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Follicle Development in Immature Spiny Dogfish (*Squalus acanthias*): Histomorphometric Analysis

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Abstract

Squalus acanthias is a commercially exploited small coastal shark, highly sensitive to overexploitation due to a low reproductive potential and a low intrinsic rate of population increase. We collected specimens caught by commercial bottom trawls from April 2005 to January 2007 in the Adriatic Sea and investigated reproductive traits of the species in ovaries of 68 immature females (total body length range: 26.1-70.1 cm). Histomorphometric analyses included measurements of the growing follicle diameters, the width of the follicular layer and the width of the zona pellucida. We analysed 2700 ovarian follicles and grouped them into four categories: small previtellogenic, medium previtellogenic, large previtellogenic and small vitellogenic follicles. The growth of follicles was positively correlated with the body size of females ($r=0.726$; $p<0.001$). We documented significant changes in the thickness of the follicular layer and zona pellucida as follicles increased in size. Small vitellogenic follicles started to appear in small proportion (around 1% of follicles) in subadult females with the minimum size of 60 cm total body length. Yolk droplets first emerged in follicles larger than 600 µm in diameter while vitellogenesis was evident in follicles ranging 1.2-2.0 mm in diameter when both follicular layer and zona pellucida were the widest (41.1 µm and 49.1 µm, respectively).

Keywords: Shark; Reproductive biology; Ovary; Mediterranean Sea; Adriatic Sea

Introduction

The spiny dogfish (*Squalus acanthias* L.) is a small coastal shark, naturally abundant and widely distributed in temperate and subartic waters. Since it is a long-lived (>40 years), late maturing (6-12 years) and slow-growing species, spiny dogfish has a low productivity and very low intrinsic rate of population increase (2-7% per year) [1,2]. Due to its life history traits, this species is extremely sensitive to perturbations in vital rates and one of the most vulnerable sharks to overexploitation. The spiny dogfish is globally listed by the IUCN Red List as Vulnerable, while in the Mediterranean Sea is classified as Endangered [3].

S. acanthias reproductive strategy includes lecithotrophic aplacental viviparity, low fecundity (1-15 pups per litter) and the longest gestation period among sharks (up to 24 months) [4,5]. The ovulated eggs are fertilised, enclosed in a protective capsule (candle) for the first 4 to 6 months; when capsule ruptures the embryos are completely dependent upon the yolk sacs for the next 16 to 17 months [5-8]. Mature females have a continuous reproductive cycle, with concurrent processes of vitellogenesis in ovaries and gestation in uterus [9]. They give birth once every two years and begin a new cycle immediately after parturition [8]. Ovarian follicles in fish are composed of an oocyte with zona pellucida, and layers of follicle and theca cells which proliferate and differentiate to form developed follicles [10].

A standardized terminology for gonadal development was recently proposed for teleosts [11] and oviparous elasmobranchs [12], while for other elasmobranch species, reproductive terms are scarce and incomplete. The process of folliculogenesis in viviparous species was investigated only in the ray *Torpedo marmorata* [13] and stingray *Urolophus jamaicensis* [14] where follicles were classified into previtellogenic and vitellogenic. Most studies on reproductive biology of *S. acanthias* were focused on sexual maturity and endocrinology of active females [9,15-17], while for immature females such studies are lacking. Studies in the Mediterranean and Black Sea basins were based on macroscopic evaluation of gonads and reproductive ducts [18-22] whereas histological analysis was only recently imposed [23].

Thus, the aim of the present study is to quantitatively describe follicle development in the ovaries of immature females of spiny dogfish and to extend the knowledge of gonadal development in this endangered species.

Materials and Methods

Spiny dogfish were collected by onboard observers on commercial bottom trawls for every month (7-10 days) from April 2005 to January 2007 in the northern Adriatic Sea, the northernmost part of the Mediterranean Sea (Figure 1). Specimens were sexed by external examination of pelvic fins and measured (Total Body Length-TBL). Following our recent findings on the reproductive biology of spiny dogfish in the Adriatic Sea [23], females smaller than 71 cm TBL were classified as immature and sampled for the present study.

We collected 68 immature females with TBL ranging from 261 to 701 mm (mean ± SD: 478 ± 114 mm). Sharks were dissected and sampled, and ovaries were fixed in 10% neutral buffered formaldehyde for a minimum of 48 h, transferred to 75% ethanol and stored at 4°C. A 1 cm^3 cross section from the middle of the gonads was dehydrated through a graded ethanol series (70%, 80%, 96% and 100%), exposed to chloroform, embedded in Paraplast embedding media (Sherwood Medical, USA) and sectioned on a rotating microtome at 6-7 µm. Sections were stained with Mayer's Haematoxylin and Eosin Y, while

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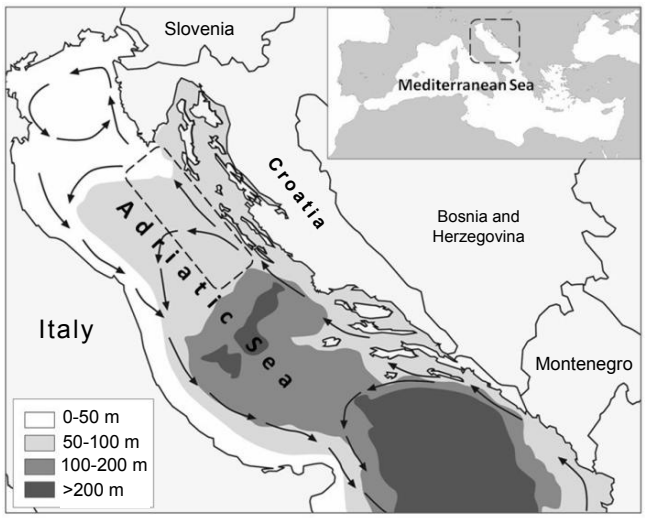

Figure 1: Study area in the northernmost part of the Mediterranean Sea, Adriatic Sea, is marked with a dotted rectangle.

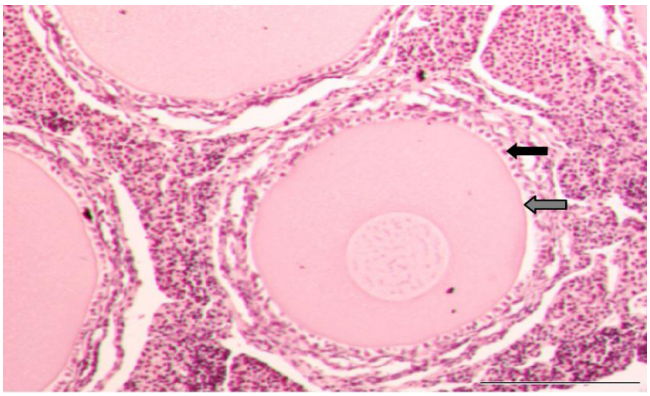

Figure 2: Light microscopy image of an ovarian follicle. The black arrow indicates the follicular layer while the grey arrow indicates the zona pellucida. HE. x100. Bar=100 µm.

some additional slides were stained with the Periodic Acid-Schiff Method (PAS) [24].

Histological slides were examined under x100 and x200 magnification through a Nikon Eclipse E600 light microscope and photographed with a Nikon DXM1200 digital camera. Histomorphometric analyses were performed on digital pictures using digital image analyser Lucia G (version 4.80, Laboratory Imaging Ltd). In total, we measured 2700 ovarian follicles. Follicular dynamics were analysed by the frequency of the size-classed follicles and the increasing follicular diameter in relation to TBL. We measured the growing diameters of follicles (without theca layers), the width of the Follicular Layer (FL) and the width of the Zona Pellucida (ZP; Figure 2). The terminology used to describe follicle development followed two papers on viviparous chondrichthyans [13,14].

In order to affirm our sampling of immature females, we created a control group composed of 15 larger follicles isolated from three mature females. These follicles were also processed for histological slides and histomorphometric analysis.

Correlation between follicle diameters and body size of animals was performed using Pearson's correlation coefficient. We grouped females according to TBL into five length-classes (100 mm size groups), from 200-300 mm to 601-700 mm TBL, and calculated the percent of occurrence for follicles of various sizes. We analysed the occurrence of follicles in relation to length-classes of females and the potential changes in the thickness of FL and ZP during follicle development by ANOVA and a *post-hoc* Hochberg GT2 test. Statistical analyses were carried out using SPSS 17.0 (SPSS Inc., USA).

Results

Ovarian follicles isolated from immature females were grouped into four size categories: (1) small previtellogenic (follicle diameter ≤300µm); (2) medium previtellogenic (301-600 µm); (3) large previtellogenic (601-1200 µm); and (4) small vitellogenic follicles (1201-2000 µm). Follicles isolated from mature animals (control group) were identified as large vitellogenic follicles and divided into two size-classes: (1) 2-5 mm and (2) 5-10 mm. These follicles were characterized by the size and the presence of numerous acidophilic yolk droplets in the cytoplasm.

Follicle diameters significantly correlated with the body size of females (Pearson's test, r=0.726; p<0.001) and a positive increase in follicle diameter was recorded for all female size-classes (Hochberg's GT2, all p<0.05). Ovaries of the smallest size-class of immature females (≤300 mm TBL) contained 96.7% of small previtellogenic follicles (≤300 µm in diameter) and only few (3.3%) medium previtellogenic follicles. Larger juvenile females (401-500 mm TBL) comprised 63.5% of small previtellogenic follicles, 34.9% of medium previtellogenic follicles, while only small proportions (1.5%) were large previtellogenic follicles. Ovaries of subadult females (601-700 mm TBL) included 45.5% of small previtellogenic follicles, 39.0% of follicles ranging 301-600 µm in diameter, 14.6% of large previtellogenic follicles and 0.9% of small vitellogenic follicles (1201-2000 µm; Figure 3).

Lipid droplets began to accumulate in the cortical regions of follicles when they reached >600 µm in diameter. The follicular layer was simple, with changes in width as folliculogenesis progressed (Figure 4). The zona pellucida was visible as an acellular line positive to the PAS reaction, while the theca was evident as a fibrous, vascularised, outer layer. Statistical analyses showed significant changes in the thickness of the FL and ZP (ANOVA, F=25.87-35.67, p<0.001; Figure 4) among analysed follicles (Table 1). In small previtellogenic follicles ZP and FL were thin layers (mean ZP width: 3.85 µm ± 0.16; mean FL width: 9.97 µm ± 5.64), while in medium previtellogenic follicles both ZP and FL increased their width (mean ZP width: 7.08 µm ± 3.81; mean FL

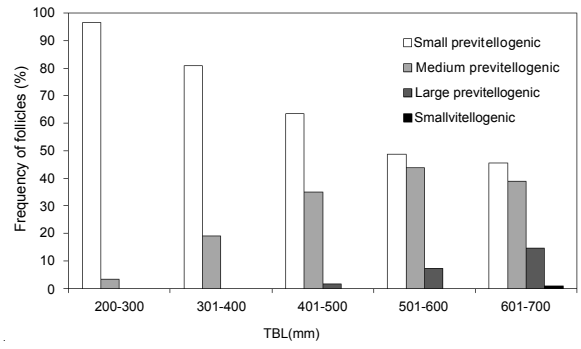

Figure 3: Frequency of ovarian follicles according to Total Body Length (TBL) of immature spiny dogfish females in the Adriatic Sea (N=68).

width: 13.13 µm ± 3.78). As follicles further increased in size, ZP and FL continued to grow and were the widest in small vitellogenic follicles (mean ZP width: 30.35 µm ± 7.93, mean FL width: 29.16 µm ± 6.65), when the maximum ZP and FL values were recorded (49.1 µm and 41.1 µm, respectively).

FL and ZP started to decrease in width in the control group, when follicles were >2 mm in diameter and were filled with yolk precursors. In follicles ranging 2-5 mm in diameter both ZP and FL displayed a decrease in width (mean ZP width: 19.72 µm ± 3.30; mean FL width: 23.92 µm ± 2.24), while in follicles ranging from 5 to 10 mm, the ZP rapidly decreased (mean ZP width: 6.97 µm ± 1.88) and the FL remained at same width (mean FL width: 24.54 µm ± 5.36).

Discussion

Although the structure of the ovary and growing ovarian follicles is similar in most fish, their development may lead to variations in fecundity and, consequently, in population biology. Since the dynamic of follicular development has not previously been reported in immature spiny dogfish, our paper presents the first quantitative description of follicle development for this endangered species.

Follicle development is a long process in spiny dogfish, since oocytes grow slowly and develop from the smallest animals (>200 mm TBL) until they are sexually active (>700 mm TBL) when females are between 12 and 15 years of age [21,25]. Small previtellogenic follicles were most frequent across all size-classes of analysed sharks. Small vitellogenic follicles were recorded only in subadult females ranging from 600 to 701 mm TBL (around 1% of follicles). It is likely that these small vitellogenic follicles will continue to develop and ovulate at the first year of mating, while the larger population of previtellogenic follicles will be recruited in the following seasons. Follicular dynamics in mature animals depends on fecundity and seasonal cycle of females. In the spiny dogfish, eggs develop and ovulate as a cohort [9] during spring months every two years, while low fecundity values (around 10 embryos in the Adriatic) [23] slowly increase as females grow and have more space to bear pups [26].

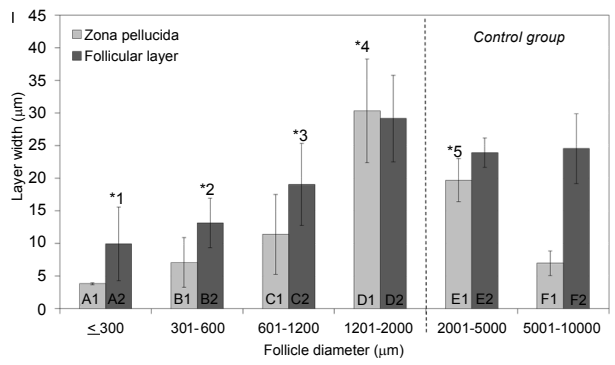

Figure 4: The width of zona pellucida and follicular layer in relationship to follicle diameter. *1-statistically significant difference ($p<0.05$) in relation to D2, E2 and F2; *2-statistically significant difference in relation to C2, D2, E2 and F2; *3-statistically significant difference in relation to D2 and F2; *4-statistically significant difference in relation to A1, B1, C1, E1 and F1. *5-statistically significant difference in relation to A1, B1, C1, D1 and F1. Control group refers to follicles isolated from sexually mature females.

The smallest recorded follicle in this study was around 21 µm in diameter, while the largest ova reached between 36 and 51 mm in the Mediterranean [19,23]. Thus, the size is increased more than 2000 times during the process of development from follicles to ovulated ova. Vitellogenesis is the main process responsible for the growth of follicles. In early vitellogenesis, follicles are characterized by the presence of little yolk globules dispersed in the peripheral cytoplasm, while larger follicles have yolk platelets of various sizes distributed throughout the cytoplasm [27]. Yolk precursors are synthesized in the maternal liver, transported through the follicle wall into the follicle and then sequestered in the yolk sac [28].

The follicular wall in chondrichthyans is composed of a single layer of follicle cells which can show changes in form and size as follicles grow. While in some species follicle cells remain uniform, they can also stratify and differentiate into two types of cells [29-31]. Lance and Callard [15] noted that follicle cells in spiny dogfish are single cuboidal cells, while Reid [32] reported that follicles >1.5 mm have a columnar or pseudostratified follicle layer. Spiny dogfish specimens from the Adriatic Sea revealed similar pattern, with single layer of follicle cells changing in shape from cuboid to columnar form. The FL cells were the widest in follicles ranging in diameter 1.2-2 mm, which is consistent with findings from Reid [32] and concomitant with the process of vitellogenesis. Since follicle cells enable transport of yolk precursors from the vascular system to the oocyte [14,33] and produce steroid hormones [15], the observed growth of the FL is attributed to the physical manifestation of these activities during vitellogenesis.

A contact between the follicle cells and the oocyte is ensured with the follicular cell projections of the oocyte and the follicle cells, which extend across the acellular layer of the ZP [34]. The ZP, also known as zona radiata, vitelline envelope or vitelline membrane, has been

Follicle diameters (µm)		Mean difference in width of layers (±SE)				
		301-600	601-1200	1201-2000	2001-5000	5001-10000
Follicular layer	≤300	3.28 ± 3.69	8.72 ± 3.73	19.19 ± 3.95**	13.95 ± 3.92*	18.06 ± 4.16**
	301-600		5.45 ± 1.23**	15.91 ± 1.79**	10.67 ± 1.72**	14.78 ± 2.22**
	601-1200			10.47 ± 1.88**	5.22 ± 1.82	9.33 ± 2.29**
	1201-2000				5.24 ± 2.23	1.14 ± 2.63
	2001-5000					4.11 ± 2.59
Zona pellucida	≤300	3.62 ± 3.87	7.33 ± 3.91	26.50 ± 4.14**	15.87 ± 4.11*	5.50 ± 4.63
	301-600		3.70 ± 1.29	22.87 ± 1.87**	12.25 ± 1.80**	1.87 ± 2.79
	601-1200			19.17 ± 1.97**	8.54 ± 1.90**	1.83 ± 2.86
	1201-2000				10.63 ± 2.34**	21.00 ± 3.16**
	2001-5000					10.38 ± 3.12*

Table 1: Changes in the width of follicular layer and the zona pellucida among compared ovarian follicles analysed in Hochberg's GT2 post hoc test. *- significance at the level 0.05; **- significance at the level 0.001.

studied in various fish where this extracellular matrix with pore-canals acts as a selective membrane. Similar to other chondrichthyan species [9,29,31,35,36], the ZP in our study was filled with mucopolysaccharides positive to the PAS reaction and was the widest in follicles ranging 1.2-2 mm in diameter, when it reached nearly 50 μm in width. It seems that ZP is the widest in small vitellogenic follicles when the cell projections seem to organize [34], while it narrows when follicles start to rapidly grow and accumulate yolk precursors. Our findings on the width of the ZP are concurrent with the study from Davenport et al. [34] who recorded the ZP width of over 70 μm in follicles with a diameter of 1-2 mm in three other shark species, and Reid [32] who reported maximum width of the ZP for spiny dogfish between 50 and 70 μm, in follicles ranging from 1.5 to 1.8 mm in diameter.

In conclusion, considering the role of the Adriatic Sea as a reproductive habitat, and the characteristic life history traits of spiny dogfish, our results contribute to the knowledge on reproductive biology of the species in the region. For future work, we would recommend ultrastructural analysis of follicles, which would enable identification of follicular cells and potentially lead to detailed classification of ovarian follicles in spiny dogfish.

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