Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Gonadal proliferation and reproductive cycle of the exotic sea squirt *Cnemidocarpa amphora* (Kott, 1992) (Pleurogona, Styelidae) sampled for the first time from the northern coast of Arabian Gulf in Saudi Arabia

Proliferação gonadal e ciclo reprodutivo da ascídia exótica *Cnemidocarpa amphora* (Kott, 1992) (Pleurogona, Styelidae) amostrada pela primeira vez na costa norte do Golfo Arábico, na Arábia Saudita

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Abstract

Specimens of *Cnemidocarpa amphora* were collected monthly from the Arabian Gulf from September 2017 to August 2018. Parts of their gonads were prepared for histological testing. The gonads' diameters varied by month. Each gonad contained many ovarian follicles with different morphologies and was surrounded by several testicular follicles. The ovarian and testicular follicles were separate, although the latter were always present near the former. Repeated measures ANOVA tests were conducted to investigate monthly changes in oocyte stages. In squirts measuring 12–13 cm in length, the gonads measured 30–50 mm from July to August; 20–25 mm from September to October; 15–20 mm from November to February; and 25–40 mm from March to June. Oogonia budded from the germinal epithelium with diameters of 20–30 µm. Previtellogenic oocytes measuring 70–120 µm were characterized by the deposition of small granules of protein around the nucleus, a continuous layer of follicular cuboidal epithelium, and scattered vacuoles in the ooplasm. The measurement of gonads and oocyte diameters were performed by image analysis (Image scope 2.3, Image Line, Inc.) and stage micrometer. The vitellogenic oocytes measured 130–220 µm and the follicular epithelium consisted of flattened and cuboidal layers. Beneath the vitelline membrane, scattered test cells appeared in the ooplasm and different granules of protein and MPS were deposited in the ooplasm. In the later phase, lipid droplets began to appear in the ooplasm. Yolk bodies formed after the impregnation of various granules together and the oocyte was ready to be shed. Before spawning, a yolk membrane appeared above the ooplasm. Post-vitellogenic oocytes, in which the homogeneity of ooplasm was restored, underwent gradual lysis and entered the atretic phase. Different stages of sperm development were present year-round in different follicles of the same squirt; hence, the testes were always mature.

Keywords: sea squirt, testicular follicle, ovarian follicles, gonad, repeated measures, ANOVA, oocyte, vitellogenesis.

Resumo

Amostras de *Cnemidocarpa amphora* foram coletadas mensalmente no Golfo Pérsico de setembro de 2017 a agosto de 2018. Partes de suas gônadas foram preparadas para testes histológicos. Os diâmetros das gônadas variaram de mês para mês. Cada gônada continha muitos ácinos ovarianos com diferentes morfologias e era circundada por vários folículos testiculares. Os folículos ovarianos e testiculares estavam separados, embora os últimos estivessem sempre presentes próximos aos primeiros. Testes ANOVA de medidas repetidas foram conduzidos para investigar mudanças mensais nos estágios oocitários. Em esguichos medindo de 12 a 13 cm de comprimento, as gônadas mediam de 30 a 50 mm de julho a agosto; 20 a 25 mm de setembro a outubro; 15 a 20 mm de novembro a fevereiro; e 25 a 40 mm de março a junho. Oogônias brotaram do epitélio germinativo com diâmetros de 20 a 30 µm. Oócitos pré-vitelogênicos medindo 70 a 120 µm foram caracterizados pela deposição de pequenos grânulos de proteína ao redor do núcleo, uma camada contínua de epitélio folicular cúbico e vacúolos dispersos no ooplasma. A mensuração dos diâmetros das gônadas e dos ovócitos foi realizada através de análise de imagem (*Imagescope 2.3*, *Image Line, Inc.)* e micrômetro de palco. Os oócitos vitelogênicos mediam 130 a 220 µm e o epitélio folicular consistia em camadas achatadas e cúbicas. Abaixo da membrana vitelina, células teste espalhadas apareceram no ooplasma e diferentes grânulos de proteína e MPS foram depositados no ooplasma. Na fase posterior, gotículas lipídicas começaram a aparecer no ooplasma. Corpos de gema formados após a impregnação de vários grânulos

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Received: April 4, 2023 - Accepted: August 24, 2023

juntos e o oócito estava pronto para ser derramado. Antes da desova, uma membrana de vitelo apareceu acima do ooplasma. Oócitos pós-vitelogênicos, nos quais a homogeneidade do ooplasma foi restaurada, sofreram lise gradual e entraram na fase atrésica. Diferentes estágios de desenvolvimento do esperma estiveram presentes o ano todo em diferentes folículos do mesmo esguicho; portanto, os testículos estavam sempre maduros.

Palavras-chave: ascídia, folículo testicular, folículo ovarianos, gônadas, medidas repetidas, ANOVA, oócito, vitelogênese.

1. Introduction

Members of Ascidiacea are termed either ascidians or sea squirts and are representatives of the subphylum Tunicata, also called Urochordata. Sea squirts attach to substrata and are sessile, vase-shaped marine organisms in the adult stage. They usually reside on ship hulls, pilings, large shells, rocks, and crabs. Some species are solitary while others live in colonies. Sea squirts are exclusively marine and are cosmopolitan in the world's seas and oceans (Swalla, 2001; Smith & Callow, 2006; Satpathy et al., 2010). They form benthic communities and constitute a major portion of the fouling fauna in many areas, although sea squirts survive poorly due to their short life span. They are either indigenous (native to definite marine ecosystem) or invasive (alien, exotic, or non-native species) (Bax et al., 2001; Connell, 2001; NIWA, 2006a, b). Non-indigenous squirts spread widely throughout temperate locations via sailing enterprises, primarily as hitchhikers on the frameworks of ships (Lambert & Lambert, 1998, Monniot & Monniot, 2001, Ayers & Waters, 2005; Davis & Davis, 2007). Anthropogenic transport has made sea squirts among the most common fouling organisms in harbors (Lambert & Lambert, 1998, 2003; Lambert, 2007). Sea squirts are a major community in the benthic marine ecosystem and contribute significantly to biodiversity through their distribution on several natural and artificial substrata across the world (Shenkar and Swalla, 2011). The animal biomass associated with sea squirts consists mainly of small invertebrates and larvae which are attracted to higher trophic levels, many of which are of commercial interest, such as crustacean and mollusca larvae (Evans et al., 2017). The epiphytic fauna is numerous and increase with depth. Many secondary metabolites have been isolated from sea squirts and applied in pharmaceutical industries to treat human diseases like anticancer, anti-inflammatory and anti-bacterial among others (Ding et al., 2019). There are also micro-fauna of ciliates, flagellates, and copepods, among others, that are important in the trophic cycle and mechanical protection. The creeping fauna that consumes sea squirts include brachyuran decapods, starfish, opisthobranchs, isopods, etc. (Hiebert et al., 2019). There are also characteristic fish fauna with different behaviors throughout the day, which include permanent residents, temporary visitors, and occasional migrants (Lveda et al., 2003). Many authors sought to investigate the origin and the role of accessory cells (the test cells and inner follicle cells) and changes in ooplasmic organelles accompanying oocyte vitellogenesis and growth in sea squirts (Kessel, 1966; Cotelle et al.,1991; Bishop, 1996; Choi et al., 2004; Mcdougall et al., 2011). The preliminary stage of oogenesis in *Ciona savignyi* has been studied by TEM (Berrill, 1975; Reverberi, 1978; Kessel and Kemp, 1983; Sugino et al., 1987; Sugino et al., 1987; Sugino et al.,, 1990). They concluded that the ovarian epithelium is composed of three distinct areas: the cell aggregate, follicular, and terminal sites. Oogonia budding starts in the cell aggregate site and previtellogenic stages move to the follicular site, where egg follicular epithelia and accessory cells form. Kawamura et al. (2011) concluded that dark cells and clear cells are present in the cell aggregate site. He proposed that the dark cells are primordial of the test cells and inner follicle cells, but then postulated that dark cells may be oogonia (NIWA, 2006c).

Cnemidocarpa amphora (Kott, 1992) is a solitary sea squirt that inhabits shallow waters in tropical seas around the world. Seasonal patterns of primary production and temperature influence the reproductive traits and growth rates of marine invertebrates (Arnaud, 1977; Dayton, 1990; Pearse and Bosch, 1991; Stanwell-Smith & Peck, 1998; Bates, 2005). The reproductive seasonality could be initiated by temperature changes in Antarctic ecosystems (Pearse, 1965; Pearse & Bosch,1991). The reproduction of sea squirts in temperate, tropical, and polar seas is influenced by seasonality (Millar, 1982; Pineda et al., 2013). Solitary species are sac-like with a huge branchial chamber. The gonads, the gut, and the heart lie alongside the branchial chamber (Kott, 1985; Monniot et al., 1991). Colony sexuality and the maturation of single zooids of the same blastogenic generation in *Botryllus schlosseri* have been studied (Sabbadin and Zaniolo, 1979). These authors concluded that, in newly set colonies, the gonads are not differentiated. Several generations have germ cells concerned with sexuality and finally, mature sperm and oocytes appear. Sea squirts are hermaphrodites, producing both eggs and sperm that are either free-spawned or brooded (Kott, 1985; Phillippi et al., 2004; Liyun Zeng et al., 2006). Most species are self-sterile, but some are self-fertile. Colonial sea squirts can reproduce sexually or asexually by budding. The colonial larvae are brooded and tend to be much larger and more advanced than those of the solitary species (Lambert and Lambert, 2003, Sahade et al., 2004; New Zealand, 2006). Gamete shedding in sea squirts varies by the geographical location of the reproductive approaches (Berrill, 1975; Millar, 1982), and spawning incidents are species-specific (Freeter, 1984). *Didemnum* sp. is always fertile on the western and northeast coasts of North America, while spawning in *Halocynthia papillosa, Microcosmus sabatieri,* and *Halocynthia roretzi* in the area northwest of Jeju Island, Korea is limited to 2–3-month intervals. *Ciona intestinalis*, *Polycarpa cryptocarpa kroboja*, *Styela plicata,* and *Styela clava* spawn over 5–8 months in the Mediterranean Sea (Lee, 1976; Yang and Lee, 1978; Chen and Dai, 1998; Michael et al., 2008; Saad, 2008). The sea squirt *C. amphora* is a stolidobranch and is considered an invasive species in the Arabian Gulf. This study is the first description of this species in the Arabian Gulf of Saudi Arabia. The adult squirts measured 1–3 cm in length and 0,5–0,7 cm in width. The oral and atrial siphons are

quadrilobed. Usually, four folds were present on each side of the pharyngeal sac. Pharyngeal stigmata were straight and longitudinal, parallel to the endostyle. This species has a characteristic shape with a narrow posterior end. It has a thick, brown tunic with irregular shallow grooves. The oral orifice occurred terminally and the atrial orifice occurred at roughly the midline of the dorsal aspect. This species exhibits prominent features of the genus *Cnemidocarpa*, with tubular, large gonads that were enclosed in a sheathing membrane with the male follicles inside the ovarian sac (Kott, 1985). Gonads in this species are variable among squirts there were 1–3 gonads on the left side of the atrial cavty, although they were 4-9 on the right side; there were branching male follicles in the mantle around the outside of the long ovarian lobules that converge on the atrial orifice. The external appearance and internal anatomy corresponded well with Kott's descriptions (Kott, 1985, 1992, 2001). The gonads were completely embedded in the mantle wall inside the atrial cavity and enclosed with the pharyngeal sac. There were 4–9 gonads on the right side and up to ten on the left side. Each gonad extended upward as a short duct and terminated in the atrial cavity near the atrial orifice. Sea squirts have short lifespans of a few months; they rapidly grow and reach sexual maturity in a few weeks (Monniot and Monniot, 1997; Castilla et al., 2004, Bourque et al., 2007; Afkhami et al., 2012).

2. Materials and Methods

2.1. Specimen collection and preservation

This study was conducted from September 2017 to August 2018 in the intertidal zone of the Arabian Gulf, Saudi Arabia. The Arabian Gulf is a shallow semi-enclosed subtropical sea surrounded by a large, arid land mass, having a water surface area of 239.00 km^2 which extends for nearly 1000 km from Shatt Al-Arab River in the northwest to Strait of Hormuz in the southeast and mean depth of about 35 m, with a maximum depth of 100 m at Strait of Hormuz (Figure 1). The invasive sea squirt *C. amphora* is brought as a hitchhiker to the Arabian Gulf on the hulls of vessels that transport goods, which often roam in the Indian Ocean. Sea squirts form benthic communities and constitute a major portion of the fouling fauna in many areas. Non- indigenous squirts are believed to have been spread widely throughout all temperate regions by shipping activities (Ayers and Waters, 2005; Davis and Davis, 2007). As a result of anthropogenic transport, sea squirts became one of the most common representatives found in fouling communities within harbors (Lambert and Lambert 1998, 2003; Lambert, 2007). The Arabian Gulf is influenced by changing monsoon winds which cause higher productivity during the monsoon periods and less productivity during the intermonsoon phases, especially during the spring intermonsoon in the western part of the basin (Schmidt et al., 2020). This change in phytoplankton abundance is also reflected in zooplankton biomass and abundance. Collections were made monthly in the same location of the Arabian Gulf during the study period by detaching sea squirts from large ocean-going vessels. Hand tools were used to dislodge the sea squirts from the ships. Snorkeling was also employed at 1–2 meters deep. The number of squirts collected during the study time was as follows: September to October n =16; November to February n =11; March to June n = 13 and July to August n =18. *C. amphora* (Kott, 1992) was found both solitary and in aggregations. This study found many small invertebrates and algae coexisted with the sea squirts. In the field squirts were active in taking in and out water from their siphons and the color of their tunic layer was light or dark brown. The collected specimens were narcotized with methanol

Figure 1. The northern coast of Arabian Gulf in Saudi Arabia. The red pen indicates the study area.

crystals, mint oil and have a waxy consistency derived from the Mint leaf through the process of distillation. This Mint Essential Oil is then frozen at a temperature of -22 °C., the tunic was opened from dorsal aspect and completely removed. Parts of the mantle containing gonads were isolated and preserved in Bouin's solution. Ethical clearance for this study was obtained from Imam Abdulrahman bin Faisal University's ethics committee.

2.2. Histology and histochemical staining

C. amphora was identified using taxonomic keys (Van Name, 1945; Berrill, 1975; Kott, 2001; Sahade et al., 2004; Inglis, et al., 2008). Pieces of the gonads were dehydrated via an ascending series of ethyl alcohol (used to dissolve other chemical substances and mixes readily with water and many organic liquids), followed by another dehydration series of tertiary butyl alcohol (as a solvent, ethanol denaturant and resistant to oxidation because the carbon atom that carries the OH group does not have a hydrogen atom attached but is instead bonded to other carbon atoms), then tertiary butanol and paraffin oil (1:1), and, finally, pure paraffin oil. All samples were then washed carefully in paraffin wax with a melting point of 54–58 °C and blocked in fresh paraplast. Sections of 5–8 µ were obtained with a microtome (RM 2235, Leica Biosystems® USA). Ehrlich hematoxylin and 0.5% eosin were used as a basic routine stain. Triple stains were used to permit differentiation of the components inside the ovary: Heidenhain's iron hematoxylin, Masson trichrome stain, Mallory triple stain, Hemalum & eosin, Alcian blue & eosin, Safranin & light green, modified Azan stain and Weigert's hematoxylin & Van Gieson stain (Table 1) (Pantin, 1948; Pearse, 1968). Histochemical stains were applied to examine the process of vitellogenesis (Table 2). To investigate monthly changes in oocyte development, roughly 1,000 oocytes (sectioned through the nucleus) per month were measured via image analysis (Image scope 2.3, Image Line, Inc.) and stage micrometer. The developmental stages of the ovaries were grouped according to previously established guidelines (Lambert, 2007) in successive stages. In the ovary, the following stages of oocyte development were recorded: oogonia, previtellogenic oocytes, vitellogenic oocytes, and atretic oocytes. To examine the different preparations investigated, a compound microscope (B. L. Scientific & Instruments®, USA) coupled with a digital camera (Leica Camera Wetzlar®,Germany) was used.

2.3. Analysis of oocyte development

Five representative ovarian follicles were chosen from the histological preparation from three squirts, and the different stages of oocyte development (oogonia, previtellogenic oocytes, vitellogenic oocytes, and atretic oocytes) were counted in each month of the year. The means of the different stages of oocyte development were calculated. All data were subjected to repeated measures ANOVA tests to compare the different stages of oocyte development during maturity. The number of oocyte stages varied by individual, population, and season; thus, the analysis revealed these interaction effects. In each season the number of the oocyte developmental stages was calculated in three squirts and the mean was estimated and subjected to statistical analysis. The repeated measures ANOVA showed means that were significantly different at $p < 0.05$ and the Tukey's multiple comparison test ($p > 0.05$) was applied to comment on the oocyte developmental stages.

3. Results

3.1. Anatomical features of C. amphora

This study aims to provide the first morphological and anatomical description of the species C. amphora living in the intertidal zone of the Arabian Gulf, Saudi Arabia, as well as to investigate the monthly changes in its gametogenesis, ovarian development, oocyte maturity, and spermatogenesis to distinguish the reproductive cycle", or similar. The gonads of *C. amphora* showed variable diameters in different months. A noteworthy observation was found during dissection. The gonads on the right side of the mantle cavity were always narrower in diameter than those of the left side during the year irrespective of fertility and fecundity. The maximal diameter was observed during July–August at 30–50 mm (right side - left side respectively) in squirts measuring 12–13 cm in length. From September–October, the gonads were 20–25 mm (right side - left side respectively); from November–February, 15–20 mm (right side- left side respectively); and from March–June, 25–40 mm (right side- left side respectively). Each gonad appeared macroscopically as numerous polygonal units, referred to as ovarian follicles according to Tucker (1942).

3.2. Development of the gonads

Each gonad appeared, in the histological sections, as many ovarian follicles with different morphologies surrounded by several testicular follicles (Figure 2a). Noteworthy observations were made while testing various histological preparations of the gonads. The testicular follicles had their own germinal epithelium. The ovarian and testicular follicles were separate entities, although the latter always occurred near the former. A convoluted germinal epithelium connected all of the follicles of a single ovary. This germinal epithelium enclosed a cavity in the direction of the mantle where the fully formed oocytes dropped from the ovarian follicle and entered the oviduct (Figure 2b). The different stages of oocyte development were observed year-round and described. Oogonia with homogenous ooplasm and large nuclei budded from the germinal epithelium with diameters of 20–30 µm. Oogonia enlarged gradually in diameter (30–60 µm), but the nucleus size remained constant and the ooplasm, homogenous (Figure 2c). Numerous mesenchyme cells migrated from the ovarian stroma and surrounded the oogonia that had already detached from the germinal epithelium, forming a discontinuous layer (Figure 2d). Testicular follicles surrounded the ovarian follicles with separate germinal epithelia (Figure 2e). Previtellogenic oocytes measuring 70–120 µm were characterized by the deposition of small granules of protein around the nucleus (Figure 2c; Table 2), the presence of a continuous

Table 1. Staining affinity of the constituents of the previtellogenic and vitellogenic oocytes of *C. amphora*.

Histological stain	Vitelline membrane		Cytoplasm		Vacuoles	Nuclear membrane		Nucleus		Nucleolus	
	Prev. oocyte	Vit. oocyte	Prev. oocyte	Vit. oocyte	Prev. & Vit. oocyte	Prev. oocyte	Vit. oocyte	Prev. oocyte	Vit. oocyte	Prev. oocyte	Vit. oocyte
Ehrlich hematoxylin & eosin	pale blue	blue	red	deep red	-ve	reddish blue	blue	pale blue	blue	deep blue	dark blue
Heidenhain's iron hematoxylin	blue	blue	red	dark red	-ve	reddish blue	blue	dark blue	blue	deep blue	deep blue
Hemalum & eosin	pale blue	blue	red	deep red	-ve	pale reddish blue	faint blue	pale blue	blue	dark blue	dark blue
Mallory triple stain	pale blue	blue	dark red	dark red	-ve	red	blue	blue	blue	pale blue	reddish blue
Masson trichrome stain	pale blue	deep blue	red	red	-ve	pale blue	dark blue	blue	blue	deep blue	dark blue
Weigert's & Van Gieson stain	reddish blue	dark blue	red	deep red	-ve	pale blue	deep blue	blue	blue	dark blue	dark blue
Alcian blue & eosin	pale blue	dark blue	red	faint red	-ve	pale red	deep red	red	blue	deep red	deep blue
Safranin & light green	yellowish green	green	green	dark green	-ve	Pale green	pale green	pale green	green	deep green	dark green
Modified Azan stain	pale blue	blue	blue	dark blue	-ve	pale blue	blue	faint blue	pale blue	dark blue	dark blue

Note: The outer & inner follicle cells and test cells of the vitellogenetic oocytes gained the same staining affinity as the vitelline membrane. -ve indicates vacuoles did not accept stains (Pantin, 1948; Pearse, 1965; Pearse, 1968 .

Table 2. Indicates the chemical nature of the yolk components in the oocytes of *C. amphora* during vitellogenesis.

Stage of oocyte	Indication	Previtellogenic oocyte ooplasm	Vitellogenic oocyte granules in ooplasm	Postvitellogenic oocyte homogenous deutoplasm
Mercury bromophenol blue (Mazia et al., 1953)*	protein	light blue	dark blue	homogenous blue
Biuret reaction (Gabe, 1976)	peptide linkage	light violet	dark violet	homogenous violet
Aqueous bromophenol blue (Mazia et al., 1953)*	acidic protein	light blue	dark blue	homogenous blue
Toluidine blue (Pearse, 1968)*	basic protein	light blue	dark blue	homogenous blue
Xanthoproteic reaction (Pearse, 1968)*	phenyl group	light orange	dark orange	homogenous orange
Millon's reaction (Baker modification) (Pearse, 1968)*	tyrosine	light pink	dark pink	homogenous pink
Ninhydrin Schiff reaction (Yasuma & Itchikawa) (Gabe, 976)*	NH_2 group	light pink	dark pink	homogenous pink
Oxidized tannin Azo method (Dixon) (Pearse, 1968)*	amino group	light pink	dark pink	homogenous pink
Sakaguchi reaction (Pearse, 1968)*	arginine	light orange	dark orange	homogenous orange
PAS (Pearse, 1968)*	mucosubstance	light red	dark red	homogenous red
Alcian blue (Pearse, 1968)*	AMS	light red	dark red	homogenous red
Alcian blue & PAS (Pearse, 1968)*	acidic & neutral MPS	light blue; orange	dark blue; orange	homogenous red
Best's carmine (Pearse, 1968)*	glycogen	light pink	dark pink	homogenous pink
Sudan black B (Pearse, 1968)*	lipids	-	dark brownish black	homogenous brownish black
Nile blue sulfate (Cain, 1947)*	neutral & acidic lipid	-	dark blue	homogenous blue
Liebermann Bürchardt (Pearse, 1968)*	cholesterol & esters	-	-	-

*Pearse (1965, 1968)

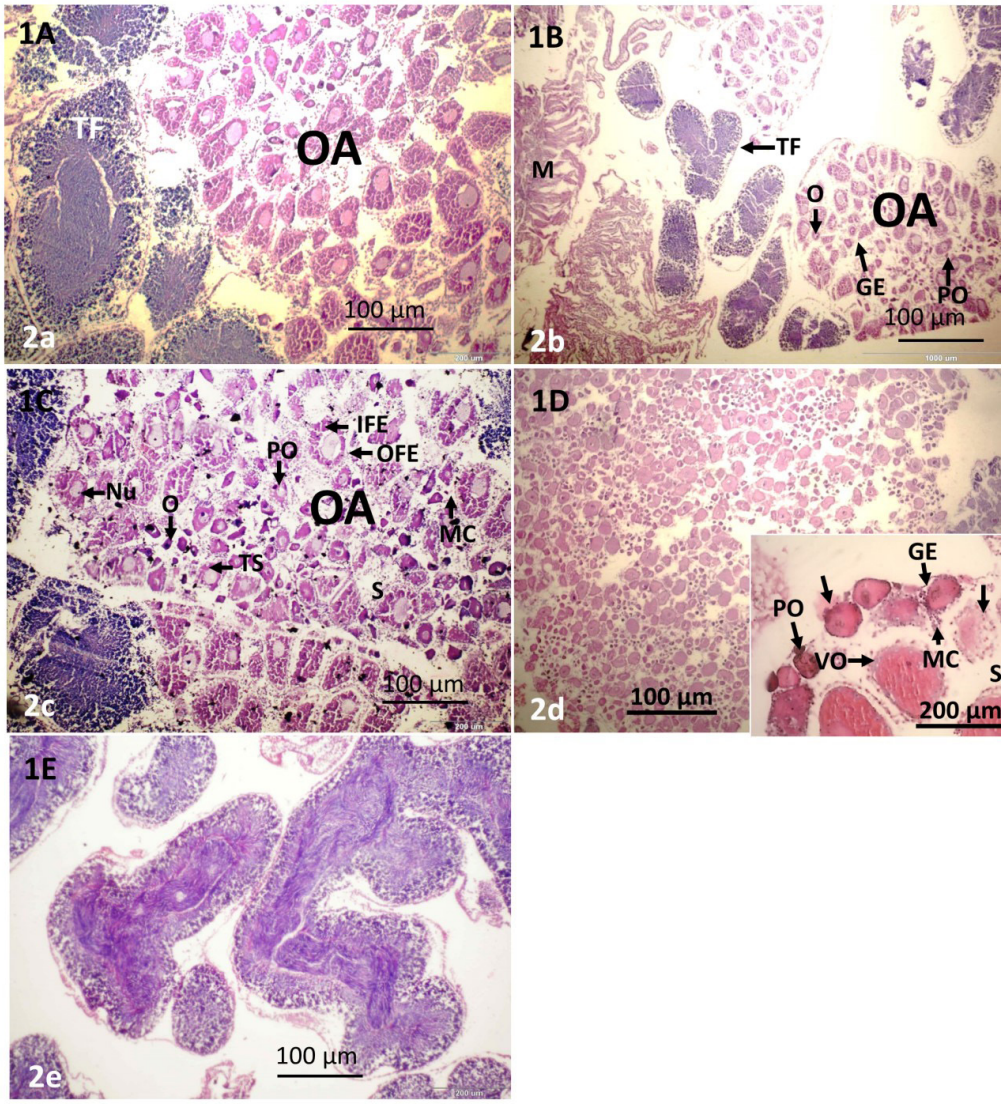


Figure 2. Photomicrographs of a transverse section of the gonad of *C. amphora* 2**a.** One ovarian acinus surrounded by many testicular follicles. Ehrlich hematoxylin and eosin stain; OA, ovarian acinus; S, ovarian stroma; TF, testicular follicle. **2b.** The convolution of the germinal epithelium. It encloses a cavity in the direction of the mantle where the fully formed oocytes drop from the ovarian acinus and enter the oviduct. Masson trichrome stain; M, mantle; GE, germinal epithelium; OA, ovarian acinus; O, oogonia; PO, previtellogenic oocyte; VO, vitellogenic oocyte. **2c.** Oogonia with homogenous ooplasm and large nucleus. Gonads in this phase enlarged gradually in diameter and retained the homogeneity of their ooplasms. Mallory triple stain; MC, mesenchyme cell; N, nucleus; Nu, nucleolus; IF, inner follicular epithelium; OFE, outer follicular epithelium; PO, previtellogenic oocyte; S, ovarian stroma; TS, test cells. **2d.** Numerous mesenchyme cells have migrated from the ovarian stroma and surrounded the oogonia that have detached from the germinal epithelium to form a discontinuous layer. 2**e.** Testicular follicles surrounding the ovarian follicles with separate entities. Tyrosine, Millon's reaction (Baker modification); AO, atretic oocyte; GE, germinal epithelium; O, oogonium; PO, previtellogenic oocyte; S, ovarian stroma; VO, vitellogenic oocyte. 2**e**. Testicular follicle. TF, testicular follicle; St, spermatogenesis; Sp, spermiogenesis.

layer of follicular cuboidal epithelium above the vitelline membrane of the oocyte, and the appearance of scattered vacuoles in the ooplasm (Figure 3a). Vitellogenic oocytes measured 130–220 µm, and the follicular epithelium divided into an outer simple squamous layer and an inner simple cuboidal layer. Beneath the vitelline membrane, scattered test cells appeared in the ooplasm and different granules of protein and mucopolysaccharides appeared in the ooplasm (Figure 3 a-h; Table 2). In the later phase, lipid droplets began to appear in the ooplasm (Figure 3e-f). Yolk bodies formed after the impregnation of various granules together, and the oocyte was ready to be shed. Before spawning, a non-cellular vitelline coat appeared over the layer of test cells. Post-vitellogenic oocytes, in which the homogeneity of ooplasm was restored, underwent gradual lysis and entered the atretic phase

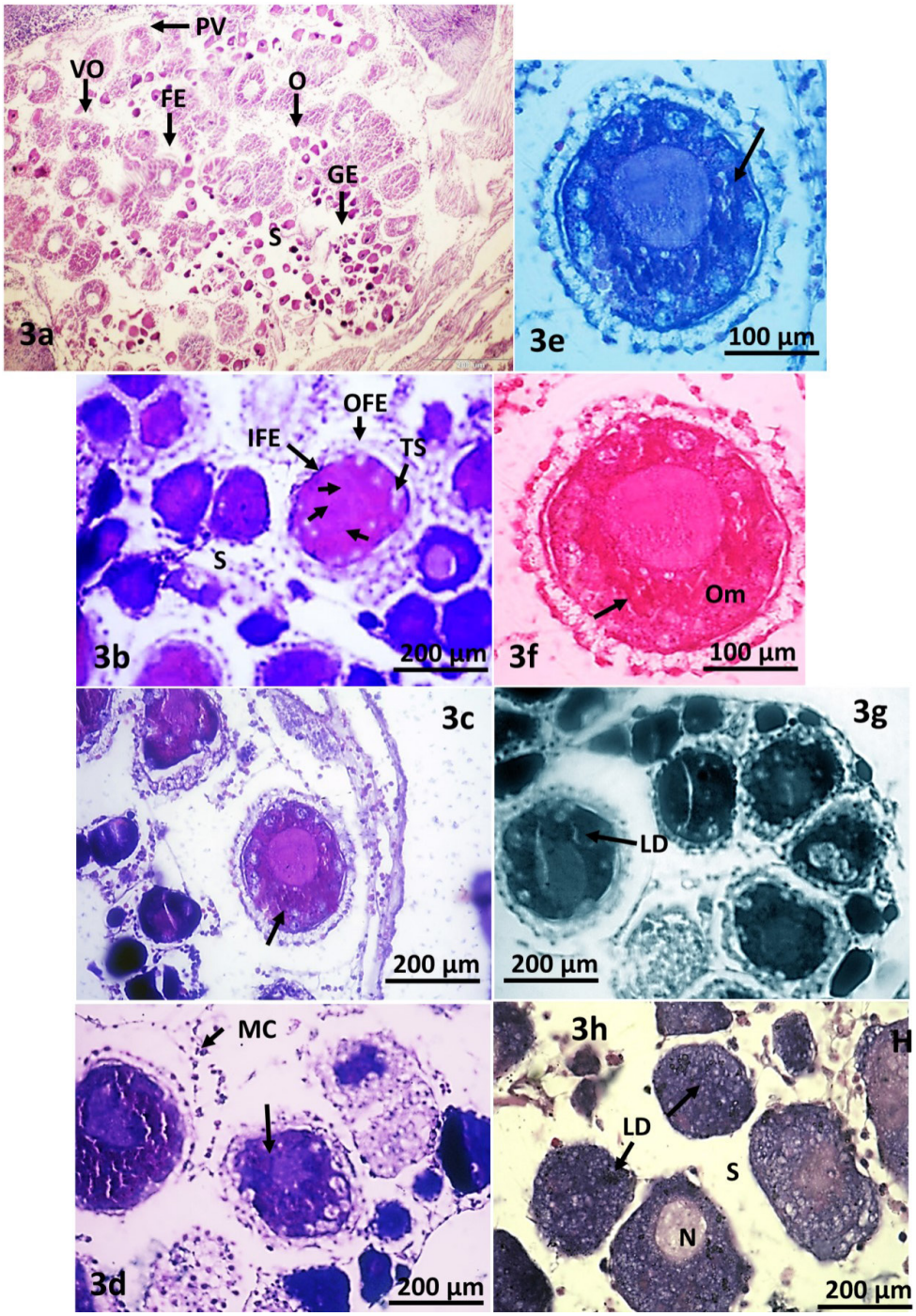


Figure 3. Photomicrographs of a transverse section of the gonad of *C. amphora* 3**a.** Previtellogenic oocyte characterized by the deposition of small granules of protein (indicated by arrows), the discontinuous layer of mesenchyme cells forming a continuous layer of follicular cuboidal epithelium above the vitelline membrane of the oocyte, and scattered vacuoles in the ooplasm. The ooplasm contained acidic protein. Aqueous bromophenol blue; FE, follicular epithelium; GE, germinal epithelium; O, oogonium; PV, peripheral vacuoles; VO, vitellogenic oocyte; S, stroma. **3b.** Vitellogenic oocyte, in which the follicular cuboidal epithelium has divided into an outer layer of simple squamous cells and an inner simple cuboidal layer underneath the vitelline membrane and scattered test cells and different granules of protein and MPS have appeared in the ooplasm (indicated by arrows); IF, inner follicular epithelium; OF, outer follicular epithelium; S, ovarian stroma; TS, test cells. **3c.** Basic protein in the ooplasm (indicated by arrows). Toluidine Blue. 3**d.** Protein in the ooplasm (indicated by arrows). Mercury bromophenol blue; MC, mesenchyme cell 3**e.** Peptide linkages in the ooplasm (indicated by arrows). Biuret reaction; IF, inner follicular epithelium; OF, outer follicular epithelium; S, ovarian stroma; TS, test cells. 3**f.** Amino groups in the ooplasm. Oxidized tannin Azo method (Dixon); Om, ooplasm. **3g.** Deposition of lipid droplets in the ooplasm; the ooplasm contains neutral and acidic lipids. Nile blue sulfate. 3**h.** Lipids. Sudan black B; LD, lipid droplets; N, nucleus; S, stroma.

(Figure 4a-c). The testicular follicles were convoluted and surrounded the ovarian follicles. They had unique germinal epithelia that were continuous in all follicles of a single testis. Different stages of sperm development (spermatogonia, primary and secondary spermatocytes, spermatids, and sperm) were present year-round in different follicles of the same squirt; hence, the testis was always mature (Figure 5a-e). It is obvious that during July – August and September – October the vitellogenic oocytes predominate the ovarian follicles and shedding of gametes occurs whereas in March – June and November – February the oogonia and the previtellogenic oocytes are numerous in the preparatory phase of oogenesis.

3.3. Repeated measures ANOVA test of oocytes during the year

The number of cells in each phase of oocyte development was counted in five representative ovarian follicles from different squirts each month using slide micrometer with objective and eye lens calibrations in micrometers. The mean for each count was calculated and subjected to repeated measures ANOVA. This analysis clarified the ovarian cycle and showed in which months the sea squirt *C. amphora* sheds oocytes. The time of the year exerted a significant effect on ovarian development.

From September–October, vitellogenic oocytes (Vit. oo.) represented 30.7% in the ovarian follicles (n=233) "[Mean ± Standard Deviation, SD]" 233±2.1; previtellogenic oocytes (Prev. oo.) and oogonia represented 26.7% and 19.8% in the ovarian follicles (n=205, n=152) 152±2.77 and 205±10.2 respectively; post-vitellogenic oocytes (Postv. oo.) 13.5% in the ovarian follicles (n=104) 104±2.1; and atretic oocytes (Atr. oo.) 9.3% in the ovarian follicles (n=72) 72±1.2 (Supplementary Material - Table 1A). The repeated measures ANOVA showed a significant difference among the means of the stages of oocyte development. Tukey's multiple comparison test showed the lowest mean difference of "-27" for oogonia *versus* Vit. oo. with $p < 0.05$ and the highest mean difference of "53.67" for Vit. oo. *versus* Atr. oo. with $p < 0.001$ (Figure 6a and Supplementary Material - Table 1A).

From November–February, oogonia represented 32.8% in the ovarian follicles (n=263) 253±26.4; Prev. oo., 21.9% in the ovarian follicles (n=168) 168±2.9; Atr. oo., 24.9% (n=192) 192±12.3; Postv. oo., 14.1% in the ovarian follicles (n=108) 108±22.3 and Vit. oo., 6.3% in the ovarian follicles (n=49) 49±33.2. The repeated measures ANOVA showed a significant difference among the means of the stages of oocyte development. Tukey's multiple comparison test showed the lowest mean difference of "-47.67" for Vit. oo. *versus* Atr. oo. with $p < 0.001$ and the highest mean difference of "48.33" for oogonia *versus* Postv. oo. with $p < 0.001$ (Figure 6b and Supplementary Material - Table 2B).

From March–June Prev. oo. the represented 35.8% in the ovarian follicles (n=232) 232±12.7; oogonia, 21.9% in the ovarian follicles (n=141) 141±11.1; Vit. oo., 19.8% in the

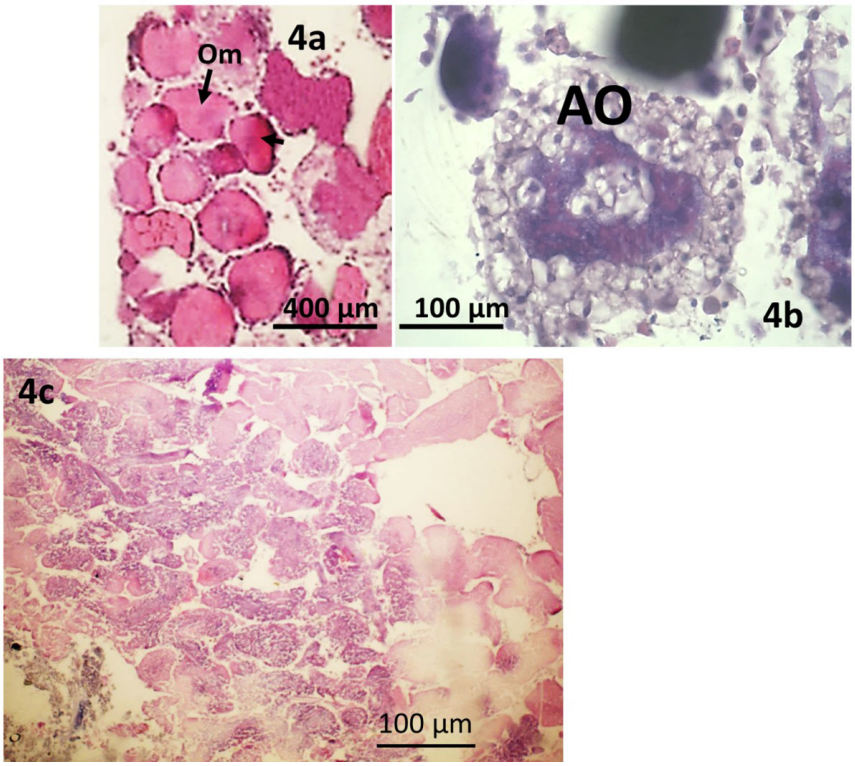


Figure 4. Photomicrographs of a transverse section of the gonad of *C. amphora* 4**a.** Post-vitellogenic oocyte in which the homogeneity of ooplasm and gradual lysis to enter the atretic phase has begun; Om, ooplasm. 4**b & 4c.** The ooplasm containing MPS, PAS, and remnants of neutral and acidic lipids. Nile blue sulfate; AO, atretic oocyte.

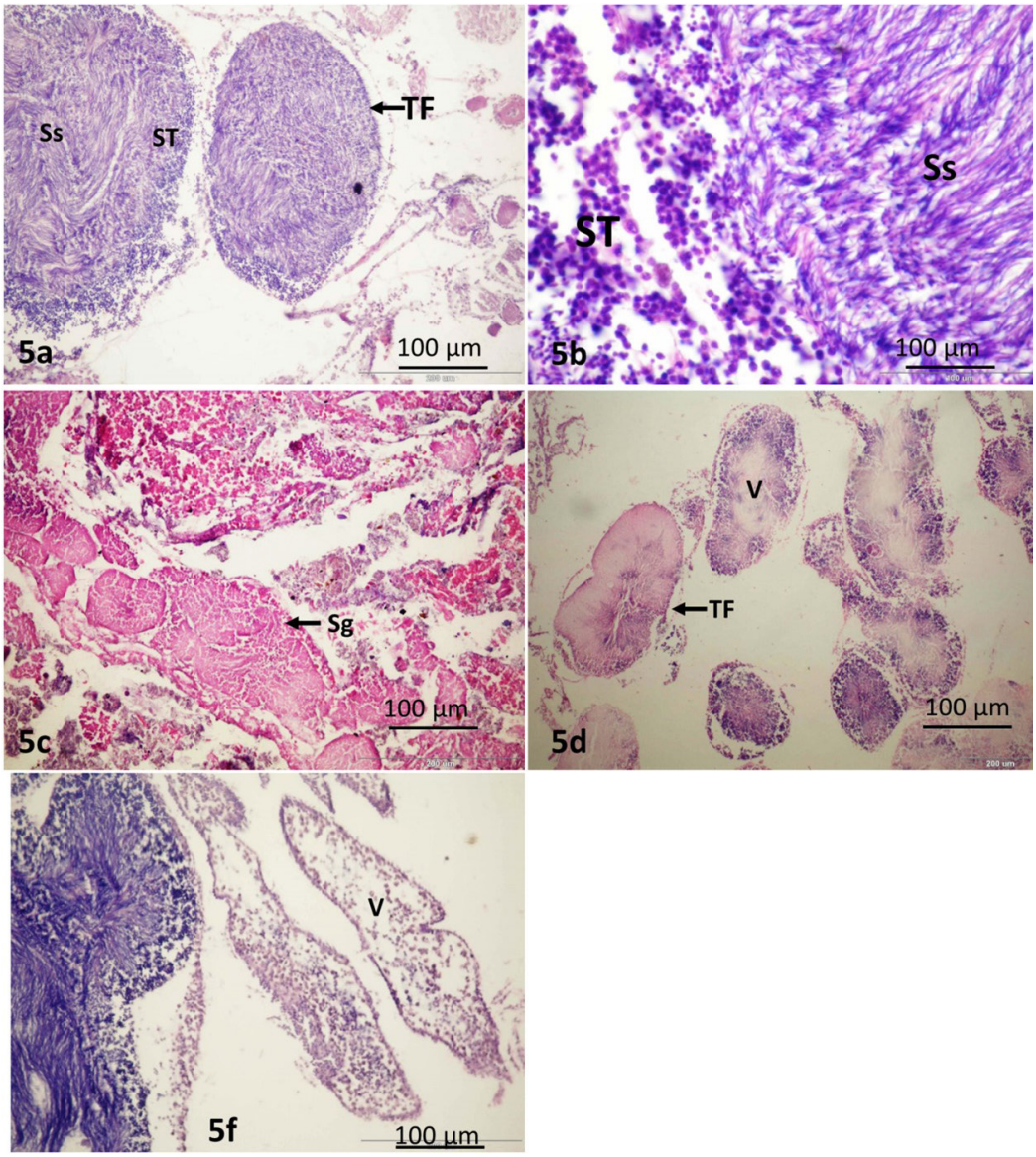
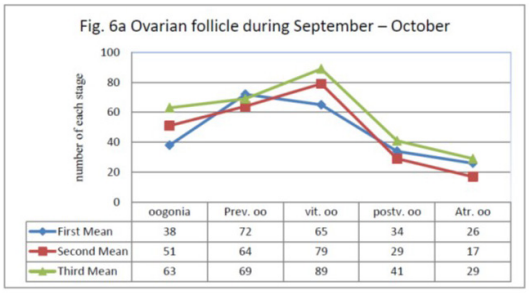


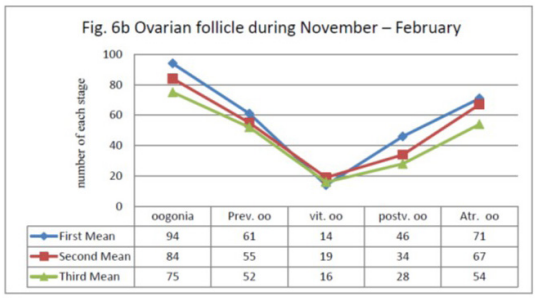
Figure 5. Photomicrographs of a transverse section of the gonad of *C. amphora* showing the different activities in testicular follicles. 5**a.** Spermatogenesis and spermiogenesis. Heidenhain's iron hematoxylin; OA, ovarian acinus; TF, testicular follicle; Ss, spermiogenesis; St, spermatogenesis. 5**b.** Spermiogenesis. Ss, spermiogenesis; St, Spermatogenesis. 5**c.** Initial vacuolization and spermatogonia. Ninhydrin Schiff reaction; Sg, Spermatogonia. 5**d & 5e.** Spent phase of testicular follicles. Oxidized tannin Azo method (Dixon); OA, ovarian acinus; V, vacuolization; TF, testicular follicle.

ovarian follicles (n=128) 128±12.1; Atr. oo., 11.7% in the ovarian follicles (n=76) 76±8.2; and Postv. oo., 10.8% in the ovarian follicles (n=70) 70±8.2. The repeated measures ANOVA showed a significant difference among the means of the stages of oocyte development. Tukey's multiple comparison test showed the lowest mean difference of "-30.33" for oogonia *versus* Prev. oo. with $p < 0.05$ and the highest mean difference of "54" for Prev. oo. *versus* Postv. oo. with $p < 0.01$ (Supplementary Material - Table 3C).

From July–August, Vit. oo. represented 56.5% in the ovarian follicles (n=287) 287±11.8; Prev. oo., 16.9% (n=85) 85±19.6; oogonia, 10.8% in the ovarian follicles (n=55) 55±19.3 ; Atr. oo., 8.2% in the ovarian follicles (n=42) 42±14.7; and Postv. oo., 7.6% in the ovarian follicles (n=39) 39±23.8. The repeated measures ANOVA showed a significant difference among the means of the stages of oocyte development. Tukey's multiple comparison test showed the lowest mean difference of "-77.33" for oogonia *versus* Vit. oo. with $p < 0.05$ and the highest mean difference of "5.33" for oogonia *versus* Postv. oo. with $p < 0.01$ (Supplementary Material - Table 4D). Figure 7 a-b and Supplementary Material - Tables 3C and 4D show the developmental stages of oocytes from March- June and July-August.

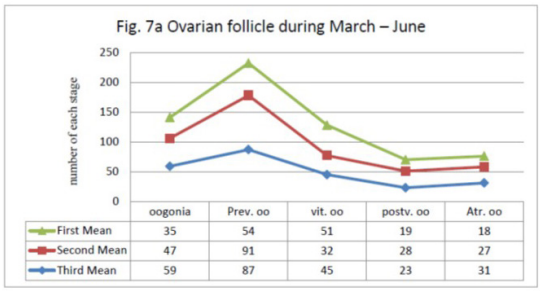


	oogonia	Prev. oo	vit. oo	postv. oo	Atr. oo
First Mean	38	72	65	34	26
Second Mean	51	64	79	29	17
Third Mean	63	69	89	41	29

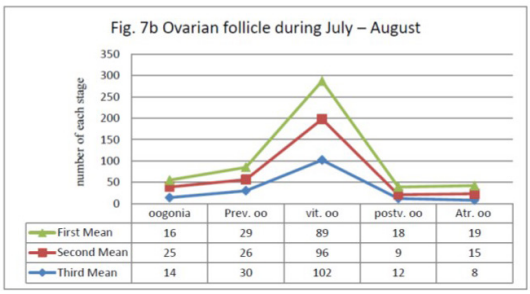


	oogonia	Prev. oo	vit. oo	postv. oo	Atr. oo
First Mean	94	61	14	46	71
Second Mean	84	55	19	34	67
Third Mean	75	52	16	28	54

Figures 6. a-b Note, "mean" indicates the total number of oocytes in all stages of maturity in one ovarian follicle divided by 6, which represents the different stages of oocyte development. oogonia; Prev. oo previtellogenic oocyte; vit. oo vitellogenic oocyte; postv. oo postvitellogenic oocyte; Atr. oo atretic oocyte



	oogonia	Prev. oo	vit. oo	postv. oo	Atr. oo
First Mean	35	54	51	19	18
Second Mean	47	91	32	28	27
Third Mean	59	87	45	23	31



	oogonia	Prev. oo	vit. oo	postv. oo	Atr. oo
First Mean	16	29	89	18	19
Second Mean	25	26	96	9	15
Third Mean	14	30	102	12	8

Figures 7. a-b Note, "mean" indicates the total number of oocytes in all stages of maturity in one ovarian follicle divided by 6, which represents the different stages of oocyte development oogonia; Prev. oo previtellogenic oocyte; vit. oo vitellogenic oocyte; postv. oo postvitellogenic oocyte; Atr. oo atretic oocyte.

The repeated measures ANOVA did not show a significant match ($p < 0.05$) between the oocyte developmental stages and the seasons. The Tukey's multiple comparison test ($p > 0.05$) showed mean differences of 0.00 for Prev. *versus* Vit. oocytes and -14.17 for oogonia *versus* Prev. and oogonia *versus* Vit. oocytes during the year (Supplementary Material - Table 5E).

4. Discussion

Understanding the seasonal reproduction of *C. amphora* in the Arabian Gulf is essential for its conservation. However, despite sea squirts' circum-global distribution and their being relatively well-studied among marine invertebrates, little information on the ecological and biological aspects of *C. amphora* around the world is available, especially regarding its migratory movements and reproductive cycle (Norkko, et al., 2002). This species lives in the waters of North and South America, Australia, and the Red Sea (Sahade, et al., 2004). It has also been brought to other marine and estuarine ecosystems. Its reproductive behavior changes according to the abiotic factors, mainly temperature, of its habitat. Information about the reproductive biology of this species in the Arabian Gulf is lacking.

C. amphora is an invasive species in the Arabian Gulf (Sanamyan, 2015). The adult squirts measured 10–13 cm in length and 4–6 cm in width. Both their oral and atrial siphons have four lobes. The squirts were compared to those identified according to Van Name (1945), Berrill, (1975), Kott, (1992), and Sahade et al. (2004). Generally, solitary sea squirts discharge their gametes into the water, where fertilization takes place. The spawning period varies not only from species to species but also by locality. This study concluded that *C. amphora* was fertile from July to October, when vitellogenic oocytes predominated. From November–February, oogonia prevailed, whereas from March–June, previtellogenic cells outnumbered the others. The reproductive biology of the common Antarctic ascidian *Cnemidocarpa verrucosa* was investigated in a shallow-water population at Potter Cove, South Shetland Islands. That study showed that gametogenesis was continuous year-round, although vitellogenesis, mature spermatocytes, and mature oocytes were mainly observed during the austral winter (Sahade et al., 2004). This study affirms that *C. amphora* is well-adapted to the Arabian Gulf as it exhibited different reproductive patterns and strategies than in its native habitat. The temperature in the Arabian Gulf is 40-50 ºC during summer, 30-35 ˚C during spring. In fall and winter it is not cold and the temperature measures about 15-25 ˚C. The daylight is higher than darkness during spring and summer. This study concludes that temperature and daylight induce gonadal activity during July – August and September – October. *Molgula manhattensis* and *Ciona intestinalis* release their eggs and sperm 1–1.5 hours before sunrise, and *Styela partita* and *Corella parallelogramma* do so in the late afternoon (Lambert, 2004). Gamete shedding in *Botryllus primigenus* occurs early in the morning, about 1 hour after dawn (Mukai and Watanabe, 1976). In general, the hermaphroditic gonads release mature oocytes before sperm (Jiang and Smith, 2005; Honegger and Koyanagi, 2008). In this study, 4–9 hermaphrodite gonads were observed on the right side of the atrial cavity and 1–5 on

the left. In *Styela plicata*, there are generally four gonads on the right side and one or two on the left, while in *Styela partita*, two hermaphrodite gonads are present on either side of the pharyngeal sac (Saad, 2002; Michael et al., 2008). *C. amphora* gonads varied in diameter and histology in different months.

The sea squirt *C. amphora* living in the Arabian Gulf was found to shed ripe oocytes from July to October. The testes were mature year-round as different testicular activities were present in different testicular follicles of the same squirt simultaneously. The literature records some environmental factors that govern the reproductive cycle of a sea squirt in a particular area (Dybern, 1965; Lambert & Brandt, 1967; West & Lambert, 1975, Mukai & Watanabe, 1976; Numakunai & Hoshino, 1980; Millar, 1982; Boyd et al., 1986; Grantham et al., 2003). In the Old Dock of Ardrossan Harbour, Ayrshire, the populations of *Ciona intestinalis*, *Ascidiella aspersa*, *Diplosoma listerianum*, and *Botryllus schlosseri* have the fastest growth in May, June, and early July, and none in winter (Kott, 1985). Kott (1985) also stated that specimens of *Ascidiella aspersa* are mature to a certain degree in this area year-round; zooids that settled in the summer grew until autumn, resumed growth the following spring, spawned in the summer of that year, and died the following winter. The population of *Botryllus primigenus* in the waters of Japan grows vigorously and develops gonads in the breeding season, from July to September (Mukai & Watanabe, 1976). Light is a crucial governing factor in the process of spawning in sea squirts. Dybern (1965) reported that spawning success depends mainly on the lightness and darkness of the day. The pleurogoneate sea squirt *Halocynthia* (*Styela roretzi*) has two breeding seasons from November to April in the Asamushi waters of Japan (Satoh et al.,, 1982). Environmental temperature also controls maturity and breeding season in sea squirts in different habitats (Dybern, 1965; Boyd et al.,1986). The first author added that in subarctic regions with temperatures below 5 °C, *Ciona* sp. attained an age of several years, while in boreal regions it lived only 1 year, and in Mediterranean, subtropical, and tropical regions, less than 1 year. This is because growth is more rapid at high temperatures than at low ones. In deeper waters, below 50–60 m, in which temperatures of 6–9 °C prevail year-round, each generation lives at least 2 years. From these data, it was concluded a greater difference between summer and winter temperatures would contribute to more distinct spawning periods. Boyd et al. (1986) stated that in areas with even temperatures year-round, reproduction may occur throughout the year. This study affirmed Dybern (1965) and Boyd et al. (1986) findings. In the waters of Japan, (Yamaguchi, 1975) proved that *Ciona intestinalis* grew to sexual maturity in 1 month in summer and 2 months in winter, while *Styela plicata* requires 2 months in summer to be mature and nearly 5 months in winter. He also found that the populations of *Leptoclinum mitsukurii* and *Botryllus violaceus* are sexually mature throughout the year. Reproduction in *Styela clava* on the Swedish Skagerrak coast starts in July and continues throughout the summer, with a peak from August–September. The degeneration of both eggs and sperm cells began in November, accelerated in the following months, and declined in February (Honegger & Koyanagi, 2008; Jørgensen and Lützen, 1997).

5. Conclusion

This study concluded that the sea squirts living in the Arabian Gulf in Saudi Arabia shed ripe oocytes from July to October. November to February could be considered a multiplication phase of oogenesis, while March to June was the growth phase. The testes are mature year-round as different testicular activities were present in different testicular follicles of the same squirt simultaneously. Our data show that *C. amphora* has adapted well to this environment and that further studies are required to ascertain its environmental impact.

C. amphora is considered a good species to study reproductive seasonality as its gonads are numerous and its ovaries and testes have separate entities. This species is introduced or has migrated to the Arabian Gulf and its reproduction has not been studied in that ecosystem. Moreover, this species is not abundant in this estuarine ecosystem compared to other ascidian species. Previous studies on this species and other ascidian species from the Mediterranean Sea and Arabian Gulf suggest ascidians as a microhabitat that may shelter and feed many small invertebrates and marine larvae. When ascidians dominate during the reproductive season, the associated microfauna dominate as well.

Acknowledgements

The authors acknowledge the Deanship of Scientific Research; the Ministry of Higher Education at Imam Abdulrahman bin Faisal University; and the Department of Zoology, Faculty of Science, Alexandria University for supporting this study.

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Supplementary Material

Supplementary material accompanies this paper.

Table 1A. Ovarian acini during September – October

Table 2B. Ovarian acini during November – February

Table 3C. Ovarian acini during March – June

Table 4D. Ovarian acini during July – August

Table 5E. Data Table oocytes in ovarian acini during the year round

This material is available as part of the online article from https://doi.org/10.1590/1519-6984.273666