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Reproductive aspects of the velvet belly *Etmopterus spinax* (Chondrichthyes: Etmopteridae), from the central western Mediterranean Sea. Notes on gametogenesis and oviducal gland microstructure

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Abstract

In this paper, the reproductive biology of the velvet belly *Etmopterus spinax* was analyzed in Sardinian waters (central western Mediterranean). This species was sexually dimorphic with females growing to a larger size than males. Marked sexual dimorphism in size was also observed along the bathymetrical gradient. Histological analysis of gonads was very useful in assigning macroscopical maturity stages. The investigation on the microstructure of the oviducal gland (OG) highlighted four morphofunctional zones with mucous and/or proteic secretions according to the zone and to their specific functions and development. Sperm in the OG was found for the first time in *E. spinax*. The localization of sperm storage tubules deeper in the OG suggested long-term sperm storage, which is in agreement with the long reproductive cycle described. This species matured late, specifically at 80.7% and 79% at the maximum observed size for females and males respectively. Mature specimens were found throughout the year with pregnant females observed in winter and autumn. Low fecundity was observed with a mean ovarian fecundity of 16.5 mature follicles.

Keywords: *Etmopterus spinax*, reproductive cycle, gametogenesis, oviducal gland, sperm storage.

Introduction

Through their evolutive history, cartilaginous fishes have remained major components of marine communities with the ability to adapt to varying selective pressures. Their success is due, in part, to biological features such as low growth rates, low egg production, and maturation late in their life cycle (Hoenig & Gruber, 1990; Hamlett *et al.*, 2005). All these features make these organisms highly vulnerable and unable to recover from any population reduction (Stevens *et al.*, 2000).

The velvet belly *Etmopterus spinax* (Linnaeus, 1758) is a small-sized deep-water squaliform, viviparous lecithotrophic (Musick & Ellis, 2005), with no maternal nutrients input, and embryos rely on the yolk sac reserves for development. It usually occurs in the eastern Atlantic Ocean, from Iceland and Norway (Compagno *et al.*, 2005), to South Africa (Compagno, 1984) including the Azores (Santos *et al.*, 1997), the Canaries (Brito *et al.*, 2002) and the Cape Verde Islands (Reiner, 1996). It is also distributed in the western and central Mediterranean Sea (Serena, 2005), including the Ionian, the lower Adriatic and the Aegean Sea (Notarbartolo di Sciara & Bianchi, 1998). This species lives mainly in the outer continental and insular shelves and upper slopes, at depths from 70 to 2000 m, but mostly between 200 and 500 m, near or well above the bottom (Compagno *et al.*, 2005).

E. spinax specimens are commonly captured and discarded as by-catch by commercial otter trawlers, making fisheries data very scarce. In the previous decades, studies on the biology of this species were carried out in the Mediterranean and North Eastern Atlantic Ocean. They focused on feeding habits and trophic interactions (Bello, 1997; Belluscio *et al.*, 2000; Santos & Borges, 2001; Fanelli *et al.*, 2009), reproduction (Vacchi & Relini Orsi, 1979; Capapè *et al.*, 2001; Cecchi *et al.*, 2004; Coelho & Erzini, 2008; Aranha *et al.*, 2009; Coelho *et al.*, 2010) and age and growth (Sion *et al.*, 2004; Gennari & Scacco, 2007; Coelho & Erzini, 2008). However, information on its general biology in the Central Mediterranean is very limited and considering the highly vulnerable life cycle of this deepwater squalid shark, there is a need for population dynamics studies on this species (Coelho & Erzini, 2008) although, at the moment, it is classified in the IUCN red list as of *Least Concern* (LC) (Coelho *et al.*, 2009).

Understanding the ecology and population dynamics of Elasmobranchs requires better appreciation of re-

productive diversity through studies on physiology, biochemistry and anatomy (Storrie *et al.*, 2008). The oviducal gland (OG), a specialized region of the anterior oviduct, is an exclusive structure of cartilaginous fishes. The biological importance of this structure, analyzing their microarchitecture and development, is closely related to the special features of the reproductive strategy of the group, the maturity stage and reproductive cycle stage (Galíndez *et al.*, 2010). Its basic function is the production of the egg jellies, candle/envelope formation, and transport of fertilized eggs and receptacle and /or sperm storage (Hamlett *et al.*, 1998). Generally, in the oviducal gland, four different zones can be recognized. They are characterized by organization and different staining affinities of the mucosa; their structure and development are closely related to the reproductive mode, the maturity stage, the reproductive season and reproductive cycle stage (Hamlett *et al.*, 1998).The external morphology, internal microstructure and histochemistry of the chondrichthyan OG have been investigated in oviparous and viviparous species (e.g. Prasad, 1948; Hamlett *et al.*, 2002). Despite the differences in egg capsules produced by these species, which range from substantial structures in oviparous species to thin diaphanous membranes in some viviparous species, the same fundamental mechanism for assembly of capsule proteins is employed (Hamlett *et al.*, 1998).

The goal of this paper is to provide the critical details necessary for the assessment of the mature proportion of the *E. spinax* population, and provide biological information concerning the reproductive biology (e.g. size at first maturity, reproductive cycle and fecundity) from the Central-Western Mediterranean Sea. For this reason, we also described the development of the reproductive tract and the gametogenesis of the velvet belly as well as the process underlying OG development from the beginning of differentiation to the spent phase, through an analysis of the secretions produced by the different zones.

Materials and Methods

Sampling

A total of 908 specimens of *E. spinax* were collected during seasonal experimental trawl surveys and during commercial hauls at depths from 260 to 1573 m on compact mud bottoms off the Sardinian coasts (Central-Western Mediterranean Sea) between June 2008 and December 2012 (Table 1).

For each individual, total length (TL) was recorded in

Table 1. Sampling dynamics, 2008–2012, represented by cruises, year, season, number of positive hauls, frequency of occurrence (for hauls at depth > 199 m), depth-range and number of analyzed individuals.

Cruises	Year	Season	Number of hauls	Occurrence (%)	Depth range (m)	Number of individuals
MEDITS*	2008	Summer	13	36.1	401-671	157
GRUND**	2008	Autumn	3	11.5	444-548	45
Commercial hauls	2009	Spring	2	-	600-620	4
MEDITS*	2009	Spring	6	42.9	390-635	30
MEDITS*	2009	Summer	9	34.6	534-730	44
CampBiol**	2009	Autumn	1	-	500	4
MEDITS*	2010	Spring	2	5.4	539-603	22
CampBiol***	2010	Autumn	2	-	600	19
Commercial hauls	2010	Autumn	1	-	600	2
Commercial hauls	2010	Winter	1	-	600	1
Deeptrawlsurvey	2011	Winter	2	28.6	1017-1573	2
Commercial hauls	2011	Winter	3	-	600	5
MEDITS*	2011	Spring	4	21.1	397-615	62
MEDITS*	2011	Summer	3	15.8	572-597	14
CampBiol***	2011	Winter	2	-	570-615	60
Commercial hauls	2012	Winter	4	-	600	34
Commercial hauls	2012	Spring	4	-	500-600	18
MEDITS*	2012	Spring	9	52.3	260-640	229
MEDITS*	2012	Summer	10	47.6	570-670	96
Commercial hauls	2012	Autumn	6	-	500	60

* Mediterranean International TrawlSurvey (Bertrand *et al.*, 2000)
** Gruppo Nazionale Demersali
*** Campionamento biologico delle catture commerciali (Data Collection Framework, Regolamento CE 199/2008)

centimetres; male claspers length (CL), the oviducal glands (OGW) and uteri width (UW) were recorded in millimetres using a gage; total mass (M), gonad mass (GM) and liver mass (LM) were recorded in grams. Specimens were sexed and the maturity stages were determined following the scales for viviparous Elasmobranchs proposed by Stehmann (2002). According to these scales, females were classified in seven stages: stage 1, immature; stage2, maturing; stage 3, mature; stage 4, developing; stage 5, differentiating; stage 6, expecting; stage 7, spent. Instead, males were classified in four stages: stage 1, immature; stage 2, maturing; stage 3 mature; stage 4, active. In females, the size of all yolk follicles (yellow) obtained by taking the minimum and maximum diameter with a caliper was also recorded (mm) from both ovaries.

Histological procedures

Several different tissue samples were dissected from gonads and oviducal glands (OGs) of a subsample of 5 specimens for each maturity stage. Transverse pieces of gonads were removed and the whole OGs were dissected and cut through the centre from the oviduct to the uterus, providing a piece of tissue representing the sagittal plane. Tissues were fixed in 5% buffered formaldehyde (0.1 M pH 7.4) and dehydrated through a progressively higher series of alcohol concentrations (70-100%), embedded in synthetic resin (GMA, Technovit 7100, Bio-Optica) and sectioned at 3.5 μm using a rotative microtome (LKB, Historange). Sections of gonads were stained with Harrys haematoxylin and eosin (H&E); sections of oviducal gland were stained with either H&E to analyze the histological structure and combined periodic acid-Schiff (PAS) and alcian blue (AB) to investigate the chemical nature of the secretions produced by the different glandular zones (PAS+ structures stained pink, AB+ stained blue and PAS + AB + stained in different intensities of purple). The histological staining protocol proposed by Cerri & Sasso-Cerri (2003) for glycol methacrylate embedded tissue sections was used.

Histological sections were observed under a binocular optical microscope (Zeiss Axioscop) and selected sections were photographed using a digital camera (Canon PowerShot G2). The different reproductive phases for females and males of the velvet belly *Etmopterus spinax*, based on macroscopic and microscopic features of the reproductive system during maturation, were described. Follicles and testicular germinal cell development stages were identified according to the scale proposed by Hamlett & Koob (1999).

Data analysis

The Kolmogorov-Smirnov (KS) two-sampled test was used to test for significant differences in the length frequencies by sex. The evolution of the mean length, for both sexes, along depth strata (100 m) was also analyzed using ANOVA test correlation (Zar, 1996).

Sex-Ratio (SR, females:males) was estimated for the whole population, considering its variation by different depth interval (100 m). The significance of deviation from the 1:1 null hypothesis was tested by the χ^2 test.

Total length and total mass relationships were calculated separately for each sex according to the power curve function $M = aL^b$ where M represents total mass and L the total length, *a* the intercept of the regression and *b* is the regression coefficient. Parameters *a* and *b* were estimated by applying logarithmic transformation (ln-transformed data). The student's *t* test was used to test the equality of regression coefficient between males and females linear regression equation (Zar, 1996).

Size at maturity (L_{50}= length at which 50% of the individuals are mature) was estimated, for males and females separately, by fitting maturity ogives to the proportion of mature individuals in each 2 cm TL size class. Non-linear least squares regression was used to estimate the parameters:

$$P = 100/(1+exp(a+(b \times TL)))$$

where P is the proportion of mature fish at TL size class and *a* and *b* are the model coefficients. L_{50} is the length at first maturity = *a/b* where *a* is the intercept and *b* is the slope of the maturity curve.

The measurements taken from the reproductive organs of females and males were analyzed by maturity stage in order to characterize the maturation process. The null hypothesis of no differences between maturity stages in the OG width was tested with ANOVA.

Reproductive seasonality was inferred through an analysis of: (1) the seasonal evolution of the percentage of maturity stages of females and males, and (2) the seasonal changes in the gonado-somatic index GSI = (GM/TM) × 100

and the hepato-somatic index HSI = (LM/TM) × 100 for the mature specimens. The seasonal variations found both in GSI and HSI were compared by analysis of variance (ANOVA). Ovarian fecundity was defined as the total number of eggs released per female during the spawning season estimated by the total number of yolked follicles (mature stage) counted in both ovaries. In pregnant females, the number of pups and relative TL were taken.

Results

Size structure

A total of 908 specimens of velvet belly *Etmopterus spinax* were sampled of which 532 were females and 376 were males. Females outnumbered males overall (SR = 0.59; $\chi^2 = 13.50$; $P < 0.05$).

Both male and female samples had a wide length range with females attaining substantially larger size than

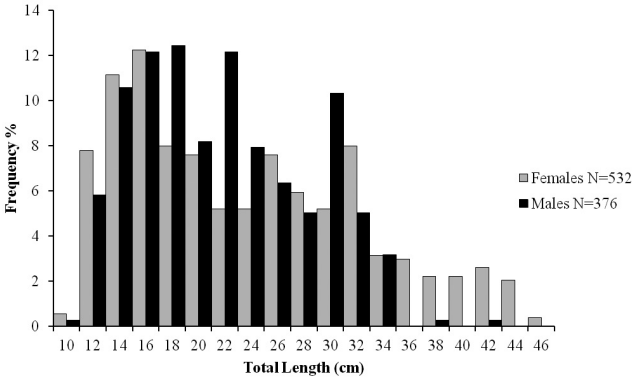

Fig. 1: Length-frequency distribution of females and males in *Etmopterus spinax*.

males. Specifically, females varied from 10 and 45.7 cm TL (22.78 ± 8.94cm, mean ± S.D.), while males ranged from 9.7 to 41.8 cm TL (20.81 ± 6.37 cm, mean ± S.D.) (Fig. 1). Results of the KS two-sample test indicated a statistically significant difference ($P < 0.05$) in length frequency distribution among sexes.

Length-mass relationships of females ($M = 0.0031 \times TL^{3.0814}$; $r^2 = 0.98$) and males ($M = 0.0036 \times TL^{3.0335}$; $r^2 = 0.97$) were significantly different (t-test, $P < 0.01$) (Fig. 2 A,B).

The mean TL of females seemed to increase with depth, with statistical differences between all bathymetric strata (ANOVA, F-ratio = 10.84 P = 0), and a significant increase of mean TL starting from 500 m of depth. In males, the variation of mean TL with depth appeared slower than for females (Table 2). Significant differences among depths were found (ANOVA, F-ratio = 4.92 P = 0.0002).

Sex ratio to depth strata highlighted a significant difference ($P < 0.05$) only between 500 and 600 m where the largest number of individuals was found, and females outnumbered males (Table 2).

Even though there was overlap in lengths of adjacent maturity stages, a clear increment in sizes with the evolution of the maturity stage in both genders was found (Fig. 3A,B). From 532 females classified into maturity stages, 86.40% were considered immature (stage 1, TL < 34.9 cm), 4.33% maturing (stage 2, TL < 41.6 cm), while only 4.34% were mature (stage 3-6, TL < 45.7) and 4.93% spent (stage 7 TL < 43.5 cm) (Fig. 3A). From 376 males, 82.88% were at immature stage (stage 1, TL < 32.7 cm), 11.14% at maturing stage (stage 2, TL < 33.5 cm), and only 5.98% at mature and active stages (stage 3-4, TL < 41.8 cm) (Fig. 3B).

The majority of mature (stage 3) and pregnant females (stages 4-6) with mature and active males (stage 3 and 4)

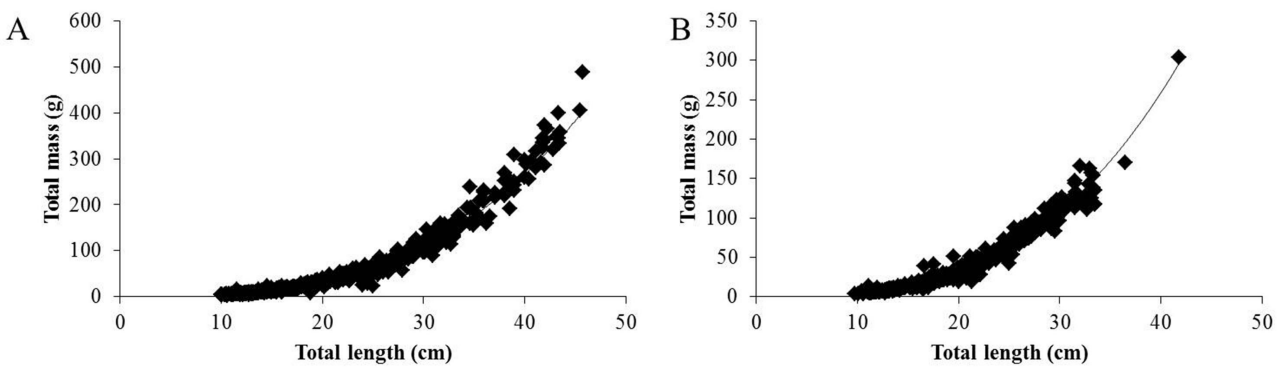

Fig. 2: Length-weight relationship of females (A) and males (B) of *Etmopterus spinax*.

Table 2. Sex ratio and mean total length of the population of *Etmopterus spinax* females and males in relation with depth strata.

Depth (m)	Total	Females	Mean TL (±S.D.)	Males	Mean TL (±S.D.)	Sex ratio	χ^2
200-300	19	10	15.98±3.6	9	14.43±2.4	0.53	0.03^{NS}
300-400	146	71	17.94±4.4	75	18.84±3.7	0.49	0.05^{NS}
400-500	63	29	17.89±5.7	34	19.09±4.3	0.46	0.2^{NS}
500-600	469	303	23.52±8.7	166	21.80±6.6	0.65	20.46*
600-700	206	118	24.80±10.5	88	21.70±7.8	0.57	2.20^{NS}
700-800	3	0		3	20.73±1.0	0	2^{NS}
1000-1100	1	1	39	0		1	0.67^{NS}
1500-1600	1	0		1	33.3	0	0.67^{NS}

NS, not significant: P > 0.05; *, significant: P ≤ 0.05; 1 df.

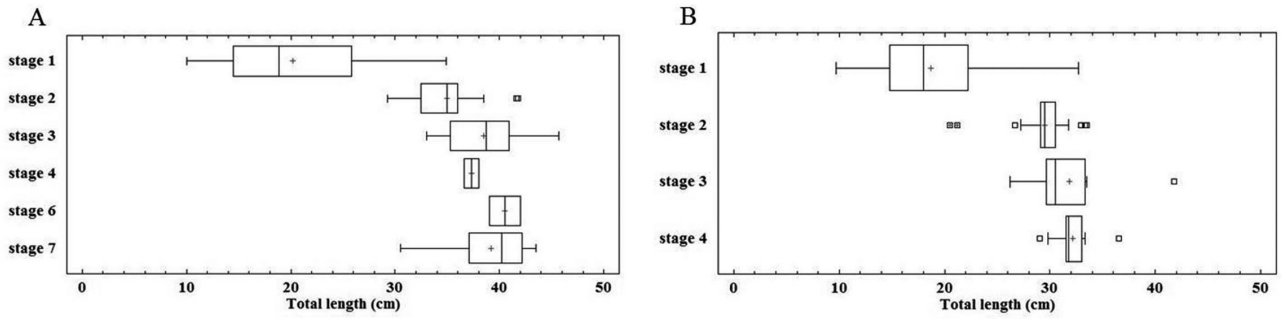

Fig. 3: Size distribution of females (A) and males (b) of *Etmopterus spinax* by maturity stage. Boxes show mean value and standard error, vertical bars show standard deviation from the mean.

were caught at depths between 500 and 700 m. One female in final pregnancy was observed at 1017 m and the only specimen caught at 1573 m was an active male.

Histology: maturity staging of the gonads

Females - ovary

In immature females (stage 1), macroscopically it was not possible to distinguish the follicles in the ovary, and the oviducal gland (OG) was not visible. Microscopically, the epigonal organ (containing different types of blood cells, mainly leukocytes as granulocytes and lymphocytes) covered most part of the ovary (Fig. 4B). The first two ovarian follicles observed were primordial and primary ones. The primordial follicles consisted of a primary oocyte (70-100 μm) surrounded by a single layer of flattened follicle cells (Fig. 4A), while the primary follicles, larger than the primordial ones (150-230 μm) showed that the follicular epithelium thickens into a columnar epithelium containing a double layer of cells (Fig. 4B).

In the maturing phase (stage 2), the ovary contained previtellogenic follicles and the oviducal glands were visible. Histologically, primordial and primary follicles were present again and for the first time previtellogenic follicles appeared. They were larger in diameter (> 300 μm) and had a more complex follicular epithelium with two kinds of cells (small and large cells) and lipid-rich inclusion began to appear and enlarge (Fig. 4C). A thin *zona pellucida* was evident between the oocyte and the granulosa cells. Sometimes, in a late maturing phase, vitellogenic follicles were present. The vitellogenesis process consisted in the formation of yolk platelets, pseudostratification of the follicular epithelium, and an increase in peripheral vascularization between the thecal layers and the follicular epithelium (Fig. 4D). Yolk droplets, first appeared in the cortical regions of the oocyte (> 1200 μm); then their size increased and they filled the entire ooplasm in mature females. In some primary, previtellogenic and at the beginning of vitellogenesis follicles, lampbrush chromosomes were observed in the nucleus (Fig. 4E).

At uterine stages (stage 4-7), the ovaries of *E. spinax* began to regress. In the histological sections of the maturing stage, in which the uteri were filled and rounded with unsegmented yolk, the ovary contents ("candle") showed the presence of primary and previtellogenic follicles surrounded by epigonal organ with some vitellogenic follicles that persisted, and atretic follicles (hypertrophic follicles in degeneration) were present. At the last uterine stage (expecting), the ovarian tissue appeared flaccid with only primary and previtellogenic follicles observed (Fig. 4F).

Oviducal gland

Macroscopically, the OG of *E. spinax* appeared simple and barrel-shaped with no lateral extensions (Fig. 5). The entire organ was composed of a mucosa, a very thin connective submucosa, a muscular layer formed by longitudinal smooth muscle fibres and a serosa.

In immature females, there was no visible differentiation of the OG from the rest of the reproductive tract. In early maturing specimens, it was structured in uniform lamellae along the entire section in which no gland tubules were present (Fig. 6A). The connective tissue was very thick and vascularized (Fig. 6B).

In a late phase of the maturing stage (or regenerating phase), it was possible to observe a slight differentiation of the distinct lamellae and to distinguish some secretory tubules. The caudal part of the OG and the uterus produced apical secretions through the epithelial secretory cells, which were sulfated acid mucins (AB+).

In mature females, the OG was fully differentiated into four zones (club, papillary, baffle and terminal). All the zones are composed of connective tissue with blood vessels and secretory tubules lined by a simple columnar epithelium with secretory and ciliated cells.

The secretory materials produced in the gland tubules from club and papillary are PAS+ and AB-, meaning that there was production of neutral mucopolysaccharides (Fig. 6C). The baffle zone had baffle plates and plateau projections (each with a blood vessel within) (Fig. 6E). Accumulation of secretory materials (PAS+ and PAS -), deep in the baffle zone (near the connective tissue), was detected.

The terminal zone (continuous with the uterus) showed a low profile of lamellae and it was lined with small and large indentations, with the epithelium producing sulfated acid mucins (AB affinity). At this stage, the connective tissue became thin. The production of the

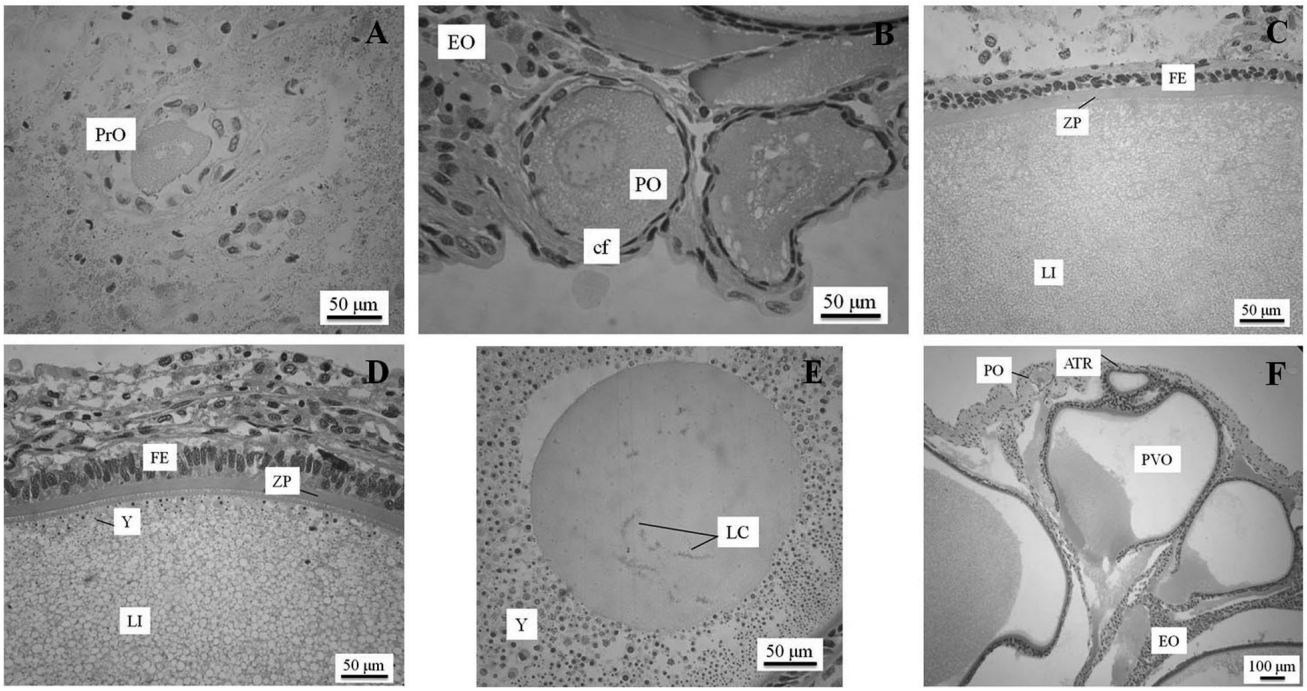

Fig. 4: The velvet belly ovary: A) Primordial follicle [H&E]; B) Primary follicle (PO) with flattened follicular cells (cf), surrounded by the epigonal organ (EO) [H&E]; Previtellogenic follicle (ZP, zona pellucida; FE, follicular epithelium; LI, lipidic inclusions) [H&E]; D) follicle at beginning of the vitellogenesis with yolk droplets (Y) in the cortical region (ZP, zona pellucida; FE, follicular epithelium; LI, lipidic inclusions) [H&E]; E) nucleus containing lampbrush chromosomes (LC) in a vitellogenic follicles (Y, yolk) [H&E]; F) ovary in the differentiating stage showing follicles in different stage of development (PVO, previtellogenic follicle; PO, primary follicle, ATR, atretic follicle) and vascularized by the epigonal organ (EO) [H&E].

secretory material decreased with the progress of pregnancy, although it continued to release the material to the gland lumen (Fig. 6D).

In *E. spinax*, sperm was identified inside the "sperm storage tubules" (SSTs) (in the terminal zone) and in the lumen of secretory tubules (in the baffle and terminal zone)(Fig. 6E,F) of mainly females assigned to maturing (regenerating) and spent stages. In certain cases, the spermatozoa were also found involved in a PAS+ matrix. The SSTs showed a simple cuboidal epithelium with ciliated and secretory cells and occurred mainly in deep recesses (Fig. 6F).

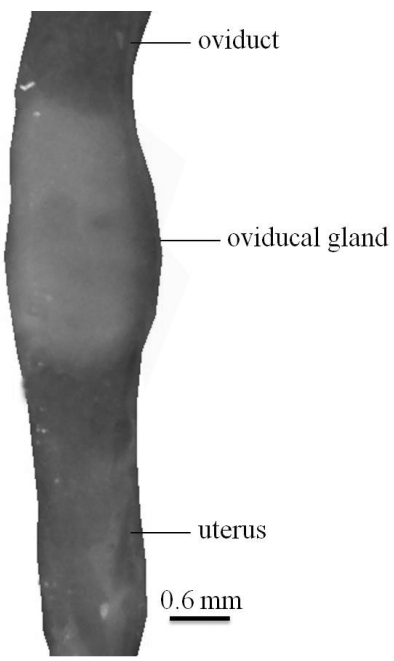

Fig. 5: External view of the oviducal gland (OG) of *Etmopterus spinax*.

Males - testis

As in all Elasmobranchs, the testis contained spheroidal lobules arranged in zones corresponding to the distinct stages of spermatogenesis. Each lobule was made of spermatocysts surrounding a central cavity. Lobules originated in the ventrolateral germinative zone and proceed diametrically to the opposite dorsal zone where spermiation occurred. The sexual phases were identified considering a plot of the proportion of the six stages (I primordial spermatogonia; II spermatogonia, III primary spermatocytes, IV secondary spermatocytes, V spermatids, VI immature spermatozoa and VII mature spermatozoa).

In immature males, the histological sections showed only spermatocysts in stage I, II and III. At maturing stage, all spermatogenic stages coexisted, including stages I-VII, but the testis was primarily occupied by spermatocytes while in mature and active testis, a greater proportion of spermatids, immature and mature spermatozoa were observed (Fig.7A-F).

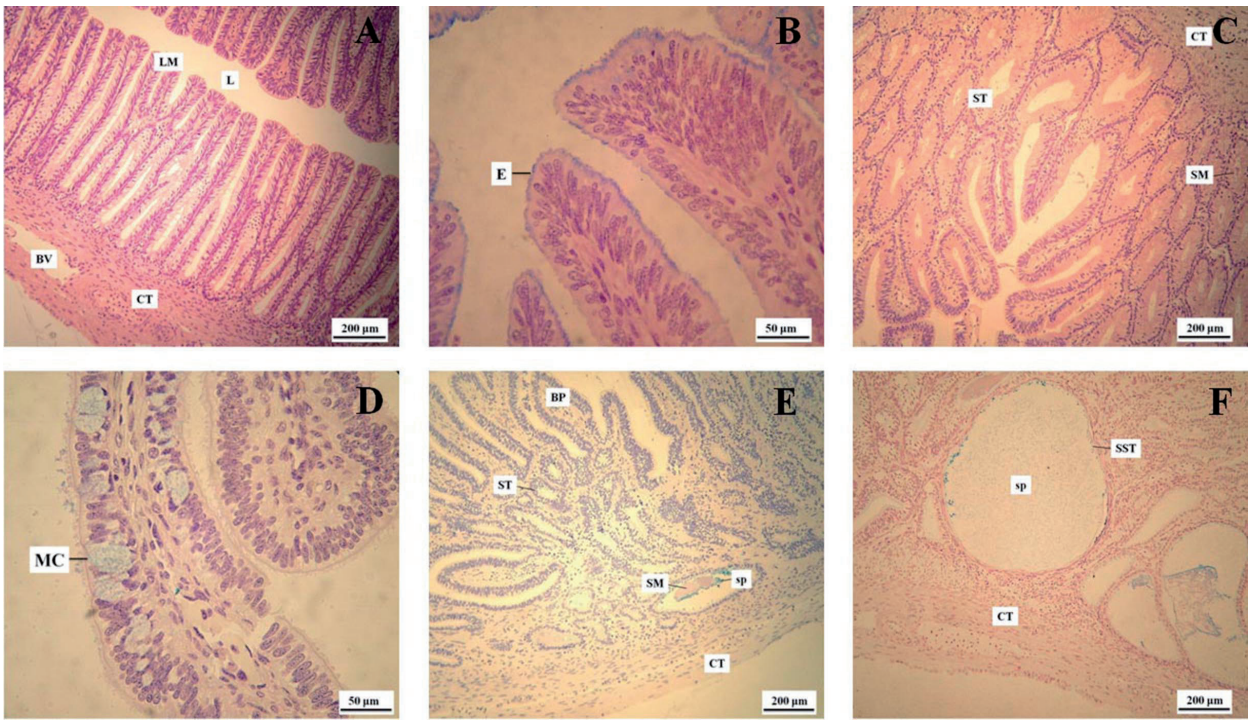

Fig. 6: Sagittal sections of the oviducal gland (OG) and uterus of *Etmopterus spinax* at the different stages of development. A) Maturing OG (LM, lamellae, L, lumen, CT, connective tissue, BV, blood vessel) [H&E], B) maturing uterus with AB-positive secretions inside the epithelial cells (E) [PAS/AB]; C) Baffle zone in a mature OG with secretory material (SM) into the secretory tubules (ST) (CT, connective tissue) [PAS/AB]; D) pregnant uterus with mucous cells (MC) AB-positive[PAS/AB]; E) sperm storage in spent OG: baffle zone (BP, baffle plate, ST, secretory tubules, SM, secretory material, CT, connective tissue; sp, sperm) [PAS/AB]; F) sperm storage in spent OG: terminal zone (SSt, sperm storage tubule; sp, sperm, CT, connective tissue) [PAS/AB].

Size at maturity

The smallest mature female was 34.3 cm TL and the smallest male was 29 cm TL. The estimated L_{50} was 36.9 cm TL (e.s. = 0.54) for females and 33.0 cm TL (e.s. = 1.18) (Fig. 8) for males, reaching maturity at 80.7% and 79% of the maximum observed size respectively.

Maturity

The width of the oviducal gland OG (OGW) increased with maturity from the maturing (OGW 1.1 - 3.5 mm) to mature stage (OGW 3.8 - 6.4 mm). In the immature stage, the OG was not differentiated, so no measurements were taken. At the pregnant phases, OGW decreased slightly (OGW 3.7 - 6.7 mm), then it increased in the spent stage. Significant statistical differences were detected between the maturing and other stages (ANOVA, F-ratio = 6.15, P = 0.0024).

In immature females, the width of uterus (UW) was relatively narrow (UW 0.01 - 2.00 mm). The UW increased from maturing (UW 0.05 - 4.00 mm) to mature females (UW 2.7 - 8.00 mm) reaching the maximum dimensions at the pregnant stages (UW 30 - 32 mm). Again, spent females showed a relatively large uterus (UW 3.5 - 5.5 mm).

In males, clasper length increased with maturity and total length, showing an accentuated increase once the specimens attained maturity.

Reproductive seasonality

A clear pattern in GSI was observed at the different maturity stages of both sexes. In females, the GSI maximum value was registered in mature condition. In pregnant specimens, GSI percentage decreased with values similar to immature ones (Fig. 9A). Significant differences among stages were found (ANOVA, F-ratio = 149.45, P = 0). In males, GSI increased from the immature to mature stage; then, it was possible to observe a small decrease in active males (ANOVA, F-ratio = 36.35, P = 0) (Fig. 9B).

The liver of *E. spinax* was relatively small reaching 11.5% and 10.3% of total body weight in females and males respectively. In females, the evolution of HSI at different maturity stages revealed a trend similar to that observed in GSI. HSI values were higher in mature females and then decreased during pregnancy (Fig. 10A). A slight increase was observed in spent females. The variations in HSI were statistically significant between maturity stage (ANOVA, F-ratio = 66.14, P = 0). In males, there was a progressive increase until the mature stage. At stage 4, the HSI values tended to decrease slightly. Significant differences were found among stages (ANOVA, F-ratio = 35.04, P = 0) (Fig. 10B).

The relative frequencies of each maturity stage by season, for both males and females, are shown in Fig.11A and B. During the sampling period, all maturity stages

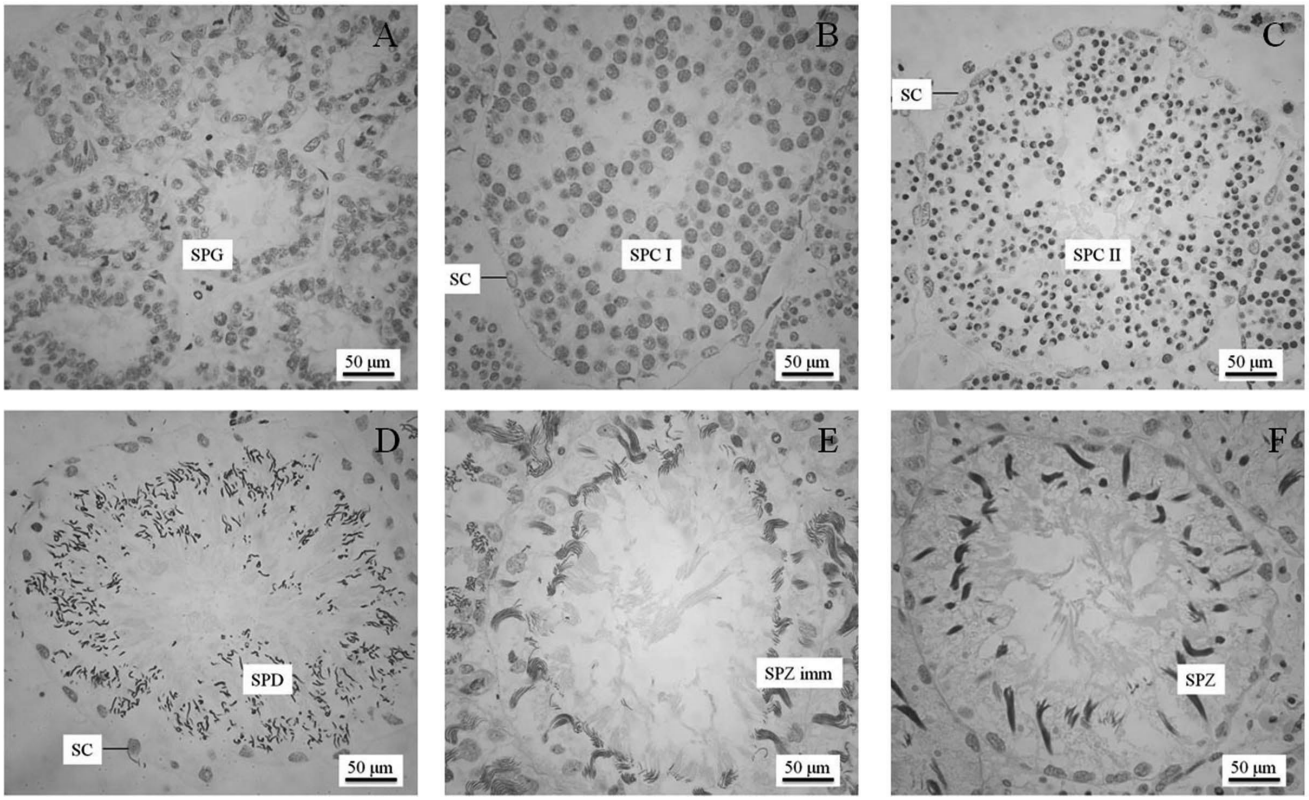

Fig. 7: The velvet belly testis (hematoxylin and eosin stain used for all panels): A) spermatogonia (SPG); B) primary spermatocytes (SPC I, SC, sertoli cell); C) secondary spermatocytes (SPC II, SC, sertoli cell); D) spermatids (SPD, SC, sertoli cell); E) immature spermatozoa (SPZ imm); F) spermatozoa (SPZ).

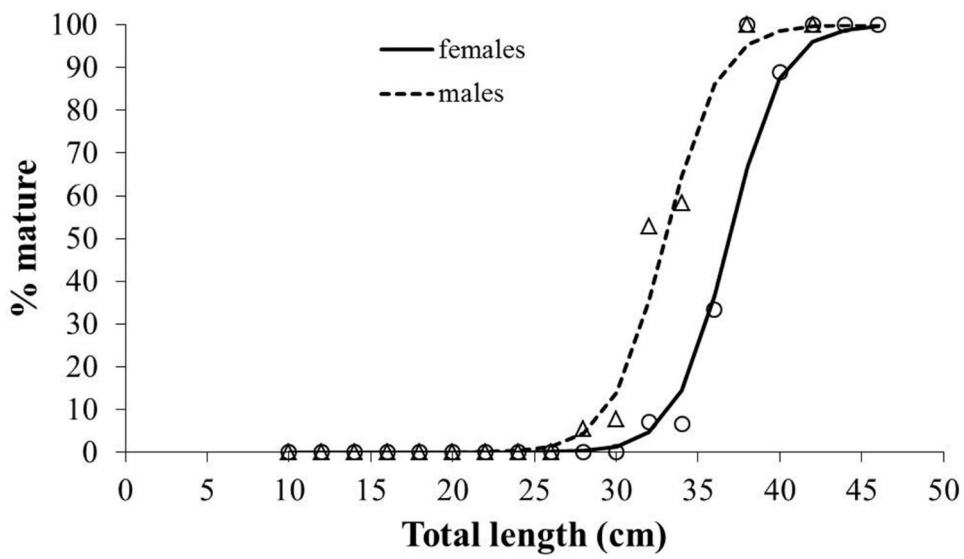

Fig. 8: Maturity ogive for females (—) and males (-----) of *Etmopterus spinax*.

were registered with some variation in their occurrence. In particular, as regards males, a predominance of immature and maturing specimens was observed throughout the year. Mature males were sampled from spring to autumn, while a higher proportion of active specimens were detected during autumn and winter (Fig. 11B). Like males, immature specimens were predominant in the female population. Furthermore, mature specimens were found mainly in autumn and winter, while pregnant individuals in spring, autumn and winter (Fig. 11A).

In general, the GSI for mature females showed maximum mean value in autumn and during the winter months. In spring and summer, these values decreased (Fig. 12A). In mature males (stage 3), the GSI values increased from spring to autumn, while in active specimens (stage 4) a peak of GSI values was observed in winter

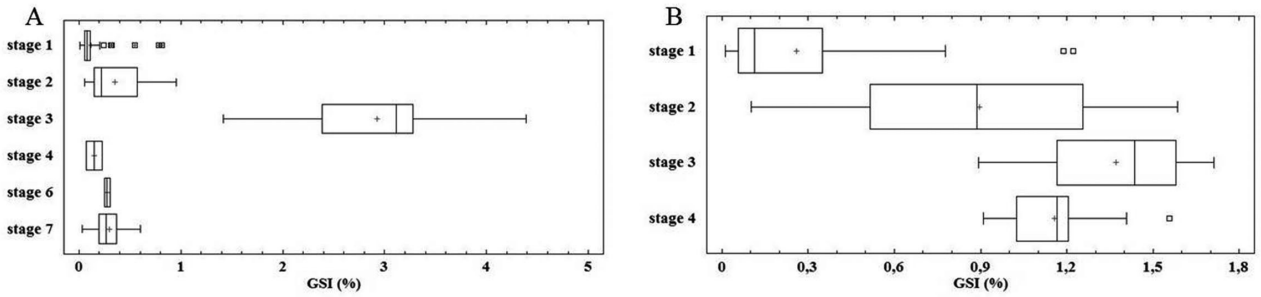

Fig. 9: Evolution of gonadosomatic index (GSI) of females (A) and males (B) of *Etmopterus spinax* by maturity stage.

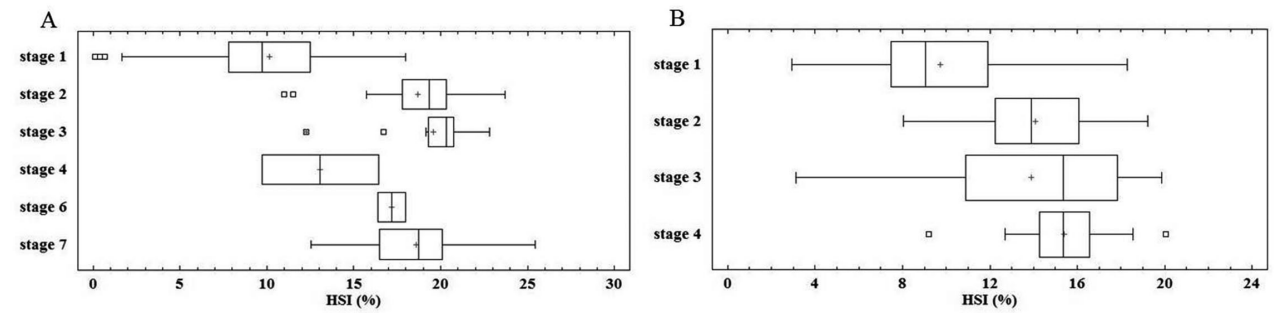

Fig. 10: Evolution of hepatosomatic index (HSI) of females (A) and males (B) of *Etmopterus spinax* by maturity stage.

months (Fig. 12B).

The seasonal evolution of mean HIS values of mature females confirmed the pattern of GSI, with the highest values in autumn and winter (Fig. 13A). In males, no specific trend in HSI was observed (Fig. 13B).

Fecundity

The ovarian fecundity in mature females varied from 6 to 27 ripe follicles (mean 16.5 ± 6.3 follicles). The diameter of vitellogenic follicles varied from 3 to 20 mm with a mean size of 9.9 mm (±3.9 mm). The pregnant females caught in final pregnancy carried from 7 to 8 completely formed embryos with a range in size between 8.3 and 11.3 cm TL.

Discussion

This study reported important information on the reproductive biology of the velvet belly *E. spinax* in the Central-Western Mediterranean Sea.

In Sardinian deep-waters, females were found to outnumber males overall as reported by Coelho & Erzini (2005). The dominance of females was also observed in the deeper part of its depth range (except for two individuals caught between 1000 and 1600 m). Several authors have already described sex frequencies differing with depth in other squalid sharks such as *E. princeps* in Icelandic waters (Jacobsodóttir, 2001). In addition, there was a positive correlation between mean total length of sexes and depth, indicating depth segregation by size. This phenomenon, called "bigger-deeper" trend, was al-

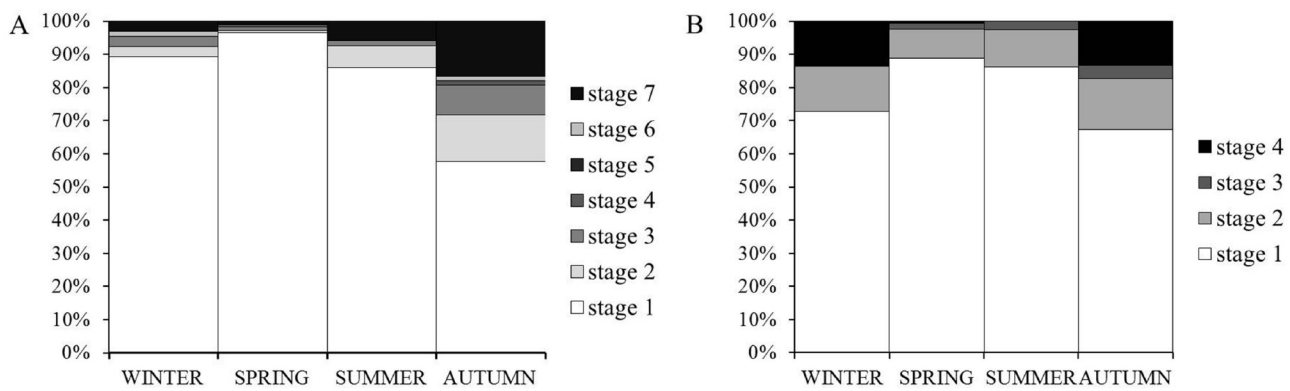

Fig. 11: Seasonal distribution of *Etmopterus spinax* females (A) and males (B) at each gonadal phase during the sampling period.

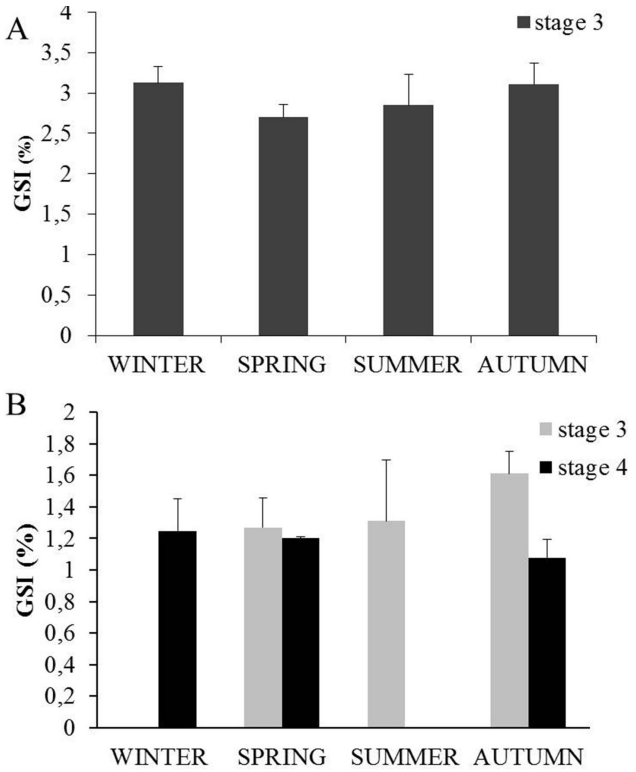

Fig. 12: Mean values of gonadosomatic index (GSI) for mature females (A) and mature and active males (B) of *Etmopterus spinax*.

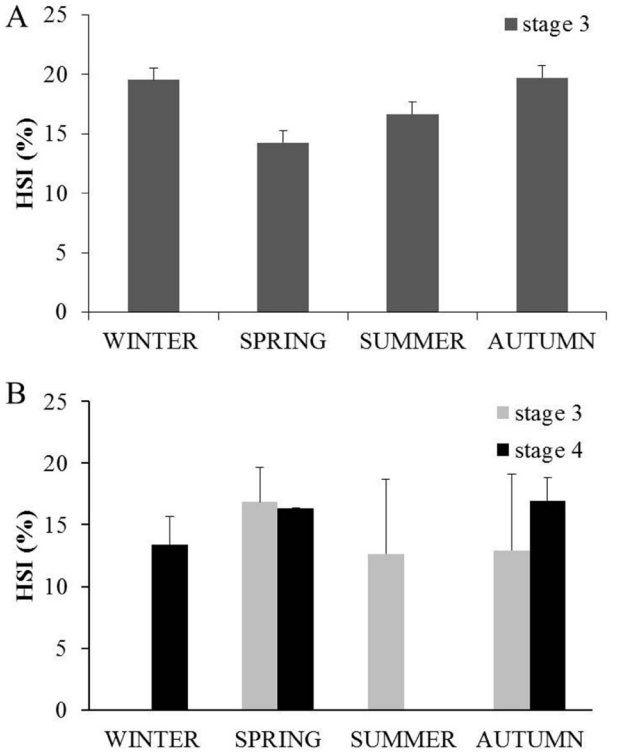

Fig. 13: Mean values of hepatosomatic index (HSI) for mature females (A) and mature and active males (B) of *Etmopterus spinax*.

ready observed for *E. spinax* by Macpherson & Duarte (1991) and Morales-Nin *et al.* (2003).

Furthermore, there were differences in the length distribution between sexes, with females attaining greater sizes than males. This sexual dimorphism is very common among other squalid sharks (e.g. Clarke *et al.*, 2001, 2002; Sion *et al.*, 2003) and is already recorded for *E. spinax* in the Tyrrhenian Sea (Cecchi *et al.*, 2004), Southern Portugal (Coelho & Erzini, 2005) and Azores (Aranha *et al.*, 2009) and also for the congenus *E. princeps* (Jacobsodóttir, 2001).

The observation of the majority of mature and pregnant females and mature and active males at the same depth (500-700 m;1017-1573 m) suggests that mating may occur in these depth ranges. A segregation of maturity stages by depth has been observed for several deep-sea squalid sharks such as *Centroscymnus coelolepis* and *C. owstoni* (Yano & Tanaka, 1988; Clarke *et al.*, 2001).

The microscopic anatomy of the ovaries and testes provided additional information for the macroscopic maturity scale used for *E. spinax*, thus confirming the assignment of the maturity stages. The analysis of histological maturity in females showed that both ovaries developed synchronously with the presence of developing follicles of various sizes. In all examined ovaries belonging to all maturity stages, most of the follicles were found during previtellogenesis. These follicles were characterized by progressive growth of the oocytes and subsequently an increase in complexity of the follicular epithelium. In addition, lampbrush chromosomes in primary, previtellogenic and vitellogenic follicles were observed. These chromosomes (intermediate structures present during the first meiotic division) were identified for the first time in shark egg cells by Rűckert (1892) and represented evidence of intense RNA transcriptional activity. Recently, they were found in the oocytes of other elasmobranchs (Wourms, 1977) such as, e.g. *Raja asterias* (Barone *et al.*, 2007) and *Sympterygia acuta* (Díaz-Andrade *et al.*, 2009). Furthermore, vitellogenesis, indicated by small droplets of yolk in the periphery of the cytoplasm (cortical region), started when oocytes had a diameter of >1200 µm. The regression of ovaries during the pregnant phases was confirmed by histological sections, which revealed the evidence of atretic and post ovulatory follicles together with previtellogenicones.

E. spinax had a diametric testis (Pratt, 1988), in which the development of spermatocysts proceeded from a germinal zone across the diameter of the gonad. In the Squaliformes, only *Squalus acanthias*, *Centroscymnus coelolepis* and *Centrophorus squamosus* showed this type of testis.

Macroscopically, the oviducal gland of *E. spinax* appeared proportionally smaller than that of the egg-laying species (e.g. *Raja* or *Scyliorhinus*, Hamlett *et al.*, 1998). It was simple and barrel shaped with no lateral extensions, similar to that of the spiny dogfish *Squalus acanthias* (Hamlett *et al.*, 1998).

The main morphology and functional features of the OG were virtually identical for all chondrichthyans (e.g. Hamlett *et al.*, 1998; Storrie *et al.*, 2008; Serra-Pereira *et al.*, 2011). The changes in structure and chemical nature of the secretions of the OG reflected their modifications during sexual maturity. Histological sections showed that females at the maturing stage did not yet have a differentiated OG with homogeneous lamellae, while a light production of mucins was observed in a regenerating OG (females that had already gone through a reproductive cycle). In mature females, secretions were mucous and/or proteic according to the zones, and to their specific functions. The chemical nature of the secretions in club and papillary zones consisted of neutral mucins, as observed in other viviparous sharks such as *Iago omanensis* (Hamlett *et al.*, 2002). The baffle zone of *E. spinax* had few transverse grooves, a common feature of viviparous species in which the egg covering is reduced to a flexible egg candle that surrounds the embryos and disappears prior to parturition (Serra-Pereira *et al.*, 2011). Terminal zone showed a low profile of lamellae and simple tubular glands embedded in loose connective tissue. During pregnancy, high amounts of mucopolysaccharides confirmed the activity of the OG, despite a slight reduction in width.

In the past, sperm storage in female sharks and rays has been largely confirmed by histological analysis (e.g. *Mustelus antarticus*- Storrie *et al.*, 2008; *Centroscymnus coelolepis* – Moura *et al.*, 2011; *Raja clavata* – Serra-Pereira *et al.*, 2011). The hypothesis concerning sperm storage in velvet belly lantern females, suggested by Coelho & Erzini (2008), has been confirmed by our histological sections of the oviducal glands. In this study, small groups of sperm were detected, densely packed as non-aligned masses in tubules of the baffle zone and in SSTs in the terminal zone (often involved in a PAS+ matrix), usually deep near the connective tissue, thus suggesting long-term storage (Pratt, 1993). In species with this type of storage and long gestation cycle, Pratt (1993) suggested that sperm could be stored for up to 15 months. Assuming that stored sperm will be used in fertilization, a long storage period must be considered, given that pregnancy might last 2-3 years in this species (Coelho & Erzini, 2008 and present study). As a consequence, the recovery of the reproductive tract for the new maturation might take a long time (vitellogenesis does not proceed in parallel with gestation). Presence of sperm in maturing females indicated that mating occurs before maturity is reached, as also reported for other aplancental viviparous species such as *M. antarticus* (Storrie *et al.*, 2008) and *C. coelolepis* (Moura *et al.*, 2011). In addition, in populations with relatively low densities, as in *E. spinax*, the storage of spermatozoa in the OG could represent an evolutionary conserved mechanism that increases the chances of successful insemination (Pratt, 1993).

In Sardinian waters, the size at first maturity in females (L_{50} = 36.9 cm TL) was higher than males (L_{50} = 33 cm TL). This pattern could be, in part, explained by the need for females to attain a larger size in order to support pups, using the energy for reproduction rather than growth, which would result in delayed onset of sexual maturity in females (Cortés, 2000). The sizes at first maturity estimated in this study were higher than those estimated for the Mediterranean by Vacchi & Relini-Orsi (1979) (Ligurian Sea), Coelho *et al.* (2010) (Alboran Sea) and for the Atlantic by Coelho & Erzini (2005) (Southern Portugal) and Aranha *et al.* (2009) (Azores) (Table 3). Only Capapé *et al.* (2001) estimated a larger size at first maturity for *E. spinax* in southern France and along the Tunisian coasts. These differences may be due to various reasons: sample size, sampling method, different rates of maturation due to different environmental conditions

Table 3. *Etmopterus spinax* estimates of lengths-at-first-maturity (L_{50}) (cm), for males (M) and females (F), ovarian and uterine fecundity and duration of the spawning and breeding season, in different geographical regions.

Geographical region	L_{50} (cm)		Fecundity		Spawning season (stage 3)	Breeding season (Stage 6)	References
Mediterranean Sea	**M**	**F**	**Ovarian**	**Uterine**			
Sardinian waters	33	36.9	6-27 (16.5±6.3)	7-8 (final pregnancy)	All year	Autumn and winter	Present study
Ligurian Sea	28-30	34	5-14	5-14	February-November	May-July, September	Vacchi & Orsi Relini, 1979
Alboran Sea	28.31	34.18	6-15 (11.06±2.29)	-	-	-	Coelho *et al.,* 2010
Tunisian coasts, South France	35	38	5-17	5-9	August, October	September, October	Capapè et al., 2001
Atlantic Ocean							
Southern Portugal	25.39	30.86	5-21 (9.94±2.61)	1-9 (final pregnancy)	All year	June	Coelho & Erzini, 2005, 2008
Azores	29.7	34.1	-	-	March-November	April-August	Aranha *et al.*, 2009

(Jones & Geen, 1977; Girard & Du Buit, 1999), or different fishing pressure (Aranha *et al.*, 2009). In addition, the different ways of assigning the mature stage, using different maturity scales, could also modify the estimation of maturity ogive.

Sexual dimorphism, in terms of size at maturity, has already been described for other congenera such as *E. granulosus* (Wetherbee, 1996), *E. princeps* (Jacobsdóttir, 2001), *E. baxteri* (Irvine *et al.*, 2006) and *E. pusillus* (Coelho & Erzini, 2005). The velvet belly *E. spinax* seemed to mature very late in its life cycle. Cortés (2000) reviewed data on 164 species of sharks and concluded that, on average, maturity in sharks begins at about 75% of their maximum size. In this study, maturity was achieved at 80.7% and 79% of their maximum observed size for females and males respectively. Our results were close to those reported for specimens of this species in another areas of the Mediterranean Sea (Alboran Sea), which matured at around 83% in contrast to data reported for Atlantic waters (at around 75% of the maximum size) (Coelho *et al.*, 2010).

In Chondrichthyes, there are two reproductive strategies: some species have a defined reproductive period while others are reproductive throughout the year (Wourms, 1977).

During the sampling period, all maturity stages were recorded. Immature females predominated during all the sampling seasons, in contrast to mature and pregnant females. Immature, mature and resting females were recorded in every season, while expecting and expectant females were caught in spring and winter respectively. This pattern was confirmed by the variation of the mean GSI values of mature females, in which a peak in autumn and winter was registered. These results were totally in disagreement with those observed in Mediterranean and Atlantic areas, part for the Tunisian coasts and Southern France (Capapè *et al.*, 2001) (Table 3). The apparent lack of large specimens in this population might indicate overexploitation, as suggested by Coelho & Erzini (2008) for Portuguese waters. In addition, the higher proportion of active males in autumn and winter suggests that mating could occur at this time, with females storing the sperm.

The highest GSI value in mature females throughout the year, in contrast with a lower percentage during the pregnant phases, indicated that the species has an alternate reproductive cycle and suggests that it takes 1 year to develop oocytes in the gonad until fertilization, as reported also by Coelho & Erzini (2008).

The change in the HSI with female maturity stages suggested that liver function is closely related to the development and/or growth of the embryos (uterine stages). The increase of HSI during ovarian development may be related to the production of vitellogenin by the liver, to be used for the development of the embryos. During pregnancy, HSI values decreased, while, at the spent phase, its percentage increased slightly because females begin to produce lipids for the next reproductive cycle. These variations have been reported for the same species by Coelho & Erzini (2008) and Aranha *et al.* (2009) and appeared common to the congenus *E. princeps* and other squalids (Clarke *et al.*, 2001; Jacobsdóttir, 2001).

Based on our results, *E. spinax* had low fecundity. As shown by Coelho & Erzini (2008), ovarian fecundity was higher than uterine fecundity, probably due in the latter case to the loss of embryos by pregnant females during the fishing process. Mean ovarian fecundity resulted higher than that observed in other areas of the Mediterranean and the Atlantic (Coelho & Erzini, 2008; Coelho *et al.*, 2010) (Table 3). This phenomenon could be related to smaller size at maturity, reported by the previous authors, given that there is a relation between female size and fecundity.

In conclusion, this paper aimed to increase the life history data available for *E. spinax* and make a valuable contribution to the implementation of basic management measures to ensure the sustainability of catches for this species. The strategy of sperm storage confirmed by us represents an important advantage for species conservation, guaranteeing the success of reproduction.

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