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Keywords: field data - gregariousness - heterologous inseminations -adult orientation - larval settlement – phototaxis - geotaxis.

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Field and Laboratory Studies on Four Species of Sea Squirts and their Larvae

Gaber Ahmed Saad [α] & Abdullah Bedeer Hussein [σ]

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Keywords: field data - gregariousness - heterologous inseminations -adult orientation - larval settlement – phototaxis - geotaxis.

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I. Introduction

The metamorphosis of the ascidian tadpole larvae has long attracted the interest of many authors. Using tadpoles of *Ciona* , *Phallusia*, *Ascidia* ,*Styela*, *Styelopsis*, *Distomus*, *Clavelina* and *Distaplia*, Berrill (1947 a & b) ; Byrne et al. (2003) concluded that ascidians spread only during larval stage and acid metabolites produced by the activity of the tail were the inducing agent of metamophosis. Moreover, two factors were identified during the metamorphosis of the ascidian tadpole, namely 'aging factor' which is the progressive exhaustion of the yolk reserves of the epidermis. The epidermis, muscles and notochord are affected. 'Nutritive exhaustion' culminates in the centripetal contraction of the epidermal envelope, a contraction that appeared to have a disruptive effect upon the tissues within (Underwood and Keough 2001). It was mentioned that adult ascidians are capable of slow-crawling (Hecht, et al. 1977; Goodbody & Fisher1974 ; Goodbody &Gibson1974). This movement involved the progressive formation of new colonies for attachment. Through light microscopy examination, Lester(1988) investigated the process of settlement and metamophosis of *Rhabdopleura nomani* (Hemichordata: Pterobranchia). These colonies were collected from Brmuda, USA. He concluded that the swimming larvae settle within 24 hrs. In the work of Cloney (1961), it was mentioned that the resorption of the tail of *Boltenia vilosa*, *Pyura haustorlo* and *Styela gibbsii* began proximally and progresses distally. The sheath at the anterior end of the notochord ruptured at the beginning of tail resorptuon. The matrix of notochord flowed into the body cavity of the trunk. The muscle cells shorten and buckle as the tail shortened and the myofibrills of these cells become disarranged. Gilbert and Raunio (1997) mentioned that there are many reasons of why ascidians are popular as research animals in developmental biology. In fact, ascidian embryos and larvae have a small number of cells, at the beginning of gastrulation, ascidian embryos contain only about 110 cells whereas amphibian gastrulae contain about10 000 cells. Ascidian tadpole larva consists of a few thousand cells and only six different tissues. Ascidian larvae develop rapidly. Swimming larvae hatched after 12-18 hrs post fertilization. Some ascidians have eggs with colored cytoplasmic regions. These regions distribute during cleavage to specific blastomeres. This feature

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allows cell fates to be followed by routine microscopy. Ascidians may provide basic information about the development of more complex chordates such as vertebrates. Ascidians have small genomes that facilitate cloning genes involved in developmental process from another point of view, there are some limitations in using ascidians as an experimental system. This is due to the fact that most of them have restricted breeding seasons and living embryos can be obtained only at certain times of the year. Moreover, genetic analysis is not available as it is in *Drosophila* for example. However, genetic approaches have been developed in some of the compound ascidians (Rinkevich and Weissman (1987). It is well-known that ascidians live solitary or colonial. In the text-books such as by Young, et al. (1988), Satoh (1994), and Gilbert & Raunio (1997), the solitary forms are commonly named 'Ascidiae simplicies' whereas those forming colonies are named 'Ascidiae compositae'. In the work of Niermann-Kerkenberg(1989), ascidians in general are referred to as 'mantle animals'.

The length of the swimming period in oviparous species can range from only few hours to several days. While in most viviparous species, it ranges from a few minutes to several hours (Byrne et al. 2003; Bullard et al. 2004). The larva swims for 6 hrs. and prepare for the onset of metamorphosis. It alters its response to light and gravity. The larva is first negative geotactic and positive phototactic. Immediately before settlement it avoided light and prefers to settle on dark or shaded surfaces. According to Cloney (1982 &1990; Hinz & Schwarzlaender 2004), the principal structures of solitary ascidian larvae are classified into 3 groups: transistory larval organs; prospective juvenile organs and larval juvenile organs. The complexities and degree of differentiation of these components vary for different taxa (Millar,1971; Hinz and Schwarzlaender, 2004). Metamorphosis involved numerous rapid morphogenitic movements and physiological changes that are initiated at the moment of settlement (Gilbert &Raunio,1997; Campbell & Donlan 2005; Dupont et al. 2006). Transistory larval organs are phagocytized or otherwise destroyed at metamorphosis whereas the prospective juvenile organs and larval-juvenile organs become the functional parts of the juvenile or oozooid. Metamorphosis began with settlement and is followed by series of coordinated morphogenetic movements that rearrange cells, tissues and organs (Calvo-Ugarteburu & McQuaid 1998; Kasper et al. 2008 ; Ross & Auge 2008; Rius et al. 2009a). The axial complex of the tail, the visceral ganglion and the sensory organs of the cerebral vesicle are destroyed and engulfed by phagocytes. The presence of adults and associated chemical factors is normally regarded as an attractor for settlement alongside conspecific adults (Koh & Sweatman 2000; Ramsay et al. 2008) or an inducer of metamorphosis (Svane et al. 1987 ; Lambert 2005, 2007; Whiteley & Bendell-Young 2007), which may cause aggregation. Phototactic and/or geotactic behavior of the larvae can determine where settlement occurs (Svane and Young 1989). For all these reasons, settlement has the capacity to influence habitat selection, determining adult distribution patterns of sedentary species (Underwood & Keough 2001). Cloney (1990) listed 10 principal events of ascidian metamorphosis: Secretion of adhesives by papillae or the epidermis of the trunk; Eversion and retraction of papillae; Resorption of the tail; Loss of the outer cuticular layer of the larval tunic; Emigration of blood cells or pigment cells; Rotation of visceral organs through an arc of about 90 degrees; Expansion of the branchial basket and elongation of the oozooid or juvenile; Expansion, elongation or reciprocation of ampullae; Reorientation of test vesicles, and expansion of the tunic; Retraction of the sensory vesicle; Phagocytosis of visceral ganglion, sensory organs and cells of the axial complex and Release of organ rudiments from an arrested state of development. The same author added that metamorphosis may be completed in seconds or minutes (papillary eversion and tail resorption) and others may take hours or days (rotation, ampullar outgrowth, phagocytosis of the axial complex. Ascidians are major contributors (Lambert 2005, 2007), and can severely modify the structure of coastal habitats by forming large aggregates (Lambert & Lambert 2003, Rius et al. 2009a). Adults live attached to hard substrata (Monniot et al. 2001) and the only motile stage is their lecithotrophic larvae, which have very limited dispersal due to their short planktonic lifespans (Svane & Young 1989). Some information is available regarding the distribution of adult ascidians in the field (Mastrototaro et al. 2008), although the settlement patterns that may explain these adult distributions are well-understood for only a few species (Svane & Young, 1989; Valiela et al., 1997; Howes et al. 2007). Many factors can influence ascidian larval behavior and settlement, including light, gravity, temperature, salinity, presence of adults or competitors, biomechanical properties and energy limitations (Svane & Young 1989; Stachowicz et al. 1999; McHenry & Patek 2004; Bennet & Marshall 2005). There has been a rapid increase in the rate of introduced non-indigenous ascidian species to many parts of the world in the past 20–40 years (Lambert, 2001). Changes in seawater temperatures due to global climate change over the last 25 years may be facilitating this spread (Young & Chia 1985; Stachowicz et al., 2002). It was noticed that the number of the individuals in the field varied according to the season and hence the number of the specimens obtained in every collection varied too. There found also seasonal variations in the size of these animals. It was observed that very small-sized specimens may be less than 5 mm in length appeared during the period from March to September.

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This study investigated the settlement patterns of larvae of four solitary ascidians found along the estuarine water of the Arabian Gulf (Saudi Arabia) namely *Ciona intestinalis* (Linnaeus, 1767), *Molgula manhattensis*, (De Kay, 1843), *Ascidella aspersa* (Müller,1776) and *Phallusia mammilata* (Cuvier,1815), which belong to different families from the enterogoneate ascidians (Kott, 1985) and are all commonly found aggregated in the field (Rius et al. 2009a, Branch et al. 2010, and personal observation). These species were chosen to include introduced species with global distributions (*Molgula manhattensis*, *Ascidella aspersa*) and two large native species (*Ciona intestinalis* and *Phallusia mammilata*) that are not known to be invasive, although congeners are recognized as invasive elsewhere (Castilla et al. 2004). All these species are important occupiers of hard substrata of coastal areas of Arabian Gulf (Branch et al. 2010). The larvae of all species had well developed statocytes and ocelli (Niermann-Kerkenberg, 1989; Lübbering, 1994; Kriegel, 1996; Hofmann, et al. 1999; Jacobs et al. 2008 and personal observation) but *Ciona intestinalis* has a highly reduced ocellus (Ohtsuki 1990), and *Molgula manhattensis* is unusual among Pyuridae in lacking an ocellus (personal observation, and see also Svane & Young 1991 for a closely related species). Thus, three species were expected to have both light and geotactic preferences, while the larvae of the remaining species were expected to respond to geotactic stimuli alone. How larval behavior determines settlement patterns in different phototactic and geotactic conditions was examined and in the presence or absence of conspecific extracts. The larval responses were compared with patterns of adult distribution in the field. Three specific hypotheses were advanced. (1) Light will influence settlement, with dark being preferred over light in species that are found in dark habitats, and the opposite for those that occur in well-lit habitats. (2) Geotactic behavior will be important in those species that have adults with clear orientation preferences. (3) Adult mantle tissue extract will have a positive effect on settlement on all the species, and will contribute to the aggregated patterns of distribution of adults.

II. Material and Methods

a) Field Study

Adult specimens of *Ciona intestinalis* (Linnaeus, 1767), *Molgula manhattensis* (De Kay, 1843), *Ascidella aspersa* (Müller,1776) and *Phallusia mammilata* (Cuvier,1815) were surveyed and sampled from four estuarine locations of the Arabian Gulf namely Al-Azezia, Southern Khobar. Northern Khobar and Ad-Dammam. They were transferred into aquaria containing see water and microorganisms to the laboratory. Immediately they are transferred to 10 liter-plastic aquaria with fresh filtered sea water, perfect aeration has been continuously carried out and suspensions of Microcell® were administrated as food. Sea water has been changed every other day. Aquaria were kept at temprature18 Ċ. At each location adult distribution and associated circumstances were quantified. To standardize conditions, all sampling took place at 12:00 am on cloudless days in January/December 2013 at depths of no more than 1 m. At each locality, 50x50 cm quadrats (n=10 per substratum orientation) were placed on horizontal hard substrata facing upwards (0-10°), downwards (170-180°), or on vertical substrata (80-100°). The number of individuals of any of the four species present and the number of individuals per clump were counted. Due to the aggregating nature of ascidians and because they were often covered by algae or other fouling organisms, Clumps were removed and brought them to the laboratory where they could be cleaned and sorted to count the number of individuals precisely. Light intensity was recorded at each sampling point by taking three random measurements within each quadrate using a photometer (Skye 177 instruments Ltd, Scientific Associates) fitted with a sensor (Quantum Sensor, Wales).

b) Laboratory Study

All laboratory experiments were conducted during the early spring of 2013 (end of August to early September) to coincide with the timing of reproductive maturity for all species: *Ascidella aspersa* and *Phallusia mammilata* mature in spring and summer (Rius et al. 2009a), *Ciona intestinalis* and *Molgula manhattensis* in spring, summer and winter (Rius et al. 2009b; Hofmann, et al. 2008 ; Saad, et al. 2010 & Saad, 2002), previous observations undertaken before and after this study to conform some results and observations.

c) Artificial heterologous insemination (Fertilization assays)

About 10 adults of each species were collected from each of the locations specified and transported in insulated containers with 20 Litre seawater to the laboratory within five hours. In the laboratory, specimens were housed in aerated seawater and maintained at room temperature (20°C). All manipulations and experiments were undertaken in filtered seawater obtained using vacuum filtration through 10-µm pore-size filters. For *Ciona intestinalis* and *Ascidella aspersa*, artificial fertilization followed the methods of Young & Chia (1985) and Hofmann, et al. 2008), which involved dissection and collection of gametes from the oviduct and sperm duct. For the remaining two species the methods of Marshall et al. (2000), modified from those of Svane & Young (1991): gametes were extracted by dissection of the ripe gonads and a mix of eggs and sperm poured through a 100-µm filter with seawater into a small beaker, so the eggs were retained by the filter, but the excess sperm and seawater passed through into the beaker. For all species cross of gametes of four

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individuals, preventing self-fertilization. Developing embryos were placed in an aerated beaker (containing 500 ml seawater) in a constant-temperature cabinet at 20°C and complete darkness. In all species, motile larvae hatched within 14 h of fertilization.

d) Experimentations

The experimental units were transparent cylindrical Perspex containers, sealed at the top and bottom with Perspex sheets and held together with an elastic band. The cylinders were 11 mm tall and 44 mm diameter with exactly the same surface area (15.205 cm^2) on the top, bottom, and lateral surfaces, thus offering equivalent surface areas for larval settlement in each of these three orientations. The containers were placed in a seawater tank for 24 hours prior to introduction of larvae, to create a biofilm, which is known to enhance settlement (Keough & Raimondi 1995). Once motile larvae of a given species were formed, they were pipetted out and placed 20 larvae per container filled with seawater (final volume 16.72 cm^3), and immersed the containers in seawater in a 200 ml beaker at 20°C for 24 hours under the experimental conditions detailed below. The Perspex chamber was subsequently dismantled in seawater, so that any unattached larvae were washed away. Three experiments were performed. The number of replicates (i.e. experimental units with 20 larvae each) per treatment and experiment varied from 3 to 10 due to variability in the number of larvae obtained. Once enough larvae were obtained in a given fertilization event, All the experiments described below in were ran parallel. The first experiment involved exposing the wells with larvae to either artificial light (47 μ mol m-2 s-1) or complete darkness (0 μ mol m-2 s-1). In the second experiment, which was modified from the approach of Jiang et al. (2005), larvae were placed in wells in which half of the top, bottom and lateral surfaces were covered by black tape (reducing the light to 0.4 μ mol m-2 s-1), while the other half of these surfaces were exposed to the same artificial light (47 μ mol m-2 s-1).

The third experiment tested the effect of adult extracts on larval settlement, and for this the general method of Svane et al. (1987) was followed, which involved dissolving mantle tissues extract in seawater. An initial concentration of 0.5 g (wet weight) of tunic, previously homogenised using a blender and filtered to eliminate the biggest fragments, was diluted in seawater to obtain a final concentration of 5 % in the experimental wells. Settlement of larvae in seawater with or without mantle tissues extract (control treatment) was then compared in complete darkness. In all three experiments, a stereomicroscope was used to count the numbers of settlers and score their orientation (top, bottom or lateral sides of the containers) after a 24-hour period.

e) Laboratory investigations on gonadal overview of Phallusia mammilata Macroscopic observation

Three specimens for each species from the seasonally collection, (the size of each species is the same), were dissected, the tunic was incised from dorsal side and entirely removed, the mantle was opened dorsally, the branchial sac was excised to expose properly the reproductive system. A part of the hermaphrodite gonad was separated. All preparations were fixed in 10% formalin.

f) Microscopic observation

The same preparations of the macroscopic ones were washed in distilled water for 24 hours, dehydration through ascending series of ethyl alcohol, followed by another dehydration series of tertiary butyl alcohol, tertiary butanol and paraffin oil (1:1), absolute paraffin oil. All preparations then washed in tissue mate or paraplast with melting point 54-58 Ċ and blocked in fresh paraplast. Sections of 5-8 μ were more or less obtained.

One specimen from each species, as a whole mount preparation, was treated like the last preparations to be examined histologically trying to investigate the relationship of varies tissues of the body to the reproductive system. The two siphons for each animal were cut to minimize the length of the specimen from one side and to obtain better embedding. A Number of stains were tried to enable differentiation of the different stages of oocyte and testicular follicles. These are Eherlich haematoxylin and eosin (Pantin,1948); Heidenhain's iron haematoxylin (Pantin,1948); Mallory triple stain (Pantin,1948); Masson's trichrome stain (Pantin,1948); Weighert's haematoxylin & Van-Gieson stain (Mahoney,1975); 6. Alcian blue countered with eosin (Pearse,1968).

Statistically the number of the previtellogenic; vitellogenic; postvitellogenic and atretic oocytes were counted in three ovarian lobules for each histological preparation of four investigated ascidian species in all seasons of the year.

g) Statistical analysis

Analysis of variance (ANOVA) is a broad group of calibrations 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 significant differences among different orientations. To evaluate among the different species the level of gregariousness found in the field, the number of individuals per clump found for each species with species as a fixed factor and adult

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orientation, surface orientation was used as a fixed factor. The number of settlers was tabulated and analyzed incorporating replicates (experimental wells), treatments (light - dark, extract-control) and position of the settlers (bottom, lateral, top). If the above-mentioned assumptions for ANOVA are not met, the Turkey`s Multiple Comparison Test, Bartlett's test for equal variances and Dunnett's Multiple Comparison Test were used for determining whether three or more independent samples originate give a clear cut differences. When this test leads to significant results, at 1 North 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. 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. However, One-way analysis of variance (ANOVA) at $P<0.0001$, Bartlett's test for equal variances, Tukey's Multiple Comparison Test, Dunnett's Multiple Comparison Test and Newman-Keuls Multiple Comparison Test at $P > 0.05$ and $P < 0.001$ were applied (Knoke and Burke 1991; Quinn and Keough 2002 ; Systat Inc., 2007 ; Dijana, et al.2012 for review).

III. Results

Solitary ascidians of this study produce a large number of eggs that are around 150 μm in ϕ. Forms with external fertilization like most phlebobranchs and stolidobranchs spawn small, rather simple sperm. Sperm from solitary ascidians can swim for extended periods and undergo hypermotility in the vicinity of conspecific eggs. In addition, ascidian eggs release factors that increase the motility of sperm and can cause the directed swimming of the sperm to the egg. The embryos of ascidians can be obtained in unlimited quantity by artificial fertilization. It is sufficient to incise an adult from the right side, cut across the oviduct and vas deferens, having previously noticed that the former contained ova, and then collect the ova and sperms as they pass out from the point of incision with a glass tube, and transfer them to a glass containing fresh sea water, and mix them well, but gently, together by stirring. Gradually the ova sink to the bottom of the glass, and in about an hour after the above operation they commence to embryogenesis. The successive cleavage furrows were described. Due to the equal distribution of the yolk granules, the egg undergoes an equal holoblastic cleavage. The first cleavage furrow is meridional resulting in 2 equal and blastomeres (after 2 hrs post fertilization. The second cleavage furrow is also meridional but perpendicular to the first one resulting in 4 blastomeres (after 2:15 hours post fertilization). The third cleavage furrow is latitudinal. It transects the first and second furrows medially resulting in 8 blastomeres (after 2:30 hours of fertilization). The fourth cleavage furrow is two meridional divisions at the same time resulting in 16 blastomeres (after 3:40 hours of fertilization). The fifth cleavage furrow is two latitudinal divisions at the same time. One in the animal hemisphere and the other in the vegitative one resulting in 32 blastomeres (after 3:41till 4 hours of fertilization). After the fifth cleave furrow, this process becomes complicated and perhaps two or three or more furrows occur at the same time. However the 64-blastomere stage was observed. The blastomeres gradually get smaller in size forming a compact sphere. After cleavage the resulting blastomeres moved to the periphery where they arrange themselves forming a continuous lamina or blastoderm. The latter encloses a concentric cavity or blastocoel. This stage of development is referred to as blastula stage (After 4 hrs. post fertilization). The blastomeres at one pole of this sphere, the vegetative pole, flatten and begin to invaginate. This invagination proceeded transforming the single celled-layer blastula into a double cell-layered gastrula (After 4:30 hrs post fertilization). Thus a new cavity appeared which increases gradually in size on the expense of the size of the segmentation cavity. The new cavity formed is referred to as gastrocoel which has an aperture opening, the atriopore.

The blastomeres surround the archentric cavity are the cells of mesendoderm while those surrounding the segmentation cavity are the ectodermal cells.

At the dorsal side of the gastrula precisely in its median portion the ectodermal cells flattens forming the so-called neural plate then invaginate. This invagination proceeded forming the neural cavity which is bordered with two neural ridges. The two neural ridges come close to each other then unite transforming the neural cavity into a neural tube with a central neurocoel and this stage represents the neurula (After 7 hrs post fertilization).

Organogenesis then begins after about 9 hours and finally the embryo inside its chorion is distinguished into an oval trunk and a long tail after 11 hours.

Hatching of the larva takes place by hatching enzyme (After 12 hours of fertilization). Three stages of development of the investigated ascidians were collected from the sea water using a micropipete and all were fixed afterwards in 4 % paraformaldhye and described. Stage 1: This larva has a long tail and an oval trunk. It swims actively and very quickly. It swims at the superficial stratum of the sea water and does not escape from the field of microscope. In other words the larva at this stage is negatively geotactic and positively phototactic. The outer covering of the larva is somewhat transparent. The trunk has two adhesive papillae at its

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proximal end, internally the alimentary tract is a simple tube having an ingestion opening and an egestion one.These two openings orifice on the dorsal side of the trunk. The nervous system is represented only by a brain vesicle having a ventrally situated statolith and a small otolith. This vesicle has an opening referred to as neuropore according to Müller and Hassel (1999.). This neuropore opens in the alimentary tract through neuroentric canal. The brain vesicle extends posteriorly in the tail region as a nerve cord with its central canal. According to Tannenbaum and Rosenbluth (1972), there are a myoneural junctions between the muscle cells and the nerve cord. This cord bears an eye at its junction with the brain vesicle. Torrence and Cloney (1975) illustrated perfectly the fine sensory neurons in the adhesive papillae in the larvae of ascidians and Tannenbaum & Rosenbluth (1972) interrested in the study of the myoneural junctions in larval ascidian tail, Underneath the nerve cord runs a rod-like notochord consisting of vacuolated cells.In addition to these structures, the trunk contains also some mesenchyme cells which later differentiate into tissue or organ rudiments. The tail is muscular and it appears morphologically that the muscle fibres are of striated type (according Mackie & Bone, 1976). There is an undulated fin covering the tail from its dorsal and ventral sides. The test cells were previously entirely embedded in the cytoplasm of the oocyte while after hatching they evaginated and cover this larval stage from all directions especially in case of the larvae of *Ascidiella aspersa* (Fig. 1). Stage II: The trunk gets larger in size. The process of phagocytosis of the tail starts in the direction from posterior to anterior. Torsion of the alimentary tract begins. At this stage of development the nervous system is still represented by the brain vesicle and the test cells decrease in number together with thickening of the outer covering of the larva. This larval stage swims always near the bottom of the sea water and escape from the field of microscope. In other words it is positively geotactic and negatively phototactic (Fig. 2). Stage III: After complete phagocytosis and resorption of the tail, the young metamorphosed ascidian is in the form of the trunk of the larval stadium. At this stage three important criteria can be observed: The tunic is already formed but still somewhat transparent with complete disappearance of the test cells from around it. Rotation of the GI is terminated. The ingestion and the egestion openings of the larva in stage II larval have distinguished into a mouth and an anus respectively. The mouth presents on the oral siphon and the anus presents on the atrial siphon. Closure of the neuropore and the brain ventricle of the larval stage now after metamorphosis differentiates into a nerve ganglion and a neural gland dorsally located to the former (Fig. 3).

a) Adult distribution

Each of the species examined exhibited differences in habitat orientation in the field (Histogram1). The morphology and the anatomy of adults and their embryology of *Ciona intestinalis*, *Ascidella aspersa*, *Phallusia mammilata* and other species were previously studied (Hofmann, et al. 2008; Saad, et al. 2002 & 2010). In both artificial and natural substrata, *Ciona intestinalis* and *Molgula manhattensis* were most abundant on illuminating surfaces, while *Ascidella aspersa* and *Phallusia mammilata* individuals densities were found in poorly illuminating surfaces. Orientation had significant effects on the density of individuals (Table 1) (ANOVA, *Ciona intestinalis*, $F = 6,847$, $p < 0.0001$, Tukey test, $p < 0.05$, Upwards > Downward, both = Vertical; *Molgula manhattensis*, $F = 14,07$, $p < 0.0001$, Tukey test, $p < 0.01$, Downwards > Upwards and Vertical; *Ascidella aspersa*, $F = 9,025$, $p = 0,0001$, Tukey test, $p < 0.05$, Upwards > Downwards, both = Vertical. In the case of *Phallusia mammilata*, $F = 13,16$, $p < 0.0001$ no significant differences among orientations was found. *Ciona intestinalis* showed Bartlett's statistic (corrected) =23,06 and R squared =6,847 and was most abundant on downward-facing surfaces, and both *Molgula manhattensis* and *Ascidella aspersa* were more abundant on downward and vertical surfaces with Bartlett's statistic (corrected) ranges from zero to 20,97 and R squared =0, 5751 - 0, 6896. *Phallusia mammilata* $F = 13,16$, $p < 0.0001$, Tukey test, $p < 0.05$, Upownwards > Downwards, both = Vertical, Bartlett's statistic (corrected) =30,03 and R squared =0,6638. Light intensities were usually highest on vertical surfaces (Histogram 1) due to the characteristics of the floating panels from where the animals were collected, Except for *Molgula manhattensis*, the only species collected from iron platforms and bottom of ships in study shore localities. Low light intensities on upward-facing surfaces for the remaining species reflected the fact that they grew on artificial substrata (panels) that were poorly illuminated due to other structures that screened them.

b) Effects of light and orientation on larval settlement

In the first experiment, results for *Ciona intestinalis* and *Molgula manhattensis* showed that settlers have an obvious difference between darkness and illumination. For the remaining species, *Ascidella aspersa* and *Phallusia mammilata* there was significant interaction of the light with the position of the settlers (Histogram 2 and Table 2). When the two factors were analyzed separately, no effect of the light/dark treatment was found (Histogram 2, Table 2 and *t*-tests on proportion of settlers: all $p > 0.05$). For the position factor, *Ciona intestinalis* showed a clear preference for settlement on top surfaces Top > Lateral = Bottom, proven through Log-likelihood and LR Chi-Square where 122.300, 2.561 and 0.369 represent top, lateral and

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bottom positions respectively, whereas the three species *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia mammilata* settled significantly more often on the bottom than elsewhere Bottom > Lateral = Top (Histogram 2, Table 2).

In the second experiment, in which the larvae had the option of settling on light or dark surfaces in the same chamber, a different picture emerged (Table 3, Histogram 3). Few number of larvae preferred top position in case of *Ciona intestinalis* regardless of light direction dark = light.

For *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia mammilata*, significant interaction was found between treatment and position, showing a marked preference for dark surfaces, and bottom orientation. It was found also that larvae of *Ascidella aspersa* and *Phallusia mammilata* continued to prefer bottom surfaces in the light but selected both bottom and top in the dark. *Molgula manhattensis* changed light preferences depending on the surface considered, but overall more larvae settled in light, and it preferred lateral surfaces in the illuminating part of the wells. These results are generally in accordance with what found in the field for adults of *Ciona intestinalis*, *Ascidella aspersa and Phallusia mammilata* (Histogram 1), all of which settled in the dark, and also for *Ascidella aspersa*, which (largely) settled in the light. The four species that displayed significant geotactic patterns in the first experiment shifted to a more random pattern in the second experiment, with two (*Ciona intestinalis* and *Molgula manhattensis*) now showing no geotactic preferences, and the other two species (*Ascidella aspersa and Phallusia mammilata*) showing greater settlement on lateral and top surfaces than previously. When the two factors are considered in the experiment (position of larval settlement and direction of light), darkness enhance larvae of *Molgula manhattensis, Ascidella aspersa and Phallusia mammilata* to prefer settlement on the bottom where bottom > lateral = top but *Ciona intestinalis* showed no significant results for this experiment.

c) *Effect of mantle tissues extract*

Ascidella aspersa showed little effect of mantle tissues extract in the water (Histogram 4, Table 4 and Turkey`s Multiple Comparison tests, $p > 0.05$). The other three species *Ciona intestinalis*, *Molgula manhattensis* and *Phallusia mammilata* showed a significant inhibition of settlement in the presence of mantle tissues extract, no extract > extract (Histogram 4, Table 4, and Turkey`s Multiple Comparison tests, all $p < 0.05$). Although in *Ciona intestinalis* the log-linear analysis revealed a significant interaction, with the extract inhibition being significant for the lateral and top surfaces only. The geotactic behavior found in the first experiment testing light/dark effects was maintained across all species in this third experiment, with the three *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia mammilata* settling preferentially on the bottom (Histogram 4). For *Ciona intestinalis*, the highest number of settlers was again on top surfaces, although in the presence of adult extract there was no significant difference between top and bottom (Table 4). For *Ascidella aspersa* there was no position effects, and for *Phallusia mammilata* there was no effect of either extract or position on settlement in the wells.

d) *Integrating field and laboratory data*

Comparing the level of aggregation and the overall abundance of individuals in the field (Histograms 1 - 5 & Tables 1 - 5), a consistent pattern emerged: the more abundant a species was in a particular orientation, the more individuals there were per clump. When analyzed, the number of individuals per clump across species, *Phallusia mammilata* showed the highest numbers (Histogram 5), but significant differences existed only between *Molgula manhattensis* and two other species *Ciona intestinalis* and *Ascidella aspersa* (ANOVA, $F = 8.075$, $p = 0.0084$, Turkey`s Multiple Comparison tests, Tukey test, *Molgula manhattensis* > *Ascidella aspersa* = *Ciona intestinalis*, $p < 0.05$). In terms of the numbers of individuals per clump in relation to orientation in the field (Histogram 5), significant differences emerged for two species (ANOVA, *Ciona intestinalis*, $F = 0.9956$, $p = 0.0802$, Turkey`s Multiple Comparison tests, Upwards greater than the other two orientations, $p < 0.05$; *Phallusia mammilata*, $F = 18.00$, $p < 0.0001$, Turkey`s Multiple Comparison tests, Downward greater than the other two orientations, $p < 0.001$).

For an overall perspective of the geotactic preference of each species, settlement data generated from the three laboratory experiments were pooled together , on the assumption that in terms of geotactic behavior, larvae in the field would encounter a combination of both phototactic stimuli and adult extracts. Setting aside *Ciona intestinalis* and *Ascidella aspersa* on the grounds that their settlement rates were too low for consideration, the mean percentage of settlers on each surface showed the same trend as the number of individuals per clump for three species (*Molgula manhattensis*; *Ascidella aspersa* and *Phallusia mammilata*), whereas *Ciona intestinalis* showed no correlation (Histogram 5).

Three trends emerged from the laboratory data (as summarised in Table 5). First, in relation to orientation, one species (*Ciona intestinalis*) tended to settle preferentially on the top, whereas three (*Molgula manhattensis; Ascidella aspersa and Phallusia mammilata*) preferred settling on the bottom in experiment 1, with almost the same pattern emerging in experiment 3. In experiment 2 the geotactic responses evident in experiment 1 were either absent or altered. *Molgula manhattensis* and *Phallusia mammilata* could be

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analyzed with respect to geotactic behavior only in experiment 3, and neither showed any preference. Second, in terms of light/dark responses, none of the four species analyzed showed any statistical preferences in experiment 1, where the larvae were held either in light or dark. However, in experiment 2, when they had a choice between dark and light, three species (*Molgula manhattensis*, *Ascidella aspersa* and *Phallusia mammilata*) displayed preference for settling in the dark, and a fourth (*Ciona intestinalis*) settled most often in the light, although this preference changed on bottom surfaces, leading to an interaction between the factors.

Third, in relation to the presence or absence of adult mantle tissues extract in the third experiment, two species (*Ascidella aspersa* and *Phallusia mammilata*) showed no response of mantle tissues extract in the water, while settlement of the other two species (*Ciona intestinalis and Molgula manhattensis*) was inhibited in the presence of mantle tissues extract.

e) Laboratory observation on the gonad of the adult stage of Phallusia mammilata

The single hermaphrodite gonad is present inside the intestinal loop (Fig. 4). The ovary appeared massive with yellowish-orange tinge in all specimens dissected in the different seasons of the year. It extended anteriorly along the dorsal side of the animal as long oviduct which runs parallel to the intestine and terminated inside the atrial cavity. The macroscopic observation of the ovary did not show any details. As the gonad was longitudinally and very superficially incised at its narrower end, a milky yellowish-colored inclusion release and this represents the sperm suspension but when incised deeper at its wider end, the oocytes are seen and appeared to be arranged linearly parallel to each other being crowded in the central lumen of the ovary. The macroscopic observations revealed that the gonad is sac-like structure which is more wider at its distal extremity and narrows gradually near the rectum then join a narrow gonoduct which was orange in color and full of rounded masses of large oocytes. These large masses of oocytes are always with orange tinge having peripheral nuclei and with dark ooplasm. The testis in gross anatomy is over-looked, however, testicular diverticulae can be observed on the outer wall of the stomach and intestine. At the anterior apex of the stomach the gonad was represented only by the testis. This male part is branched and ramified into several testicular diverticulae. They were attached on the outer wall of the stomach and surrounded with the outer atrial wall being dominant and in excess inside the intestinal loop. The germinal epithelium of all diverticulae is in continuation as a single lamina. Underneath the simple cubical germinal epithelium the different stages of spermatozoa development could be seen. Along the dorsal side of the animal and parallel to the rectum these branches of testis join each other forming a common vas deferens which open in the atrial cavity. The ovary is a saccular structure lied under the testis and more posteriorly oriented. It is surrounded with the outer atrial wall externally. The ovary in the macroscopic observation seemed to be one part but histologically the germinal epithelium marked the external boundary of the ovary and gave off internal branches that unite each other (Fig. 4). The germinal epithelium divided the ovary internally into lobules. In each lobule the different stages of oocyte development are observable. Both the sperm duct and oviduct are much longer and have no relation with the mantle. The vas deferens attached along the intestine and the oviduct lied on its top. Different seminiferous tubules (testicular diverticulae) of the same animal showed different histological appearance i.e., the process of spermatogenesis and spermeogenesis (spermeohistogenesis) were well observed in all histological preparations taken in the different seasons of the year. This means that the testis is always mature all the year round. At a high magnification there are some very small rounded cells darkly stained and scattered in between the spermatozoa. They are larger in size than the cells in the spermatid layers. The different stages of oocyte development were described and counted in three ovarian lobules taken from three different squirts in each season of the year.

f) Squirts collected during spring

i. *First ovarian lobule*

Small-sized oocytes (50-70 μm) were arranged along the germinal epithelium (Figs. 5-6). These masses of small oocytes gained blue stain with different histological dyes. The small oocytes near the germinal epithelium are surrounded by very small rounded haemocytes which are randomly scattered inside the matrix of the ovary (Fig. 8). The small oocytes were present in aggregates inside the lobules of the single ovary. The ooplasm was homogenous without granulation and vacuolated, nucleus was pale while nucleolus seemed darkly stained. Each small oocyte was bounded externally with a darkly stained cell membrane and a single layer of follicular epithelium. There was another kind of oocytes scattered randomly inside the lobules (Fig. 9). They are large-sized (80- 100 μm), having dark granulated ooplasm, The small rounded cells around the small oocytes are completely absent in the vicinity of these large ones. These masses of large oocytes are follicular. At the periphery of ooplasm scattered test cells were observed. These large oocytes are always oval-shaped.

There is another type of large follicular oocytes (110-130 μm) having granulation only at the periphery of ooplasm which was darkly stained, while in the central region of ooplasm lacked granulation and this region is pale stained completely similar to the staining affinity of the ooplasm of the small oocytes. The matrix of the

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ovary was of connective tissue and blood vessels. See Histogram 6 & Figs. 4-9 for review.

ii. *Second & third ovarian lobules*

The same ovarian appearance of the first preparation but the ovary contains variable number of follicular oocytes. The internal lobulation of the ovary was well-marked. The internal folded germinal epithelium divided the lumen of the ovary into compartments. These internal compartments contained the different stages. Some atretic large-sized oocytes (160 – 180 μm) were observed.

g) Squirts collected during summer

i. *First & second ovarian lobules*

Small oocytes (50 - 70 μm) were arranged at the periphery of the ovary near the germinal epithelium and each one was surrounded externally with small rounded cells. Each small oocyte was bounded with a dark cell membrane, ooplasm was pale stained, homogenous without granulation but vacuolated, nucleus was pale while the nucleolus was darkly stained. Large follicular oocytes (170 - 180 μm) were observable (Fig. 10). They were distributed randomly inside the ovary and having dark granulated ooplasm and chorion. The outer follicular squamous cells were elevated over the inner follicular cells. The nucleus was pale while the nucleolus was darkly stained. Another type of oocytes can be seen inside the ovary. This oocyte was larger in size, oval-shaped and completely decayed ruptured inclusions inside, without definite outer membrane (Fig. 11). It seems to be undergone autolysis. The matrix of the ovary was more homogenous than that of the ovary of squirts in the preceding period. See Histogram 6 & Figs. 10-11 for review.

ii. *Third ovarian lobule*

Very small rounded oocytes (30 -50 μm) were observable attached to the germinal epithelium. Moderately-sized oocytes (80 -100 μm) were scattered randomly inside the lobules of the ovary and they were surrounded externally with very small cells. In this preparation , large-sized masses of oocytes were observed. These oocytes were rounded and rarely oval shaped. The ooplasm was dark stained and strongly granulated. The granules were darker than the ooplasm itself. At the periphery of each oocyte there were a dark chorion, a pervitelline space, test cells arranged at the periphery of cytoplasm, an inner and an outer follicular epithelia, The nucleus was always pale while the nucleolus is darkly stained. Atretic large-sized oocytes were present. They haven't a definite shape, their outer follicular epithelium was discontinuous, Moreover inside the ooplasm many vacuoles were present. The matrix was crowded with connective tissue, haemocytes and decayed contents.

h) Squirts collected during autumn

i. *First ovarian lobule*

The lobulated ovary was compact and completely full of oocytes. Very small oocytes presented near the germinal epithelium surrounded with very small cells. Moderately-sized oocytes were distributed randomly. Large follicular oocytes were found and distributed randomly. Atretic oocytes are in excess. The matrix was full of connective tissue, small cells surround the small and moderately-sized oocytes (haemocytes) and decayed contents. See Histogram 6 & Figs. 4-11 for review.

ii. *Second & third ovarian lobules*

The lobulated ovary was compact and full of oocytes at different stages of development. The only difference between these two ovaries and these described in the first preparation was that the germinal epithelium here was stretched because of the huge amount of oocytes inside the ovary.

i) Squirts collected during winter

i. *First, second & third ovarian lobules*

Small-sized oocytes were rare near the germinal epithelium. Full follicular oocytes (170 – 180 μm) were distributed randomly inside the matrix. Each small or follicular oocyte was surrounded with very small-sized cells (haemocytes), rounded or oval follicular oocytes were present in excess. Atretic large-sized oocytes were observable. See Histogram 6 & Figs. 4-11 for review.

IV. Discussion

Many benthic marine invertebrates reproduce by releasing sperm into the sea (free-spawning), but the amount of time that sperm are viable after spawning may have different consequences for fertilization, depending on the type of free-spawner. In egg-broadcasting marine organisms, gamete age is usually assumed to be irrelevant because of the low probability of contact between dilute sperm and egg. However, direct dilution effects might be reduced in egg-brooding free-spawners that filter dilute sperm out of the water column, and sperm longevity may play a role in facilitating fertilization in these taxa. To a large extent, the range of conditions where adults of each species occurred in the field correlated well with the behavior of the larvae in the laboratory. *Ciona intestinalis* is a common fouling species in sheltered marine and harbours (Dybern, 1965 ; Monniot et al. 2001; Lambert & Lambert 2003), where it is found in relatively dark places on the lower surfaces of substrata. This study concluded that *Ciona intestinalis* and *Molgula manhattensis* were most abundant on poorly illuminating surfaces, while *Ascidella aspersa* and *Phallusia mammilata* individuals densities were found in poorly illuminating and well illuminating surfaces. Orientation had significant effects on the density of individuals. Moreover, no significant

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differences were found among settlement orientations in *Ciona intestinalis*. Correlated with this, its larvae showed preferences to settle on beneath the upper surface of the experimental wells. Both *Molgula manhattensis* and *Ascidella aspersa* were more abundant on downward and vertical surfaces. *Phallusia mammilata* preferred to settle Upownwards > Downwards, both = Vertical. The field study concluded that low light intensities on upward-facing surfaces for the remaining species *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia mammilata* reflected the fact that they grew on artificial substrata (panels) that were poorly illuminated due to other structures that screened them. Light intensities were usually highest on vertical surfaces due to the characteristics of the floating panels from where the animals were collected, *Ciona intestinalis* lives on well-illuminating upper or lateral surfaces and its larvae settled on the bottoms or sides of wells and preferred light conditions when settling on the sides. *Molgula manhattensis, Ascidella aspersa* and *Phallusia mammilata* adults displayed clear preferences for dark surfaces, and accordingly their larvae preferred dark conditions and upward-facing surfaces. *Ciona intestinalis* exhibited no habitat preference in the field and no preferential geotactic or phototactic larval responses. When the phototactic preference for dark places and geotactic behavior in those species with clear orientation preference were supposed, emphasising the importance of settlement in determining adult distribution patterns, with three of the four species displaying larval behavior that was in agreement with field observations. In addition, this study showed how the biotic factor examined (presence or absence of mantle tissues extract) and the two abiotic factors (phototaxis and geotaxis) can play an integrated role in determining settlement patterns, providing insight into how such factors may influence adult distribution in the field. In the first experiment, when larvae were held under either light or dark conditions, geotactic preferences drove larval behavior. However, in the second experiment, when larvae had the option of choosing between shaded and light conditions, three species clearly preferred to settle on dark surfaces. Our results are in accordance with the general statement that shading facilitates the dominance of hard substrata by sessile invertebrates while well-illuminating surfaces lead to algal-dominated communities (Miller and Etter 2008). For those species settling in the dark, this might incidentally lead to settlement among adult conspecifics, where light is reduced in the shade of adults, ultimately contributing to a gregarious distribution.

An interesting result of the second experiment was that few number of settlers on less illuminating surfaces in case of *Ciona intestinalis*. On the contarary, in case of *Molgula manhattensis, Ascidella aspersa* and *Phallusia mammilata*, no significant interaction was found between treatment and position. *Molgula manhattensis* changed light preferences depending on the surface considered, but overall more larvae settled in light, and it preferred lateral surfaces in the illuminating part of the wells. Moreover, the three last species showed a marked preference for dark surfaces, and no significant preference for any orientation. It was found also that larvae of *Ascidella aspersa* and *Phallusia mammilata* continued to prefer bottom surfaces in the light but selected both bottom and top in the dark. These results are generally in accordance with what found in the field for adults of *Ciona intestinalis*, *Ascidella aspersa* and *Phallusia mammilata*, all of which settled in the dark, and also for Ascidella aspersa, which (largely) settled in the light. The four species that displayed significant geotactic patterns in the first experiment shifted to a more random pattern in the second experiment, with two (*Ciona intestinalis* and *Molgula manhattensis*) now showing no geotactic preferences, and the other two species (*Ascidella aspersa* and *Phallusia mammilata*) showing greater settlement on lateral and top surfaces than previously. A considerable observation noticed in the laboratory that larvae of all species altered their geotactic behavior from that displayed in the first experiment, showing a more haphazard geotactic settlement distribution or alteration of preferences in the second experiment. These results contrast with what has previously been found for the tadpole larvae of another solitary ascidian (*Ascidia mentula*) and for the planulae of a scyphozoan, in which the larvae did not alter their negative geotactic behavior across a range of light conditions. This study suggests that during settlement, time of day and weather conditions (which can alter light conditions) may greatly influence larval behavior. Most species of ascidians are invasive (M. Rius, C.L. Griffiths and X. Turon, in preparation) and have succeeded in establishing populations worldwide (Lambert & Lambert 2003; Barros et al. 2009). The fact that there were no settlement preferences in either of these species may indicate that they can successfully settle under a range of conditions and on a range of surfaces, increasing the likelihood of their colonising new localities. Young and Braithwaite (1980) have shown that *Styela montereyensis*, like *Styela plicata* and *Ascdilla aspersa*, shows no discrimination with respect to light or substratum type. Similarly, Young and Chia (1985) failed to find any settlement preferences in six other solitary ascidian species that were exposed to different light regimes. In this study it was found that strong patterns in the terms of light/dark responses, none of the four species analyzed showed any statistical preferences in experiment 1, where the larvae were held either in light or dark. However, in experiment 2, when they had a choice between dark and light, three species (*Molgula manhattensis, Ascidella aspersa* and *Phallusia mammilata*) displayed preference for settling in the dark,

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and a fourth (*Ciona intestinalis*) settled most often in the light, although this preference changed on bottom surfaces, leading to an interaction between the factors. In relation to orientation, one species (*Ciona intestinalis*) tended to settle preferentially on the top, whereas three (*Molgula manhattensis; Ascidella aspersa* and *Phallusia mammilata*) preferred settling on the bottom in experiment 1, with almost the same pattern emerging in experiment 3. In experiment 2 the geotactic responses evident in experiment 1 were either absent or altered. *Molgula manhattensis* and *Phallusia mammilata* could be analyzed with respect to geotactic behavior only in experiment 3, and neither showed any preference. Perhaps the ocelli on the trunk play a role in response to light intensities in the ascidian larvae.

This contrasts with the behavior of the larvae of a closely related species that lacks photoreceptors, *M. exasperatus*, which displays no light sensitivity or preferences (Svane & Young 1991). Both conspecific attraction and gregarious behavior have been identified as driving forces for the distribution of many organisms (Alonso et al. 2004; Gautier et al. 2006). This study concluded that in relation to the presence or absence of adult mantle tissues extract in the third experiment, *Ascidella aspersa* showed little effect of mantle tissues extract in the water while the other three species *Ciona intestinalis* , *Molgula manhattensis* and *Phallusia mammilata* showed a significant inhibition of settlement in the presence of mantle tissues extract. Similar to these findings, the percentage of metamorphosis of the solitary ascidian *Molgula citrina* decreases when its larvae are exposed to conspecific tunic homogenate (Dupont, et al. 2006). This has implications for understanding how prior invasions might affect further colonization. This study showed also that settlement was not promoted by the presence of adult extracts. However, it is possible that the adult extracts acted as a repellent because they signaled damaged tissues of a conspecific; but other authors using adult extracts have found that the presence of extracts induced metamorphosis (Svane et al. 1987), so this study has found the same result for two studied species *Ascidella aspersa* and *Phallusia mammilata*. This study suggests that the gregarious distribution of adults observed in the field are unlikely to be explained by larval attraction to adult extracts, but may be the result of settlement being concentrated in habitats characterised by particular physical conditions. For many other marine species, physical factors seem to be stronger factors for settlement than chemical attraction by conspecific adults (Berntsson et al. 2004). Sometimes these preferred physical conditions such as light intensity and hydrodynamic conditions may coincidentally be associated with the presence of adults, or even created by adults, leading indirectly to aggregations. For instance, a baffle effect of created by aggregations of adults (Eckman, 1983) may enhance the settlement of new larvae and protect the juveniles, thereby increasing their survival. However, more needs to be learnt concerning the mechanisms driving the effect of conspecific adult attraction and further experiments using gregarious ascidians have the potential to provide important insights. In confined environments, such as harbours and marine environment, where invasive ascidians are highly successful, the specific biological features of each species such as larval movement and offspring retention, the particular hydrodynamics of the location (Havenhand & Svane 1991) and adequate conditions for settlement (as shown in this study) may play important roles in influencing species distributions and the success of introduced populations. For example, *Ciona intestinalis* is widespread in estuarine conditions in harbours and successfully colonizes the culture ropes of mussel farms, with important economic impacts (Robinson, et al. 2005), as also in northeast American coastal waters (Ramsay, et al. 2008). Biotic and chemical factors, other than those arising from conspecific adults, may determine aggregated settlement of ascidians in the field (Hadfield & Paul 2001). However, results of this study favour the view that the aggregated distribution of the solitary ascidians considered reflects responses to abiotic rather than biotic factors, although there is always the possibility that complex biotic interactions, such as competition or facilitation, occur during juvenile and adult stages, as it has been demonstrated in other gregarious organisms (Rius & McQuaid 2009). There is a need to further study the mechanisms that determine gregarious distribution in invasive species. Comparisons of species performance and biology across both introduced and native ranges could be enlightening (Bossdorf, et al. 2005). Concepts such as conspecific and kinship attraction, and gregarious behavior should be incorporated to the study of the distribution of invasive species, as they might be key features for our understanding of the viability and success of these populations.

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Histograms

Histogram 1. Adult distribution in the field, indicated as the mean density of individuals, and mean light intensity (μ mol m-2 s-1) in relation to surface orientation. Error bars denote + 1 SE. Note differences in scales of y-axes

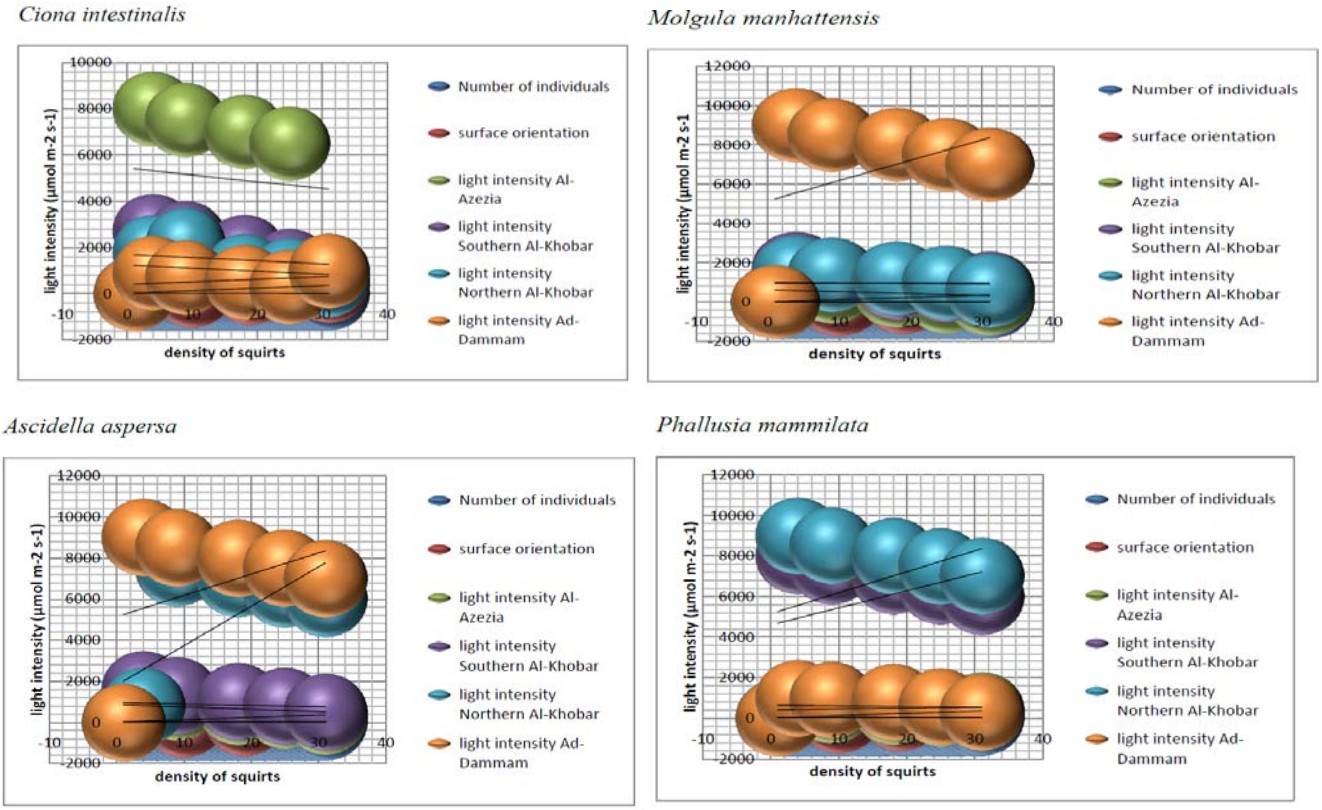
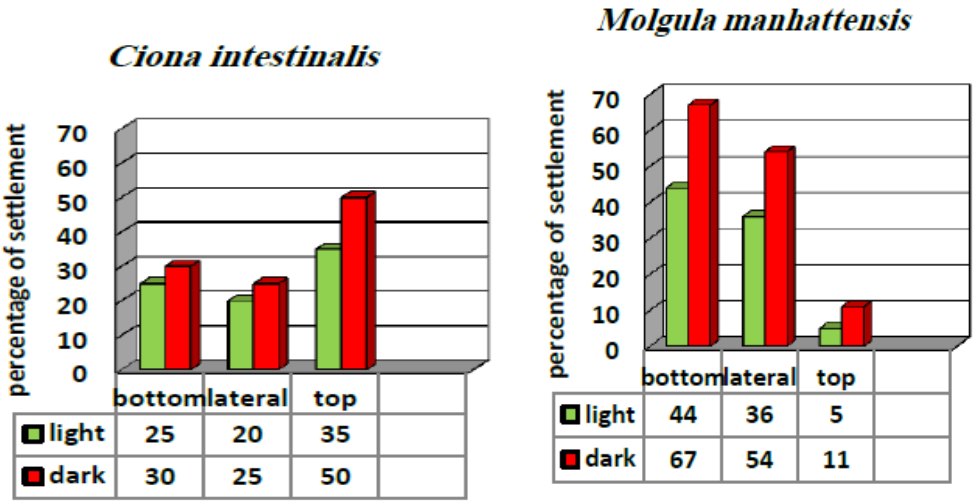


Fig. 1 : Phase contrast photomicrograph of a whole mount of a hatching larval stage of Ciona intestinalis (beginning of differentiation of the trunk and tail regions)

Histogram 2. Mean percentage settlement in relation to orientation (bottom, lateral or top) and treatment (light - green bars, dark - red bars) in the 1st experiment, in which larvae were held either in the dark or in the light. Error bars denote + 1 SE. Note differences in scales of y-axes



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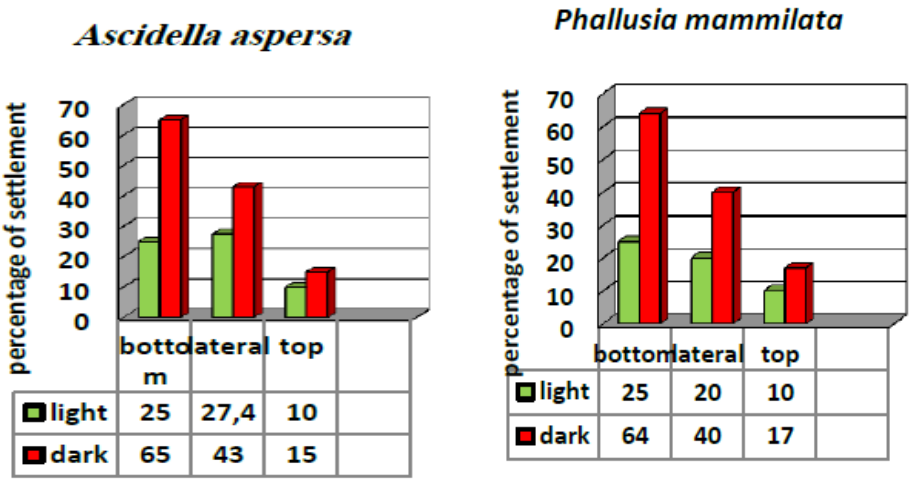

Fig. 2 : Phase contrast photomicrograph of a whole mount of a larval stage of Ciona intestinalis (Early tail resorption is indicated by an arrow)

Histogram 3. Mean percentage settlement in relation to orientation (bottom, lateral and top) and treatment (light - green bars, dark - red bars) in the 2nd experiment, in which larvae had the choice of settling in light or dark positions of the same wells. Error bars denote + 1 SE. Note differences in the scales of y-axes.

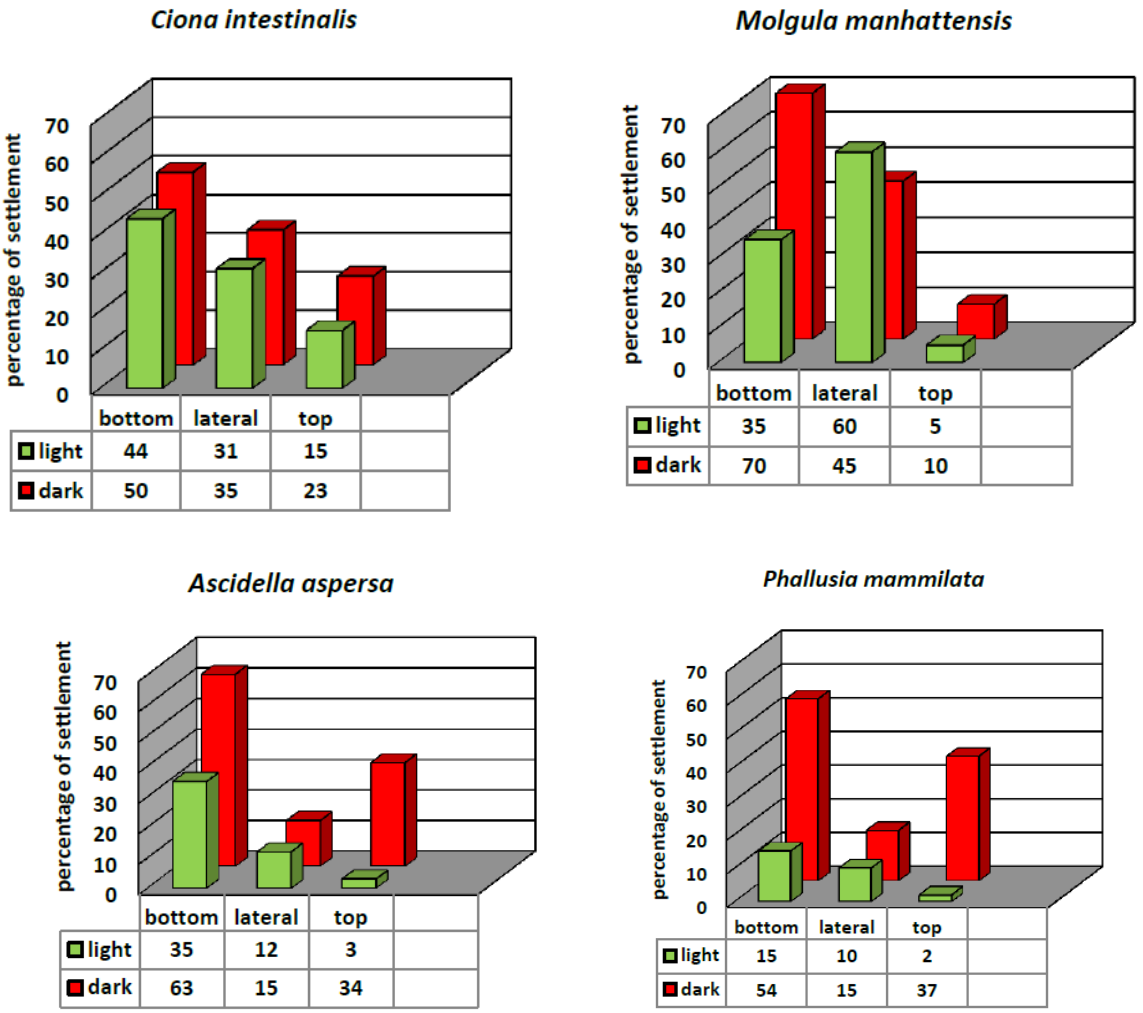

Fig. 3 : Phase contrast photomicrograph of a whole mount of a late metamorphosed larval stage of Ciona intestinalis

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Histogram 4. Mean percentage settlement with respect to orientation (bottom, lateral and top) and treatment (control - blue bars, mantle tissues extract - brown bars) in the 3rd experiment, in which larvae were held in wells either with or without adult extract. Error bars denote + 1 SE. Note differences in the scales of y-axes.

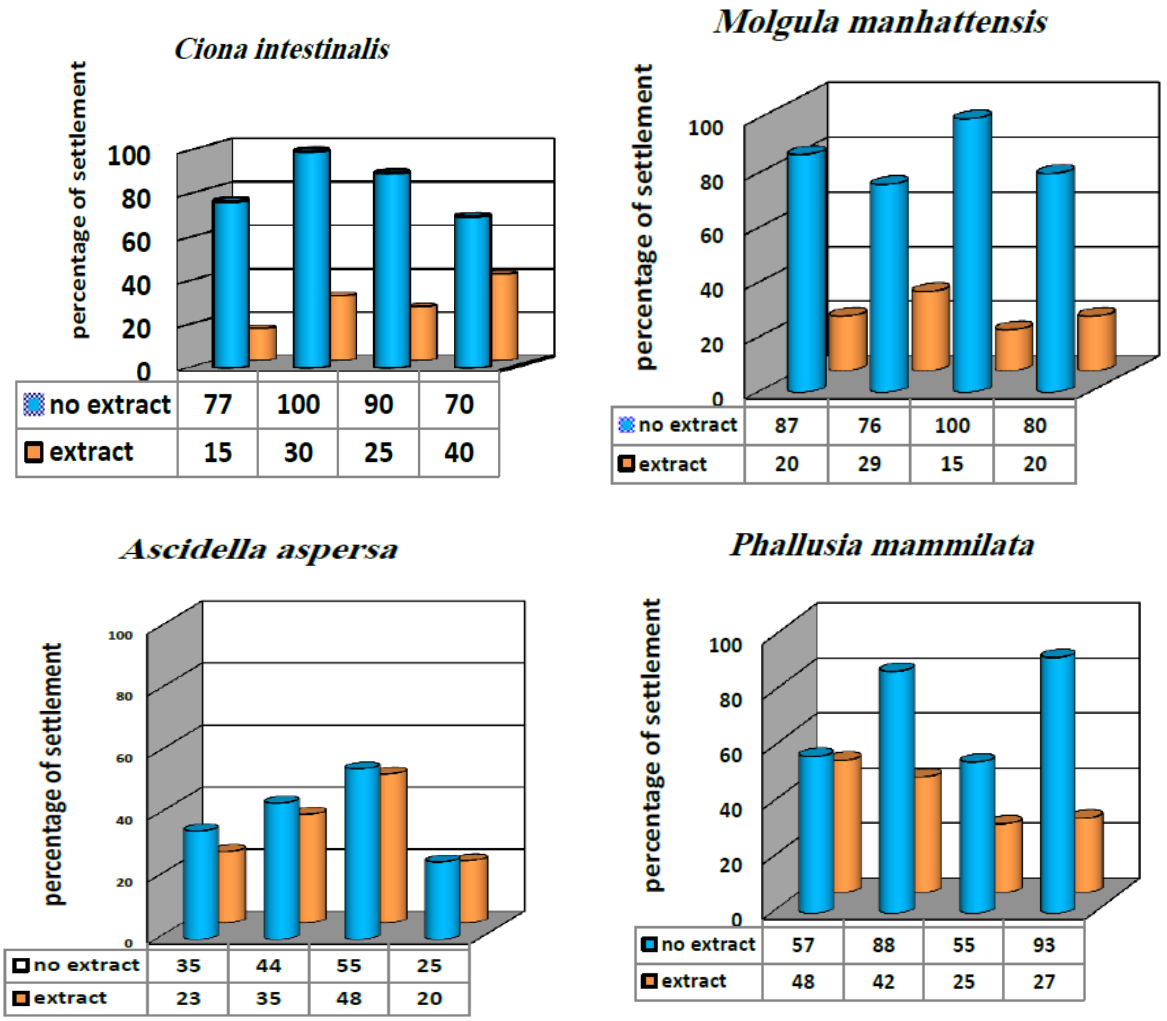
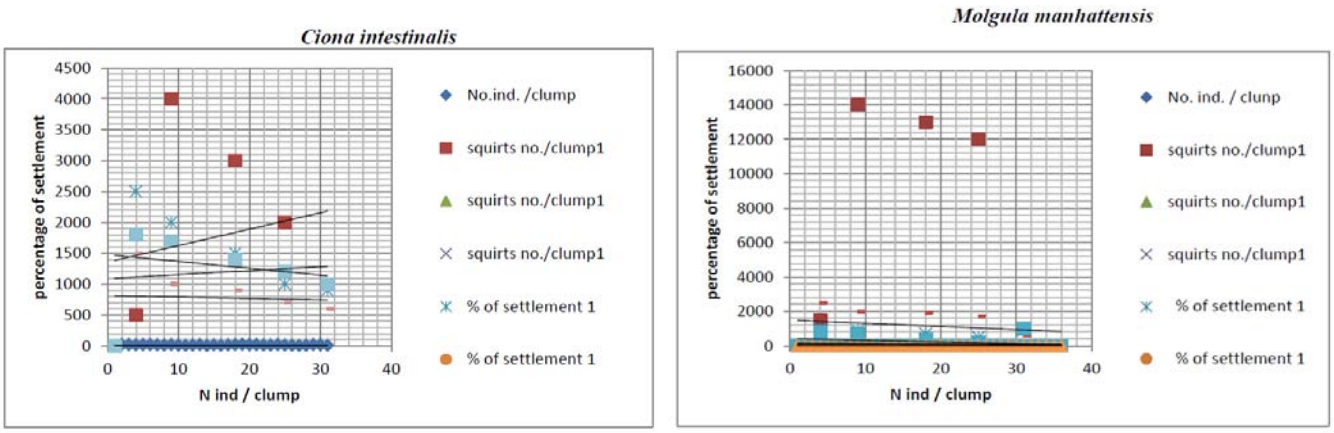


Fig. 4 : Photomicrograph of a transverse section of Phallusia mammilata showing the gonad. The squirt was collected during summer

Histogram 5. Mean percentage of settling larvae per clump from all the experiments, in relation to orientation. Error bars denote +1 SE. Note differences in scales of y-axes



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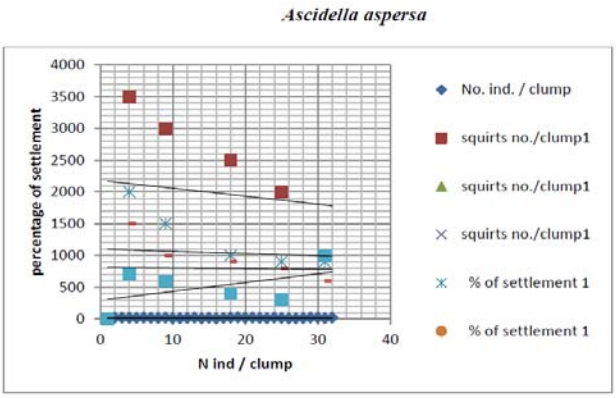
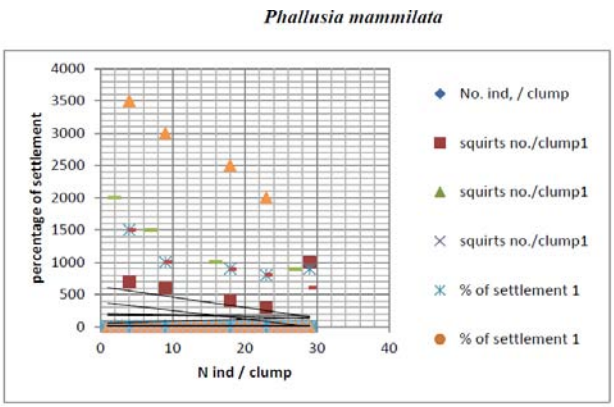
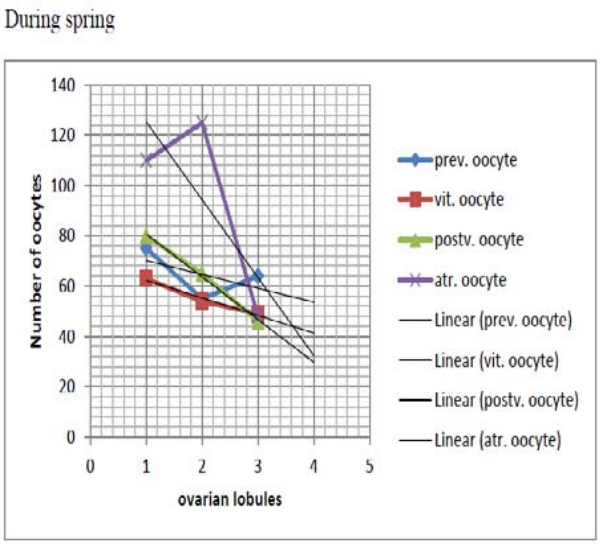
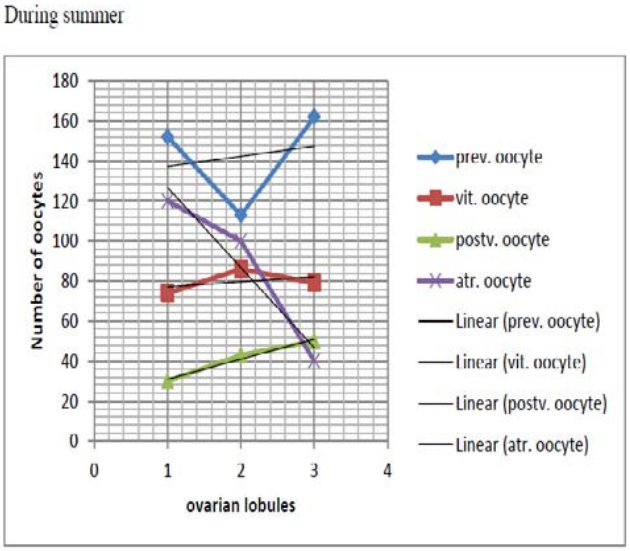


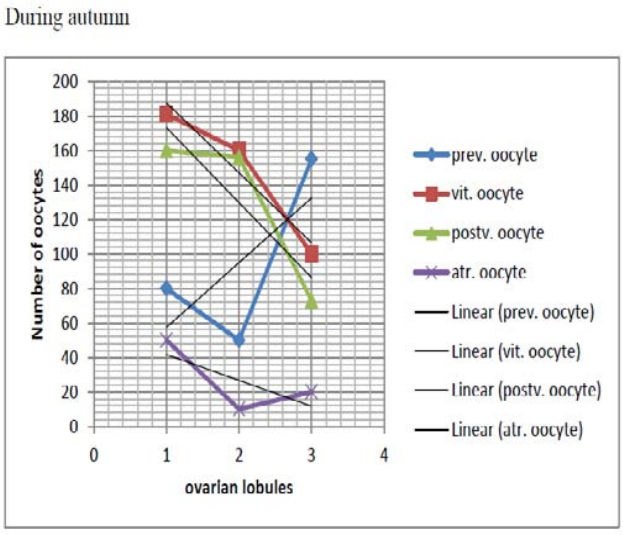


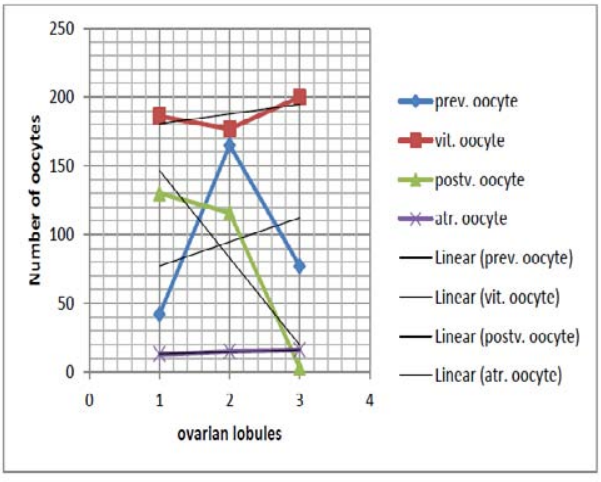
Figs. 5-6 : Photomicrograph of a transverse section of Phallusia mammilata ovary showing germinal epithelium and previtellogenic oocytes. The squirt was collected during spring

Histogram 6. Mean percentage of oocyte developmental stages of *Phallusia mammilata* along the four seasons of the year. Error bars denote +1 SE. Note differences in scales of y-axes









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Tables

Table 1 : Comparison of squirts density in the field, Note: One-way analysis of variance and Bartlett's test for equal variances were applied

Statistical analysis		*Ciona intestinalis*	*Molgula manhattensis*	*Ascidella aspersa*	*Phallusia mammilata*
One-way analysis of variance	P value	0,0023	P<0.0001	0,0006	P<0.0001
	Are means signif. different? (P < 0.05)	Yes	Yes	Yes	Yes
	Number of groups	4	4	4	4
	F	6,847	14,07	9,025	13,16
	R squared	0,5067	0,6896	0,5751	0,6638
Bartlett's test for equal variances	P value	P<0.0001	P<0.0001	P<0,0001	P<0.0001
	Do the variances differ signif. (P < 0.05)	Yes	Yes	Yes	Yes

Table 2 : Tukey's Multiple Comparison Test explaining mean percentage of settlement in relation to squirts orientation towards light direction

Ciona intestinalis				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
bottom vs lateral	6,5	1,019	P > 0.05	-20.29 to 33.29
bottom vs top	26,13	4,094	P > 0.05	-0.6672 to 52.92
bottom vs bottom	25,75	4,036	P > 0.05	-1.042 to 52.54
lateral vs top	19,63	3,076	P > 0.05	-7.167 to 46.42
lateral vs bottom	19,25	3,017	P > 0.05	-7.542 to 46.04
top vs bottom	-0,375	0,05877	P > 0.05	-27.17 to 26.42
Molgula manhattensis				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
bottom vs lateral	35	5,286	P < 0.05	5.015 to 64.99
bottom vs top	44,67	6,747	P < 0.01	14.68 to 74.65
bottom vs bottom	-10	1,51	P > 0.05	-39.99 to 19.99
lateral vs top	9,667	1,46	P > 0.05	-20.32 to 39.65
lateral vs bottom	-45	6,797	P < 0.01	-74.99 to -15.01
top vs bottom	-54,67	8,257	P < 0.01	-84.65 to -24.68
Ascidella aspersa				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
bottom vs lateral	15,63	1,391	P > 0.05	-31.54 to 62.79
bottom vs top	25,25	2,248	P > 0.05	-21.91 to 72.41
bottom vs bottom	8,375	0,7456	P > 0.05	-38.79 to 55.54
lateral vs top	9,625	0,8569	P > 0.05	-37.54 to 56.79
lateral vs bottom	-7,25	0,6455	P > 0.05	-54.41 to 39.91
top vs bottom	-16,88	1,502	P > 0.05	-64.04 to 30.29
Phallusia mammilata				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
bottom vs lateral	37,33	3,035	P > 0.05	-18.38 to 93.04
bottom vs top	51,33	4,173	P > 0.05	-4.376 to 107.0
bottom vs bottom	6,667	0,542	P > 0.05	-49.04 to 62.38
lateral vs top	14	1,138	P > 0.05	-41.71 to 69.71
lateral vs bottom	-30,67	2,493	P > 0.05	-86.38 to 25.04
top vs bottom	-44,67	3,631	P > 0.05	-100.4 to 11.04

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Table 3 : Tukey's Multiple Comparison Test explaining mean percentage of settlement in which larvae had the choice of settling in light or dark positions in the same wells of the tissue culture plates of the experiment

Ciona intestinalis				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
dark vs light	8	2,508	P > 0.05	-6.446 to 22.45
dark vs dark	12,17	3,814	P > 0.05	-2.279 to 26.61
dark vs light	14,5	4,546	P < 0.05	0.05427 to 28.95
light vs dark	4,167	1,306	P > 0.05	-10.28 to 18.61
light vs light	6,5	2,038	P > 0.05	-7.946 to 20.95
dark vs light	2,333	0,7315	P > 0.05	-12.11 to 16.78
Molgula manhattensis				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
dark vs light	20,33	6,686	P < 0.01	6.559 to 34.11
dark vs dark	10,33	3,398	P > 0.05	-3.441 to 24.11
dark vs light	23,17	7,617	P < 0.01	9.392 to 36.94
light vs dark	-10	3,288	P > 0.05	-23.77 to 3.774
light vs light	2,833	0,9316	P > 0.05	-10.94 to 16.61
dark vs light	12,83	4,22	P > 0.05	-0.9411 to 26.61
Ascidella aspersa				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
dark vs light	4,667	1,207	P > 0.05	-12.84 to 22.17
dark vs dark	-11,33	2,932	P > 0.05	-28.84 to 6.171
dark vs light	9,833	2,544	P > 0.05	-7.671 to 27.34
light vs dark	-16	4,14	P > 0.05	-33.50 to 1.504
light vs light	5,167	1,337	P > 0.05	-12.34 to 22.67
dark vs light	21,17	5,477	P < 0.05	3.663 to 38.67
Phallusia mammilata				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
dark vs light	7,333	1,775	P > 0.05	-11.38 to 26.05
dark vs dark	-14,33	3,469	P > 0.05	-33.05 to 4.382
dark vs light	7,833	1,896	P > 0.05	-10.88 to 26.55
light vs dark	-21,67	5,243	P < 0.05	-40.38 to -2.951
light vs light	0,5	0,121	P > 0.05	-18.22 to 19.22
dark vs light	22,17	5,364	P < 0.05	3.451 to 40.88

Table 4 : Tukey's Multiple Comparison Test explaining mean percentage of settlement in which larvae were held in wells either with or without adult mantle tissues extract

Ciona intestinalis				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
no extract vs extract	10,5	3,372	P > 0.05	-3.601 to 24.60
no extract vs no extract	-7,333	2,355	P > 0.05	-21.43 to 6.768
no extract vs extract	11	3,533	P > 0.05	-3.101 to 25.10
extract vs no extract	-17,83	5,728	P < 0.05	-31.93 to -3.732
extract vs extract	0,5	0,1606	P > 0.05	-13.60 to 14.60
no extract vs extract	18,33	5,888	P < 0.05	4.232 to 32.43
Molgula manhattensis				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
no extract vs extract	21,17	5,109	P < 0.05	2.402 to 39.93
no extract vs no extract	4,167	1,006	P > 0.05	-14.60 to 22.93
no extract vs extract	20,33	4,908	P < 0.05	1.568 to 39.10
extract vs no extract	-17	4,103	P > 0.05	-35.76 to 1.765
extract vs extract	-0,8333	0,2011	P > 0.05	-19.60 to 17.93
no extract vs extract	16,17	3,902	P > 0.05	-2.598 to 34.93
Ascidella aspersa				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
no extract vs extract	5,333	3,314	P > 0.05	-1.956 to 12.62
no extract vs no extract	-2	1,243	P > 0.05	-9.289 to 5.289

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no extract vs extract	6	3,728	P > 0.05	-1.289 to 13.29
extract vs no extract	-7,333	4,556	P < 0.05	-14.62 to -0.04421
extract vs extract	0,6667	0,4142	P > 0.05	-6.622 to 7.956
no extract vs extract	8	4,971	P < 0.05	0.7109 to 15.29
Phallusia mammilata				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
no extract vs extract	5,333	3,314	P > 0.05	-1.956 to 12.62
no extract vs no extract	-2	1,243	P > 0.05	-9.289 to 5.289
no extract vs extract	6	3,728	P > 0.05	-1.289 to 13.29
extract vs no extract	-7,333	4,556	P < 0.05	-14.62 to -0.04421
extract vs extract	0,6667	0,4142	P > 0.05	-6.622 to 7.956
no extract vs extract	8	4,971	P < 0.05	0.7109 to 15.29

Table 5 : Tukey's Multiple Comparison Test explaining mean number of squirts per clump in the field and settling larvae of all experiments in relation to orientation

Ciona intestinalis				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
squirts no./clump1 vs % of settlement 1	433,3	1,085	P > 0.05	-1147 to 2014
squirts no./clump1 vs squirts no ./clump2	966,7	2,42	P > 0.05	-614.1 to 2547
squirts no./clump1 vs % of settlement 2	566,7	1,419	P > 0.05	-1014 to 2147
% of settlement 1 vs squirts no ./clump2	533,3	1,335	P > 0.05	-1047 to 2114
% of settlement 1 vs % of settlement 2	133,3	0,3339	P > 0.05	-1447 to 1714
squirts no ./clump2 vs % of settlement 2	-400	1,002	P > 0.05	-1981 to 1181
Molgula manhattensis				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
squirts no./clump1 vs % of settlement 1	6250	4,503	P < 0.05	757.0 to 11740
squirts no./clump1 vs squirts no ./clump2	5467	3,939	P > 0.05	-26.32 to 10960
squirts no./clump1 vs % of settlement 2	6400	4,612	P < 0.05	907.0 to 11890
% of settlement 1 vs squirts no ./clump2	-783,3	0,5644	P > 0.05	-6276 to 4710
% of settlement 1 vs % of settlement 2	150	0,1081	P > 0.05	-5343 to 5643
squirts no ./clump2 vs % of settlement 2	933,3	0,6725	P > 0.05	-4560 to 6426
Ascidella aspersa				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
squirts no./clump1 vs % of settlement 1	950	2,935	P > 0.05	-331.3 to 2231
squirts no./clump1 vs squirts no ./clump2	1200	3,707	P > 0.05	-81.27 to 2481
squirts no./clump1 vs % of settlement 2	1500	4,634	P < 0.05	218.7 to 2781
% of settlement 1 vs squirts no ./clump2	250	0,7723	P > 0.05	-1031 to 1531
% of settlement 1 vs % of settlement 2	550	1,699	P > 0.05	-731.3 to 1831
% of settlement 3 vs % of settlement 4	300	0,9267	P > 0.05	-981.3 to 1581
Phallusia mammilata				
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
squirts no./clump1 vs % of settlement 1	-350	1,714	P > 0.05	-1149 to 449.5
squirts no./clump1 vs squirts no ./clump2	-509,1	2,836	P > 0.05	-1212 to 193.7
squirts no./clump1 vs % of settlement 2	-2250	9,856	P < 0.001	-3144 to -1356
% of settlement 1 vs squirts no ./clump2	-159,1	0,8863	P > 0.05	-861.9 to 543.7
% of settlement 1 vs % of settlement 2	-1900	8,322	P < 0.001	-2794 to -1006
squirts no ./clump2 vs % of settlement 2	-1741	8,43	P < 0.001	-2549 to -932.4

Table 6 : Tukey's Multiple Comparison Test explaining mean percentage of oocyte developmental stages of *Phallusia mammilata* along spring season

Table Analyzed				
Data Table-% of oocyte developmental stages along autumn season.				
One-way analysis of variance				
P value	0,2516			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	4			

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F	1,66			
R squared	0,3837			
ANOVA Table	SS	df	MS	
Treatment (between columns)	2624	3	874,8	
Residual (within columns)	4215	8	526,8	
Total	6839	11		
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
prev. oocyte vs vit. oocyte	9,333	0,7043	P > 0.05	-50.68 to 69.35
prev. oocyte vs postv. oocyte	1	0,07546	P > 0.05	-59.02 to 61.02
prev. oocyte vs atr. oocyte	-29,67	2,239	P > 0.05	-89.68 to 30.35
vit. oocyte vs postv. oocyte	-8,333	0,6288	P > 0.05	-68.35 to 51.68
vit. oocyte vs atr. oocyte	-39	2,943	P > 0.05	-99.02 to 21.02
postv. oocyte vs atr. oocyte	-30,67	2,314	P > 0.05	-90.68 to 29.35

Table 7 : Tukey's Multiple Comparison Test explaining mean percentage of oocyte developmental stages of *Phallusia mammilata* along summer season

Table Analyzed				
Data Table--% of oocyte developmental stages along summer season.				
One-way analysis of variance				
P value	0,0079			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	8,228			
R squared	0,7552			
ANOVA Table	SS	df	MS	
Treatment (between columns)	15690	3	5231	
Residual (within columns)	5086	8	635,8	
Total	20780	11		
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
prev. oocyte vs vit. oocyte	62,67	4,305	P > 0.05	-3.264 to 128.6
prev. oocyte vs postv. oocyte	101,3	6,961	P < 0.01	35.40 to 167.3
prev. oocyte vs atr. oocyte	55,67	3,824	P > 0.05	-10.26 to 121.6
vit. oocyte vs postv. oocyte	38,67	2,656	P > 0.05	-27.26 to 104.6
vit. oocyte vs atr. oocyte	-7	0,4809	P > 0.05	-72.93 to 58.93
postv. oocyte vs atr. oocyte	-45,67	3,137	P > 0.05	-111.6 to 20.26

Table 8 : Tukey's Multiple Comparison Test explaining mean percentage of oocyte developmental stages of *Phallusia mammilata* along autumn season

Table Analyzed				
Data Table- % of oocyte developmental stages along autumn season.				
One-way analysis of variance				
P value	0,0394			
P value summary	*			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	4,506			

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R squared	0,6282			
ANOVA Table	SS	df	MS	
Treatment (between columns)	25470	3	8491	
Residual (within columns)	15080	8	1884	
Total	40550	11		
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
prev. oocyte vs vit. oocyte	-52	2,075	P > 0.05	-165.5 to 61.51
prev. oocyte vs postv. oocyte	-34,67	1,383	P > 0.05	-148.2 to 78.84
prev. oocyte vs atr. oocyte	68,33	2,726	P > 0.05	-45.18 to 181.8
vit. oocyte vs postv. oocyte	17,33	0,6916	P > 0.05	-96.18 to 130.8
vit. oocyte vs atr. oocyte	120,3	4,801	P < 0.05	6.824 to 233.8
postv. oocyte vs atr. oocyte	103	4,11	P > 0.05	-10.51 to 216.5

Table 9 : Tukey's Multiple Comparison Test explaining mean percentage of oocyte developmental stages of *Phallusia mammilata* along winter season

Table Analyzed				
Data Table-% of oocyte developmental stages along winter season.				
One-way analysis of variance				
P value	0,0139			
P value summary	*			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	6,747			
R squared	0,7167			
ANOVA Table	SS	df	MS	
Treatment (between columns)	45550	3	15180	
Residual (within columns)	18000	8	2251	
Total	63560	11		
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
prev. oocyte vs vit. oocyte	-93	3,396	P > 0.05	-217.0 to 31.05
prev. oocyte vs postv. oocyte	11,67	0,426	P > 0.05	-112.4 to 135.7
prev. oocyte vs atr. oocyte	80	2,921	P > 0.05	-44.05 to 204.0
vit. oocyte vs postv. oocyte	104,7	3,821	P > 0.05	-19.38 to 228.7
vit. oocyte vs atr. oocyte	173	6,316	P < 0.01	48.95 to 297.0
postv. oocyte vs atr. oocyte	68,33	2,495	P > 0.05	-55.71 to 192.4

Legends

Fig. 7 : Photomicrograph of a transverse section of *Phallusia mammilata* ovary showing previtellogenic and early stage of vitellogenic oocytes. The squirt was collected during summer.

Fig. 11 : Photomicrograph of a transverse section of *Phallusia mammilata* ovary showing atretic oocytes. The squirt was collected during late winter.

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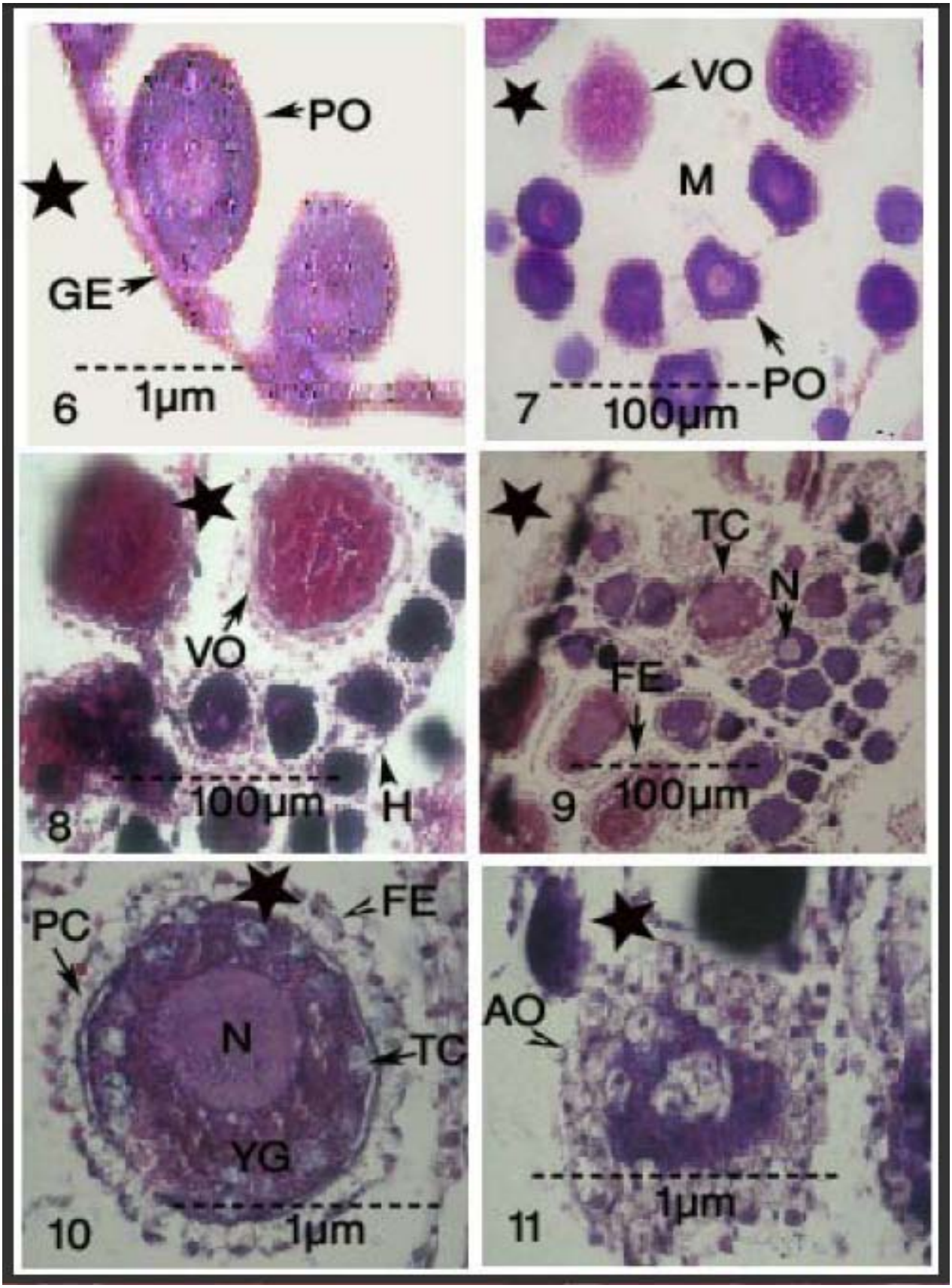

Figs. 8-9 : Photomicrograph of a transverse section of *Phallusia mammilata* ovary showing vitellogenic oocytes. Note, follicular epithelium and test cells were developed. The squirt was collected during autumn.

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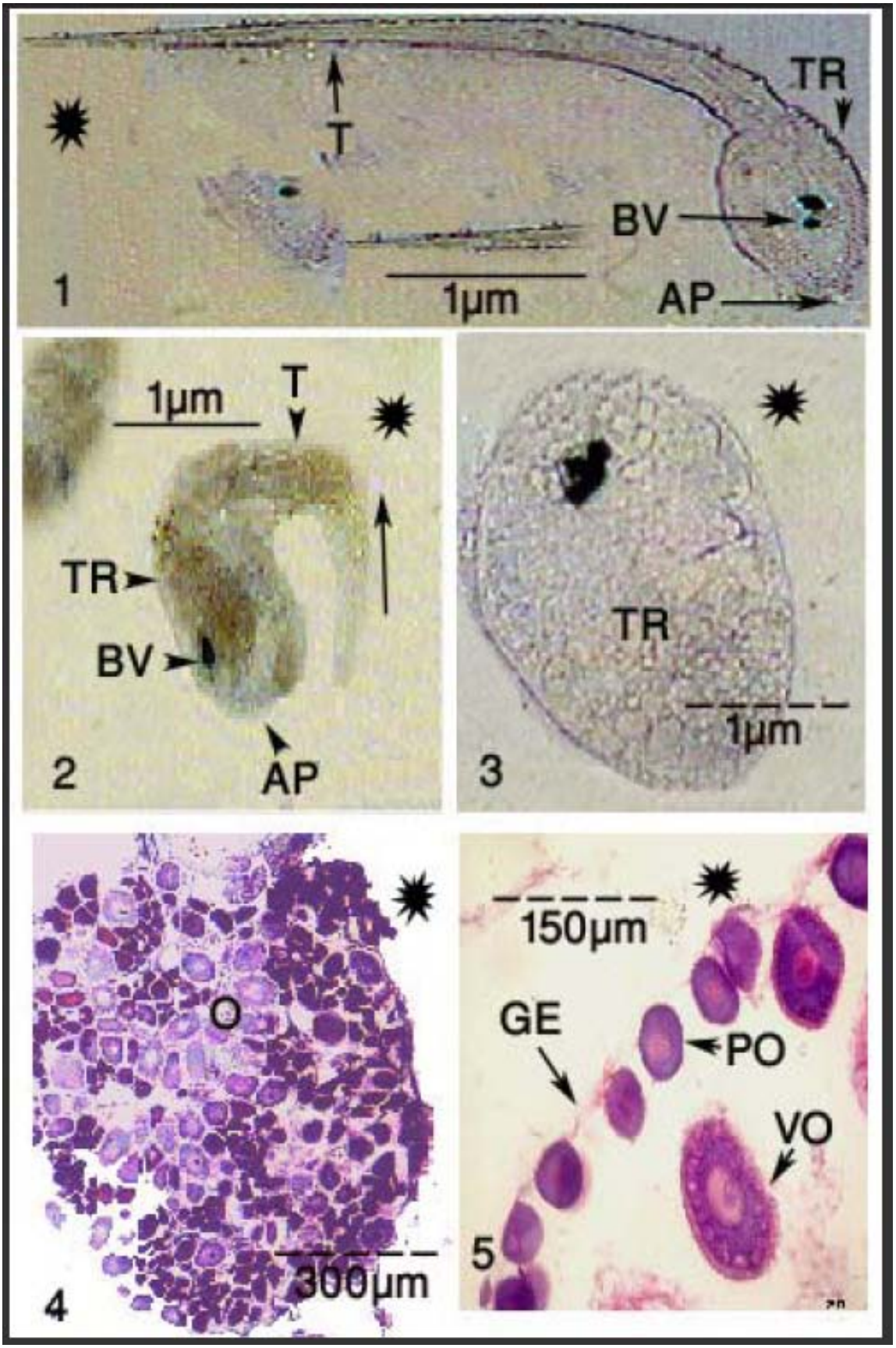

Fig. 10 : Photomicrograph of a transverse section of *Phallusia mammilata* ovary showing late stage of vitellogenic oocytes. Note, follicular epithelium, test cells and pervitelline space were developed. The squirt was collected during winter.

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Figs. 11 : Photomicrograph of a transverse section of *Phallusia mammilata* ovary showing atretic oocytes. The squirt was collected during late winter

Abbreviations

AO	Atretic Oocyte
AP	Adhesive Papillae
BV	Brain Vesicle
FE	Follicular Epithelium
HC	Haemocyte
O	Ovary
PO	Previtellogenic Oocyte
T	Tail
TR	Trunk
VO	Vitellogenic Oocyte
GE	Germinal Epithelium
M	Matrix
PS	Pervitelline Space
M	Martix
TC	Test Cell
N	Nucleus
YG	Yolk Granules

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