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Histology and Morphology of The Epigonal Organ with Special Reference to the Lymphomyeloid System in *Rhinobatos rhinobatos*

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Abstract

The present study is the first trial to describe the structure of the lymphomyeloid epigonal organ of the *Rhinobatos rhinobatos*. The morphological structure of the epigonal organ was similar in males and females and it was classified as stingray type. This organ possessed erythrocytes, thrombocytes, lymphocytes and two distinct types of granulocytes (eosinophils and neutrophils). Basophils were not detected in this study. Thrombocytes were variable in shape and lacked visible granules and some of them were spindle-shaped. Erythrocyte histology was similar to that previously described in birds, reptiles, amphibians, teleost and elasmobranchs. Granulocytes were probably stored within the lymphomyeloid epigonal organ and entered the circulation on maturation.

Keywords: *Rhinobatos rhinobatos*, Epigonal organ, Lymphomyeloid system, Histology.

Rhinobatos rhinobatos'ta Lenfomyeloid Sisteme Özel Epigonal Organın Histolojisi ve Morfolojisi

Özet

Bu çalışma *Rhinobatos rhinobatos*'un epigonal organının lenfomyeloid yapısını tanımlayan ilk çalışmadır. Epigonal organın morfolojik yapısı erkek ve dişilerde benzer ve stingray tip olarak sınıflandırılmıştır. Bu organ eritrosit trombosit lenfosit ve belirgin iki tip granülosit (eozinofil ve nötrofil) içermektedir. Çalışmada bazofillere rastlanmamıştır. Trombositler farklı şekillerde, görülebilir granülleri olmayan ve bazıları iğ şeklinde tespit edilmiştir. Eritrosit histolojisi kuşlarda, sürüngenlerde, kurbağalarda, kemikli ve diğer kıkırdaklı balıklarda daha önceden tanımlanmış histolojilerle benzer bulunmuştur. Granülositler muhtemelen lenfomyeloid epigonal organ içinde depolanmakta ve olgunlaşma sürecinde dolaşıma girmektedirler.

Anahtar Kelimeler: *Rhinobatos rhinobatos*, epigonal organ, lymphomyeloid system, histology.

Introduction

Elasmobranch fishes, such as sharks, skates, rays and chimaeras lack lymphoid nodes and are bone marrowless (Fänge and Mattisson, 1981; Mattisson and Fänge, 1986; Walsh *et al.*, 2002; 2006). Epigonal organ has been described as bone marrow in some elasmobranch fish. It was found to be absent in holocephalians, another subclass of Chondrichthyes (Stahl, 1967). Zapata *et al.* (1996) studied the structure of the epigonal organ in elasmobranch fishes and stated that epigonal organ has the capacity of production of blood stem cells. Walsh and Luer (2004) later studied the histology of the epigonal organ and described the blood cells. Blood cells were originated from the spleen and thymus as in other

vertebrates but included unique organ associated with gonads (epigonal organ) and oesophagus (leyding organ) (Mattisson and Fänge, 1982; Walsh and Luer, 2004).

A histological study of the epigonal organ of *Mustelus schmitti* (narrow nose smooth hound shark) was performed by Galíndez and Aggio (2002). They described lymphocytes macrophages and reticular cells and suggested that this species can be used as a model for immunological studies. Recently, a medium enriched by shorth term culture of bonnethead shark (*Sphyrna tiburo*) epigonal cells has been shown to inhibit the growth of mammalian tumor cell lines, including fibrosarcoma, melanoma, B-cell lymphoma, T-cell leukemia, pancreatic cancer, ovarian cancer and three breast carcinoma cell lines (Walsh *et al.*,

2006). Therefore it is extremely important, to investigate epigonal organ cells as a source of novel tumor cell inhibitors for possible implications to human health (Walsh *et al.*, 2006).

The common guitarfish (*Rhinobatos rhinobatos*) is widely distributed in sub-tropical regions of the Eastern Atlantic Ocean and in the Mediterranean (Bauchot, 1987; Başusta *et al.*, 2008). There has been very few studies on this species regarding to its immunological system. Morphological structure of the epigonal organ of *R. rhinobatos* was briefly described by Çek *et al.* (2009). To our best knowledge, there is no other studies on epigonal organ of *R. rhinobatos*. Therefore, the present work gives detailed information on morphology and histology of the epigonal organ of common guitarfish in Iskenderun Bay, Eastern Mediterranean. This paper can also be considered as an initiative to regulate the potential for future immunological studies.

Materials and Methods

Sampling

Specimens of *R. rhinobatos* were collected monthly from catches made with trawl and long fishing-line by fishermen (20-100 m depth), in several area of the Iskenderun Bay, located North East Mediterranean sea (35°33'-36°13' E; 36°18'-36°55' N) (Figure. 1). Throughout the study, regulations and guidelines concerning animal ethics were followed. The epigonal organ was estimated from 18 male and 42 females, measuring between 41cm and 147 cm in total body length that were sampled during the study period. Study was performed from May 2009 to May 2010. The samples were removed withing 24 hours. The epigonal organ of 5 specimens were used for the histological analysis, montly samples were taken. Male and female *R. rhinobatos* from which the

epigonal organs were sampled (Figure 2a).

Histological Procedures

The epigonal organ was dissected from all female and male specimens for each month and they were directly fixed in 10% neutral buffered formalin (prepared in phosphate buffered saline modified for use on elasmobranch tissue, 10 Mm NaH₂PO₄, 450 Mm NaCl, pH 7.4). Testes from all males from each month except June were also fixed. A cross section (thickness 5µm) from the centre of each testis was fixed in formalin, dehydrated in graded ethanol, embedded in paraffin, and stained with hematoxylin and eosin (MERCK) for histological examination (Gelsleichter *et al.*, 2002; Çek *et al.*, 2009). All slides were examined under light microscope (CH-2 Olympus-Japan) to describe the blood cells and the overall morphology of the epigonal organ.

Results

Macroscopic Observation of the Epigonal Organ

The epigonal organ of the *R. rhinobatos* was bilateral, running ventrally of the gonads and its development was very variable, from small masses on the surface of the gonads to a large organ at the posterior part of the gonads (Figure 2b). This organ extends from the liver to the rectal gland completely surrounding both, testes and ovaries. In most observed samples, the posterior part of the epigonal organ was more developed than the anterior part. The morphological structure of the epigonal organ was similar in males and females (Figure 2b). The colour of the epigonal organ was whitish and in some samples, pale colour was also observed. In very few samples redishbrown colour was macroscopically observed.

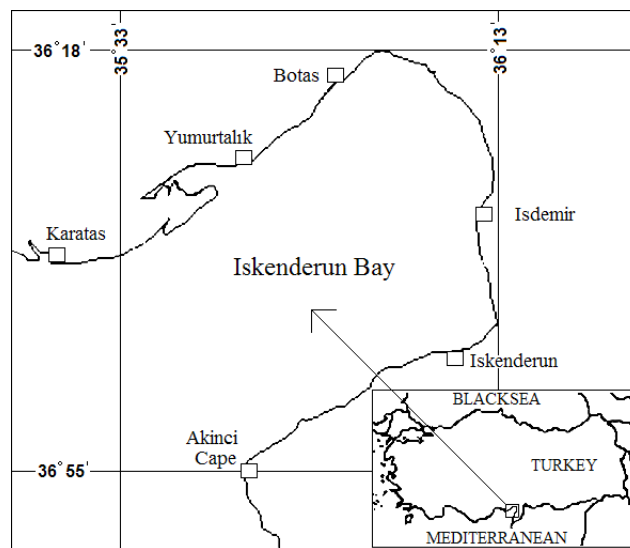


Figure 1. Map of the Iskenderun Bay showing the study sites (Akinci, Isdemir, Botaş, Karataş), Turkey.

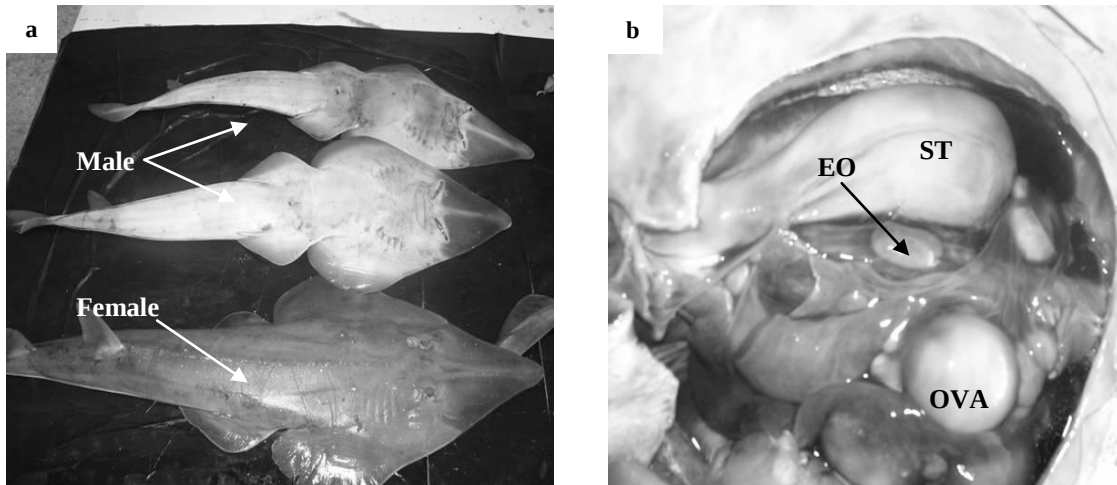


Figure 2. Overview of studied species a) ventral view of males and dorsal view of a female b) Ventral view of the epigonal organ in female *R. rhinobatos*. ST: Stroma, EO: Epigonal Organ, OVA:Ovarium.

Histological Observation of the Epigonal Organ

The highly vascularized epigonal organ enclosed the ovary and testes. Spermatogonia and oogonia cells were present in the epigonal organ of the *R. rhinobatos*.

Corpora atretica and corpora lutea were also embedded in the epigonal organ. After ovulation many oogonia were detected near the post - ovulatory follicles and some oogonia were embedded in the epigonal organ (Figure 3a, 3b). The ovaries of *R. rhinobatos* were enclosed by epigonal organ and were divided into two lobes by the connective tissue of the capsule and they were located in the anterior region of the body cavity where they were suspended from the dorsal wall by the mesovaria. In *R. rhinobatos*, the anterior part of the genital ridge turned into the gonad and the posterior part developed into lymphohaemopoietic tissue, the epigonal organ. This organ had Y shaped structure in the abdomen. Its development was variable from tiny cell masses at its anterior pole to a huge organ, which extended from the liver to the rectal gland completely surrounded and covered the testis and/or ovaries. Cells in the epigonal organ were undifferentiated lymphomyeloid cells (Figure 3c). The epigonal organ was covered by the peritoneum. It consisted of large amounts of leukocytes, in various stages of development, many blood vessels were filled with erythrocytes and also contained large amounts of lymphocytes (Figure 3d).

Erythrocytes

Erythrocytes were thin ovoid and elliptical in shape and possessed a nucleus in the center of the cell. The erythrocytes were clearly detected throughout of the epigonal organ. These cells were ovoid in shape and possessed a nucleus in the center. The nucleus was round to slightly oval and stained dark blue or

purple. The cytoplasm of the erythrocytes was abundant and stained a pale, orange red. Vacuoles were frequently visible in the cytoplasm of mature erythrocytes (Figure 4a, 4b).

The nucleus was ovoid in shape. The diameter of erythrocytes varied from 5 to 13 μm . The erythrocyte population in the epigonal organ of the *R. rhinobatos* was composed mostly of mature erythrocytes (Figure 4b).

Mitotic activity of the erythrocytes was frequently detected in the epigonal organ, supporting the notion that replication as well as maturation of erythrocytes occurred regularly in the epigonal of *R. rhinobatos* (Figure 4a, 4b)

Lymphocytes

The diameter of the lymphocytes ranged from 7 to 14 μm . They were clearly detected by their dark blue grey nucleus and a lack of granules in their narrow sparse cytoplasm (Figure 4c, 4d). The cytoplasm was light blue in colour. It was very common for the nucleus to be located at the periphery of the cell and often had a slightly indented appearance (Figure 4c, 4d). The amount of cytoplasm varied between small and large lymphocytes. In other word the ratio of nucleus to cytoplasm was typically high in lymphocytes but very often varied with stage of maturity. As lymphocytes mature, the nucleus occupied an increasingly greater proportion of the cytoplasm. Although lymphocytes were characteristically round in shape in some sections, irregular cytoplasmic projections were also clearly detected.

Thrombocytes

The thrombocytes were usually oval or spindle with projections of the cytoplasm in one or both ends

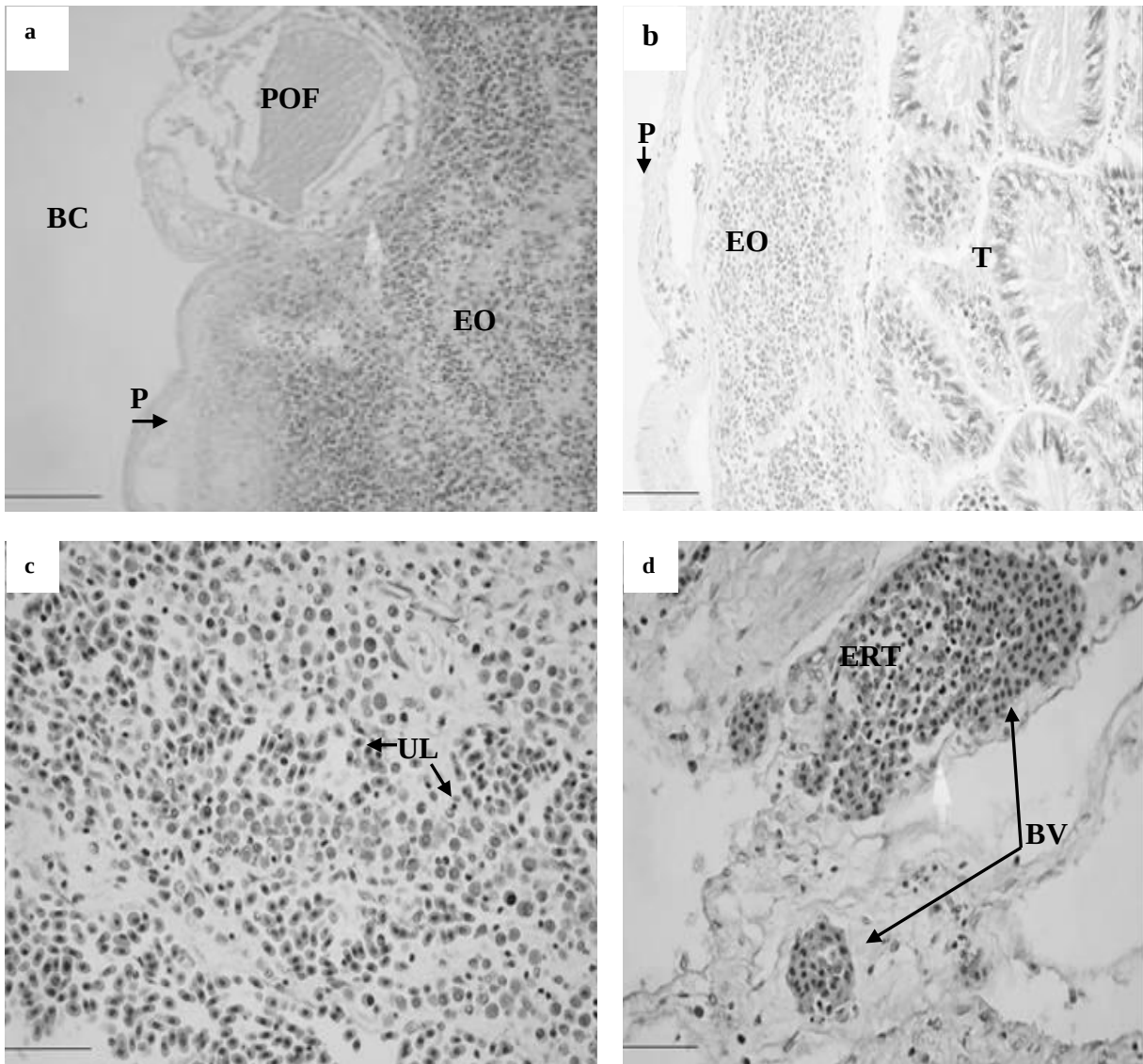


Figure 3. A portion of the cross- sectioned *R. rhinobatos* epigonal organ, a) the epigonal organ surrounding the female gonads at the post ovulated stage, b) Epigonal organ surrounding the testis at various stages of development, c) Undifferentiated lymphomyeloid cells in the epigonal organ, d) Blood vessels filled with erythrocytes in the epigonal organ. A and B Scale bar= 25µm, C Scale bar= 75 µm, D Scale bar= 50 µm. P: Peritoneum, POF: Post Ovulatory Follicles, BC: Body cavity, BV: Blood vessels, EO: Epigonal Organ, ERT: Erythrocytes, UL: Undifferentiated lymphomyeloid cells. Stained with hematoxylin and eosin (HE).

of the cell (Figure 4c, 4d). The cell had a small amount of cytoplasm and a large oval nucleus with a granular chromatin network. Nevertheless they were variable in shape and lacked visible granules. Some thrombocytes were round ranging in size from 3 µm to 10 µm with a purple stained nucleus (Figure 4c, 4d). Some thrombocytes were spindle- shaped with the average of 5 µm width and 9µm length. The cytoplasm of the thrombocytes were clear and colorless to pale blue and was only visible as a faint rim around the nucleus.

Granulocytes

Granulocytes were termed by the staining

characteristic of the cytoplasmic granules and two distinct type of granulocytes were detected. The first type was the neutrophils that ranged in size from 8 to 14 µm in diameter. In same samples the nucleus of the neutrophil granulocytes had two lobes, but in others three-lobed was detected.

Eosinophils were clearly identifiable with pink round granules in the cytoplasm. The colour of the nucleus was purple and very often the nucleus was bi-lobed (Figure 5). The average diameter of the eosinophils were 10 ± 1 µm.

The nucleus of neutrophil and eosinophil was very often eccentric multi-lobed. It possessed two or three lobes with a coarse, clumped chromatin that stained blue or purple and was often more noticeable

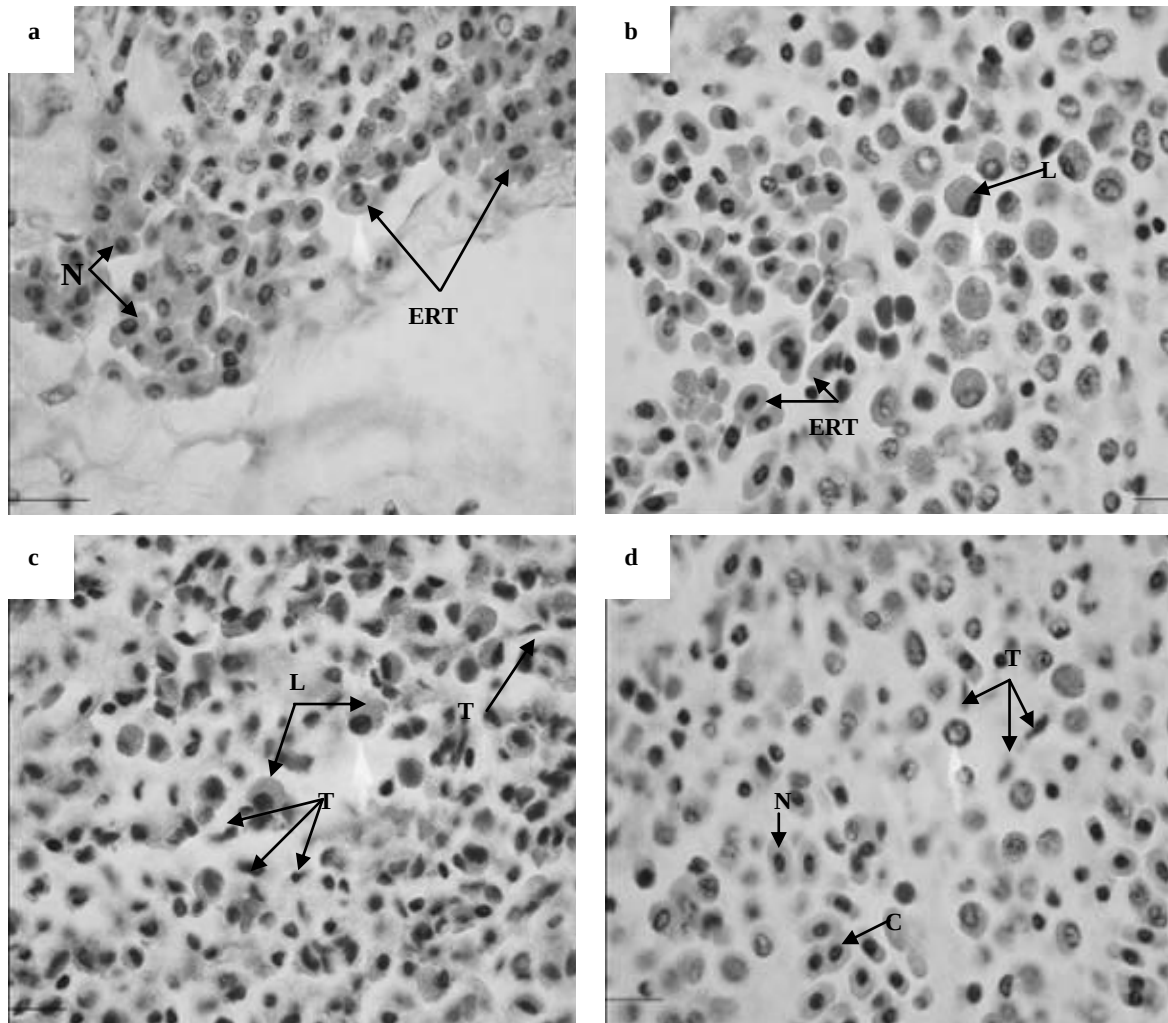


Figure 4. Photomicrographs of the epigonal organ showing different types of the blood cells. a) Erythrocytes with central nucleus and cytoplasm around the nucleus, b) Erythrocytes and lymphocytes, c) Lymphocytes and thrombocytes, d) Thrombocytes. A, Scale bar= 50 μ m, B,C,D, Scale bar=75 μ m, N: Nucleus, C: Cytoplasm, ERT: Erythrocyte, L: Lymphocytes, T: Thrombocytes, Stained with hematoxylin and eosin (HE).

than the nuclei of neutrophil (Figure 5).

Discussion

Elasmobranch fish represent a unique animal model potentially harboring novel products with relevance to public health. One of these products is squalamine, an aminosterol derived from shark liver first reported for its significant antibiotic activity (Moore *et al.*, 1993). Further studies revealed that, squalamine inhibited angiogenesis (angiogenesis is the fundamental process by which persistent, unregulated blood vessels are formed) and solid tumor growth and blocked the mitogen-induced proliferation and migration of endothelial cells, preventing tumor neovascularization (Sills *et al.*, 1998).

Second one, is a compound isolated from shark cartilage, Neovastat (AE-941) has shown antiangiogenic and antitumor activities in animals

(Davis *et al.*, 1997) and humans (Berbari, 1999). Recently, epigonal organ cells (epigonal conditioned medium, by short-term cultures of epigonal cells) has been shown to inhibit the growth of mammalian tumor cell lines, including fibrosarcoma, melanoma, B-cell lymphoma, T-cell leukemia, pancreatic cancer, ovarian cancer and three breast carcinoma cell lines (Walsh *et al.*, 2006). Comprehensive studies on blood cells of epigonal organ of *R. rhinobatos* are extremely valuable.

In the present study it was found that the epigonal organ was composed of sinuses reminiscent of mammalian bone marrow, but with the absence of adipose cells. Similar results were also obtained by Fänge and Pulsford (1983) in the dogfish, *Scyliorhinus canicula*, Walsh and Luer (2004) in some elasmobranch species and Old and Huveeners (2006) in three species of sharks. The epigonal organ consisted a stroma of fibrocytes and collagenous

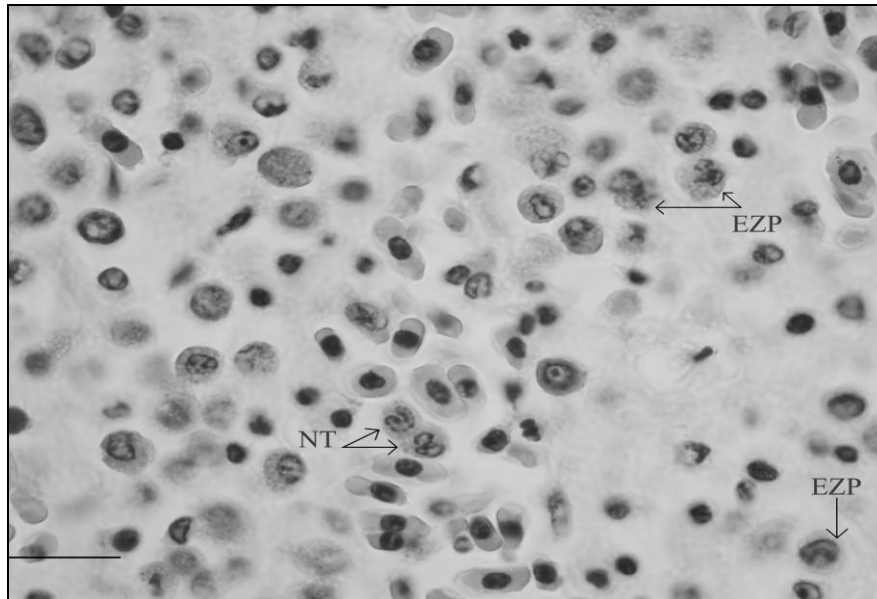


Figure 5. Photomicrograph of the epigonal organ, showing neutrophil and eosinophil granulocytes. Arrows show EZP: Eosinophil and NT: Neutrophil. Scale bar= 75 µm.

fibres arranged between sinusoidal blood vessels, which harbored masses of mature and developing granulocytes and a variable amount of lymphocytes. This founding was similar to that of Zapata *et al.* (1996), Galindez and Aggio (2002), Walsh *et al.* (2006), Çek *et al.* (2009). Galindez and Aggio (2002) studied the epigonal organ in *Mustelus schmitti* and pointed out that the organ was located more caudal than in other selachian species. For this reason they proposed the name of epigonal organ should be changed to subgonal organ. In the present investigation the ratio of the epigonal organ to the gonad were very variable during the reproductive cycle of *R. rhinobatos*. At the full maturation stage in females the epigonal organ was hardly visible with naked eye, but were unlikely to disappear completely. In another words, the degree of contact of the epigonal organ with the gonads were somewhat different from one sampe to another but it always surrounded the testis and ovaries continuing caudally to the posterior margin of the gonads. Therefore our study suggest that the name epigonal should stay the same for *R. rhinobatos*. The results on the position of the epigonal organ were similar to that of Çek *et al.* (2009).

Honma *et al.* (1984) classified the epigonal organ into three types. 1) In smoothhound type, the epigonal organs were connected to the posterior part of gonads; 2) in stingray type, the epigonal organs envelop entirely the ventral surface of both gonads and the part of the organs reach and cover the dorsal surface; 3) in cloudy dogfish type, the epigonal organ covers the gonads and the tip of epigonal organ extended and reached to the level of the rectum.

The present study described the epigonal organ for male and female *R. rhinobatos*. On the basis of

morphological and histological observations of the present study the epigonal organ of *R. rhinobatos* might be classified as the second type (stingray type). Nevertheless, further comprehensive studies on the epigonal organ are needed. Old and Huveneers (2006) described the blood cells of the epigonal organ of Wobbegong sharks. They were found similarities and differences in several morphological characteristics of wobbegong shark blood cells compared to those of other elasmobranchs and teleosts. Walsh *et al.* (2006) studied on bonnethead shark (*Sphyrna tiburo*) and pointed out that, the epigonal cells inhibit the growth of mammalian tumor cell lines. This organ produced both lymphocyte and granulocyte lineages that were proved to be unique to elasmobranch fishes (Walsh and Luer, 2004; Old and Huveneers, 2006).

The present study described various blood cell types in the epigonal organ based upon histology. These cells included eosinophil granulocytes, lymphocytes, thrombocytes and erythrocytes. The morphological structure and overall appearance of these cells in the epigonal organ of *R. rhinobatos* were similar to that observed in some other elasmobranchs (Walsh and Luer, 2004), Wobbegong sharks, *Orectolobus species* (Old and Huveneers, 2006), skate *Leucoraja erinacea* (Lutton and Callard, 2008). In these species including *R. rhinobatos* the epigonal organ was directly associated with the gonads.

Different terminology is used for fish blood cells by different authors. This inconsistency in the identification of elasmobranch blood cells is particularly evident for the granulocytes. Walsh and Luer (2004) have described elasmobranchs as having two, three or four different types of granulocytes using light microscopy. Their study was

comprehensive and helped to standardize the terminology and minimize the confusion. The present study described granulocytes and other blood cells based on their size, diameter, staining pattern with hematoxylin and eosin (HE) but didn't use electron microscopy and functional tests. Two types of granulocytes were distinguished. Granulocytes with round pink granules were called eosinophils and were similar in appearance to those termed eosinophils by Old and Huveneers (2006). The neutrophil granulocytes had lightly stained granules and were most similar to those of wobbegong sharks, *Orectolobus species* (Old and Huveneers, 2006).

Basophils were not detected in the present study. It seemed that basophils were absent in most elasmobranch species studied (Arnold, 2005; Old and Huveneers, 2006). Basophils were described by Luer et al. (2004). Nevertheless their classification wasn't convincing. Because, in many studies basophils were not detected and most workers believe them to be absent (Arnold, 2005; Old and Huveneers, 2006). We also have some doubt on description of basophils by Luer. Luer (2004) counted basophils and found out that 0-1% of white blood cells were basophils without given any photomicrographs of these cells. We also tried to describe basophils and used different staining techniques but, were not able to define them, frankly speaking were not sure whether the cells we were observing was basophils or heterophils. Basophils were described in a very recent study by Dove et al, (2010). In their study photomicrographs of basophils were given even in that study we were not convinced that described cells by Dove et al, (2010) were basophils, since basophils and heterophils were very alike in their histological appearance.

The erythrocytes described in the present study were similar to those reported for birds, reptiles, amphibians, teleost fish species and elasmobranchs (Lutton and Callard, 2008; Claver and Quaglia, 2009).

The thrombocytes in this study were similar in morphological appearance to those described by Walsh and Luer (2004) in bull shark *Carcharhinus leucas* and in wobbegong sharks by Old and Huveneers (2006).

In conclusion *R. rhinobatos* has an epigonal organ, but mammals, birds, reptiles, amphibians and also teleost fishes don't possess. This organ is a unique accessory immunological structure in the common guitarfish. It was composed of lymphoid tissue and seemed to be involved in leukocyte formation. But to date no studies were done on the blood cells of the epigonal organ of *R. rhinobatos*. For this reason further studies using more accurate and reliable techniques such as using antibodies, functional cell assays, electron microscopy and the development of *R. rhinobatos* specific cell markers will permit a more accurate identification of the blood cells in the epigonal organ of *R. rhinobatos*.

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