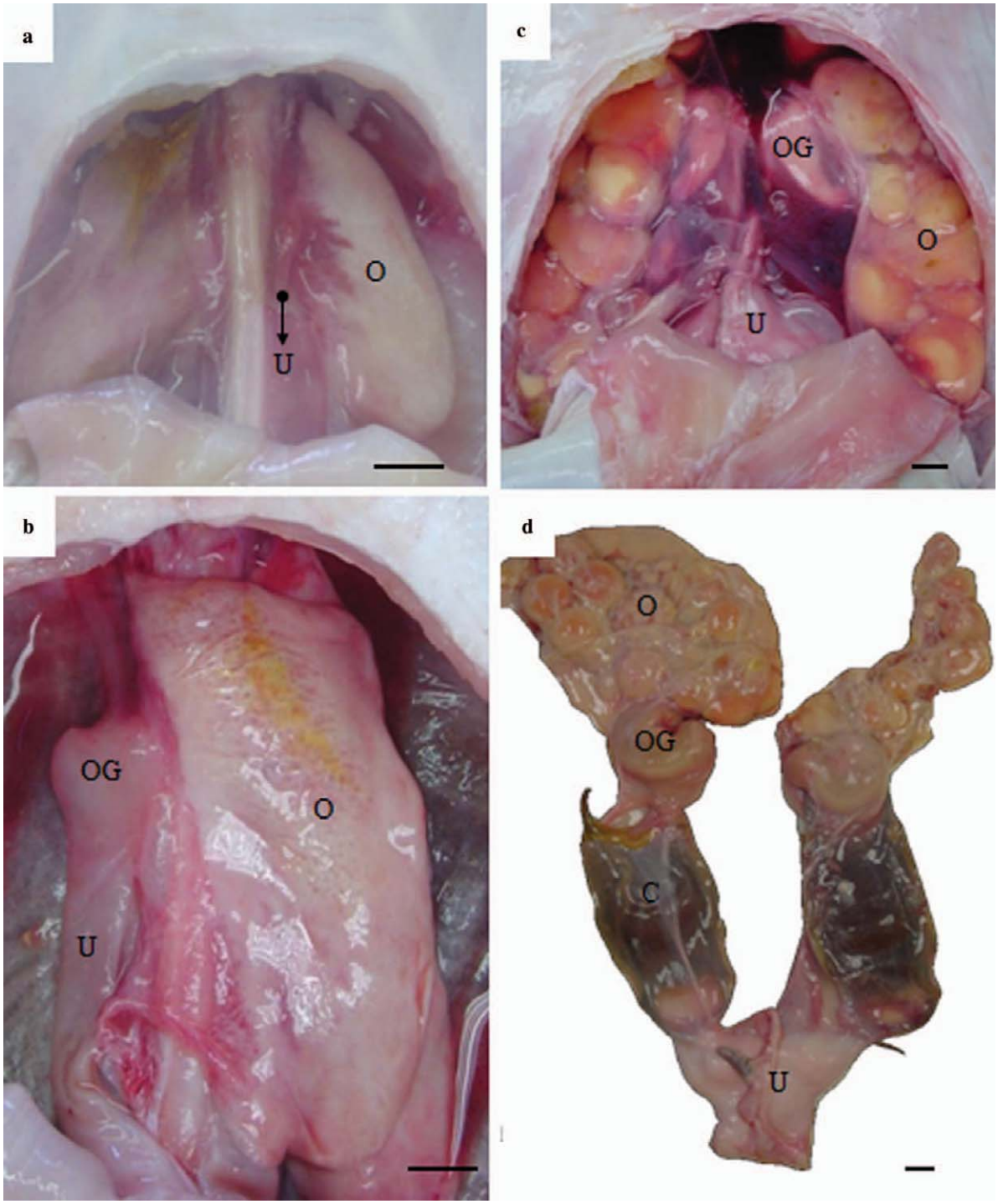


FIGURE 1. Macroscopic reproductive phases in female thornback rays: **(a)** immature (O = ovary; U = uterus), **(b)** developing (OG = oviducal gland), **(c)** spawning capable, and **(d)** actively spawning subphase (C = egg capsule).

ovaries. Such a phenomenon is not expected to occur in this phase based on what is known for teleosts (McMillan 2007). However, regarding the long reproductive cycle of thornback rays and all other oviparous elasmobranchs, in immature females the follicles seem to undergo a premature somatic growth of the oocyte and proliferation of follicular epithelium cells without transformation of the oocyte internal structure. Since this could only be detected with histology, the macroscopic criterion was maintained as a main character to classify immature females.

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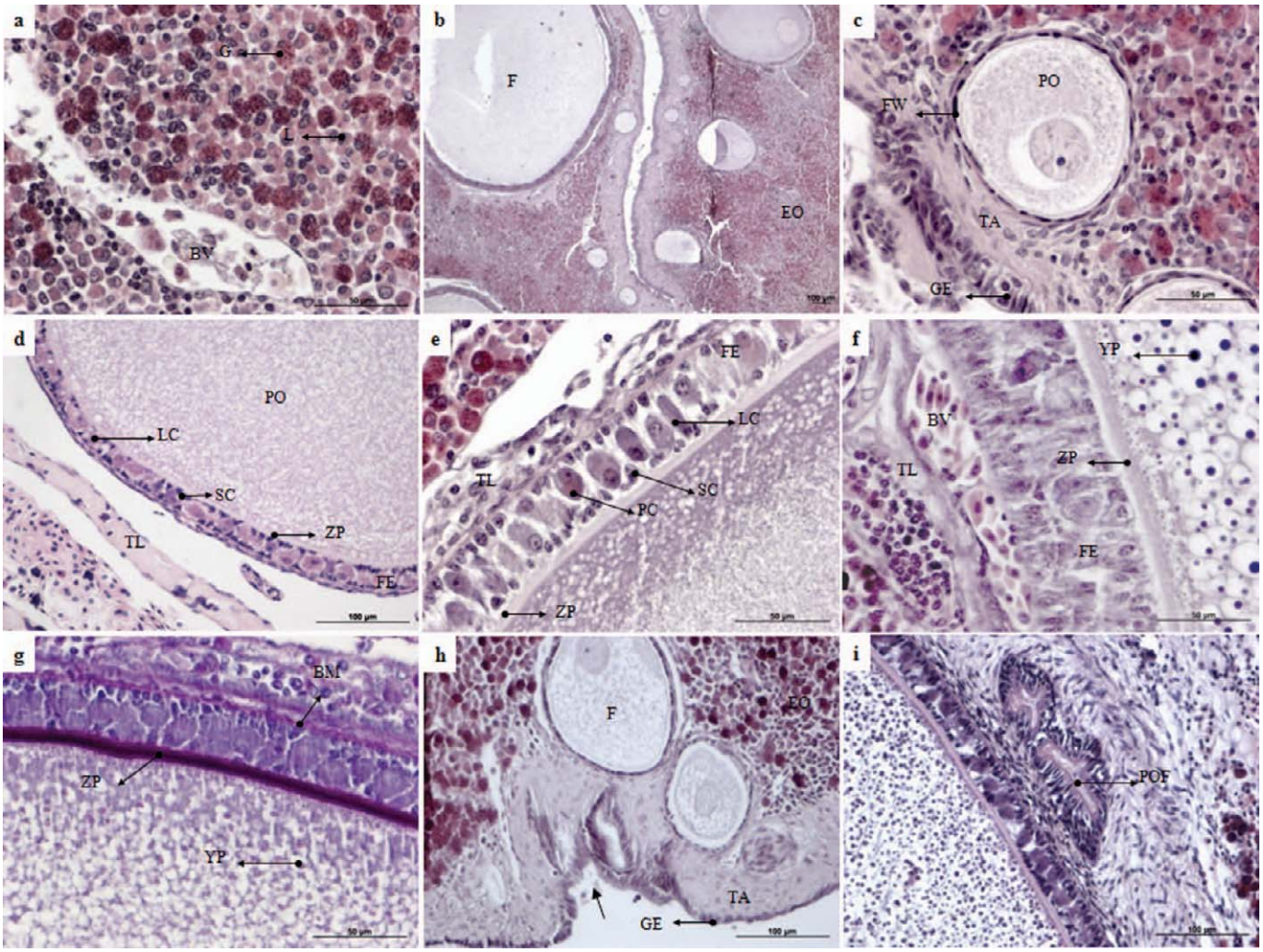


FIGURE 2. The thornback ray ovary: **(a)** highly vascularized epigonal organ (BV = blood vessel) containing granulocytes (G) and lymphocytes (L; hematoxylin and eosin [H&E]; scale bar = 50 μm); **(b)** ovary in the developing phase, showing follicles (F) in different stages of development, surrounded by the epigonal organ (EO; H&E; scale bar = 100 μm); **(c)** primordial follicle (105 μm in diameter) composed of a primary oocyte (PO) surrounded by squamous cells; follicular wall (FW) is attached to the germinal epithelium (GE) and tunica albuginea (TA; H&E; scale bar = 50 μm); **(d)** primary follicle (790 μm in diameter) composed of a PO surrounded by the zona pellucida (ZP), a pseudostratified columnar follicular epithelium (FE) containing small cells (SC) and large cells (LC), and more externally by the thecal layers (TL; H&E; scale bar = 50 μm); **(e)** previtellogenic follicle (1,031 μm in diameter) composed of an oocyte surrounded by the ZP; the FE containing SC, pyriform cells (PC), and LC; and more externally by the TL (H&E; scale bar = 50 μm); **(f)** vitellogenic follicle (3,480 μm in diameter) with visible yolk platelets (YP) inside the cytoplasm, thicker ZP and FE, and vascularized TL (H&E; scale bar = 50 μm); **(g)** vitellogenic follicle (4,100 μm in diameter; BM = basement membrane; periodic acid–Schiff positive [PAS+]; scale bar = 50 μm); **(h)** deformations on the GE (H&E; scale bar = 100 μm); and **(i)** postovulatory follicles (POF) in a developing female (H&E; scale bar = 100 μm).

The developing phase occurs in females between 300 and 700 mm total length. In contrast to what is known for teleosts (e.g., Tyler and Sumpter 1996; Jalabert 2005), in elasmobranchs the developing phase is not a fast process; it seems to last at least 1 year in sharks (e.g., Costa et al. 2005; Ebert et al. 2006), skates, and rays (e.g., Ebert 2005; Coelho and Erzini 2006; Moura et al. 2007; Ebert et al. 2008a, 2008b; Frisk and Miller 2009) and lasts up to 6 years in the thornback ray (Serra-Pereira et al. 2008). This long period of maturation is a major feature of all elasmobranchs (Frisk et al. 2001). A number of significant changes occur during the maturation process. Macroscopically, ovaries initially contain only previtellogenic follicles (<4 mm) and the oviducal gland starts to differentiate from the uterus as a white-colored, bean-shaped structure (Figure 1b). At the end of this phase, the ovaries develop large vitellogenic follicles (<15 mm) and the oviducal glands near full development, very similar to the subsequent reproductive phase. The subdivision of the developing phase, although facultative, allows for a better idea of the reproductive phase by differentiating between a female that is just starting to develop and a female that has almost reached the spawning capable phase. The term "early developing" should be used for females with ovaries containing only white follicles

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less than 2 mm in diameter and oviducal glands that are absent or beginning to form (whitish). "Mid-developing" should refer to females with ovaries containing yellow follicles less than 8 mm in diameter (commonly less than 30 follicles) and developing oviducal glands. Lastly, "late developing" should be used for females containing ovaries with a great quantity of yellow follicles (commonly more than 30 follicles) less than 15 mm in diameter and oviducal glands that are completely formed.

In the developing phase, primordial and primary follicles persist in the ovary. Previtellogenic follicles, which are larger in size (diameter > 1 mm) and have thicker follicular epithelia, are observed. In addition to small and large cells, previtellogenic follicles also contain pyriform and round cells with lipid-like substances in the epithelium (Figure 2e). Small cells are known to grow into large cells, which subsequently transform into pyriform cells (Andreuccetti et al. 1999). The pyriform cell apex connects with the oocyte, forming an intercellular bridge through which cytoplasmic constituents are transferred into the oocyte (Andreuccetti et al. 1999). This type of follicle development from primordial to vitellogenic follicles is similar to that described for oviparous elasmobranchs (e.g., Andreuccetti et al. 1999; Barone et al. 2007) and viviparous elasmobranchs (e.g., Prisco et al. 2002, 2007). In teleosts, the early developing previtellogenic phase corresponds to that in which cortical alveolar oocytes are formed, which is a main difference from elasmobranchs since this type of follicle never occurs in elasmobranch ovaries (e.g., Lutton et al. 2005; Barone et al. 2007; McMillan 2007).

Ovarian follicles (~2.5 mm in diameter) begin the vitellogenesis process (Figure 2f), which consists of the formation of yolk platelets, pseudostratification of the follicular epithelium, and an increase in peripheral vascularization between the thecal layers and the follicular epithelium. The peripheral vascularization is related to the transport of yolk precursors into the oocyte (Andreuccetti et al. 1999). In the thornback ray, the basement membrane, zona pellucida, and yolk platelets were markedly stained with PAS (Figure 2g). Vitellogenesis seems to start in follicles at around a similar size in rajids (e.g., Barone et al. 2007) and other elasmobranchs (Prisco et al. 2002, 2007).

The pair of oviducal glands starts to differentiate in the developing phase. The gland tubules form from the lumen and expand to the entire oviducal gland. In the late developing phase, the oviducal gland is fully developed and all of the secretory zones are distinguished (Serra-Pereira et al., in press). The uterus is composed of a very broad lamina propria (connective tissue) and is slightly vascularized. Internally, the uterus structure is arranged into invaginations and covered by simple columnar epithelium (Figure 3a, b).

Thornback ray females in the spawning capable phase are observed year-round in Portuguese waters. These females possess ovaries filled with follicles in different development stages, including large vitellogenic follicles greater than 15 mm in diameter. Also, their oviducal glands are completely formed and their uteri are enlarged (Figure 1c). The maximum follicle diameter in the thornback ray is 40 mm. The maximum follicle diameter observed in the ovaries seems to be related to the maximum size of the species. Smaller rajid species, such as the cuckoo ray *Leucoraja naevus* (du Buit 1976) and the argus skate *Dipturus polyommata* (Kyne et al. 2008), attain a maximum follicle size less than 30 mm in diameter. Species with maximum sizes similar to that of the thornback ray also attain similar follicle diameters (e.g., eyespot skate *Atlantoraja cyclophora*; Oddone and Vooren 2005), whereas larger species tend to have larger follicles, around 50–60 mm in diameter (e.g., longnose skate *Raja rhina*; Ebert et al. 2008b). Based on follicle composition after ovulation, the thornback ray may be a batch spawner. Further studies should be developed in this field to clarify the type of fecundity in the thornback ray and other rajid species.

Deformations of the germinal epithelium were observed in various cross sections along the ovary in all reproductive phases (Figure 2h). These deformations seem to relate to detachment of larger follicles from the periphery and subsequent movement inside the ovary and cannot be related to ovulation since they occurred prior to the spawning phase. In this study, histological slides with follicles larger than 6 mm were not analyzed. However, Andreuccetti et al. (1999) observed large follicles, close to ovulation size, in the starry ray. In those follicles, the follicular epithelium was reduced in thickness compared with previous stages, and only a few small cells and scattered large cells persisted. Pyriform cells disappeared by apoptosis, and round cells were reduced in size and disappeared prior to ovulation.

In the spawning capable phase the oviducal gland possesses tubules filled with secretions, including within the lumen (Serra-Pereira et al., in press). Sperm bundles were observed at the interior of the female's oviducal gland (Serra-Pereira et al., in press). The uterus showed an increase in invaginations (Figure 3c). The simple epithelium changed into undulating surface epithelium composed of ciliated cells with basal elongated nuclei and secretory cells with apical globulous nuclei (Figure 3d). Vascularization increased both near the folds and near the external surface of the uterus. The blood vessels reached the tip of each fold, as shown in Figure 3 (d, e). In this reproductive phase, the uterus produced secretions through the epithelial secretory cells, which were sulfated acid mucins (AB positive; Figure 3e) and neutral mucins (PAS positive; Figure 3f).

The actively spawning subphase corresponds to all of the uterine phases (i.e., active, advanced, and extruding) described by Stehmann (2002), which are collectively associated with capsule formation (Figure 1d). Thornback rays in the actively spawning subphase are observed year-round in Portuguese waters. In other areas, the spawning season is also extended, occurring between February and September in UK coastal waters (Holden et al. 1971; Holden 1975) and between May and December in the southeastern Black Sea (Demirhan et al. 2005). A continuous spawning reproductive strategy seems to be the most common strategy among rajids (e.g., Richards et al. 1963; du Buit 1976; Walker 1999; Oddone and Vooren 2005) and some

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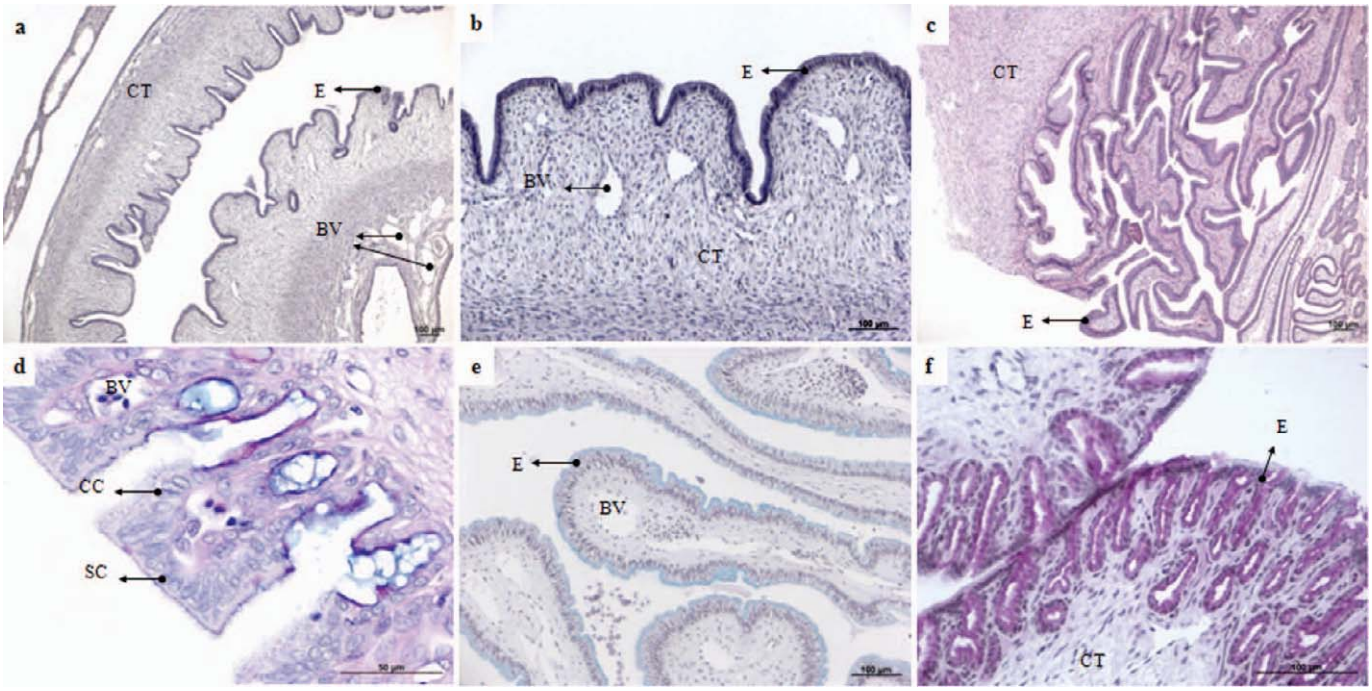


FIGURE 3. The thornback ray uterus: **(a)** immature uterus composed of vascularized (BV = blood vessel) connective tissue (CT) covered by simple columnar epithelium (E; hematoxylin and eosin [H&E]; scale bar = 100 μm); **(b)** surface detail of an immature uterus (H&E; scale bar = 100 μm); **(c)** spawning capable uterus with longitudinal folds (H&E; scale bar = 100 μm); **(d)** longitudinal fold detail, showing the two types of cells, ciliated cells (CC) and secretory cells (SC; combined periodic acid–Schiff [PAS] and alcian blue [AB], PAS/AB; scale bar = 50 μm); **(e)** spawning capable uterus with AB-positive secretions inside the epithelial cells (PAS/AB; scale bar = 100 μm); and **(f)** spawning capable uterus with PAS-positive secretions inside the epithelial cells (PAS/AB; scale bar = 100 μm).

cat sharks (Compagno et al. 2005). However, it is important to note that not all adult females were observed in actively spawning condition at the same time, so an asynchrony within the population must occur.

The actively spawning subphase is identified by the presence of egg capsules in the uterus. The ovulation of follicles occurred at a diameter of around 30 mm. After being fertilized, the egg was surrounded by the following series of envelopes produced by the oviducal gland: (1) sulfated acid and neutral mucins that were secreted by the club zone (hydrodynamic support); (2) a second layer of jelly that was secreted by the papillary zone and composed of sulfated acid and neutral mucins; (3) a third layer of jelly that consisted of sulfated acid mucins secreted by the papillary zone (lubricant and bounding layer); (4) a hard, proteic egg envelope that was secreted by the baffle zone; and (5) surface hairs (chemically similar to the capsule) coated with mucous secretions that cover the exterior of the capsule (sulfated acid mucins) and that were produced by the terminal zone (Serra-Pereira et al., in press). The chemical nature of the different egg envelopes is similar among oviparous species (Hamlett et al. 2005). In the actively spawning subphase, the uterus possesses highly vascularized folds with ciliated microvilli and branched tubular glands that produce sulfated acid and neutral mucins (AB positive and PAS positive, respectively; Figure 3d–f). Due to its structure and secretions, the uterus has a great contribution to the capsule surface structure and chemistry (Hamlett et al. 2005), facilitating biochemical processes for capsule sclerotization, including provision of oxygen and absorption of water (Koob and Hamlett 1998; Hamlett et al. 2005). The whole process from capsule formation to oviposition is rapid. In the thornback ray, it may last 1–2 d (Holden et al. 1971; Ellis and Shackley 1995).

Postovulatory follicles (Figure 2i) were only observed in females that were initially assigned to the developing phase—characterized by ovaries with follicles smaller than 1 mm and an enlarged oviducal gland and uterus. This combination of characteristics represented 30–40% of the females after attaining the size at first maturity. Since postovulatory follicles should not be found in the developing phase and should instead be observed after spawning (Brown-Peterson et al. 2011), it is suggested that these females could in fact be in a regressing phase. A regressing phase has already been described in the following rajids: the starry ray, based on the presence of postovulatory follicles in females with small oviducal glands (Barone et al. 2007); the thornback ray, based on the cessation of egg laying from October to January (Holden 1975); and adult female Aleutian skate *Bathyraja aleutica*, Commander skate *B. lindbergi*, and whitebrow skate *B. minispinosa* with inactive and atrophied ovaries (Ebert 2005). In other oviparous elasmobranchs, a regressing phase must also occur, especially in those species with short spawning seasons (Compagno et al. 2005). In

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species with continuous spawning, the regressing phase seems to occur at an individual level (Oddone and Vooren 2005). In the thornback ray and in other oviparous elasmobranchs (e.g., Barone et al. 2007), small, white previtellogenic follicles persist in the ovaries across all reproductive phases, apparently acting as a follicle reserve that may contribute to future spawning episodes.

Although not identified in the present study, the regenerating phase should be considered as a reproductive phase. Since in oviparous elasmobranchs the reproductive tract does not seem to regress to a phase where only primary growth follicles are found in the ovary (Brown-Peterson et al. 2011), the regenerating phase could be used to classify adult females that are entering a new cycle of follicle growth. In this phase, females have ovaries full of small follicles and enlarged oviducal glands and uteri. A regenerating period was already described in the eyespot skate based on gonadosomatic index values and the presence of females with white follicles in length classes where vitellogenesis and egg deposition occurred (Oddone and Vooren 2005). In that study, the regenerating phase was termed the "resting period." Further investigation will be needed to better characterize this phase.

In summary, the reproductive terminology used in teleosts seems to be adaptable to oviparous elasmobranch females. However, some differences in the characterization of the different phases should be taken into account as follows. In the immature phase, follicles in a more advanced stage than primary growth oocytes can be found in immature female oviparous elasmobranchs (i.e., "primary follicles," in which some proliferation of the thecal and follicular epithelium cells is observed). Gamete development seems to be more extended in time, which could be related to the longer reproductive cycles and higher longevity of these species. The developing phase in oviparous elasmobranchs is longer in duration and is characterized by the occurrence of previtellogenic follicles with major differences from those identified in teleosts, specifically the cortical alveolar oocytes (Brown-Peterson et al. 2011), which are absent from elasmobranchs. During the spawning capable phase in oviparous elasmobranchs, no hydrated oocytes are observed. For oviparous elasmobranchs in the actively spawning subphase, after being fertilized the ovulated egg is surrounded by a series of mucins and by a proteic capsule secreted by the oviducal gland, the activity of which must be triggered by a complex hormonal regulation (Hamlett et al. 2005). Further histological analysis should be made to better characterize the regressing and regenerating phases in female oviparous elasmobranchs.

Male Thornback Rays

Testes of male thornback rays have a lobular surface and are surrounded by the epigonal organ (Figure 4). Similar to the condition in females, the epigonal organ is a thin layer surrounding the male gonad. The development of sperm in each lobe is radial (i.e., developing from the germinal zone in the center to the periphery of the lobe) and diametric (i.e., developing across the testis from the dorsal surface to the ventral surface). This type of development leads to a compound testes according to Pratt's (1988) nomenclature. The main reproductive structure is similar among all rajid species and oviparous sharks (Stehmann 2002).

In immature males, the claspers are flexible and shorter than the pelvic fins (Figure 5a). Testes are homogeneous or have small lobules in the dorsal surface (Figure 4a). The sperm ducts—the epididymis and vas deferens—are very thin and can hardly be differentiated with the naked eye. Microscopically, spermatogenesis starts both from the germinal zone or germinal papilla located in the center of the lobe and dorsally in the testis (Figure 6a, b). Following the spermatocyst classification of Parsons and Grier (1992), at this reproductive phase only spermatocysts of stages I (primordial germ cells or gonocytes), II (spermatogonia), and III (primary spermatocytes) were observed (Figure 6c–e). Spermatocysts are spherical units composed of germ cells and Sertoli cells surrounded by an acellular basal lamina (Figure 6d). In all elasmobranchs, the germ cells of only a single developmental stage are associated with a Sertoli cell at any given time and then degenerate after the development is complete (Stanley 1966). Stage I spermatocysts, which are located beneath the coelomic epithelium in the germinal zone, consist of loosely organized gonocytes or primary spermatogonia, some of which are already bound to a basement membrane (Figure 6c). In stage II spermatocysts, the spermatogonia and Sertoli cells divide and the spermatocysts enlarge; the spermatogonia are aligned beneath the basement membrane, and the Sertoli cell nuclei also start to migrate to the periphery of the spermatocyst, surrounding a central lumen (Figure 6d). Stage III spermatocysts consist of primary spermatocytes resulting from the first meiotic division of spermatogonia; primary spermatocytes have large nuclei and fill the entire spermatocyst, and Sertoli cells remain in the periphery (Figure 6e).

As in females, the relatively long maturation process of oviparous elasmobranchs is translated in early male gamete development during the immature phase, since more advanced stages than primary spermatogonia are observed (Brown-Peterson et al. 2011). This fact has also been described in other elasmobranchs, such as the Portuguese shark *Centroscymnus coelolepis* and leafscale gulper shark *Centrophorus squamosus* (Girard et al. 2000). In these deepwater species, males that were macroscopically classified as immature showed more advanced stages (secondary spermatocysts and spermatids) than primary spermatocysts when analyzed microscopically.

In the developing phase, claspers are enlarged but still flexible. Claspers are longer than pelvic fins (Figure 5b), and the number of lobules in the testes increases (Figure 4b). Microscopically, all of the spermatocyst stages coexist, including stages I–VII. Stages I–III are defined above. Stage IV spermatocysts are formed from a division of primary spermatocytes into secondary spermatocytes, containing small, round nuclei and condensed chromosomes (Figure 6f). Stage V consists of spermatids, which are produced after the second meiotic

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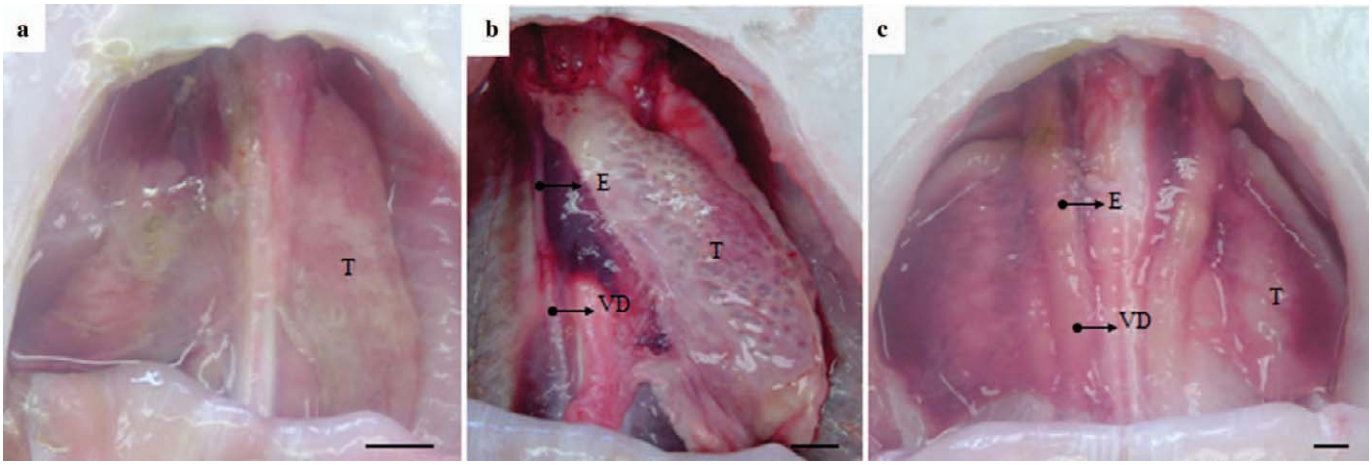


FIGURE 4. Macroscopic reproductive phases in thornback ray males: **(a)** immature (T = testis), **(b)** developing (E = epididymis, VD = vas deferens), and **(c)** spawning capable.

division of secondary spermatocytes; the spermatids show elliptical nuclei and emerging flagella and are separated and unorganized inside the spermatocyst (Figure 6g). In stage VI (early spermatozoa), spermatids that have undergone spermiogenesis are transformed into more elongated immature spermatozoa, which form loose bundles with heads facing the basement membrane and tails projecting toward the lumen (Figure 6h). Stage VII consists of mature spermatozoa that are organized in tight bundles associated with Sertoli cells arranged in the periphery (Figure 6i). Both the epididymis and vas deferens are already visible and start to coil as maturation advances (Figure 4b).

In the spawning capable phase, male thornback rays have claspers that remain rigid upon reaching maturity (i.e., due to their hard cartilages) and attain their maximum length (Figure 5c). The testes are completely formed (Figure 4c) and are filled with lobules containing all of the spermatocyst stages but a greater proportion of stages V–VII (spermatids to mature spermatozoa) than was observed in the previous phase. If no differences exist among elasmobranchs, the distribution of spermatocysts in this species should be homogeneous across the gonad (Maruska et al. 1996). The mature spermatozoa exit the testes via efferent ducts. The sperm is then transported through the epididymis and vas deferens to the seminal vesicles and the claspers. The epididymis (Figure 7a, b) shows a simple columnar epithelium that is folded into villosities, each containing a blood vessel inside. In the lumen, spermatozoa are arranged in bundles surrounded by sparse liquid, which is probably secreted by the Sertoli cells. The vas deferens (Figure 7c, d) is composed of a simple columnar, ciliated epithelium that is also folded into villosities; not all of these villosities contained a blood vessel. The lumen was filled with seminal liquid containing abundant sperm bundles

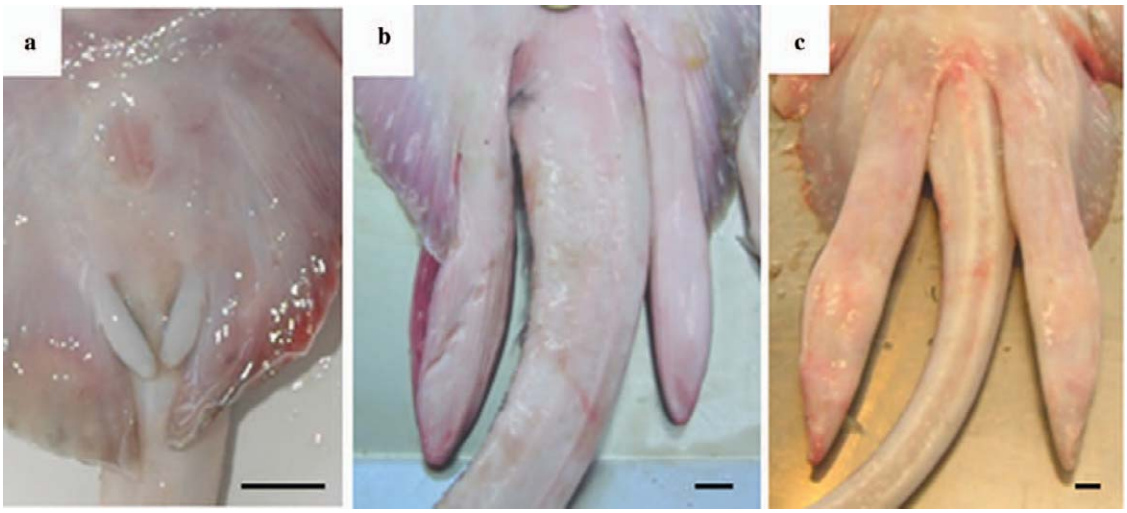


FIGURE 5. External reproductive phases based on clasper growth in thornback ray males: **(a)** immature, **(b)** developing, and **(c)** spawning capable.

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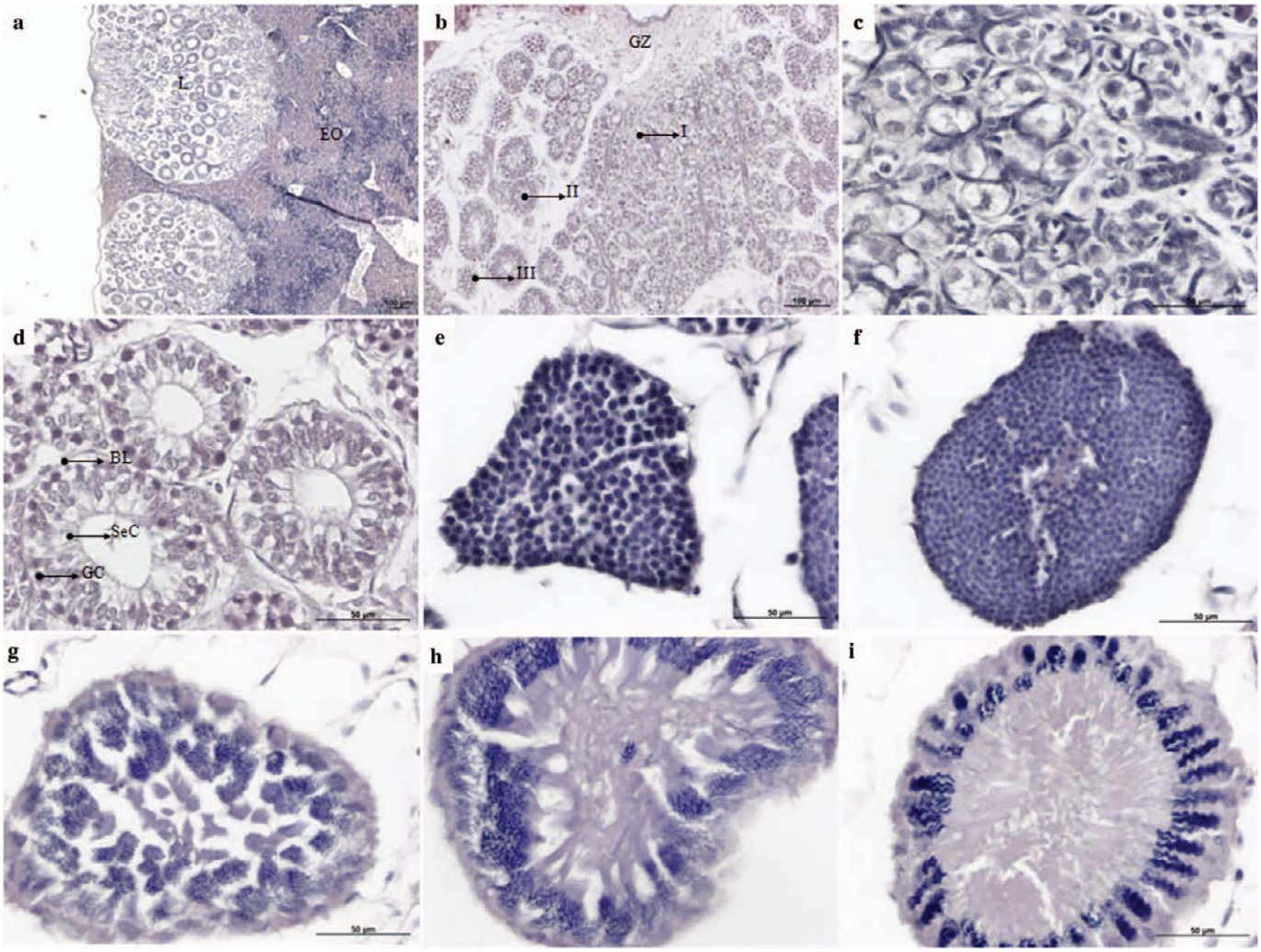


FIGURE 6. The thornback ray testis (hematoxylin and eosin stain used for all panels): **(a)** immature testis with small lobules (L) starting to differentiate, surrounded by the epigonal organ (EO; scale bar = 100 μm); **(b)** first stages of spermatogenesis in a lobe from an immature testis, starting from the germinal zone (GZ; stage I = gonocytes, stage II = spermatogonia, stage III = primary spermatocytes; scale bar = 100 μm); **(c)** stage I (gonocyte; scale bar = 50 μm); **(d)** stage II (spermatogonia) composed of germ cells (GC) and Sertoli cells (SeC) surrounded by an acellular basal lamina (BL; scale bar = 50 μm); **(e)** stage III (primary spermatocyte; scale bar = 50 μm); **(f)** stage IV (secondary spermatocyte; scale bar = 50 μm); **(g)** stage V (spermatid; scale bar = 50 μm); **(h)** stage VI (immature spermatozoa; scale bar = 50 μm); and **(i)** stage VII (mature spermatozoa; scale bar = 50 μm).

and round structures similar to primary spermatocytes (Figure 7d).

Males are assigned to the actively spawning subphase based on the appearance of the claspers. The internal appearance of the claspers is reddish and swollen after copulation; the sperm ducts are completely filled such that when sliced, the sperm spills out of the ducts. As in teleosts, the actively spawning subphase cannot be distinguished through histology (e.g., Brown-Peterson et al. 2011).

There was no evidence of regressing and regenerating phases in male thornback rays. In other words, males with hard, enlarged claspers with regressing gonads or those that appeared reproductively inactive were not observed. In fact, males seem to not exhibit a reproductive cycle but rather progress from the immature phase to the spawning capable phase one time and then remain in the spawning capable phase for the rest of their lives. This suggests that active spermatogenesis is always occurring in males once they have reached sexual maturity, which is completely different from what is known for teleosts. In other elasmobranchs, such as the starry ray (Barone et al. 2007), males with large claspers and small testes were described to occur, and these characteristics would correspond to the regressing phase. Therefore, although the regressing phase was not observed in the thornback ray, it should be considered a reproductive phase for male elasmobranchs so that it can be applied to the species in which it occurs.

In summary, the reproductive terminology used in teleosts seems to be adaptable to oviparous elasmobranch males.

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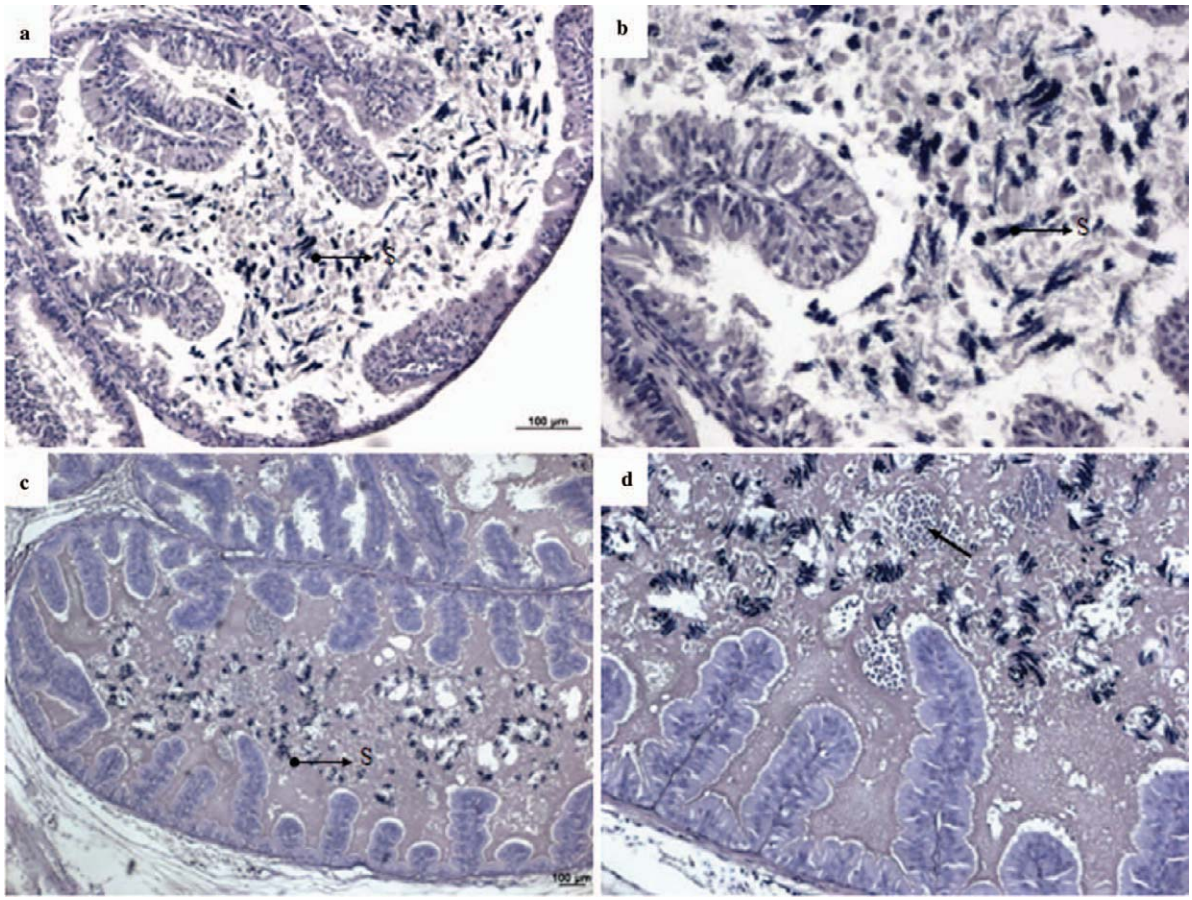


FIGURE 7. Sperm ducts in thornback ray males (hematoxylin and eosin stain, scale bar = 100 µm for all panels): **(a)** epididymis in the spawning capable phase, showing sperm bundles (S) surrounded by Sertoli cell material; **(b)** detail of a villosity in the epididymis; **(c)** vas deferens in the spawning capable phase, exhibiting sperm bundles (S) surrounded by a dense seminal liquid; and **(d)** detail of the vas deferens, showing ciliated villosities, sperm bundles, and structures similar to primary spermatocytes (arrow) dispersed in the lumen.

However, the following differences should be taken into account. In skates, spermatogenesis occurs in seminiferous follicles arranged inside lobules, each containing a germinal epithelium. This is a distinct organization from that found in most teleosts, where the testes are arranged in elongated, branching seminiferous tubules instead of follicles and lack a permanent germinal epithelium (Matty 1985). Spermatogenesis seems to be triggered earlier in development for oviparous elasmobranchs than for teleosts, since primary spermatocysts are observed in the testes of immature thornback rays and more advanced stages of spermatocyst are observed in immature males of other elasmobranch species. For the spawning capable phase, oviparous elasmobranchs demonstrate the occurrence of internal fertilization, in which the sperm is released inside the female through claspers instead of being released into the sea through short gonoducts. Once a male elasmobranch attains maturity, entry into the regressing phase apparently does not occur in some species (e.g., the thornback ray), whereas in other species the regressing phase has been observed.

In conclusion, the reproductive terminology adopted from Brown-Peterson et al. (2011) proved to be adequate when applied to females and males of the thornback ray as an example of an oviparous elasmobranch. A direct application of previous terminologies was accomplished. A summary of the main macroscopic- and microscopic-scale features of each reproductive phase as described in the present study is presented in Table 2. A more detailed description considering particular features of other oviparous elasmobranch species as well as an adaptation of the same terminology to viviparous species should be pursued by future studies. The need for a more detailed terminology that includes regressing and regenerating phases has already been mentioned by other authors (e.g., Ebert et al. 2008b), since the common misclassification of adult elasmobranch specimens as developing (i.e., classifying an individual that had already spawned at least once as "immature" for the purposes of fitting maturity ogives rather than as "mature") could lead to overestimation of the length at 50% maturity and to biased estimates of a population's reproductive potential and growth (Ebert et al. 2008b).

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TABLE 2. New proposal for a reproductive terminology for oviparous elasmobranchs, as applied to skates, based on the new terminology by Brown-Peterson et al. (2011) and the reproductive phases proposed by Stehmann (2002). The macroscopic- and microscopic-scale features of both females and males are presented for each phase. Microscopic-scale features were based on thornback ray reproductive development; the measurements presented are specific for thornback rays.

	Females		Males	
Phase	Macroscopic scale	Microscopic scale	Macroscopic scale	Microscopic scale
Immature	Ovaries small, whitish, and homogeneous; undistinguishable ovarian follicles. Oviducal gland not visible. Uterus threadlike and narrow.	Ovary with primordial follicles (<0.3 mm) and primary follicles (0.3–1.0 mm) connected to the germinal epithelium and tunica albuginea. Uterus composed mainly of connective tissue covered by simple columnar epithelium with some invaginations; some blood vessels present.	Claspers flexible and small, shorter than or as long as the pelvic fins. Testes small, sometimes with lobules already visible. Sperm ducts straight and threadlike.	Testes containing spematocysts of stages I, II, and III only.
Developing*	Ovaries enlarged with small follicles of different sizes, sometimes restricted to the anterior part of the ovary. Developing oviducal gland; enlarged uterus. *A subdivision could be considered: Early developing: ovary with only white follicles less than 2 mm; oviducal gland not visible. Mid-developing: ovary with yellow follicles smaller than 8 mm (commonly fewer than 30 follicles); developing oviducal gland. Late developing: ovary with a great quantity of yellow follicles (commonly more than 30) with a diameter less than 15 mm; oviducal gland completely formed.	Ovary with primordial, primary, previtellogenic, and vitellogenic follicles (<15 mm). Oviducal gland can show only the beginning of gland tubule formation or can be completely formed, with differentiation of the four secreting zone, depending on the level of maturation. Beginning of secretion production in the oviducal gland and uterus. Uterus more invaginated and vascularized.	Claspers still flexible, extended longer than the tip of the pelvic fin. Testes enlarged with developing lobules. Sperm ducts beginning to coil. Males with only one of these features should also be classified under this phase.	Testes containing spematocysts in all stages. Sperm ducts start to differentiate villosities. No sperm is observed inside the ducts.

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Spawning capable	Large ovaries with large, yolked follicles that can reach around 40 mm in diameter. Oviducal gland and uterus fully developed.	Follicles in all stages can be observed in the ovary. Secretions present in the tubules of the oviducal. Uterus highly invaginated, showing longitudinal folds that produce secretions to the lumen.	Claspers enlarged, longer than the pelvic fins; fully formed and rigid. Testes greatly enlarged, filled with developed lobules and often reddish in color. Sperm ducts tightly coiled and filled with sperm.	Testes containing spematocysts in all stages. Stages V–VII are more abundant than in the developing phase. Sperm ducts composed of villosities full of seminal liquid, more dense in the vas deferens. Sperm bundles observed in the lumen.
Actively spawning subphase	Ovaries and oviducal gland similar to the spawning capable phase. Egg capsule present in the uterus and may or may not be attached to the oviducal gland. Capsules may be in early production or fully formed, hardened, and dark; capsules present in one uterus or both uteri.	Postovulatory Follicles (POFs) can be present in the ovary. Oviducal gland tubules full of secretion materials. Secretions also present in the gland lumen.	Clasper glands reddish, dilated, and swollen. On pressure, sperm flowing in the sperm ducts and out of the cloaca.	Same as spawning capable.
Regressing	Large ovaries with follicles not occupying the entire surface. Few large vitellogenic follicles may be present. Oviducal gland completely formed; expanded uterus. Can be mistaken for the developing phase.	Follicles in all stages can be observed in the ovary; POFs present in the ovary; Oviducal gland completely formed but without secretions in the lumen. Uterus completely formed and with production of secretions.	Claspers, longer than the pelvic fins, fully formed, and rigid (similar to spawning capable stage). Shrunken testes with few visible lobules.	Not analyzed.
Regenerating	Ovaries full of small vitellogenic follicles; enlarged oviducal glands and uterus. Can be mistaken for the developing phase.	Not analyzed.	This phase is not known to occur in males of oviparous elasmobranchs.	

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