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RESEARCH ARTICLE

INVESTIGATIONS ON GAMETOGENESIS AND GONADAL INDICES IN THE VEINED SQUID *DORYTEUTHIS FORBESII* (STEENSTRUP, 1856) (CEPHALOPODA -LOLIGINIDAE)

[1, 2]Abdullah Bedeer Hussein and *[1, 3]Gaber Ahmed Saad

[1]Department of Biology, College of Medicine, Dammam University, Saudi Arabia, KSA
[2]Department of Zoology, Faculty of Science, Mansoura University, Mansoura, Egypt
[3]Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt

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ABSTRACT

Knowledge of the maturation process is vital to understand the relationship between spawning stock andrecruitment. The purpose of the present study was to validatethe reproductive cycle of *Doryteuthis forbesii* using histological and statistical procedures. The changes in gonad during sexual maturation permitted the determination of different histological stages for oogenesisin 12 phases and spermatogenesis in 7 phases. This species spawns throughout the year with two peaks during spring and summer. During oogenesis the oocyte becomes progressively more elongated, with a subsequent narrow elongation of the apical zone forming the oocyte peduncle. Finally the oocytes turn smooth (chorion becomes the outer surface when the follicular cells disintegrate) and free, finally reaching the oviducts. The spermatogonia and the primary spermatocytes are difficult to distinguish from each other, both being relatively large cells with a large, distinct nucleus. The first meiotic division occured, and the primary spermatocytes become secondary spermatocytes. A second meiotic division occurred immediately afterwards, so the secondary spermatocytes become spermatids. The transformation of the spermatids to spermatozoa occurs by a process of differentiation known as spermiogenesis. Morphologically, this differentiation involves the transformation of the cell from a round to an elongate shape. The spermatozoa are grouped around the lumen, towards the centre. As spermatozoa matured and released from the testis they passed directly to the spermaduct which is surrounded by complex accessory glands. The first glands inactivate the spermatozoa and others cover the sperm mass with different secretions to form the spermatophore. Spermatophores usually accumulate in the Needham sac. Spawning season was determined by calculating the different stages of oocyte development in the four seasons of the year using three methods. One Way Analysis of Variance (ANOVA) with $p<0.05$ and Newman-Keuls Multiple Comparison Test with $P < 0.01$and $P > 0.05$ were applied to compare the different stages of oocyte development in the four seasons of the year. The gonadosomatic index (GSI) for both sexes and the nidamental gland–somatic index (NSI) for the females, were calculated and commented.This study provided evidence suggesting that *Doryteuthis forbesii* is an intermittentspawner.

INTRODUCTION

The reproductive biology of different cephalopods has been the subject of many research investigations (**Gabr *et al*., 1999; Gabr and Riad, 2008; Leporati *et al*., 2008; Poveda *et al*., 2009**). These systems are highly complex structures with ducts, glands, and storage organs. Fertilized eggs are embedded in one or more layers of protective coatings produced by the oviducal and nidamental glands and generally are laid as egg masses. Egg masses are benthic in Loliginidae and Octopodidae and pelagic in Ommastrephidae. Development of embryos is direct, without true metamorphic stages and hatchlings undergo gradual changes in proportions during development. The term 'paralarva' was first introduced by **Young and Harman (1988)** for the early stages of cephalopods between hatchling and subadult, which differ morphologically and/or ecologically from the older stages. Cephalopods show great resilience to environmental change by having short lives, high turnover of generations, asynchronous growth and maturation, and extended spawning (**Boyle *et al*., 2002**). This gives the populations the potential to exploit available food resources and overcome short adverse environmental episodes moreefficiently (**Katsanevakis and Verriopoulos, 2006**). It is documented that populations of shelf species fluctuate enormously. They may decrease sharply from one year to thenext, but yet survive and flourish in later years (**Clarke, 2007**).

Corresponding author:[1, 3]Gaber Ahmed Saad,
[1]Department of Biology, College of Medicine, Dammam University, Saudi Arabia, KSA.
[3]Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt.

Cephalopod populations are extremely vulnerable to large adverse fluctuations of biological and physical variables, in particular during the paralarval phase. To minimize this vulnerability and provide some temporal stability (**O'Dor, 1998; Pierce *et al*., 2008**), many cephalopod populations are composed at any given time, of several groups of similar age, cohorts or microcohorts. This population dynamics results from the interaction of the temperature and the productivity of seasonal cycles with the hatching season and subsequent animal growth (**Grist and des Clers, 1999;Pierce *et al*., 2005**).Temperature is one of the main factors inducing geographic variability of reproductive and growth parameters in cephalopods (**Moreno *et al*., 2002**). Males of *Loligo vulgaris* attain greater lengths (640 mm ML) than females (540 mm ML), with the maximum values recorded on the Saharan Bank (**Raya, 2001**). The life cycle may be completed within approximately one year, with maximum lifespans of 15 months recorded in western Iberia (**Moreno *et al*., 1996; Rocha and Guerra, 1999**). *Loligo vulgaris* is a terminal spawner, but oocyte maturation and egg-laying occur in separate batches during the spawning period (**Rocha *et al*., 1996; Rocha and Guerra, 1999**). Fecundity has been estimated at between 10 150 and 42 000 eggs (**Baddyr, 1988; Coelho *et al*., 1994; Guerra and Rocha, 1994; Laptikhovski, 2000; Pierce *et al*., 2005**). Mean spawning age is ten months, and mean age-at-maturity is nine months. Hatching time significantly influences mean age-at-maturity, which is higher in winter cohorts than in summer cohorts (**Moreno *et al*., 2005**). The spawning season is shorter in the northern part of the distribution (**Moreno *et al*. 2002; Sifner and Vrgoc, 2004**). Elsewhere, spawning occurs throughout the year, with two main peaks between November and June, although these occur earlier in more southern latitudes and earlier in the Atlantic than in the Mediterranean (**Baddyr, 1988; Coelho *et al*., 1994; Guerra and Rocha, 1994; Moreno *et al*., 1994, 2002; Rocha, 1994; Bettencourt *et al*., 1996; Villa *et al*., 1997; Raya *et al*., 1999; Belcari, 1999a;Pierce *et al*., 2008**).

Oogenesis in cephalopods involves a highly coordinated differentiation of the oocyte and follicular epithelium. (**Selrnan and Arnold, 1977**). Studies in cephalopods (**O'Dor and Wells, 1973; Richard and Dhainaut, 1973; Bottke, 1974; Arnold and William-Arnold 1976**) have revealed that during oogenesis the follicular cells become highly differentiated secretory cells, but the exact nature and product have not been fully known. The structure of the egg capsules is similar in cephalopods as described by **Jecklin (1934)**, but the way these capsules are deposited is different. In *L. vulgaris*, the egg capsules are always suspended in dense clusters from overhanging substrates (**Boletzky 1983, 2004**), whereas in *D. opalescens*, they are deposited on (generally soft) bottoms (**Fields 1965**). An open question remains with regard to the actual making of an egg capsule by the spawning female: Is the spirally coiled string of eggs embedded in oviducal jelly released along with the outer capsule, the material of which is produced by the nidamental glands, or is the outer capsule released first and the egg string 'pumped' into it, as described by **Zeidberg (2008 &2011)**?In neritic loliginid, *octopus* and sepiolid females lay their eggs in capsules/clusters attached to hard substratum or branched sessile organisms on the sea bottom, while in ommastrephids and most other oceanic squids the eggs are laid into large masses that drift submerged in the open sea (**Jereb and Roper, 2005**).

As spermatozoa mature and are released from the testis they pass directly to the spermaduct which is surrounded by a complex of accessory glands. The first glands inactivate the spermatozoa and others cover the sperm mass with different secretions to form the spermatophore (**Drew, 1919**). Spermatophores usually accumulate in the Needham sac. Each spermatophore is therefore analogous to a single egg laying of a female squid. Usually only a portion of the spermatophores from the Needham sac is transferred to any female. Males of the most primitive cephalopods (*octopus* of the suborder Cirrata) produce sperm packets rather than spermatophores. In each of these packets spermatozoa are positioned with their tails towards the centre and heads towards the periphery (**Aldred *et al*., 1983**). In *octopus* of the Arqonautoidea superfamily only one spermatophore is formed in the spermducts. This spermatophore "bursts" in the Needham sac and its contents are transferred to the seminal reservoir on a specialized arm called the hectocotylus. The hectocotylus detaches during copulation and is inserted into the female mantle cavity (**Nesis, 1982 &1987**).

In primitive mussels (Monoplacophora and others) the male reproductive system is organized and functions similarly to that of female *octopus*, i.e. sperm accumulate in sexual coelom and are released into the spermduct at spawning. In primitive prosobranch molluscs (*Littorina*) the male sexual system functions similarly to that of female squid, i.e. sperm accumulate in the enlarged part of the spermduct. In higher prosobranchs (*Ptenoglossa*), spermatozoa are formed into special structures called spermatozeugmae which are able to move actively (**Chukchin, 1984**). In some higher gastropods (pulmonate molluscs - Stylommastophora), spermatophore formation takes place in the sexual viae and accumulate in the Needham sac which is similar to what occurs in some cephalopod males. In higher gastropods, sperm is transferred to the female cloaca by a special copulatory organ, which is either a body wall protrusion or a modified tentacle. In cephalopods, the distal part of the Needham sac has slightly muscular walls and forms what is termed a "penis" which serves not for internal fertilization but for sperm transfer. This transfer is either directly to a female (for example, in squid (*Onyehoteuthis* sp.) with a long penis or in most cases to the hectocotylized part of a ventral arm in squid (*Ommastrephes* sp. and others) with a short penis.

Little is known about the seasonal variation of sexual maturity of cephalopods inhabiting the Arabian Gulf. Study of information on maturation and spawning of a chosen and available species of cephalopods will contribute to the knowledge of its general biology, population dynamics, and management of the stocks. Therefore, the current study was designed to address the lack of information regarding the reproduction biology of *Doryteuthis forbesii* in its distribution in the Arabian Gulf (Saudi Arabia). The work presents the first detailed study of seasonal variation in sexual maturity in reference to the histological and statistical composition of the ovary and testis in the four seasons of the year. The study

documented the spawning season of the species under investigation and compared different morphometric characteristics of these animals and their contributions in the reproduction process. There is high variability in the annual abundance of theveined squid under investigation, which suggests that environmental effects on populations tend to be both pronounced and transient. This reflects the short life, rapid and labile growth, maturation patterns, and the lack of overlap between generations

MATERIALS AND METHODS

Identification of the squid under investigation

The veined squid *Doryteuthis*(*Loligo*) *forbesii* (**Steenstrup, 1856), (Hanlon, 1978; Koslow and Allen, 2011**) has been identified according to **Graham*et al.* (2010**) and Zeidberg *et al*. (2011) as follows: Cornea present. Rhomboidal fins of length ca. 75 % of dorsal ML, posterior borders lightly concave. Arms with two rows of suckers. Suckers on the manus of thetentacular club are subequal in size; sucker rings with 13-18 sharp conical teeth. Unlike *L. vulgaris*, it lacks enlarged medial suckers on the tentacular club (suckers in all four rows are fairly similar in size). In males, the left ventral arm (IV) ishectocotylized in its distal third by modification of suckers into long papillae thatgradually decrease in size distally. Simple funnel-locking cartilage. Diagnosisadapted from **Roper *et al.* (1984)**. *Doryteuthis forbesii* is a neritic occurring in coastal waters andthe continental shelf including the North Sea, Red Sea extending into the Mediterranean.

Animals

Specimens of the veined squid *Doryteuthis forbesii* (**Steenstrup, 1856**) (**Hanlon, 1978; Koslow and Allen, 2011**) were sampled regularly twice every month at depth 1- 2 meters during the four seasons in the year (2012) along the northern estruarine harbour of the Arabian Gulf - Saudi Arabia. Each collection was transported in well-aerated sea water to the laboratory. Few amount of magnesium sulphate has been added in the aquaria as a relaxing agent. Individuals of each collection were used to prepare sections of ovaries and testes for histological and statistical studies.

Microscopic observation

Isolated parts of ovaries and testes from specimens of each collection were fixed in neutral 10 % formalin or Bouin. These isolated parts have been washed in distilled water for 24 hours, dehydration through ascending series of ethyl alcohol, alternated by another dehydration series of tertiary butyl alcohol (used as a softening agent), then tertiary butyl alcohol and paraffin oil (1:1), absolute paraffin oil. All preparations then washed carefully in tissue mate (melting paraplast) with melting point 54-58 °C and blocked in fresh paraplast. Sections for histology with thickness of 5-6µ were somewhat successfully carried out. For the routine histological study, the sections were stained with Ehrlich haematoxylin and eosin stain. A number of triple stains (Table 1) were tried to enable the differentiation of the tissues (**Pearse, 1968, 1980 and Pantin, 1948**). For microscopic examination, Ortholux Leintz Wetzler Stereoscope microscope with 100, 250 and 400 magnification capacities with Lighthouse 250 with external light source of Schott KL 1500 was used. The Camera used was a full automatic microscope Camera for research and laboratory purposes. The selected sections of the ovaries and testes were then photographed to give a somewhat complete overview for the process of gonadal maturation in the veined squidunder investigation.

Statistical analysis

Analysis of variance (ANOVA) is a broad group of techniques for identifying and measuring different sources of variation within the data set. It consists of a set of procedures by which a variance of the random variable is broken down by certain sources of variation of its value. With the components of variance, depending on the sources, one can conclude if there is a significant difference between the values of dependent variable for different levels of the observed factor variables. In the present study, a one-way analysis of variance is used to compare the different stages of oocyte developmental stages in the four seasons of the year. If the above-mentioned assumptions for ANOVA are not met, the Newman-Keuls Multiple Comparison Test is used for determining whether four or more independent samples originate give a clear cut differences. When this test leads to significant results, at least one sample differs from the others. A principal component analysis is a standard tool in modern data analysis. It is a simple, nonparametric method for extracting relevant information out of confusing data sets. Principal component analysis is concerned with the interpretation of the variance and covariance structure of the original set of variables through a small number of their linear combinations (**Dijana *et al.*, 2012 for review**).

The general objectives of principal component analysis are data reduction and interpretation. In order to reduce the number of variables. For more details about methodology of calibrations see (**Dijana, *et al*., 2012 for review**).For each individual, sex was determined by checking thepresence of the left arm IV hectocotylized (modified arm)typical for males (**Richard 1976**). The indices of reproductivestatus (**Joy 1989; Pierce *et al*., 2005**) were calculated for malesand females in each season. The gonadosomatic index (GSI) for both sexes and the nidamental gland–somaticindex (NSI) for the females, were calculated as follows: GSI = 100 X GW /(BW-GW) and NSI = 100 X NGW / (BW-NGW),where GW is the gonad weight, BW is the body weight, and NGW is the nidamental gland weight. Besides indices, maturity stages was assigned to the specimen collected in different seasons based on the color of accessory nidamental glands and size of the gonads according to **Lipinski and Soule (2007)**. The 3 maturity stages are immature, with small gonads and white accessory nidamental glands; maturing, with orange accessory nidamental glands; and fully mature, with large gonads and pink accessory nidamental glands.

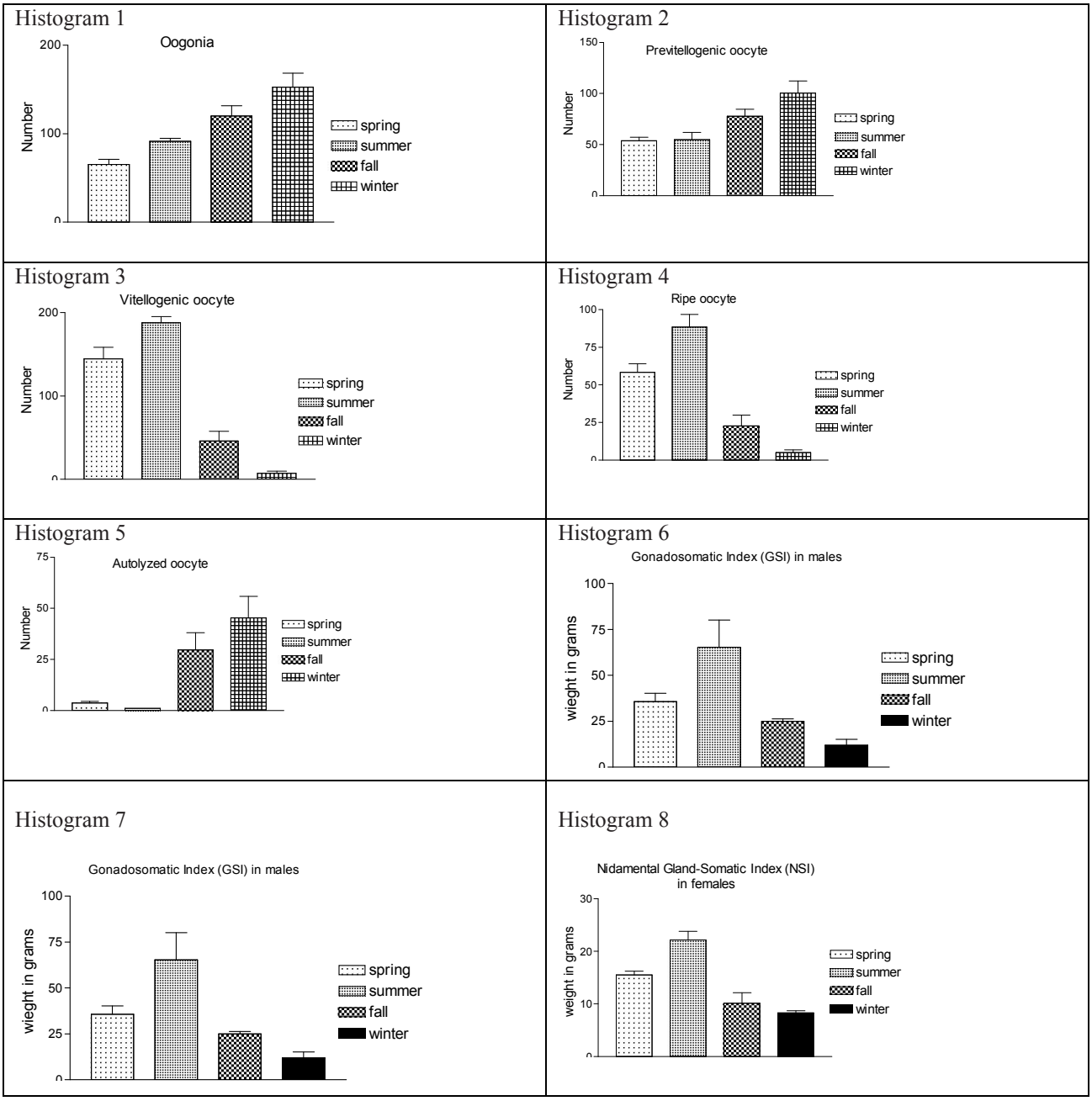
RESULTS

Female reproductive system and oogenesis

Macroscopically, the ovary of the veined squid is a translucent and lobulated mass lying in the mantle cavity towards the posterior of the visceral mass (Plate 1, Fig. 1).

Table 1. Morphological nature of the cytoplasm and the nucleus of the oocyte of the veined squid *Doryteuthis forbesii*at its different stages of development

Stages of oocyte development	Oogonia		Previtellogenic oocyte		Vitellogenic oocyte		Ripe oocyte	
StainReferenes	Cytoplasm	Nucleus	Cytoplasm	Nucleus	cytoplasm Largegranules	Nucleus	Yolk material (homog-enous)	Nucleus
Ehrlich haematoxylin and eosin Pearse 1968	red	blue	blue	deep blue	opaque blue	deep blue	blue	deepblue
Heidenhain's iron haematoxylin Pearse 1968	red	blue	red	deep blue	opaque blue	deepblue	blue	deepblue
Mallory triple stain Pearse 1968	red	deep blue	dark red	deep blue	opaque blue	deepblue	red	deepblue
Masson trichrome stain Pearse 1968	red	deep blue	dark red	deep blue	opaque blue	deepblue	red	deepblue
Weigert's haematoxylin & Van Gieson stain Pearse 1968	red	deep blue	dark red	deep blue	opaque blue	deepblue	red	deepblue



Histograms 1-5 showing oocyte developmental stages in the four seasons of the year
Histograms 6-7 showing Gonadosomatic Index (GSI) in males and females respectively in the four seasons of the year
Histograms 8 showing Nidamental Gland–Somatic Index (NSI) in females in the four seasons of the year

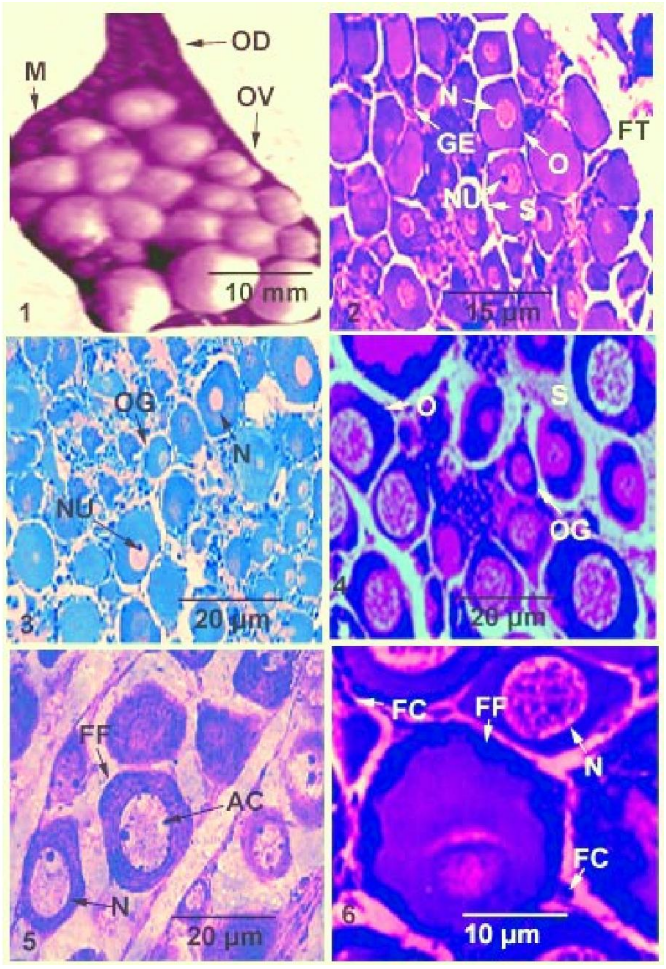


Plate 1: Fig 1. To Fig. 6.

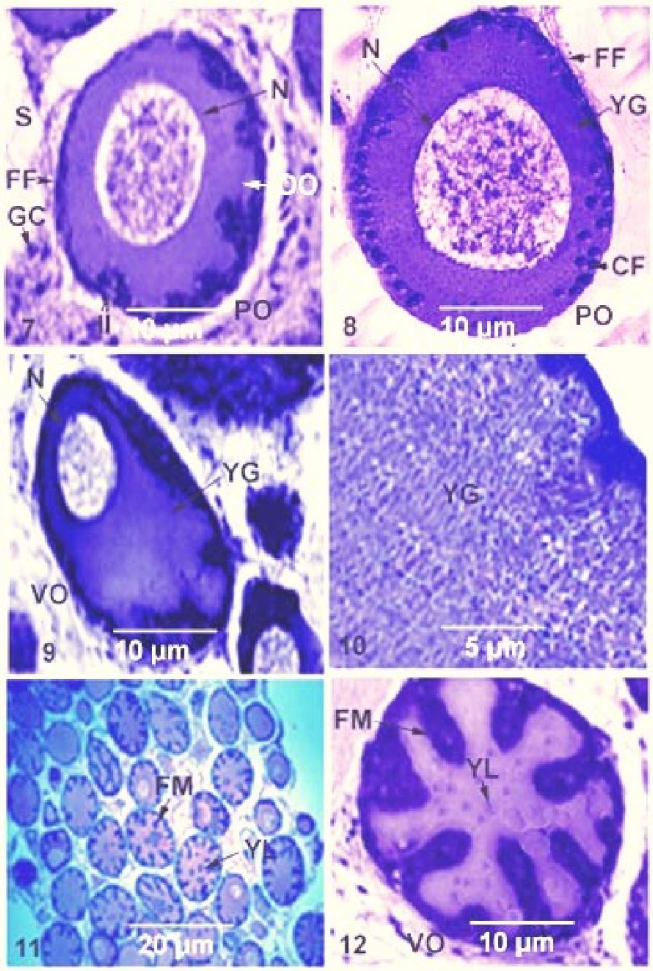


Plate 2: Fig. 7 to Fig. 12

It is associated with a pair of white nidamental glandslocated anterior to the gills and a pair of red-spotted accessory nidamental glands. Its size is variable depending on the season of the year. The oviduct exits the left side of the ovary and extends anteriorly. It swells enormously to become the large, white oviducal gland, just posterior to the left branchial heart. The oviduct extends from the oviducal gland to open into the mantle cavity via the female gonopore located anterior to the left branchial heart. There may be a cluster of bristle-like, white spermatophores attached to the dorsal mantle wall on the left side of the mantle cavity. If present, they will be medial to the left gill and anterior to the opening of the oviduct. Histologically, the ovary is surrounded by a thick fibrous tissue which emerged projections or trabeculae within the ovary, where the germ cells and developing oocytes formed in the ovarian stroma (Fig. 2, Plate 1). The distal and proximal oviducts have smooth muscle fibersparallel to each other, with elongated central core. Oviductal glands are constituted by various glandular tissues, which have a folding arrangement. These tissues have a contrasting colour, which probably could be related to the type of substance they secrete. In the ovary, different histological features were observed in the different seasons of the year. However, the maturity stages were dealt separately: 1- the ovarywas white and small hardly to be visible, the oogoniawere small rounded cells of 5 to 14 mm diameter and without visible ooplasm. These cells are still attached to the germinal epithelium (Fig. 3, Plate 1).2-The immature ovary is small in size; ova minute, opaque,embedded in the ovarian tissue. Nidamental glands small in size. Oogonia predominatedand attached to the ovarian stroma, oocyte in developing statecan also be observed (Fig. 4, Plate 1). The oogonia were spherical withlack of follicle cells. The oviducts and oviductal glands arefairly developed.3-Maturing ovary size increasedand occupied nearlyhalf of posterior bodycavity. Ovaare visible. Nidamentalglands enlarged.An oocyte at this stage measures from 20 to 60 mm, with a nucleus size/cell size ratio of 50 %to 90 %. At this stage oocytes show aggregated chromatin in the nucleus (Fig. 5, Plate 1). In the earliest stages they are similar to the oogonia, with a thin layer of cytoplasm surrounding the nucleus; this layer becomes thicker as the growth process continues.4-In initial folliculogenesis, pseudostratified follicular epithelium or secondary follicle were formed that consisted of flat cells. Some ova have polyhedral shapes. Oocytes are associated with one or several follicle cells and each contains various spherical nucleoli in the nucleus. Follicle cells have an oval shape, are smaller than the oocytes and are located on the connective tissue. At this stage oocytes range from 25 to 170 mm, with a nucleus size/cell size ratio of 26 % to 92 % (Fig. 6, Plate 1). 5-In final folliculogenesis, initial invagination process of the oocyte membrane and the cells lining it.

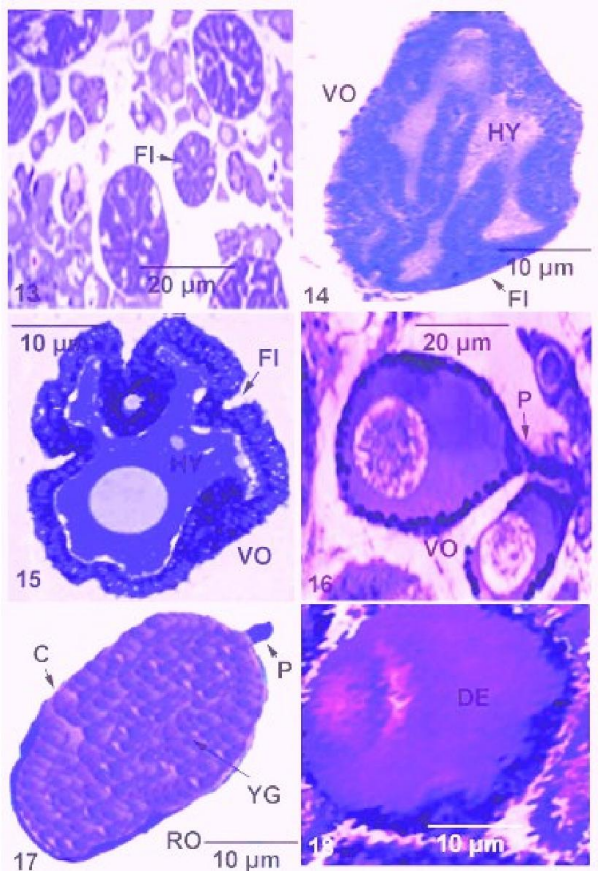


Plate 3: Fig. 13 to Fig. 18

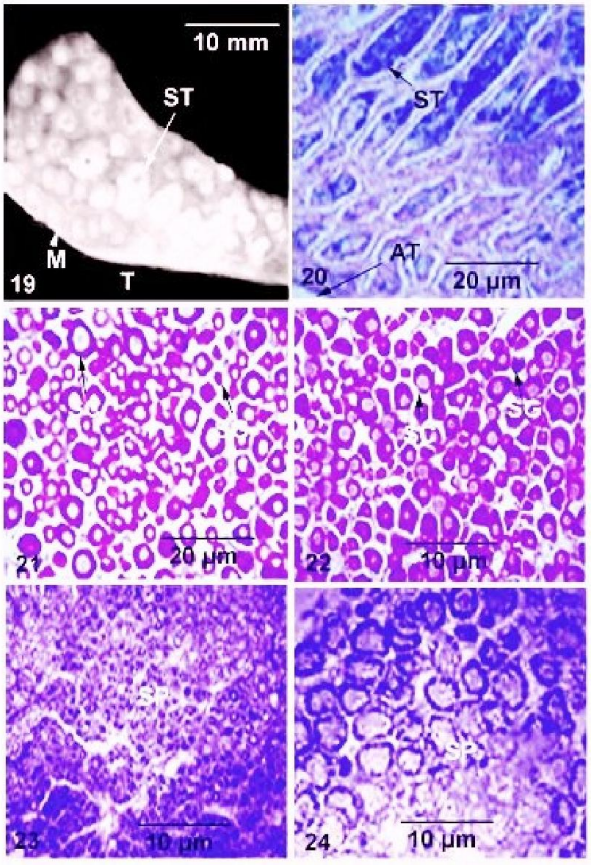


Plate 4: Fig. 19 to Fig. 24

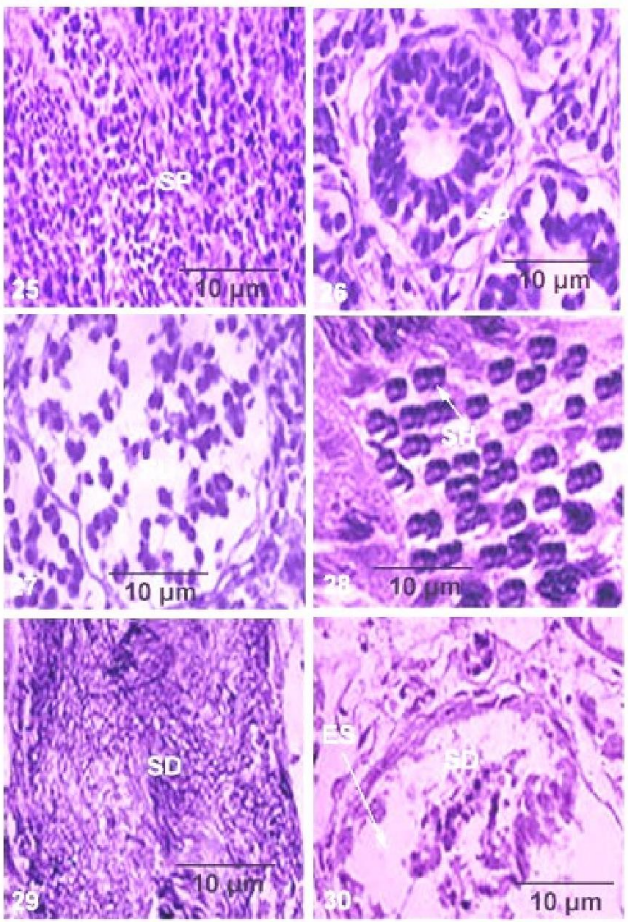


Plate 5: Fig. 25 to Fig. 30

The stroma is confined to the cortical region of the ovary and the oogonia are difficult to observe because of the massive oocyte growth. The oocyte is surrounded by a layer of flat follicle cells. This is the beginning of the previtellogenic phase. The oocyte diameter varies between 35 and 700 mm. The portion of the cell occupied by the cytoplasm is larger than that occupied by the nucleus (the nucleus size/cell size ratio ranged from 10 % to 75 %) (Fig. 7, Plate 2). The follicle cells change shape to become cuboidal in the terminal phase of this stage. In the terminal phase two layers of follicular cells are observed, the inner one consisted of cuboidal cells, the outer one of flat follicular cells. At this stage the cells have one distinct spherical nucleolus in the nucleus (Fig. 8, Plate 2). 6- In previtelogenesis, the oocytes with deep invaginations in their membrane, which is surrounded by follicular cells. Lipid inclusions can be observed and the nuclei are not easily observed.7-In early vitelogenesis, yolk granules are seen inside the eggs and their size increased considerably compared to earlier stages. Invaginations remain deep in the follicles (Figs. 9-10, Plate 2). The follicle epithelium initiates the oocyte embedding by intensive multiplication of the follicle cells, with a subsequent displacement of the nucleus to the polar zone of the cell. Oocyte size increases to a maximum diameter of 140 to 800 mm, with a nucleus size/cell size ratio of 10 % to 60 %. There is an initiation of nucleoli degeneration and the first production of yolk globules (Figs. 11-12, Plate 2). 8- In final vitelogenesis, the appearance of the yolk is homogeneous, granules are not seen. The follicle invaginations are shallow and very close to each other (Figs. 13-15, Plate 3). 9-In mature phase, most of the oocytes have been ovulated from their

ovarian follicle and the peduncle separated from the germinal epithelium (Fig. 16, Plate 3). The chorion is formed and the eggs are ready to be fertilized and spawned. Mature ovary is very prominent with plenty of translucent eggs in oviducts and occupies entireposterior mantle space. Nidamental glands was whitish cream and attained maximum size. Accessory nidamental glands coloured with a combination of yellow and orange. The follicular epithelium is active in vitellogenesis and the formation of a chorion. 10- in ripe phase, the follicular folds are being displaced towards the periphery of the oocyte by the formation of yolk. Oocyte sizes range between 150 and 3500 mm (with peduncle) (Fig. 17, Plate 3). The cytoplasm is completely filled with yolk granules, the whole being surrounded by a chorion. 11- in spawning phase, the follicular layer becomes thin and finally degenerates, leaving a mature oocyte which is ready for ovulation. The sizes of oocytes vary between 1900 and 2600 mm. 12- in spentphase, degenerating eggs were observed and the nidamentalglands appeared like shrunken sacs. Condition of animal poor and oocyte reabsorption took place (Fig. 18, Plate 3).

Male reproductive system and Spermatogenesis

Macroscopically, the test is oval shaped and in an immature state is white-yellow; when the degree of maturation increases it turns into a whitish colour (Fig. 19, Plate 4). The sperm duct is a very thin organ, is less than 2 mm in diameter and is morecoiled and compacted. When extended, its length is very similar to the total body length. The spermatophoral gland lies on the left of the visceral mass just posterior to the left branchial heart. The proximal segment of the gland is wide at this point, then the gland coils, decreases its width, instead the distal segment, is a small tube extended into the proximalends of the accessory spermatic gland. It is the storagesite for spermatophores. Histologically, the testis is wrapped in an albuginea tunica of connective tissue and made up of numerous seminiferous tubules, where spermatogenesis takes place (Fig. 20, Plate 4). In each seminiferous tubule several developmental stages may be present simultaneously. Sperm maturation issequentially from the tubular basement membrane to the centralregion, where they are discharged into the spermatic duct, which consists of a simple epithelium of ciliated cubic cells, surrounded by connective tissue (Fig. 21, Plate 4). Through this duct, sperm reached the spermatophoric gland that has a tubular shape with foldsinside. The proximal and distal region of the gland consist of one pseudostratified ciliated epithelium, with very highcolumnar cells that has a bulbous dilation in contact withthe lumen; and a pseudostratified ciliated epithelium with unicelular glands and no bulbose dilatations. Themiddle section of the gland is formed by a number ofsecretory units composed of ciliated columnar cells with basal nuclei. Thereafter, the spermatophore inmaturing state passes through the proximal portion of the accessory spermatic gland. In the testis, different histological features were observed in the different seasons of the year. Maturity stages were identified based on cell types and their proportions, abundance of connective tissue, immature spermatophores present in the glands and mature spermatophores. However, the maturity stages were dealt separately: 1-in immature phase, seminiferous tubules are small but well defined and delimited by connective tissue. Spermatogoniapredominate, although there are few spermatocytes atthe center of the tubulae (Fig. 22, Plate 4). 2-inmaturing phases, spermatogonia, spermatocytes, spermatids can be observed and there is abundant sperm in the lumen of the seminiferous tubules (Fig. 23, Plate 4). 3- All cell types are present, with a huge amount of sperm in the center of the tubulae (Fig. 24, Plate 4). 4-There are sperms in the spermatic duct and there are spermatophores in the accessory spermatic and spermatophoric gland. 5-All cell types are present, but spermis the most abundant (Figs.25-26, Plate 5). Sperm is frequently found in the spermatic duct forming spermatophores in the spermaticgl and and the accessory spermatic gland (Fig. 27, Plate 5). 6-Abundant maturespermatozoa have been expelled from the testis, stored in spermatophores which are passed to Needham'ssac (Fig. 28, Plate 5). 7- in sperm deterioration phase, the seminiferous tubules have alimp look and deteriorated. Empty spaces can be seen, indicating that spermatozoa have been expelled from the testis (Figs. 29-30, Plate 5). The present study concluded that the number of the different stages during oogenesis and spermatogenesis differed significantly according to the season of collection.

Statistical analysis

One Way Analysis of Variance (ANOVA) with $p<0.05$ and Newman-Keuls Multiple Comparison Test with $P < 0.01$and $P > 0.05$ were applied to compare the different stages of oocyte development in the four seasons of the year. For each season, three ovaries in serial histological preparations were applied. It was observed that oogonia, previtellogenic and atretic oocytes predominated the ovary during fall and winter although few number of vitellogenic and ripe oocytes were found. During summer and spring the ovary was crowded with vitellogenic and ripe oocytesal though few number of oogonia, previtellogenic and atretic oocytes were found (see Histograms 1-5 and Tables 2-8).For each individual, sex was determined by checking the presence of the left arm IV hectocotylized typical for males (**Richard 1976**). The indices of reproductive status (**Joy 1989; Pierce *et al*., 2008**) were calculated for males and females in each season. The gonadosomatic index (GSI) for both sexes and the nidamental gland–somaticindex (NSI) for the females, were calculated as follows: GSI = 100 X GW/(BW-GW) and NSI = 100 X NGW / (BW-NGW), where GW is the gonad weight, BW is the body weight, and NGW is the nidamental gland weight. Besides indices, maturity stages was assigned to the specimen collected in different seasons based on the color of accessory nidamental glands and size of the gonads according to **Lipinski and Soule (2007).** The 3 maturity stages are immature, with small gonads and white accessory nidamental glands; maturing, with orange accessory nidamental glands; and fully mature, with large gonads and pink accessory nidamental glands. It was observed that GSI had its peaks in males and females of summer and spring collections while during fall and winter it showed a lesser values especially in females (see Histogram 6-8; Tables5-9). NSI showed a peak during summer and another shorter peak during spring. It had a considerable value during fall and winter (Histogram 8; Table 4).According to the morphology of ovary, gonadosomatic index (GSI), Nidamental Gland–Somatic Index (NSI)and percentageof oocyte at different stage, it can be concluded that the veined squid under investigations reproduces throughout the year with two obvious peaks during summer and spring.

Table 2. Stages of oocyte development in the four seasons of the year

Oocyte maturation phases	animal	Spring	Summer	Fall	Winter
Oogonia	1st animal	73	98	104	174
	2nd animal	69	87	113	162
	3rd animal	54	89	143	122
Previtellogenic oocyte	1st animal	55	65	91	96
	2nd animal	47	58	74	82
	3rd animal	59	41	68	123
Vitellogenic oocyte	1st animal	164	201	54	12
	2nd animal	152	187	23	7
	3rd animal	118	175	61	3
Ripe oocyte	1st animal	68	92	21	2
	2nd animal	59	101	36	5
	3rd animal	48	72	11	8
Autolyzed oocyte	1st animal	2	1	43	65
	2nd animal	4	1	32	42
	3rd animal	5	1	14	29

Table 3. Gonadosomatic Index (GSI) in males in the four seasons of the year

NSI = 100 X NGW / (BW-NGW)	animal	Spring	Summer	Fall	Winter
Body Weight (BW)	1st animal	28	30	24	20
	2nd animal	26	32	22	19
	3rd animal	25	29	27	16
Gonad Weight (BW)	1st animal	7	9	5	3
	2nd animal	8	12	4	2
	3rd animal	5	14	6	1
Gonadosomatic Index (GSI)	1st animal	33.3	42.8	26.3	17.6
	2nd animal	44.4	60	22.2	11.7
	3rd animal	29.4	93.3	26.5	6.6

Table 4. Gonadosomatic Index (GSI) and Nidamental Gland–Somatic Index (NSI) in females in the four seasons of the year

GSI = 100 X GW /(BW-GW) and NSI = 100 X NGW / (BW-NGW)	animal	Spring	Summer	Fall	Winter
Body Weight (BW)	1st animal	24	26	19	14
	2nd animal	22	25	18	12
	3rd animal	21	26	17	13
Gonad Weight (BW)	1st animal	5	6	3	2
	2nd animal	4	8	3	1
	3rd animal	3	7	2	2
Nidamental Gland Weight (NGW)	1st animal	3	5	2	1
	2nd animal	3	4	2	1
	3rd animal	3	5	1	1
Gonadosomatic Index (GSI)	1st animal	26.3	30	18.7	16.6
	2nd animal	22.2	47	20	9
	3rd animal	16.6	36.8	13.3	18.1
Nidamental Gland–Somatic Index (NSI)	1st animal	14.2	23.8	11.7	7.6
	2nd animal	15.7	19	12.5	9
	3rd animal	16.6	23.8	6.2	8.3

Tables 5 -9. One-way analysis of variance and Newman-Keuls Multiple Comparison Test of oocyte developmentalstages in the four seasons of the year

Table Analyzed Table 5			
Data Table-Oogonia			
One-way analysis of variance			
P value	0,0019		
P value summary	**		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	13,1		
R squared	0,8309		
ANOVA Table	SS	df	MS
Treatment (between columns)	12710	3	4236
Residual (within columns)	2586	8	323,3
Total	15290	11	
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
spring vs winter	-87,33	8,413	P < 0.01
spring vs fall	-54,67	5,266	P < 0.05
spring vs summer	-26	2,505	P > 0.05
summer vs winter	-61,33	5,909	P < 0.01
summer vs fall	-28,67	2,762	P > 0.05
fall vs winter	-32,67	3,147	P > 0.05

Table Analyzed Table 6			
Data Table-Previtellogenic oocyte			
One-way analysis of variance			
P value	0,0097		
P value summary	**		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	7,677		
R squared	0,7422		
ANOVA Table	SS	df	MS
Treatment (between columns)	4412	3	1471
Residual (within columns)	1533	8	191,6
Total	5945	11	
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
spring vs winter	-46,67	5,84	P < 0.05
spring vs fall	-24	3,003	P > 0.05
spring vs summer	-1	---	P > 0.05
summer vs winter	-45,67	5,715	P < 0.01
summer vs fall	-23	---	P > 0.05
fall vs winter	-22,67	2,836	P > 0.05

Table Analyzed Table 7			
Data Table-Vitellogenic oocyte			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	72,37		
R squared	0,9645		
ANOVA Table	SS	df	MS
Treatment (between columns)	63400	3	21130
Residual (within columns)	2336	8	292
Total	65730	11	
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
winter vs summer	-180,3	18,28	P < 0.001
winter vs spring	-137,3	13,92	P < 0.001
winter vs fall	-38,67	3,919	P < 0.05
fall vs summer	-141,7	14,36	P < 0.001
fall vs spring	-98,67	10	P < 0.001
spring vs summer	-43	4,359	P < 0.05

Table Analyzed Table 8			
Data Table-Ripe oocyte			
One-way analysis of variance			
P value	P<0.0001		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	33,99		
R squared	0,9272		
ANOVA Table	SS	df	MS
Treatment (between columns)	12440	3	4146
Residual (within columns)	976	8	122
Total	13410	11	
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
winter vs summer	-83,33	13,07	P < 0.001
winter vs spring	-53,33	8,363	P < 0.001
winter vs fall	-17,67	2,77	P > 0.05
fall vs summer	-65,67	10,3	P < 0.001
fall vs spring	-35,67	5,593	P < 0.01
spring vs summer	-30	4,704	P < 0.05

Table Analyzed Table 9			
Data Table-Autolyzed oocyte			
One-way analysis of variance			
P value	0,0045		
P value summary	**		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	9,931		
R squared	0,7883		
ANOVA Table	SS	df	MS
Treatment (between columns)	4089	3	1363
Residual (within columns)	1098	8	137,3
Total	5187	11	
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
summer vs winter	-44,33	6,554	P < 0.01
summer vs fall	-28,67	4,238	P < 0.05
summer vs spring	-2,667	0,3943	P > 0.05
spring vs winter	-41,67	6,16	P < 0.01
spring vs fall	-26	3,844	P < 0.05
fall vs winter	-15,67	2,316	P > 0.05

Table 10. Gonadosomatic Index (GSI) in males in the four seasons of the year

Table Analyzed Table 10			
Data Table-Gonadosomatic Index (GSI) in males			
One-way analysis of variance			
P value	0,008		
P value summary	**		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	8,213		
R squared	0,7549		
ANOVA Table	SS	df	MS
Treatment (between columns)	4657	3	1552
Residual (within columns)	1512	8	189
Total	6168	11	
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
winter vs summer	-53,4	6,728	P < 0.01
winter vs spring	-23,73	2,99	P > 0.05
winter vs fall	-13,03	---	P > 0.05
fall vs summer	-40,37	5,086	P < 0.05
fall vs spring	-10,7	---	P > 0.05
spring vs summer	-29,67	3,738	P < 0.05

Table 11. Gonadosomatic Index (GSI) in females in the four seasons of the year

Table Analyzed Table 11			
Data Table-Gonadosomatic Index (GSI) in females			
One-way analysis of variance			
P value	0,0046		
P value summary	**		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	9,835		
R squared	0,7867		
ANOVA Table	SS	df	MS
Treatment (between columns)	983,6	3	327,9
Residual (within columns)	266,7	8	33,34
Total	1250	11	
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
winter vs summer	-23,37	7,01	P < 0.01
winter vs spring	-7,133	2,14	P > 0.05
winter vs fall	-2,767	---	P > 0.05
fall vs summer	-20,6	6,18	P < 0.01
fall vs spring	-4,367	---	P > 0.05
spring vs summer	-16,23	4,87	P < 0.01

Table 12. Nidamental Gland-Somatic Index (NSI) in females in the four seasons of the year

Table Analyzed Table 12			
Data Table- Nidamental Gland-Somatic Index (NSI) in females			
One-way analysis of variance			
P value	0,0003		
P value summary	***		
Are means signif. different? (P < 0.05)	Yes		
Number of groups	4		
F	21,85		
R squared	0,8912		
ANOVA Table	SS	df	MS
Treatment (between columns)	350,8	3	116,9
Residual (within columns)	42,81	8	5,351
Total	393,6	11	
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	P value
winter vs summer	-13,9	10,41	P < 0.001
winter vs spring	-7,2	5,391	P < 0.05
winter vs fall	-1,833	1,373	P > 0.05
fall vs summer	-12,07	9,035	P < 0.001
fall vs spring	-5,367	4,018	P < 0.05
spring vs summer	-6,7	5,017	P < 0.01

DISCUSSION

Cephalopods are marine invertebrates and are often called the chameleon of the sea for their incredible ability to camoflage themselves. They are considered to be the most intelligent of the marine organisms. This study revealed that the ovary is a translucent, lobulated mass lying in the posterior end of the visceral mass of the gonocoel. Its size is variable in the different seasons of the year. The oviduct exits the left side of the ovary and extends anteriorly. It swells enormously to become the large, white oviducal gland, just posterior to the left branchial heart. The oviduct extends from the oviducal gland to open into the mantle cavity via the female gonopore

located anterior to the left branchial heart. There may be a cluster of bristlelike, white spermatophores attached to the dorsal mantle wall on the left side of the mantle cavity. During oogenesis the oocyte becomes progressively more elongated, with a subsequent narrow elongation of the apical zone forming the oocyte peduncle. Finally the oocytes turn smooth (chorion becomes the outer surface when the follicular cells disappear) and free, finally reaching the oviducts. The process of oocyte maturation in *Octopus vulgaris* is similar to that in other cephalopods, as described in previous works. It is shown that the stage number and measurement of oocytes observed by several authors is nearly similar during oogenesis, for different species of cephalopods. The number of discrete stages considered by each author to describe the maturation process appears to be dependent on the different criteria used, as was noted by **Juanicó (1983).** The present study concluded that changes in the ovary during sexual maturation permitted the determination of different histological stages for oogenesis in 12 phases. In previous studies on cephalopods, **Roa (1988)** identified 6 stages in *Loligo duvaucelii*, **Hixon, (1983)**and **Butler, *et al.* (1999**) identified 6 stages in ***Loligo opalescens*, Ines, *et al.* (2002**) identified 6 stages in *Octopus vulgaris*,Olgaç and **Mehmet (2007**) identified 6 stages in *Sepia officinalis* and **Lopez-Peraza*et al.* (2013)** identified 8 stages in *Octopus rubescens*. The reproductive systems of male cephalopods have long been studied because of their morphological and functional intricacies. The reproductive system consists of a single testis and accessory organs. The changes in the testis during sexual maturation permitted the determination of different histological stages for spermatogenesis in 7 phases. In previous studies on cephalopods, most authors identified 5-6 stages in *Loligo opalescens, Loligo duvaucelii, Octopus vulgaris*and *Octopus rubescens* (**Hixon, 1983; Roa, 1988; Butler*et al.*, 1999; Ines, *et al.*, 2002; Olgaç and Mehmet, 2007; Lopez-Peraza*et al.*, 2013**). Sperm produced in the testis pass through accessory organs and are packaged into spermatophores, each of which contains millions of densely packed spermatozoa.

During copulation the male uses a specialized, hectocotolized arm to pass spermatophores into the mantle of the female, where they ejaculate and release the male gametes. The male reproductive tract, spermatophores and spermatophoric reaction in the giant octopus of the North Pacific, *Octopus dofleini* have been well documented (**Mann *et al.*, 1970**). Past studies of cephalopod spermatogenesis have been limited to the light microscope level. **Franzen (1955, 1967)** reviewed spermiogenic events and sperm structure in several cephalopods and discussed the significance of spermiogenesis as a systematic tool within the class. **Austin *et al.* (1964)** gave a brief description of sperm of the squid *Loligo pealei* using phase-contrast microscopy. The present study showed that spermatogonia and the primary spermatocytes are difficult to distinguish from each other, both being relatively large cells with a large, distinct nucleus. The first meiotic division occurs, and the primary spermatocytes become secondary spermatocytes. A second meiotic division occurs immediately afterwards, so the secondary spermatocytes become spermatids. The transformation of the spermatids to spermatozoa occurs by a process of differentiation known as spermiogenesis. Morphologically, this differentiation involves the transformation of the cell from a round to an elongate shape. The spermatozoa are grouped around the lumen, towards the centre. As spermatozoa mature and are released from the testis they pass directly to the spermaduct which is surrounded by a complex of accessory glands. The first glands inactivate the spermatozoa and others cover the sperm mass with different secretions to form the spermatophore. Spermatophores usually accumulate in the Needham sac.

A comparison of oogenesis and spermatogenesis illustrates that maturation occurs earlier in males than in females. The males became mature at a smaller size and probably a younger age than the females. The testis attains a relatively large size 2–3 months before the ovary begins to enlarge, while in the females, the ovary, the oviduct glands and the oviducts enlarge greatly 3–4 months before spawning (**Mangold, 1963a**). In *O.vulgaris* mating frequently occurs when the females are immature. The spermatophores can be transferred to the immature females where spermatozoa are stored in the spermatheca of the oviducal gland. In Illex argentinus the earlier start of maturation in males also occurs but in this case both sexes conclude functional maturity simultaneously. This simultaneous maturation appears to correspond to the absence of seminal receptacles in Illex, which would otherwise permit precocious copulation and storage of sperm in immature females, until the initiation of spawning (**Arkhipkin and Laptikhovsky, 1994**). **Coelho *et al.* (1997)** reports that in *Illex illecebrosus*, for the oocyte and spermatozoa to be mature at the same time, the mature spermatozoa must be stored longer in the males than the mature oocytes in the females, and that this might permit more than one period of spermatophore transfer. The development of tubules and spermatids seems to be much more rapid in O. vulgaris than in *Loligo vulgaris* reynaudii as described by Sauer and **Lipinski (1990**) and in *Illex illecebrosus* as described by **Coelho *et al.* (1997).** The same appears to be true of the degenerationprocess. Sauer and **Lipinski (1990**) did not observe any degenerative process in males of *Loligo vulgaris reynaudii*.

Loligo forbesii is commonly assumed to have an annual life cycle, with pulses of recruitment in April and August-November (**Lum-Kong *et al.*, 1992; Collins *et al.*, 1995, 1997; Belcari, 1999b; Pierce *et al.*, 2008**), although some individuals may reach an age of ca. 18 months (**Rocha and Guerra, 1999**). The present study revealed that reproduction period of *Doryteuthis forbesii*inhabiting the Arabian Gulf covers the whole year with two peaks observed in GSI values. First of these was in spring and the second was in summer. Mature females were more frequent in these months. Similar results of the present study concluded that the oogenesis of pink cuttlefish ovaries is asynchronous **(Rocha *et al.*, 2001**) and spawning likely is intermittent, as reported by **Laptikhovsky *et al.* (2003)** for S. *officinalis*. Because both mature females and recruitment of MLs of 20–30 mm could be found in the Aegean Sea year round, it could be conclude that spawning happens in any season. Dynamics of GSI values indicate 2 spawning peaks (spring and fall). Similar results were reported from the western Mediterranean Sea by **Mangold-Wirz (1963b)** and **Jereb and Roper (2005)**. Mature male *S. orbignyana* specimens from the Aegean Sea generally have many more spermatophores than their counterparts from the western Mediterranean (**Mangold-Wirz, 1963a**). It has been supposed

that the higher number of spermatophores is necessary to fertilize due to the higher fecundity of females. The ripe egg size found in this study (6.7–8.3 mm) was similar or slightly smaller than that in the western Mediterranean (7.0–9.0 mm) (**Mangold-Wirz, 1963a&b; Jereb and Roper, 2005**). *Loligo forbesii* is semelparous, displaying "intermittent, terminal spawning", in which the females lay eggs in batches and die shortly after spawning (**Rocha *et al*., 2001**). Many studies describe an extended spawning season, although it is unclear how long an individual continues to spawn (**Guerra and Rocha**, **1994; Moreno *et al*., 1994; Collins *et al*., 1995**). Female *L. forbesii* produce only a few thousand (up to 23 000) eggs in their lifetime, with larger females producing more eggs. Clusters of eggs are normally attached to substrata that include algae, shells, rock crevices, nets, ropes, creels, and other fishing gear that remains submerged and undisturbed. There are few reports of eggs from offshore waters, but this may be because the squid spawn over rocky substratum, making spawning areas largely inaccessible to trawling (**Holme, 1974; Lordan and Casey, 1999**). Cephalopods seem to respond to environmental variation both actively (e.g. by migrating to areas with more favourable environmental conditions for feeding or spawning) and passively (e.g. by variations in growth and survival according to conditions experienced, and by passive migration with prevailing currents). Environmental effects on early life stages can affect post-recruit, life-history characteristics (growth and maturation rates) as well as adult distribution and abundance.In *Loilgo duvallceli*, the spawnmg activity was recorded throughout the year in males and females. All the stages of maturity could be observed in both the sexes. However the abundance of mature individuals showed a peak during spring. A secondary peak during summer could be observed with spawning activity at its peak. This may be due to the fact that in the cephalopods, light acts as a limiting factor in the maturation of the gonad. **Wells and Wells (1969)** were able to show that the sexual maturation in *octopus* is under the hormonal control produced by the optic gland, seen as small subpedunculate lobes at the back of the supra-opthalmic veins **(Frosch, 1974)**.

The histological studies have revealed that the squid oocytes undergo atresia. Atresia involves degeneration and resorption of oocytes, the processes that are not only important in the reproductive cycle of squid but also in declining the reproductive capability of the population on the spawning grounds. In terms of estimating reproductive capability in squid, atresia certainly lowers the number of maturing eggs in the ovaries, so decreasing effective fecundity.

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