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Original Article

Ovarian maturatıon stages in Arabian carpet shark, *Chiloscyllium arabicum* Gubanov, 1980 (Orectolobiformes, Hemiscylliidae) during spring and autumn in the Persian Gulf

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Abstract: The present study aimed to assess the ovarian tissue structure and plasma levels of the hypophysial-gonadal hormones, including 17-β estradiol, progesterone, GTH-I and GTH-II of the Arabian carpet shark, *Chiloscyllium arabicum* inhabiting the Persian Gulf in spring (April to July) and autumn (August to December). In this regards, a total of 60 *C. arabicum* female specimens were collected from the Bahrakan Creek, in the north of the Persian Gulf. Fish were bleed after euthanization and biometrical characters and levels of 17-β estradiol, progesterone, GTH-I and GTH-II were measured. Fish were then dissected and samples were taken from the ovary and fixed in Bruin's solution for 48 hrs. Histological sections were prepared using routine histological techniques and stained with hematoxylin and eosin. Based on the results, four different developmental stages were observed in the ovary in spring, including stage I, stage II (primary oogenesis), stage III (mediate oogenesis), and stage IV (final oogenesis). However, only three first stages were detected in autumn samples. The plasma levels of the studied hormones were higher in spring. Based on the results, spring (especially mid-April to mid-July) is the reproduction season of *C. arabicum* in the Persian Gulf.

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Introduction

The reproductive system of female sharks includes a pair of ovaries and passageways for eggs. Similar to other vertebrates, ovaries have three major functions viz. producing sex cells, accumulation of yolk, and synthesis and secretion of steroid hormones (Koob and Callard, 1999). In sharks, ovaries are positioned in the anterior part of the body cavity and hung by mesovarium from the abdomen roof (Koob and Callard, 1999). Like teleost, the hypothalamic–pituitary-gonadal (HPG) axis is the most important axis of controlling reproduction in the sharks. Hypothalamus secretes a variety of hormones and neurotransmitters (such as gonadotropin-releasing (GnRH) and dopamine-releasing (DA)) and then regulates the production and secretion of the pituitary gonadotropic hormones, which in turn, affect gonads to release the steroid hormones (Tostivint, 2011). Two types of gonadotropin hormones are secreted from pituitary anterior lobe of several elasmobranchs which are similar structurally to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Querat et al., 2001). Similar to other vertebrate, gonadal steroids such as progesterone, androgen and estrogen play important role in the reproductive process of elasmobranchs (Lutton et al., 2005). In female elasmobranchs, androgen and estrogen are primarily produced by granulose and theca cells of the ovarian follicles; however the granulosa cells can also produce progesterone (Lutton et al., 2005).

There are 26 shark species in the Persian Gulf (Rima et al., 2014). Arabian carpetshark, *Chiloscyllium arabicum* is a member of carpet sharks inhabiting the coral reefs, wetlands, rocky shores, mangrove forests and river mouth. It can be found in the depth of 3-100 m, in the Persian Gulf to Western India Ocean, Oman Sea, and India Ocean. They have been reported to be abundant in the spring and summer

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in the Persian Gulf, Oman Sea and India Ocean (Compagno, 2002).

There is a little information available regarding various biological aspects of sharks such as their reproductive system. However, *C. arabicum* is a proper model for laboratory studies because of its small size and tame and non-aggressive mood. Therefore, the present study aimed to investigate the reproduction features of *C. arabicum* such as the reproductive time, morphological and histological structure of ovary, levels of steroid (17 beta-Estradiol (E2) and Progesterone (P4)) and gonadotropin hormones (GtHI and GtHII) during its reproduction and rest seasons.

Materials and Methods

A total of 60 mature females of *C. arabicum* were caught from the Bahrakan Creek using trawl net in autumn (September to December 2015) and spring (April to July 2016) (30 fish/season) and transported alive to the laboratory (Fig. 1).

Blood and tissue sampling: The fish were euthanized by 2-phenoxy ethanol (0.35 ml^{-1}) and blood samples were collected from the caudal vein after measurement of their weight and total length. Then, the blood samples were centrifuged at 6000 rpm for 10 min, plasma was separated and frozen at -80°C for further analysis. Fish were dissected after bleeding and ovaries were detached and weighted. The samples were taken from the ovaries and fixed in Bouin's solution for 48 hours before histological processing.

Histological and histometrical study: Tissue samples were washed in 70% ethanol for 24 hours and then dehydrated in ethanol series and embedded in paraffin using tissue processor apparatus (Tissue tek-rotary, RX 11B, Japan). Histological sections of 5-6 µm thickness were prepared using a rotary microtome (Leica, RM2245, Germany) and stained by hematoxylin and eosin (Eagderi et al., 2013). Stained sections were studied under the light microscopy, and digital images were taken using Dino Capture software (FDP2, New Taipei City, Taiwan). For histometric analysis of the ovaries in both seasons, 10 tissue sections from each sample were studied and

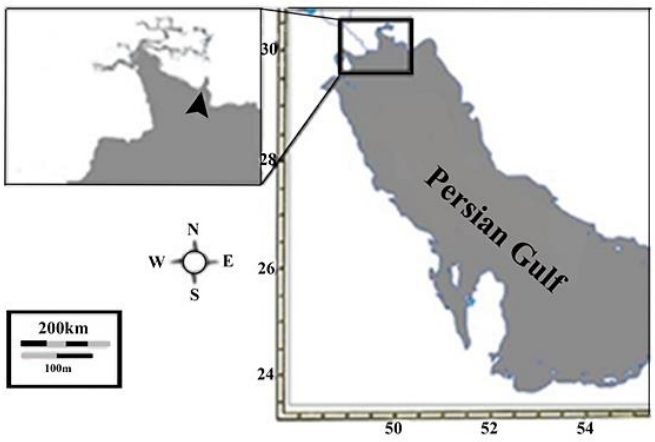

Figure 1. Sampling area in the Bahrakan Creek at the north west of the Persian Gulf.

number of the ovarian follicles in different developmental stages was counted. The thickness of follicular wall and follicular diameter also were measured.

Ganado-somatic index: The fish weight (g) and gonad weight (mg) were measured in both seasons to calculate the gonado-somatic index (GSI) according to Roff (1983) as following:

GSI = (Weight of ovaries/Total body weight) × 100

Steroid hormone assay: Plasma levels of E2 and progesterone were measured using the radioimmunoassay (RIA) method according to Rinchard et al. (1993). Briefly, 50-100 µl of standards, controls and plasma samples were moved into microtubes coated by polyclonal rabbit antibodies. 500 µl of 125I-labelled E2 (radioactivity 170 kBq, Immunotech, France), and 1 ml of 125I-labelled Progesterone (Radioactivity 185 kBq, Immunotech, France) tracer were added to microtubes and incubated in a water bath. The tubes were washed in phosphate buffer saline three times and radioactivity was counted using a gamma counter (LKB gamma counter/France).

Gonadotropin hormone assay: The plasma levels of GTHI and GTHII hormones were measured by commercial kits (Immunotech, France) using the radioimmunoassay (RIA) method described by Van Der Kraak et al. (1983).

Statistical analysis: All data were represented as means ± Standard Error (SE). The normality of data was controlled using Shapiro-Wilk test. Then,

Table 1. Biometrical data of *Chiloscyllium arabicum* collected from the Bahrakan Creek in spring and autumn.

	Spring	Autumn
Weight (gr)	986.7±133.88	672.5±87.67
Length (cm)	73.7±5.18	55.6±4.5

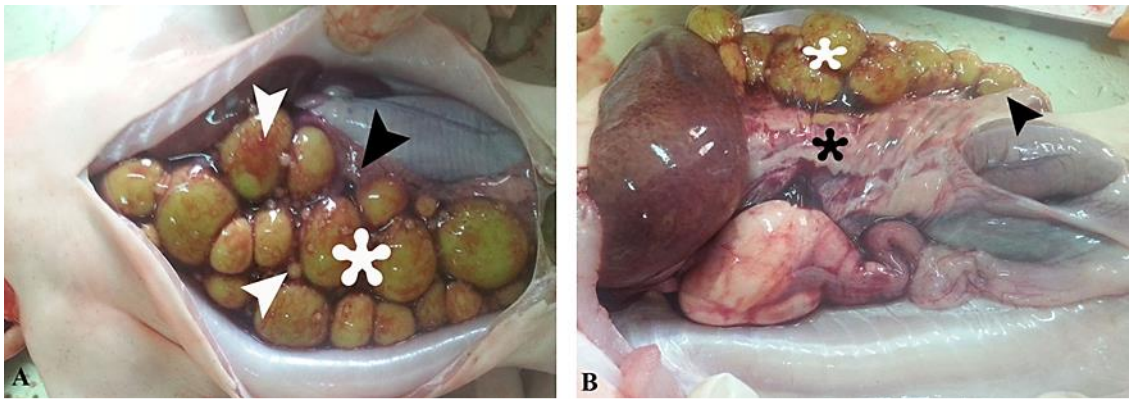

Figure 2. The position and morphology of the ovary in *Chiloscyllium arabicum*; ovarian follicle (white arrowhead), epigonal organ (black arrowhead), and mesentery (black star).

independent-sample student T-test was used to determine the significant difference among data obtained in spring and autumn. The significant level of $P<0.05$ was accepted. All analyses were performed in SPSS 16.0 software.

Results

The average weight and total length of female were significantly smaller during autumn season (Table 1). *Chiloscyllium arabicum* possesses a pair of large yellow oval ovaries located in the ventral part of the epigonal organ and hung by mesentery in the anterior part of the abdominal cavity (Fig. 2). Follicles at various developmental stages were observed in the ovaries in spring and autumn confiming the asynchronous ovary in the Arabian carpetshark (Fig. 2). In spring, the ovary occupies a great volume of the abdominal cavity. Follicles were recognizable separately with naked eye (Fig. 3), although follicles were significantly larger in spring ($P<0.05$, Fig. 6).

The structure of ovary in *C. arabicum* consists of the stromal connective tissue with many blood vessels and ovarian follicles at different stages of growth (Fig. 4). Epigonal organ was a vessels-abundant lympho-myeloid tissue, consisting various blood cells (leukocytes especially granulocytes were dominant). This organ is positioned around the ovarian follicles (Fig. 4). The ovary is covered by a cell layer i.e. germinal epithelium and a fibrous and dense connective tissue so-called tunica albuginea (Fig. 4).

The ovarian follicles include one oocyte and surrounding membranes in both spring and autumn, at various developmental stages. Meanwhile, tubular glands i.e. oviducal glands were detected among ovarian follicles (Fig. 4). Oviduct secretes egg shell to cover the released oocytes. In spring, sperm was found inside the follicles (Fig. 4). The wall of ovarian follicles was thicker in spring ($P<0.05$, Figs. 4, 6). Follicles had also larger diameter in spring; then, they might be torn during preparation, therefore full image of mature follicle could not be prepared (Fig. 4). Developmental stages of the ovarian follicles were as following in spring:

Stage I: No ovarian follicles were formed and a group of the primordial germ cells (oogonia) were observed. Oogonia were spherical or ovoid in shape (409.04-519.83 μm diameters). A nucleus with dense chromatin was seen at the center of oogonia.

Stage II (Early Oogenesis): The primary follicles (631.23-784.06 μm diameters) include oocytes (nucleus with 106.08-175.36 μm diameters in center or between center and perimeter of oocyte) covered by zona radiata and a layer of the follicular cells. Inner and outer theca layers consisted of wide cells and collagen fiber, respectively were observed around the follicles.

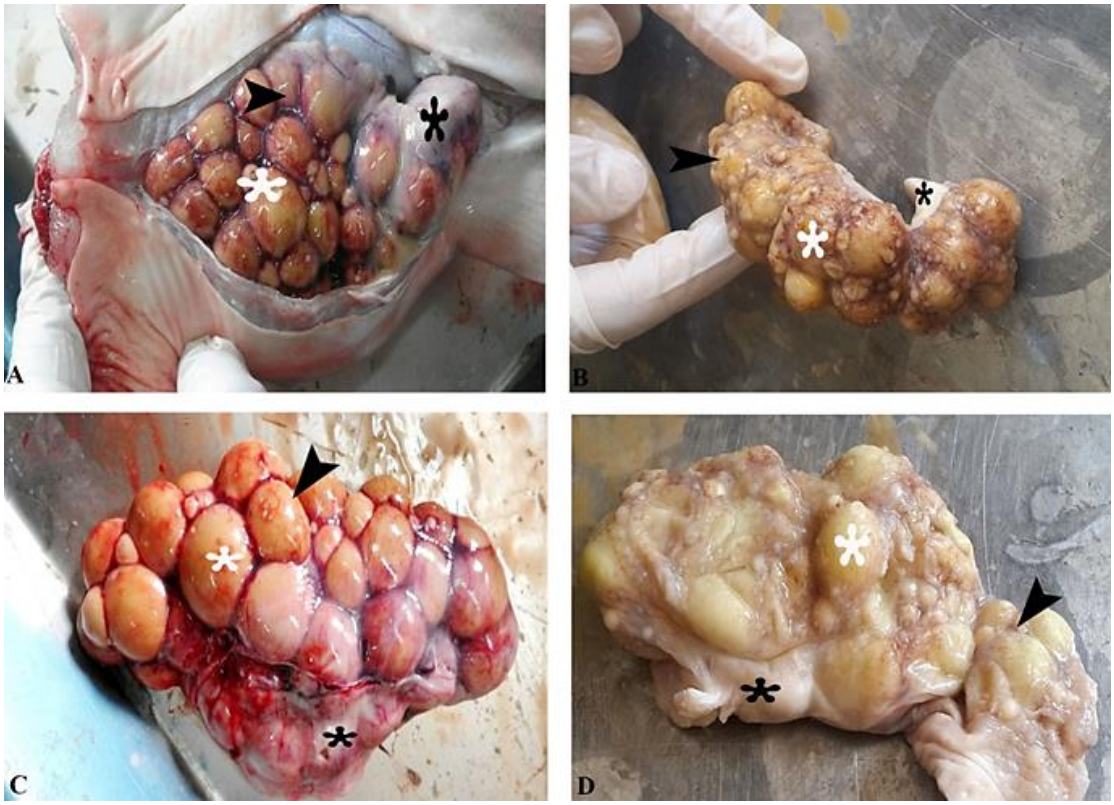

Figure 3. The anatomical structure of the ovary in *Chiloscyllium arabicum* in spring **(A, C)** and autumn **(B, D)**. ovary (white star), ovarian follicles (black arrowhead), and epigonal organ (black star).

Stage III (Middle Oogenesis): The growing follicles with 1027.32-2914.64 µm diameters were the most follicles detected in the ovary. The increase in size of these follicles was mainly because of the vitellogenesis (yolk deposition). Many yolk granules in the oocyte cytoplasm, a nucleus in the periphery of the oocyte, thicker zona radiata and thicker follicle membrane (comprising of the granulosa cells) were characteristics of the ovarian follicles in this stage.

Stage IV (Late Oogenesis): The growing follicles (3147.45-3566.62 µm diameters) with many yolk granules were observed. Meanwhile, lipid vacuoles were appeared inside the oocyte and they become large. Zona radiata was very thick. Granulosa cells were cylindrical in shape around oocyte and sperm was observed in some follicles (Fig. 4).

A large amount of follicles in stages I (85.21-120.18 µm diameter) and II (160.28-236.64 µm diameter) and a little number of the ovarian follicle in stage III (213.33-660.72 µm diameter) were found in the ovary of *C. arabicum* in autumn. No ovarian follicle at stage IV was detected in this season (Fig. 5).

The ovarian follicles in autumn were primarily immature (previtelloginic stages) while those observed in spring were mainly mature (Fig. 6). The mean of diameter and wall thickness of the ovarian follicles was significantly higher in spring (P<0.05, Fig. 6). The weight of the ovaries was significantly higher in spring and it had close correlation with the fish's weight. The weight of the ovaries and percentage of the gonadosomatic index (GSI) was higher in spring (P<0.05, Fig. 6).

The results showed a significant difference between the amounts of steroid (17 beta-Estradiol (E2) and Progesterone (P4)) and gonadotropin hormones in plasma of female sharks in autumn and spring. The plasma level of these hormones were significantly higher in spring (P<0.05, Fig. 7).

Discussion

Since the fishes have scheduled reproductive behavior, morphohistological study of the gonads may precede the study of maturity procedure. Thus, structural and morphological changes in the oocyte

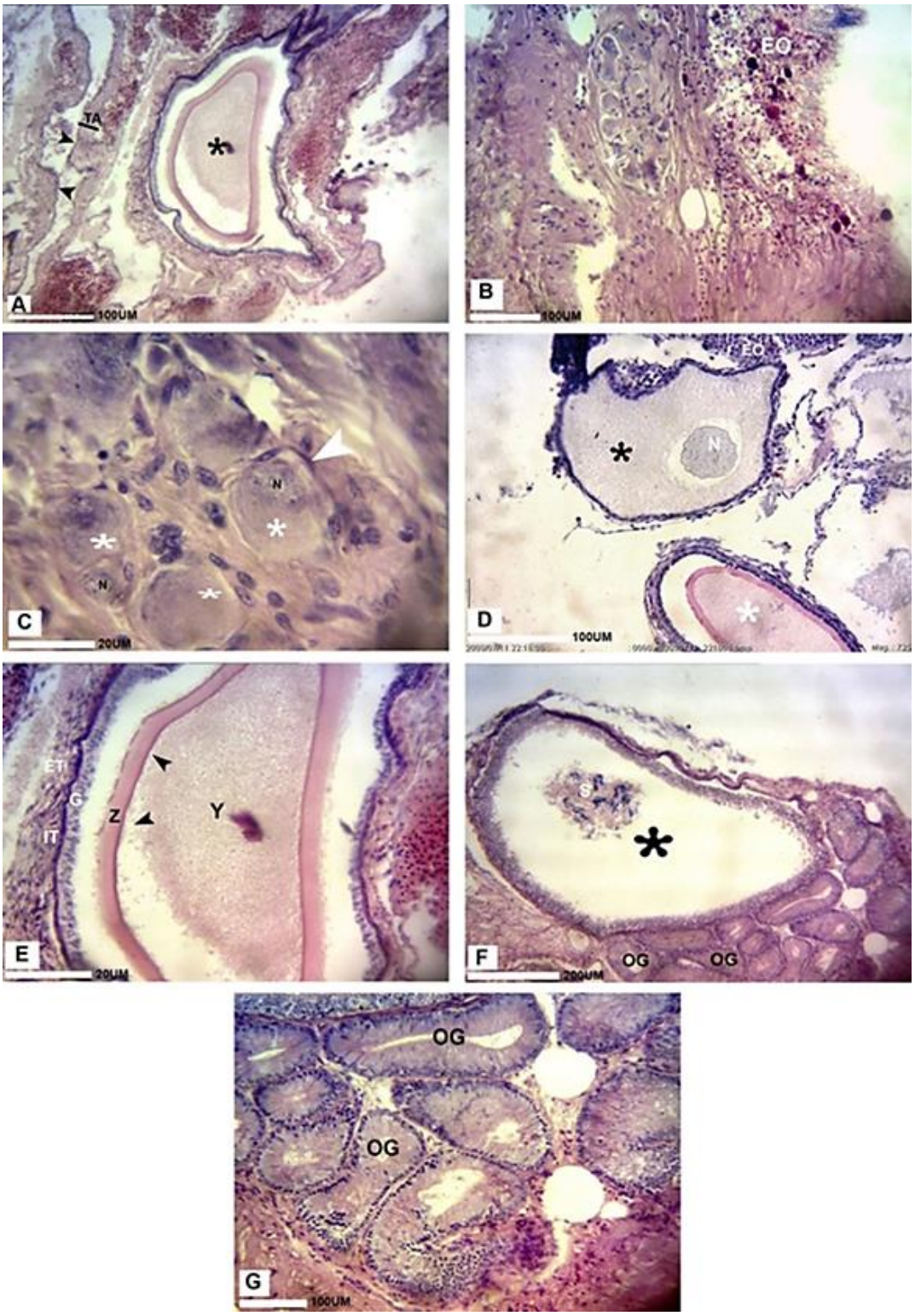

Figure 4. Representative photomicrograph of the ovarian tissue structure in *Chiloscyllium arabicum* in spring. **(A)** germinal epithelium (black arrowhead), tunica albuginea (TA), ovarian follicle (black star); **(B)** epigonal organ (EO), oogonium (white arrowhead); **(C)** oogonium (white star), oogonial nucleus (N), oogonial squamous epithelial cell (white arrowhead); **(D)** ovarian follicle at stage I (black star), nucleus (N), ovarian follicle at stage II (white star), epigonal organ (EO); **(E)** ovarian follicle at stage III: yolk granules (Y), zona radiata (Z), granolosa cells (G), Inner theca layer (IT), outer theca layer (ET); F. ovarian follicle at stage IV (black star), oviducal gland (OG), sperm (S); G. oviducal gland (OG); A,B,D,G (H&E;×725), C, E (H&E;×2900), F (H&E;×290).

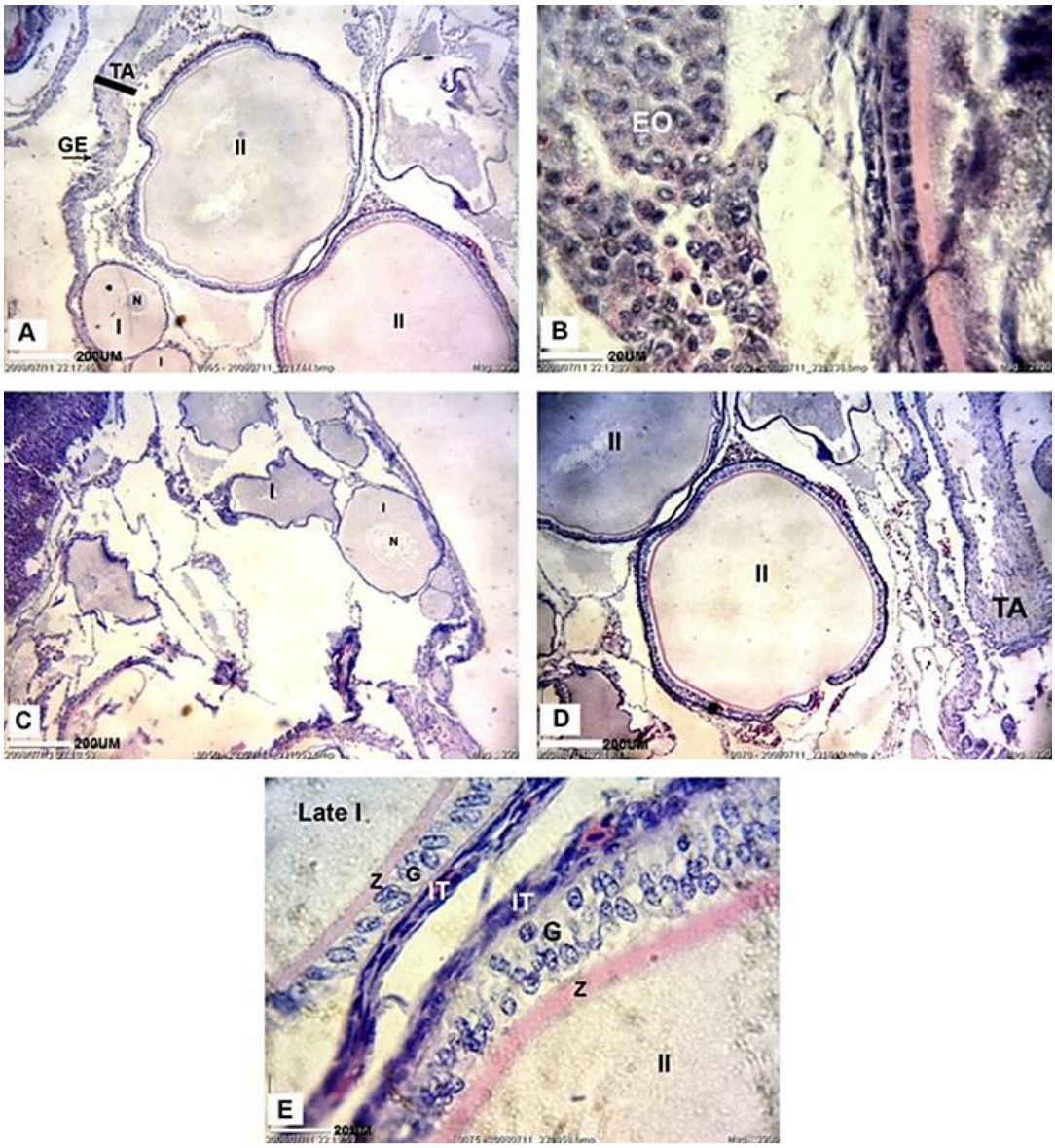

Figure 5. Representative photomicrograph of the ovarian tissue structure in *Chiloscyllium arabicum* in autumn. **(A)** germinal epithelium (GE), tunica albuginea (TA), ovarian follicle at stage II (II), nucleus (N), ovarian follicle at stage I (I); **(B)** epigonal organ (EO); **(C)** nucleus (N), ovarian follicle at stage I (I); **(D)** ovarian follicle at stage II (II), tunica albuginea (TA); **(E)** the wall of ovarian follicles at stage I and II, zona radiata (Z), granolosa cells (G), Inner theca layer (IT); A,C,D (H&E, 290X), B, E (H&E, 2900X).

and ovary could be used to determine the various stages of maturity (Khalatbari, 2013). Follicular cells are appeared around oocytes and change in shape during follicular development. In the present study, these cells were in cuboidal shape in the previtellogenic follicles and cylindrical at the end of vitellogenic stage. The theca layer was observed in both previtellogenic and vitellogenic follicles in the ovary of *C. arabicum*. Sperm was found inside a number of ovarian follicles in *C. arabicum*. In this regard, Chen and Liu (2006) reported that a delay is occurred between the maturation and fertilization in *C. plagiosum* is mainly due to the sperm storage in the ovarian follicles of females.

Conde-Moreno and Galvan-Magana (2006) reported that only one ovary of shortfin mako shark, *Isurus oxyrinchus* is active and ovarian follicles are presented at the four developmental stages: in the first stage a group of oogonia were observed; in the early oogenesis, the primary follicles with zona radiate and follicular cells are detected; in the middle oogenesis, the diameter of follicles increase due to yolk disposition; and in the late oogenesis, the diameter of follicles and thickness of zona radiate are more than

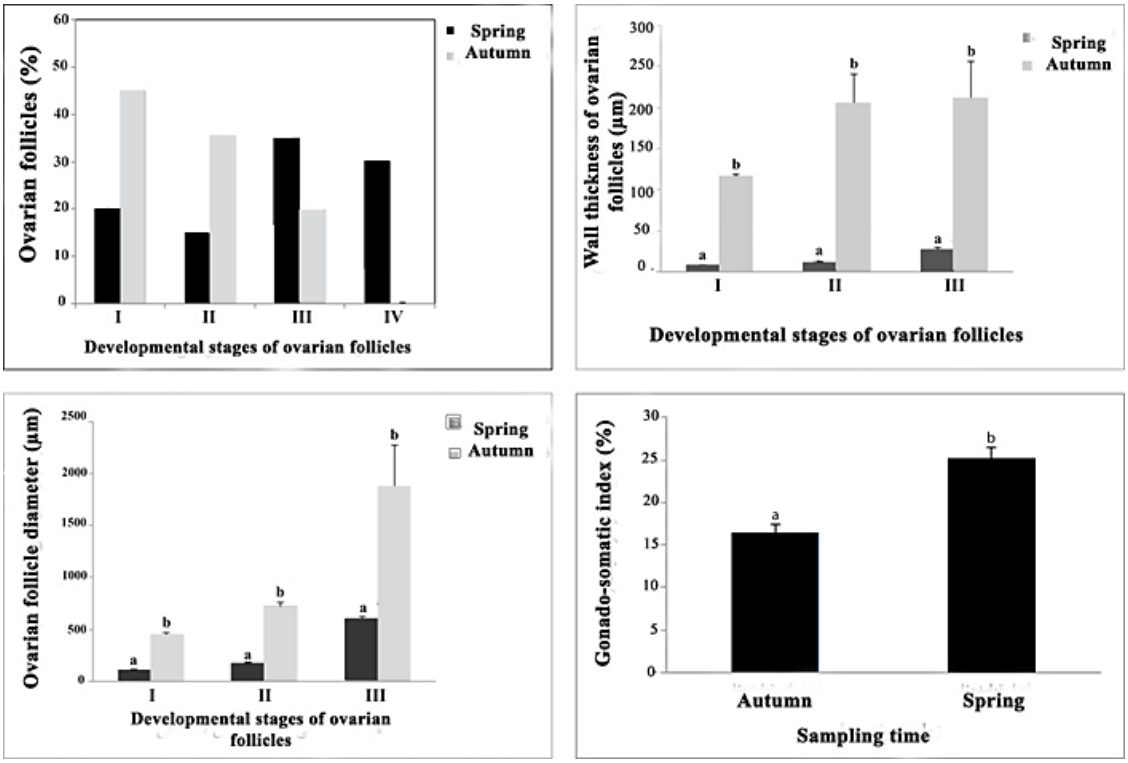

Figure 6. The percent of ovarian follicles at different developmental stages, diameter and wall thickness of the ovarian follicles and GSI amount in *Chiloscyllium arabicum* in spring and autumn. Data are represented as mean±SE. The letters indicate the significant difference between seasons (P<0.05).

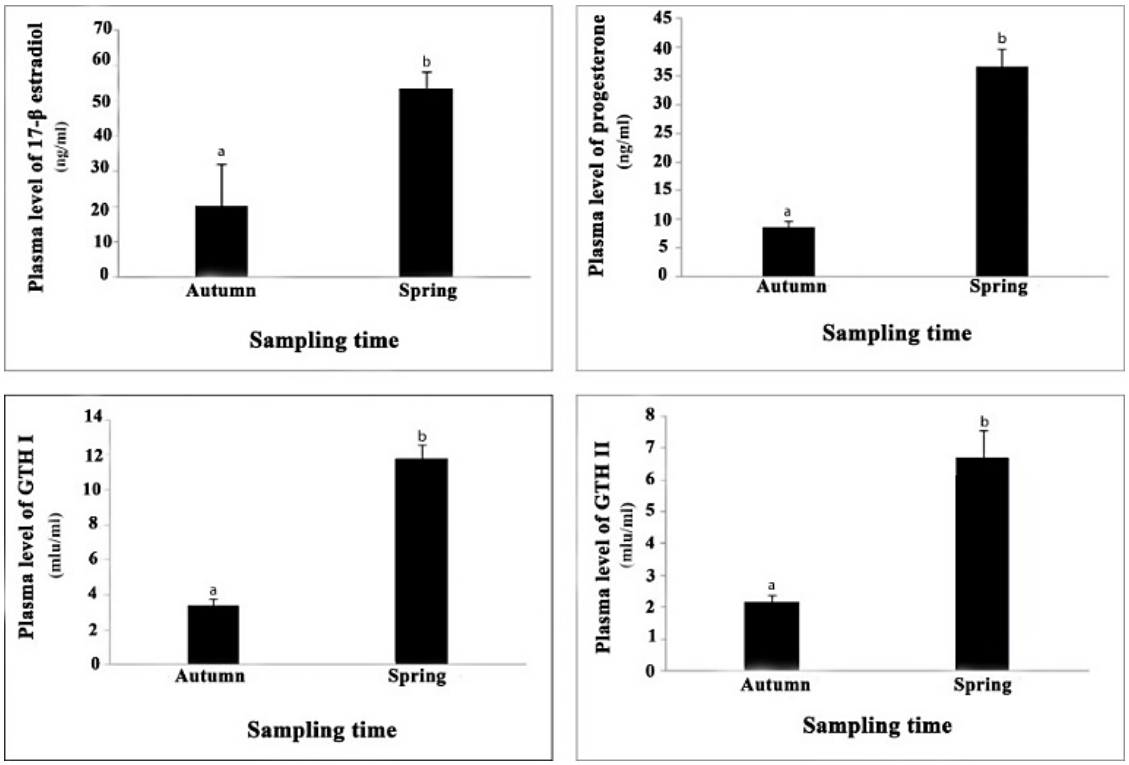

Figure 7. The plasma level of E2, P4, GTHI and GTHII hormones in *Chiloscyllium arabicum* in spring and autumn. Data are represented as mean±SE. The letters indicate the significant difference between seasons (P<0.05).

previous stages and sperm present inside some follicles. In the present study, similar developmental stages of the follicles were observed.

The gonadosomatic index (GSI) is considered as

the most conventional method to determine the growth of gonads and spawning season (Biswas, 1993). According to the results, the spring is the reproduction season of *C. arabicum*. In spring, the body weight and weight of sharks' ovaries were higher. Increasing the gonadosomatic index of *C. arabicum* may be as the result of increased number of the vitellogenic and/or watered follicles in the final stage (Lee and Yang, 2002). Watered follicles absorb the water during the developmental process and increase the weight of ovary two to four times (Lee and Yang, 2002). Abasi et al. (2003) stated that changes in GSI are closely related to the number of eggs and their size.

In the present study, increase in the size of the follicles was the most salient sign of follicle growth. Barone et al. (2007) found that the ovary was asynchronous in *Raja asterias*, in which, the developing follicles with various sizes were detected in the ovary and the largest follicles were observed in the vitellogenic stage. The results of the present study were in agreement with these findings.

The gradual increase in thickness and complexity of zona radiate represent its function in transferring the necessary materials. Vitellogenesis, egg maturity and hatching are complex processes that need the transfer of raw materials to oocyte and active synthesis. Mackie (2000) and Samira et al. (2008) pointed out that diameter of the follicles increases in the reproduction season. Erfani Majd et al. (2015) also reported that diameter of the zona radiata reaches its maximum in the mature follicles in *Hypophthalmichthys molitrix*.

It has been well-documented that GnRH secretion from the hypothalamus stimulates the GTH secretion form the pituitary and consequently secretion of steroid hormones from the gonads (Omoto et al., 2005). GTH-I controls the estradiol synthesis by ovarian follicular cells and vitellogenin absorption by follicles (Omoto et al., 2005). Meanwhile, the oocyte maturation is directly started under GTH-II control by means of a progestin so-called induced maturation steroid made in the follicular cells (Jalabert, 2005). Based on the studies on salmonids that have reproductive stages with a specific time difference, it was reported that GTH-I is the mediator of vitellogenesis, and GTH-II regulates the final maturation of oocytes (Tyler et al., 1991). Seasonal changes of the gonadotropin levels in *Carassius auratus* have indicated that synthesis and secretion of GTH-I and GTH-II occur simultaneously for fishes with asynchronous ovaries (Sohn et al., 1999). Mateos et al. (2003) reported that the level of GTH-I in pituitary of striped bass increase as the GSI increased. These results were in agreement with the results of the present research. Gomez et al. (1999), Sohn et al. (1999) and Melamed et al. (2000) reported that synthesis and secretion of GTH-II increased during gametogenesis and spawning. Similar results were found in *C. arabicum*.

Conflict of interest statement

The authors report no conflict of interest.

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چکیده فارسی

مراحل بلوغ تخمدانی در ماهی گربه کوسه عربی *Chiloscyllium arabicum* Gubanov, 1980 (Orectolobiformes, Hemiscylliidae) طی بهار و پاییز در خلیج فارس

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چکیده:

مطالعه حاضر با هدف ارزیابی ساختار بافتی تخمدان و سطوح پلاسمایی هورمون‌های هیپوفیزی-گنادی شامل ۱۷-بتا استرادیول، پروژسترون، گنادوتروپین I و گنادوتروپین II در ماهی گربه کوسه عربی خلیج فارس *Chiloscyllium arabicum* در طی بهار (انتهای اسفند تا خرداد) و پاییز (شهریور تا آذر) صورت پذیرفت. در این راستا، ۶۰ قطعه ماهی *C. arabicum* از خور بحرکان واقع در شمال خلیج فارس جمع‌آوری شد. پس از بیهوش نمودن و انجام زیست‌سنجی، خونگیری از ماهیان انجام شد. نمونه‌های خونی سانتریفوژ شده و سطوح هورمون‌های ۱۷-بتا استرادیول، پروژسترون، گنادوتروپین I و گنادوتروپین II اندازه‌گیری شد. پس از خونگیری، ماهیان تشریح شده و نمونه‌های بافتی از تخمدان آن‌ها برداشت و در محلول بوئن به‌مدت ۴۸ ساعت تثبیت گردیدند. مقاطع بافتی با استفاده از روش‌های معمول بافت‌شناسی تهیه و با استفاده هماتوکسیلین و ائوزین زنگ‌آمیزی شدند. چهار مرحله تکاملی مختلف در تخمدان ماهیان صید شده در فصل بهار مشاهده شد: مرحله I، مرحله II (اووژنز اولیه)، مرحله III (اووژنز میانی) و مرحله IV (اووژنز نهایی). تنها سه مرحله اول تکاملی در تخمدان ماهیان صید شده در پاییز مشاهده شدند. سطوح پلاسمایی هورمون‌های مورد مطالعه حاضر در ماهیان صید شده در فصل بهار بیشتر بود. براساس نتایج به فصل بهار (به‌ویژه اواخر اسفند تا اوایل خرداد) فصل تخمریزی این گونه در خلیج فارس می‌باشد.

کلمات کلیدی: گربه کوسه عربی، تخمدان، ۱۷-بتا استرادیول، پروژسترون، گنادوتروپین.