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Hcl Binary System

Methoxy Alkane Frankincense

Highlights

Sea Squirts and their Larvae

Metal Complexes Synthesis

Discovering Thoughts, Inventing Future

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First Reporting on the Chemistry and Biological Activity of a Novel Boswellia chemotype: The Methoxy Alkane Frankincense

By Prabodh Satyal & Robert S. Pappas
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Abstract- Two oleogum resin essential oils (from two different seasons: fall and summer), of *Boswellia spp.* (collected from Somalia), were obtained by hydrodistillation and analyzed by GC-MS. Out of 147 peaks, components were identified among the two essential oils accounting for 100%, and 99.7% of the oils, respectively. The two essential oils were dominated by the 1-methoxy alkane [octyl methyl ether (5.5-11.7%), and decyl methyl ether (30.6-54.9%)], α-pinene (0.3-11.5%), sabinene (2.1-7.2%), and α-bourbonene(1.7-5.7%). This is the first report of a methoxy alkane chemotyped frankincense essential oils as well as the first reporting of the natural occurance of decyl methyl ether (methyoxy decane) and octyl methyl ether (methyoxy octane). Monoterpenes chiral distributions were also measured and it was found that both of the oils have same enantiomeric ratio. Large chemical variation was attributed to seasonal variation. The essential oil harvested on fall season had also exhibited notable antimicrobial activities [*Aspergillus niger* (MIC = 39 μg/mL), *Candida albicans* (MIC = 78 μg/mL), *Bacillus cerreus* (MIC = 78 μg/mL), *Staphylococus aureus* (MIC = 78 μg/mL), and *Escherichia coli* (MIC = 78 μg/mL)], the essential oil also showed pronounced cytotoxic activities (100% kill on MCF-7 cells at 100 μg/mL).

Keywords: *decyl methyl ether, frankincense, cytotoxicity, antimicrobial, enantiomeric distribution, methoxy alkane.*

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FIRSTREPORTINGONTHECHEMISTRYANDBIOLOGICALACTIVITYOFANOVELBOSWELLIACHEMOTYPETHEMETHOXYALKANEFRANKINCENSE

Strictly as per the compliance and regulations of :

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Prabodh Satyal [α] & Robert S. Pappas [σ]

Abstract- Two oleogum resin essential oils (from two different seasons: fall and summer), of *Boswellia spp.* (collected from Somalia), were obtained by hydrodistillation and analyzed by GC-MS. Out of 147 peaks, components were identified among the two essential oils accounting for 100%, and 99.7% of the oils, respectively. The two essential oils were dominated by the 1-methoxy alkane [octyl methyl ether (5.5-11.7%), and decyl methyl ether (30.6-54.9%)], α-pinene (0.3-11.5%), sabinene (2.1-7.2%), and α-bourbonene (1.7-5.7%). This is the first report of a methoxy alkane chemotyped frankincense essential oils as well as the first reporting of the natural occurance of decyl methyl ether (methyoxy decane) and octyl methyl ether (methyoxy octane). Monoterpenes chiral distributions were also measured and it was found that both of the oils have same enantiomeric ratio. Large chemical variation was attributed to seasonal variation. The essential oil harvested on fall season had also exhibited notable antimicrobial activities [*Aspergillus niger* (MIC = 39 µg/mL), *Candida albicans* (MIC = 78 µg/mL), *Bacillus cereus* (MIC = 78 µg/mL), *Staphylococus aureus* (MIC = 78 µg/mL), and *Escherichia coli* (MIC = 78 µg/mL)], the essential oil also showed pronounced cytotoxic activities (100% kill on MCF-7 cells at 100 µg/mL).

Keywords: decyl methyl ether, frankincense, cytotoxicity, antimicrobial, enantiomeric distribution, methoxy alkane.

I. Introduction

Boswellia spp (also known as frankincense or Olibanum) is one of the most popular essential oils in aromatherapy from the Bursearaceae family. word frankincense is derived from an ancient French word which means "Franc = pure + enens=incense" and is mentioned frequently in many sacred text including the Bible. Frankincense essential oil is obtained from a variety different species of the genus *Boswellia*. The most prevalent species in today's world market are *B. carterii (Somaliland), B. serrata (India), B. sacra (Oman), B. frereana (Somaliland)* and *B. papyrifera (Ethiopia).* The bark of the *Boswellia* tree is filled with oleo-gum-resin reservoirs. When the reservoirs are penetrated, a milky juice is excreted onto the bark. When the milky juice becomes exposed to air it begins to harden producing the oleogum resin. [Tucker, 1986]. The resin typically contains between 5 and 15% essential oil [Mertens, et al., 2009]. Frankincense oil is used medicinally in many cultures still today. For example, frankincense essential oil has been claimed to contain chemical components present that aid in the removal of scars and stretch marks, in addition to having antibacterial and antifungal properties [Mikhaeil, et al.,2003]. The oil has also been studied in reference to pharmaceutical properties as well as clinical trials [Vuuren, et al., 2010].

Frankincense resin is traditionally used in treatment of inflammation, wound healing, skin diseases, urinary tract infections, etc. Its application in medicines and cosmetics product formulation are increasing daily.

For a number of years there has been considerable controversy [Paul, et al., 2011, Woolley, et al., 2012] in identifying the correct botanical name of certain frankincense species. Paul et al have proposed a simple TLC method for identification of three olibanum resins on the basis of biomarker compounds. According to their findings, the presence of incensole and incensyl acetate confirms the presence of *Boswellia papyrifera*, caryophyllene oxide confirms *Boswellia carterii* and/or *Boswellia sacra*, *Boswellia serrata* has neither incensole acetate nor caryophyllene oxide, but has a remarkable amount of serratol present in addition to trace amount of incensole. It has been claimed for some time that *B. sacra* is actually the same as *B. carterii* [Thulin and Warfa, 1987], but the enantiomeric studies by Woolley et al [Wooley, et al., 2012] as well as the finding of this laboratory give strong evidence that they are indeed different species.

The current study was conducted to report the unique chemical composition (first time presence of alkyl methyl ether as the natural volatile component in an essential oil as well as first reporting of its presence in nature in general), enantiomeric distribution, and biological activities of the oleogum resin essential oil of *Boswellia spp* from Somalia.

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Picture 1 : Methoxy alkane chemotyped *Boswellia* resin in collected in Northern Somaliland

II. Essential Oils Composition

The Frankincense oil was obtained in 1-1.5% yields, a notably lower oil yield than the typical resin from Somaliland. A total of 147 compounds were identified, accounting for about 100% of compound identification (see Table 1). The essential oils contained α-pinene (0.3-11.6%), sabinene (2.1-7.2%), octyl methyl ether (5.5-11.7%), decyl methyl ether (30.6-54.9%), and α-bourbonene (1-5.7%). These results are qualitatively different than any result previously published in a significant review paper [Mertens, et al., 2009; Niebler & Buttner, 2016; Niebler et al., 2016]. On analyzing twenty commercial essential oil samples (mostly carterii, neglecta, sacra, thurifera, frereana) from South Africa, revealed those oils were composed of α-pinene (2.0-64.7%), α-thujene (0.3-52.4%), β-pinene (0.3-13.1%), myrcene (1.1-22.4%), sabinene (0.5-7.0%), limonene (1.3-20.4%), p-cymene (2.7-16.9%), and β-caryophyllene (0.1-10.5%) [Vuuren et al. 2010]. None of the samples exhibited the presence of methoxy alkane (octyl methyl ether, decyl methyl ether) even in trace quantities. Frankincense the North east region of Somalia has also been studied and according to this report α-pinene (10.3-37.7%), α-phellandrene (12.2-41.8%), limonene (6.4-19.6%) were the major components [Vuuren, et al., 2010]. Presence of 9.84% decyl methyl ether (Table-4) in dichloromethane extract indicated natural occurrence of decyl methyl ether in *Boswellia* spp.

It has been shown that enantiomeric distribution is important in properly identifying frankincense species [Woolley et al., 2012], so on our enantiomeric studies of two resin samples harvested at different times show similar enantiomeric distribution from each other as shown in table 3.

In addition to genetic variation, other factors such as age, vegetative cycle stage, climate, season, soil composition, etc. are among several things responsible for the considerable variation in essential oil compositions [Satyal, et al., 2012]. Based on the observed chemical composition, this variety of Somaliland frankincense may be treated as a distinct and novel chemotype.

III. Cytotoxicity, Antimicrobial Activity

Frankincense oil showed notable biological activity on all of the tested microorganisms (Table 3): *Bacillus cereus* (MIC = 78 μg/mL), *Staphylococcus aureus* (MIC = 78 μg/mL), *Escherichia coli* (MIC = 78 μg/mL) and *Aspergillus niger* (MIC = 39 μg/mL). Several previous studies have reported antibacterial and antifungal [Vuuren et al 2010] activities for *Frankincense* essential oils, consistent with our results. α–pinene and

decyl methyl ether, especially in synergy with other essential oil components, are likely responsible for the antimicrobial activities as shown in the table 3.

Frankincense essential oil demonstrated notable *in-vitro* cytotoxic activity against MCF-7 breast tumor cells (100% kill at 100 µg/mL). Note that decyl methyl ether, α-pinene, and β-pinene, are not appreciably cytotoxic, either alone (Table 3).

Table 1 : Chemical Composition of methoxy alkane chemotyped Frankincense EO from Somaliland

RI[1]	Compounds	%[2a] Fall	%[2b] Summer
821	Hexyl methyl ether	TR	ND
863	n-Hexanol	TR	ND
881	2-Butyl furan	TR	ND
900	Nonane	TR	ND
919	Hasheshene	0.10	ND
921	Tricyclene	0.03	ND
924	α-Thujene	2.01	0.38
932	α-Pinene	11.55	0.34
941	Thujadiene	0.05	ND
948	Camphene	0.37	ND
951	Thuja-2,4(10)-diene	0.21	ND
953	α-Fenchene	0.02	ND
971	Sabinene	7.20	2.1
976	β-Pinene	0.56	0.04
982	Hept-5-en-2-one <6-methyl->	0.02	ND
988	Myrcene	0.26	0.04
1003	Octanal	ND	0.02
1004	Pseudolimonene	0.10	ND
1006	α-Phellandrene	0.87	0.06
1008	3-Carene	0.19	ND
1011	Hexyl acetate	ND	0.01
1014	1,4-Cineole	0.02	0.01
1016	α-Terpinene	0.49	0.17
1023	*p*-Cymene	1.58	0.61
1025	Octyl methyl ether	5.53	11.72
1028	Limonene	1.45	0.13
1030	β-Phellandrene	1.16	ND
1031	1,8-cineole	0.11	ND
1034	(*Z*)-β-Ocimene	0.13	0.04
1036	2,5-dimethylcyclohexanol A	0.05	0.03
1042	2,5-Dimethylcyclohexanol B	ND	0.06
1045	(*E*)-β-Ocimene	0.13	ND
1057	γ-Terpinene	0.88	0.31
1066	*o*-Cymenene	TR	0.04
1069	Octanol	0.09	0.39
1075	2-Decanol methyl ether	0.21	0.41
1080	Unidentified	ND	0.07
1084	Terpinolene	0.19	0.05
1089	*p*-Cymenene	0.10	0.03

RI[1]	Compounds	%[2a] Fall	%[2b] Summer
1099	Linalool	0.19	0.09
1101	2-Nonanol	TR	0.04
1112	4,8 Dimetyl-nona-1,3,7-triene	0.13	0.46
1117	β-Thujone	TR	ND
1118	3-Octyl acetate	TR	0.01
1121	*cis*-Non-3-en-1-ol, methyl ether	TR	ND
1123	*cis*-*p*-Menth-2-en-1-ol	0.06	ND
1126	Methyl nonyl ether	1.37	2.21
1128	Hexyl propanoate	0.11	0.07
1138	*trans*-Sabinol	TR	ND
1139	*trans*-Pinocarveol	0.22	ND
1140	*cis*-Verbenol	0.12	ND
1142	Camphene hydrate	ND	0.02
1144	*trans*-Verbenol	0.27	ND
1149	α-Felandren-8-ol	0.13	ND
1156	Sabina ketone	TR	ND
1158	β-Pinene oxide	TR	ND
1159	*trans*-Pinocamphone	TR	ND
1168	α-Phellandrene epoxide	TR	ND
1170	*p*-Mentha-1,5-dien-8-ol	0.32	ND
1171	n-Nonanol	ND	0.05
1179	Terpinen-4-ol	1.27	0.47
1185	*p*-Cymen-8-ol	0.07	0.02
1191	Hexyl butanoate	0.17	0.14
1194	α-Terpineol	0.24	0.02
1206	*cis*-Decen-9-ol methyl ether	0.34	0.34
1207	*trans*-Piperitol	TR	ND
1208	(2*E*,4*E*)-Methyl dodeca-2,4-dienoate	ND	0.03
1209	Octyl acetate	0.15	0.58
1214	*trans*-Decen-9-ol, methyl ether	0.26	0.4
1218	*cis*-Decen-1-ol, methyl ether	0.37	0.74
1222	trans-Decen-1-ol, methyl ether	ND	0.06
1227	Decyl methyl ether	30.65	54.91
1242	*cis*-Dec-2-en-1-ol, methyl ether	0.72	1.17
1248	Linalyl acetate	TR	ND
1252	Piperitone	ND	0.02
1262	*trans*-Dec-2-enal	ND	0.02
1271	Decanol	0.63	2.1
1283	Bornyl acetate	0.27	ND
1319	*cis*-Undecen-1-ol, methyl ether	0.11	0.09
1322	Methyl decanoate	0.00	0.03
1326	Undecyl methyl ether	0.20	0.29
1345	α-Cubebene	0.59	0.32
1367	α-Ylangene	0.37	0.28
1374	α-Copaene	1.68	1.74

RI[1]	Compounds	%[2a] Fall	%[2b] Summer
1382	α-Bourbonene	5.72	1.69
1385	Hexyl hexanoate	0.89	0.21
1387	β-Elemene	1.12	0.24
1390	Sativene	0.07	0.05
1393	1,5-di-epi-α-Bourbonene	0.13	ND
1402	*cis*-Caryophyllene	0.07	0.02
1405	α-Gurjunene	0.20	0.11
1408	Decyl acetate	0.09	1.18
1416	β-Ylangene	0.43	0.12
1417	*trans*-Caryophyllene	0.83	0.72
1428	β-Copaene	0.75	0.22
1442	α-Aromadendrene	0.29	0.07
1447	*cis-Muurola-3,5-diene*	0.13	0.13
1453	α-Humulene	0.32	0.18
1458	Alloaromadendrene	0.20	0.17
1466	β-Aromadendrene	0.15	0.08
1470	*cis*-Cadina-1(6),4-diene	0.11	0.07
1473	*trans*-Cadina-1(6),4-diene	0.73	0.64
1476	*cis*-4,10-epoxy-Amorphane	TR	0.03
1479	Germacrene D	1.52	1.23
1483	Heptylhexanoate	ND	0.02
1487	β-Selinene	0.48	0.07
1489	*trans*-Muurola-4(14),5-diene	0.24	0.19
1493	α-Selinene	0.41	0.23
1496	α-Muurolene	0.31	0.21
1500	γ-Amorphene	0.06	0.03
1503	β-Dihydroagarofuran	0.04	0.03
1511	δ-Amorphene	0.20	0.16
1513	Cubebol	0.30	0.45
1516	δ-Cadinene	1.09	0.82
1520	Tridecyl methyl ether	0.38	0.5
1522	Methyl dodecanoate	TR	ND
1527	Kessane	0.06	0.07
1530	*trans*-Cadine-1,4-diene	0.06	0.05
1534	Isokessane	0.07	0.07
1539	Italicene ether	ND	0.02
1539	α-Calcorene	0.02	0.02
1546	α-Elemol	0.20	0.22
1557	Dodecanoic acid	ND	0.06
1559	*cis*-Muurol-5-en-4-α-ol+*trans*-Nerolidol	0.69	0.88
1565	Zierone	ND	0.02
1568	Caryophyllenol	0.05	0.11
1570	*trans*-Tridec-2-en-1-al	0.06	0.11
1576	Germacrene-D-4-ol	ND	0.02
1579	Caryophyllene oxide	TR	ND

RI[1]	Compounds	%[2a] Fall	%[2b] Summer
1592	Viridiflorol	0.11	ND
1602	Ledol	TR	0.03
1606	1,10 di-*epi*-Cubenol	0.24	0.16
1611	8-*epi*-γ-Eudesmol	1.10	1.38
1625	1-*epi*-Cubenol	TR	0.03
1641	Cubenol	0.11	0.03
1641	epi-α-Cadinol	ND	0.01
1644	δ-Cadinol	TR	0.03
1652	Unidentified Sesquiterpineol	ND	0.26
1653	α-Eudesmol	0.20	0.15
1673	Bulnesol	TR	0.06
1696	Tridec-4-en-1-yl acetate	ND	0.02
1791	α-Phellandrene dimer	0.05	ND
1950	*3E*-Cembrene A	0.49	0.31
1995	α-Pinacene	0.09	0.06
2007	Verticilla-4(20),7,11-triene	0.33	0.14
2131	Neocembrene A	0.14	0.12
2142	Incensole	0.79	0.43
2144	Serratol	0.41	0.55
	Total identified	100%	99.74%

Note: Where RI[1] Retention Index determined to a series of n-Alkanes on DB-5 column; compounds are listed in order of elution (Increasing RI), %[2a] and %[2b] refers to Percent of total oil collected in fall season and percent of total oil collected in summer season respectively. "TR" indicates trace components (<0.01%), and "ND" indicates non detected compounds on the provided conditions.

Table 2 : Enantiomeric Distribution of Frankincense EO collected in fall and summer season from Somalia

Monoterepene Components	Fall	Summer
(1R)+-α-thujene: (1S)-(-)-α-thujene	43 to 57	36 to 64
(1R,5R)-(+)-α-pinene: (1S,5S)-(–)-α-pinene	83 to 17	76 to 34
(4R)-(+)-limonene :(4S)-(-)-limonene	73 to 27	65 to 35
(4S-(+)-terpinen-4-ol : (4R)-(-)-terpinen-4-ol	81 to 19	71 to 29
(4S)-(+)-sabinene :(4R)-(-)-sabinene	93 to 7	98 to 2

Table 3 : Biological activities of *Frankincense* essential oils and major essential oil components

Bioassay	EO	Methoxydecane	α-Pinene	β-Pinene
MCF-7 cytotoxicity (% kill at 100 µg/mL)	100±1.6	80±10.6	16.8±2.6	30.4±7.7
Antimicrobial (MIC, µg/mL)				
Bacillus cereus	78	78	1250	1250
Staphylococcus aureus	78	313	1250	1250
Escherichia coli	78	78	1250	1250
Aspergillus niger	39	313	156	156
Candida albicans	78	313	625	313

Table 4 : Chemical composition of dichlromethane extract of methoxy alkane chemotyped frankincense resin from Somalia

RI[a]	Compounds	%[b]
924	α-Thujene	0.26
932	α-Pinene	0.26
971	Sabinene	1.08
1024	*p*-Cymene	0.13
1025	Octyl methyl ether	3.34
1057	γ-Terpinene	0.06
1075	2-Decanol, methyl ether	0.09
1112	2-Methyl-6-methylen-octa-1,7-dien-3-one	0.11
1126	Methyl nonyl ether	0.39
1209	Octyl acetate	0.06
1214	*cis*-Decen-1-ol, methyl ether	0.07
1218	*trans*-Decen-1-ol, methyl ether	0.11
1227	Decyl methyl ether	9.84
1242	*cis*-Dec-2-en-1-ol, methyl ether	0.21
1271	Decanol	0.47
1375	α-Copaene	0.36
1383	α-Bourbonene	0.35
1408	Decyl acetate	0.24
1419	*trans*-Caryophyllene	0.11
1474	*trans*-Cadina-1(6),4-diene	0.18
1480	Germacrene D	0.37
1494	*epi*-Cubebol	0.07
1514	Cubebol	0.47
1517	δ-Cadinene	0.2
1520	Tridecyl methyl ether	0.17
1547	Elemol	0.32
1558	Dodecanoic acid	0.41
1561	Prenopsan-8-ol	0.75
1570	*2E*-Tridecen-1-al	0.16
1608	1,10-di-*epi*-Cubenol	0.18
1613	5-*epi*-7-*epi*-α-Eudesmol	2.48
1652	8-epi-γ-Eudesmol	0.67
1654	α-Eudesmol	0.36
1674	iso-Bulensol	0.1
1952	*3E*-Cembrene A	1.36
1996	α-Pinacene	0.43
2009	Verticilliol	0.72
2133	Neocembrene A	7.1
2145	Incensole	43.39
2147	Serratol	20.23
2253	Incensole oxide A	0.2
2263	Incensole oxide B	1.04
2269	Isoincensole	0.46
2293	Isoincensole oxide	0.35
2350	Cembra-2,7,11-trien-4,5-diol isomer	0.29
	Total Identified	100%

IV. Experimental

a) Plant Material

Oleo-gum-resin were collected from the city Ufeyn (10.6500° N, 49.7500° E, 470 m above sea level) in the Puntland region of Somalia for two times of year [2014 April 20th and 2015 September 9th].

The voucher specimen of resin collected plant was stored in, Somalia. Local botanist has identified this resin as *Boswellia carterii,* but on the basis of previously published articles, it did not match chemical composition, so throughout of this article, we mention it as Frankincense or Boswellia spp to avoid controversy in taxonomy. The air-dried sample (250 g) were hydrodistilled using a Clevenger Apparatus for 4 hours to yield a translucent, yellow essential and colorless oil. The essential oil was stored at room temperature until analysis was carried out.

In addition to steam distillation, this resin was also extracted with methylene chloride and analyzed by GC/MS (see Table-4) in order to confirm the presence of the methoxy alkane components in the resin itself. This experiment was done to avoid any possible arguments against these components occurring naturally, ruling out the possibility that the methoxy alkanes are arising from a secondary reaction occurring during the distillation process.

b) Gas Chromatographic-Mass Spectral Analysis

The essential oil of Boswellia was analyzed by GC-MS using a Shimadzu GCMS-QP2010 Ultra operated in the EI mode [(electron energy = 70eV), scan range = 3.0 scans/sec], and GCMS Solution software. The GC column was Zebron ZB-5MS fused silica capillary column with a (5% phenyl)-polymethyl siloxane stationary phase a film thickness of 0.25 mm. The career gas was helium with a column head pressure 80 psi and flow rate of 1.37 ml/min. Injector temperature was 250°C and the ion source temperature was 200°C, increase in temperature rate 2°C/min to 260°C. The GC oven temperature program was programmed for 50°C initial temperature, increase in rate 2°C/min to 260°C. A 5% w/v solution of the sample in CH_2Cl_2 was prepared and 0.1 μL was injected in splitting mode (30:1). Identification of the oil components was based on their retention indices determined by reference to a homologous series of n-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature [Adams], and stored in the MS library.

c) Chiral Gas Chromatographic-Mass Spectral Analysis

Chiral analysis of the essential oils was performed on a Shimadzu GCMS-QP2010S operated in the EI mode [(electron energy=70eV), scan range = 3.0 scans/sec]. GC equipped with a RestekB-Dex 325 capillary column (30 m×0.25 mm ID×0.25 _m film). Oven temperature was started at 50◦C, and then gradually raised to 120◦C at 1.5 ◦C/min. The oven was then raised to 200◦C at 2◦C/min and held for 5 min. Helium was the carrier gas and flow rate was maintained at 1.8 ml/min. Samples were diluted 3% w/v with CH_2Cl_2 and then a 0.1 μL sample was injected in a split mode with a split ratio of 1:45.

d) Antimicrobial Screening

The essential oil was screened for antimicrobial activity against Gram-positive bacteria, *Bacillus cereus* (ATCC No. 14579) and *Staphylococcus aureus* (ATCC No. 29213); Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798). Minimum inhibitory concentrations (MICs) were determined using the microbroth dilution technique [Satyal et al., 2013]. Dilutions of the crude extracts were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 μL of 1% w/w solutions of crude extracts in DMSO plus 50 μL CAMHB. The extract solutions were serially diluted (1:1) in CAMHB in 96-well plates. Organisms at a concentration of approximately 1.5×10^8 colony-forming units (CFU)/mL were added to each well. Plates were incubated at 37°C for 24 hours; the final minimum inhibitory concentration (MIC) was determined as the lowest concentration without turbidity. Geneticin® was used as a positive antibiotic control; DMSO was used as a negative control. Antifungal activity against *Aspergillus niger* (ATCC No. 16888) was determined as above using YM broth inoculated with *A. niger* hyphal culture diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control.

e) Cytotoxic Activity

Human MCF-7 breast adenocar-cinoma cells (ATCC No. HTB-22) [Satyal et al, 2014] were grown in a 3% CO_2 environment at 37°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per liter of medium, 15mM of Hepes, and buffered with 26.7 mM $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per well. The volume in each well was 100 µL. After 48 h, supernatant fluid was removed by suction and replaced with 100 µL growth medium containing 1.0 µL of DMSO solution of the essential oil (1% w/w in DMSO), giving a final concentration of 100 µg/mL for each well. Solutions were added to wells in four replicates. Medium controls and DMSO controls (10 µL DMSO/mL) were used. Tingenone [Satyal et al, 2015] was used as a positive control. After the addition of compounds, plates were incubated for 48 h at 37°C in 5% CO_2; medium was then removed by suction, and 100 µL of fresh medium was added to each well. In order to establish percent kill rates, the MTT assay for cell viability was carried out [Satyal et al., 2012]. After colorimetric readings were recorded (using a Molecular Devices SpectraMAX Plus

microplate reader, 570 nm), average absorbances, standard deviations, and percent kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated.

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Behavior Classification Physical/Chemistry PA66/HCℓ Binary System Utilized to Obtain Separation Membranes

By Jocelei Duarte, Venina dos Santos & Mara Zeni
Caxias do Sul University

Abstract- The dissolution of polymers in solvents is an interest area of polymers science and technology due to their applications in films and membranes. The starting point for the preparation of polymer membranes by phase inversion is having a thermodynamically stable solution. In this work, the thermodynamic behavior of polyamide 66 (PA66) solutions in formic acid (FA) and in hydrochloric acid (HCℓ), was studied to evaluate its physical/chemistry classification. The polymer systems may be divided into five classes (types), which incorporate polymer-low molecular liquid systems, ranging from true solutions to dispersions of polymers in liquids. The polyamide 66/formic acid (PA66/FA) binary system is considered a true solution. Several experiments were performed to verify if the polyamide 66/hydrochloric acid (PA66/HCℓ) binary system has all characteristics of true solution. The analysis showed that the PA66/HCℓ binary system presents all characteristics of true solution, including the final morphology of the membranes that are similar obtained membranes by PA66/FA binary system.

Keywords: polymer-low molecular liquid systems, true solution, PA66/HCℓ binary system.

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Behavior Classification Physical/Chemistry PA66/HCℓ Binary System Utilized to Obtain Separation Membranes

Jocelei Duarte [a], Venina dos Santos [a] & Mara Zeni [a*]

Highlights

- The thermodynamic equilibrium is reached ($\Delta\mu = 0$ or $= \mu_1$) the solution becomes homogeneous.
- The PA66/HCℓ solution was frozen and thawedand the solution remained homogeneous.
- The Gibbs free energy of PA66/HCℓ system indicates to be more favorable to PA66/FA system.
- In PA66/HCℓ system dipole-dipole interactions occur, justifying the behavior similar to polyelectrolyte.
- The PA66 solution in HCℓ behaves as a true solution.

Abstract- **The dissolution of polymers in solvents is an interest area of polymers science and technology due to their applications in films and membranes. The starting point for the preparation of polymer membranes by phase inversion is having a thermodynamically stable solution. In this work, the thermodynamic behavior of polyamide 66 (PA66) solutions in formic acid (FA) and in hydrochloric acid (HCℓ), was studied to evaluate its physical/chemistry classification. The polymer systems may be divided into five classes (types), which incorporate polymer-low molecular liquid systems, ranging from true solutions to dispersions of polymers in liquids. The polyamide 66/formic acid (PA66/FA) binary system is considered a true solution. Several experiments were performed to verify if the polyamide 66/hydrochloric acid (PA66/HCℓ) binary system has all characteristics of true solution. The analysis showed that the PA66/HCℓ binary system presents all characteristics of true solution, including the final morphology of the membranes that are similar obtained membranes by PA66/FA binary system.**

Keywords: polymer-low molecular liquid systems, true solution, PA66/HCℓ binary system.

I. Introduction

The population world growth, as well as their level of requirement, both in quality and quantity, led the industry to produce ever more food, medicament, clothing and all kinds of durable goods and nondurable needed, directly and indirectly, to man. Facing this reality, new technologies have emerged to meet this demand and requirements of the population, in this context, Membrane Separation Processes (MSP) emerged as an alternative based on clean technologies, with its modular features and low energy.

The main advantage of the MSP is the separation occurs without phase change of components, different from the classical separation processes such as distillation. So, besides being economically more advantageous in a energy lower cost, the MSP can be used in very common in areas biomedical separation of thermolabile substances in juice concentration, where the use of thermal processes alter the organoleptic properties, among others[1]. Another advantage of this process is simplicity of operation, compact equipment, easy to mix with other processes and an increase in the production scale [2].

The boundary for industrial and commercial growth of MSP was the development of asymmetric membranes of cellulose acetate by researchers Loeb and Sourirajan (1961), using the phase inversion technique. These membranes are characterized by a fine surface layer compact containing pore or not (called skin or to player) responsible for the selectivity, supported by a porous structure which ensures the mechanical resistance to skin and offers little resistance to transportation. As the thickness of the skin is very thin (less than 1μm), resistance to transport through such membranes is low compared to other dense homogeneous membranes prepared from the same material [3].

In MSP often are used polymeric membranes and polyamide 66 (PA66), one of the polymers used for this application [4]. These are mainly obtained by the technique of phase inversion, since this technique allows controlling important variables that govern the morphology of these membranes thus improving process performance [5-6].

A variety of separation membranes are available on the market today for various applications. Distinct separation processes require membranes with different characteristics. In microfiltration and ultra filtration processes where filtration occurs by size difference, porous membranes that act as sieves are used. Processes such as gas separation and pervaporation, where the transport mechanism and selectivity, involves gas diffusion and solubility, require dense membranes.

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In the literature there is a large number of works addressing studies both of the thermodynamics of polymer solutions, as kinetics of mass transfer, formation of the filter skin and the sublayer of the polymeric asymmetric membranes [7].

Knowledge of the thermodynamic properties of a ternary system is relevant to understand and predict the membranes formation [8]. Many researchers have studied the morphology and properties of polyamide membranes [4,9–11] as well as the physicochemical interactions between components in the polymer solution and thermodynamic properties non-solvent (1) / solvent (2) / polymer (3) systems[12–15].

The study of the behavior of polymer solutions require a thermodynamic approach exhibit different aspects of those usually employed in the study of solutions formed by only low molecular mass components [16]. The size and shape of the macromolecules introduce changes in the thermodynamic behavior as well, show large deviations from ideality. From this, it is possible to relate the solubility properties of the components forming part of the system to obtain PA66 in formic acid and/or hydrochloric acid membranes, using water as non-solvent.

a) True Solutions of Polymers

i. *Specific Properties of True Solutions*

According to Tager[17], a true solution is a multi component system which has the following characteristic features: (a) affinity between components; (b) spontaneous formation; (c) constant concentration in time; (d) homogeneity, i.e., presence of only one phase e (e) thermodynamic stability.

Second Lucas et al.[16], the components may mix in any proportion (complete miscibility) or in definite proportions (partial miscibility). If the external conditions (pressure and/or temperature) do not vary, the true solution concentration remains constant in time. A true solution is a system consisting of a single phase. The solution phase may be separated from the other phases (precipitate, vapor or solution) by an interface, but within the solution itself there is no interface, i.e., the system is homogeneous.

The most important feature of a true solution is its thermodynamic stability or equilibrium state. A system is called thermodynamically stable if its formation is accompanied by a decrease in the Helmholtz free energy (at constant volume and temperature) or by a decrease in the Gibbs free energy - ΔG (at constant pressure and temperature). The Gibbs free energy decreases to a definite equilibrium value which does not change subsequently with time[16-17].

ii. *Dissolution and Swelling of Polymers*

According to Schuld & Wolf [18], a polymer does not dissolve in all liquids. In some cases, the polymer and the low molecular liquid (solvent) possess mutual affinity, while in others no such affinity exists. In the former case, the formation of a true solution may be expected, but, in the latter case only a colloidal solution can be formed. The polymer solutions formed spontaneously have all the characteristics of true solutions, including reversibility, equilibrium, swelling before dissolving, high viscosity and slow diffusion. All these features are due to the great difference in size between the particles of the components mixed [19].

Second Canevarolo[20], before dissolving the polymer "swells", i.e., absorbs the low molecular liquid, increasing in mass and volume. Swelling inevitably involves a change in polymer structure, which sharply increases the volume of the samples. Swelling may be limited (it is interaction of the polymer with a low molecular liquid in which the polymer chains are not completely separate from each other) or unlimited (where dissolution occurs spontaneously).

For Tager[17], the degree of swelling can be determined only for polymers with limited swelling, because upon unlimited swelling the polymer itself begins to dissolve and the mass of the sample decreases. Sometimes a phenomenon called negative swelling is observed in polymers, involving decrease rather than increase of sample mass in time.

Polymers may absorb low molecular substances not only from the liquid, but also from the gaseous phase; if the polymer swells on a liquid, it will swell on its vapors as well[16].

Describe the influence of thermodynamic properties in water / hydrochloric acid (HCℓ) / polyamide 66 (PA66) ternary systems with the morphology of the PA66 membranes; starting by characterization of PA66 in HCℓsolutions has motivated this work.

II. Experimental

a) Materials

The polymer used for the solutions and membranes preparation it is the polyamide 66 (PA66) commercial supplied by Alfa Chem. Co.(Brazil), in pellets.

The solvents used in the solutions and membranes preparation were fuming hydrochloric acid (HCℓ) with purity of 37-38% and formic acid (FA) with purity 98-100%, both supplied by Merck (Brazil).

The non-solvent used in the membranes preparation was distilled water. The information of the PA66 and the solvent properties are listed in Table 1.

Table 1: Characteristic data of the solvents and polymer investigated

Materials	Molar volume ($cm^3 \cdot mol^{-1}$)	Molar mass ($g \cdot mol^{-1}$)	Density ($g \cdot cm^{-3}$)
PA 66	10,642.20	11,600[a]	1.22[b] e 1.09[c]
HCℓ (37-38%)	30.67	36.5	1.19
FA (98-100%)	37.7	46	1.22

a = viscosimetric average molar mass (M_v)
b = PA66 density solid (arranged)
c = PA66 density in solution (amorphous)
Source: BRANDRUP & IMMERGUT, 1989

b) Methods

PA66 was dried for 2 hours at 90°C prior to each procedure performed [21].

i. *Preparation of asymmetric flat sheet membranes*

To obtain asymmetric flat sheet membranes two different casting solutions were prepared: PA66 / formic acid / water and PA66 / hydrochloric acid / water, by the phase inversion method(PI) [2].

PA66/HCℓand PA66/FA solutions were prepared using 20 g of PA66 dissolved in 100 cm^3 of each solvent for both systems, at controlled room temperature 25 ± 2°C and at atmospheric pressure (1 atm), under magnetic stirring for 2h. The magnetic stirrer was isolated with glass wool.

The homogeneous casting solutions were cast on glass plate with a knife casting of approximately 200 µm, after was taken solvent evaporation under controlled temperature depending of the solvent used, then immersed in the coagulation bath. The membranes preparation conditions are shown in Table 2.

Table 2 : PA66 Membrane spreparation conditions

	Evaporation conditions		PI* bath conditions	
Membranes	Time (mim)	Temperature (°C)	Time (mim)	Temperature (°C)
FA	10	20	120	25±2
HCℓ	60	60	120	25±2

**PI = phase inversion*

The prepared membranes were washed with distilled water to remove residual solvent and maintained at temperature of 25 ± 2°C for drying. The membranes were called as FA and HCℓ, according use of formic or hydrochloric acid respectively, and them were dried for 24 h at 60°C and characterized by Scanning Electron Microscopy (SEM).

ii. *Scanning Electron Microscopy (SEM)*

Analysis by Scanning Electron Microscopy (SEM) of the surface and the cross section of the membranes were performed on Shimadzu Model SSX550 microscope. Samples were fractured in N_2 liquid at –196°C and metalized by means of a sputtering with a thin gold layer prior to its characterization.

iii. *Solutions preparation and techniques to prove be the polyamide 66 / hydrochloric acid system a true solution*

a. *Affinity between components*

To observe the affinity between components, a solution was prepared using PA66 (20 g) and placed in flat-bottomed flask, to which was added 100 cm^3 hydrochloric acid (HCℓ).

b. *Spontaneous formation*

In order to demonstrate do not be necessary the use of heat or other external force for dissolving the polymer, PA66 (20 g) solution was prepared and placed in double-jacket reactor to which was added 100 cm^3 of hydrochloric acid (HCℓ). In the inner part of the double-jacket was adapted a thermocouple to impart the bath temperature. In contact with the solution, a thermometer measured the temperature of the same during the dissolution process. The system was coupled in a thermostatic bath (25°C).

This experiment was also performed under the same conditions, but with isolated system with glass wool and vacuum in the double-jacket to prevent the heat transfer between the system and the environment.

c. *Constant concentration in time*

To investigate the precipitation possibility (phase separation) of the solution by temperature, PA66 (20 g) solution in 100 cm^3 HCℓ, was frozen in liquid nitrogen(-196°C). Once frozen, the solution was allowed to stand at controlled room temperature (25 ± 2°C) and, when liquid again placed under stirring.

PA66 solutionin HCℓ was placed in double-jacket reactor coupled to temperature controller at -5°C (Frigomix), under magnetic stirring for 2h. After this time, the temperature was raised to 96°C, still under stirring.

d. *Homogenity*

An aliquot of PA66/HCℓ [PA66 (20 g) in 100 cm^3 HCℓ]solution, was placed on a glass coverslip and examined under an optical microscope Carl Zeiss

AxioScope A1 to analyze the presence of interface within the solution.

e. *Thermodynamic stability*

For to measurethe most important feature of a true solution that is its thermodynamic stability or equilibrium state[16-17], for the PA66/HCℓ and PA66/FAbinary systems, the Gibbs free energy of mixing(ΔG_m) was calculated by the following equation (1):

$$\frac{\Delta G_m}{RT} = x_1 ln\varphi_1 + x_2 ln\varphi_2 + g_{12}(\varphi)x_1\varphi_2 \qquad (1)$$

where φeχ are volume fraction and molar fraction of components; T is the temperature (K) e R is the gas constant. The g_{12} parameter can be considered a free energy term containing contributions enthalpy and entropy[18]. The Gibbs free energy of mixing was calculated as a function of volume fraction of polymer for both solvents.

iv. *Determination degree of swelling*

In order to determine the degree of swelling of PA66, were placed 100 cm^3 of solvent (HCℓ) in flat-bottomed flask. In the mouth flask was adapted polyester fabric, with enough closed mesh to allow the passage of solvent vapor. On the screen a few PA66 pellets was placed. The flask was kept under magnetic stirring for 5h. This method was adapted from Tager[17].

III. Results and Discussion

a) *Polymer solution*

i. *Specific Properties - True Polymer Solutions*

According to Papkov e Iovleva[22], all systems can be divided into five classes (true solutions containing predominantly solvent; plasticized systems; gels; systems with complete phase separation and polymer dispersions) that comprise polymer with low molecular liquid systems, ranging from true solutions to polymer dispersions in liquids. The low molecular liquid terminology is adopted for solvents, thinners, non-solvents, plasticizers and liquid dispersion media.

Following the concepts of Papkov and Iovleva[22], formic acid (FA) is considered a low molecular liquid (46 $g \cdot mol^{-1}$) when compared with polyamide 66 (11,600 $g \cdot mol^{-1}$), this system (PA66/FA) is considered by Lin et al. [9] as a true solution.

In order to check if the PA66/HCℓ system displays all the characteristics of true solution such as: affinity between components; spontaneous formation; constant concentration in time; homogeneity and thermodynamic stability, several experiments were performed.

ii. *Affinity between components with spontaneous formation of solution*

To prove affinity between components PA66/HCℓ, the polyamide 66 (PA66) was placed in contact with hydrochloric acid (HCℓ)(Figure 1a)for 2h under magnetic stirring, and the polymer molecules absorbed the solvent, the polymer chains diffused slowly, acquiring mobility and making the homogeneous solution, as can be seen in Figure 1b.

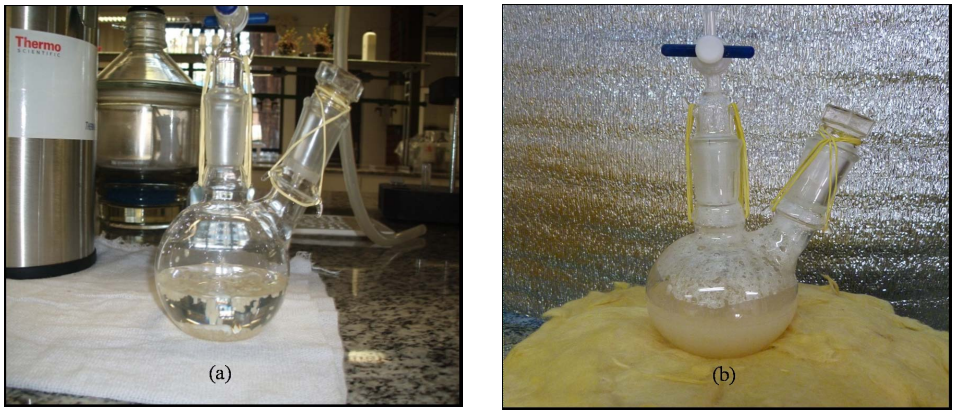

Figure 1: Homogeneous solution of PA66 in HCℓ after testing affinity between components with spontaneous solution formation

According to Tager[17], form true solutions when there is affinity, i.e., there is interaction between components; them disperse spontaneously in direct contact, without the use of heat and/or pressure. Second Lucas et al. [16] this is because the chemical potential of the pure solvent (μ_0) is larger than the chemical potential of the solvent in the solution (μ_1), and when the thermodynamic equilibrium is reached ($\Delta\mu = 0$ or $= \mu_1$) the solution becomes homogeneous. This is corroborated by Gundert & Wolf [23] who claim that HCℓ and FA are good solvents for polyamide at room temperature and atmospheric pressure.

iii. *Constant concentration in time and homogeneity*

Also according Tager[17], if the external conditions (pressure and temperature) do not vary, the concentration of a true solution remains constant over time. In studies by Mulder [2], in most cases, polymer/solvent solutions are characterized by an upper critical solution temperature (UCST), or when cooled (T_0 to T_1) induce phase separation, altering the concentration.

From tests carried out it was observed that when the PA66/HCℓ solution was frozen and thawed, there was no phase separation, the solution remained homogeneous without the presence of precipitates or interface within the solution, as can be seen in Figure 2, only with the presence of air bubbles due to magnetic stirring.

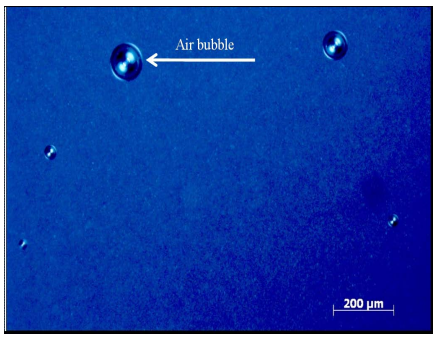

Figure 2: Optical microscopy of the PA66/HCℓ solution - 20 g/100 cm³ (10x)

iv. *Thermodynamic stability*

Second Tager[17]and Lucas et al. [16], a system is called thermodynamically stable if their formation is accompanied by a decrease in the Gibbs free energy of mixing - ΔG_m (constant pressure and temperature), which reduces to a definite value of equilibrium which does not change with time.

So that PA66/HCℓ and PA66/FA binary systems form a homogeneous system (one phase), the Gibbs free energy is negative, as can be seen in Figure 3.The Gibbs free energy of mixing was calculated as a function of volume fraction of polymer for both solvents. The PA66/HCℓ system indicates be more favorable in all concentrations compared to PA66/FA system as values obtained fromΔG_m.

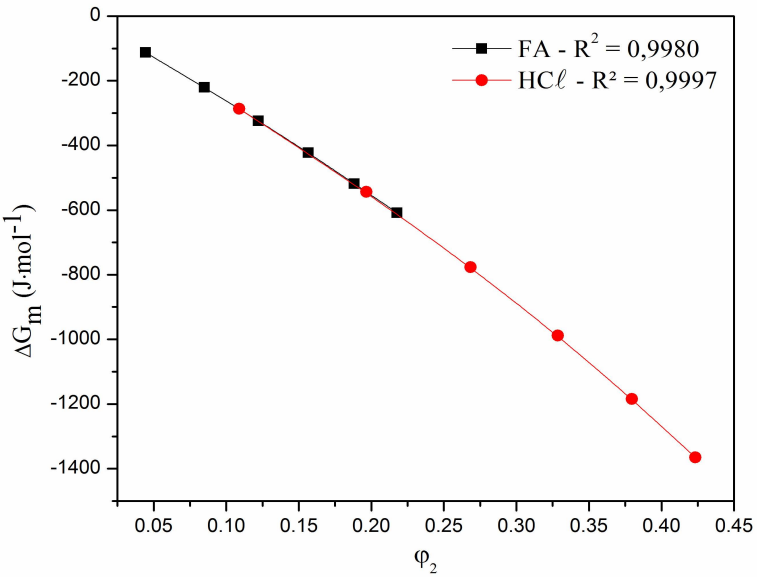

Figure 3: Gibbs free energy of mixing (ΔGm) for PA66/FA and PA66/ HCℓbinary systems as a function of volume fraction of PA66 (φ_2), which is different between systems depending on the concentration of the solvents

The HCℓ (37-38%) is a solution and does not a pure solvent, indicating the presence of water in the PA66 solution, while FA is purity solvent with 98-100%. In the PA66/FA system occur hydrogen bonds between polymer and formic acid, which explains the Gibbs free energy to be negative; in the PA66/HCℓ system dipole-dipole interactions occur, which are also strong interactions. What likely explain the energy of mixing PA66/HCℓ be more negative with respect to PA66/FA system is the acid strength (Ka = acidity constant).

While the HCℓ Ka = 1 x 10^{7}[24-25], formic acid Ka = 1, 77 x 10^{-4}[25], justifying the similar behavior to the polyelectrolyte solution[26-27].

v. *Dissolution and swelling degree of polymers*

According to results obtained for the specific properties of true solutions of the PA66/HCℓ system, confirmed that this is a polymer with low molecular liquid system (HCℓ = 36, 5 g·mol^{-1}) and also is it a true solution, such as PA66/FA system [9]. Being PA66/HCℓ spontaneous system, requiring no external force (pressure and/or temperature) to dissolve the polymer, it was investigated the swelling degree, and according to Lucas et al. [16], if the polymer swells in contact with the low-molecular liquid, also swell in contact with its vapor. The Figure 4 shows the swelling test of the PA66pellets in contact with HCℓ vapor.

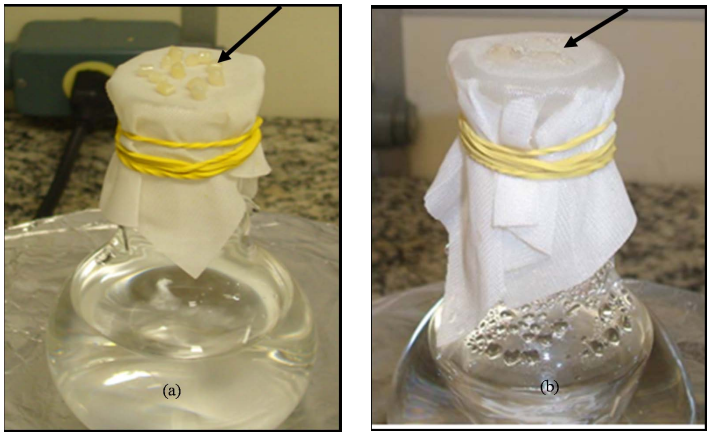

Figure 4 : Dissolution test of the PA66pellets in contact with HCℓ vapor: (a) before and (b) after the test

After 5h the PA66 in contact with HCℓ vapor showed unlimited swelling, i.e. dissolved before swell (Figure 4b). Tager[17] attributed this behavior to interaction between polymer/solvent and solvent strength.

b) Morphological analysis by Scanning Electron Microscopy (SEM) of PA66films

The surface and cross section of the PA66 films were analyzed by scanning electron microscopy (SEM).

The upper surfaces of the films showed dense layer, nonporous, homogeneous and composed of polygonal grains, as can be seen in Figure 5.

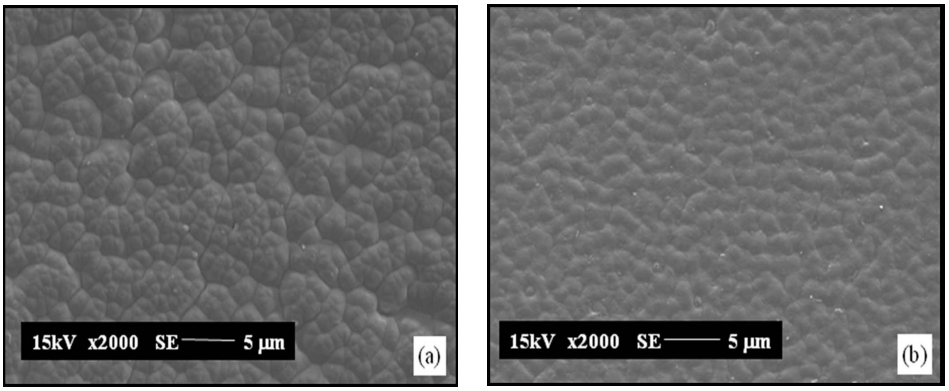

Figure 5: Micrograph (SEM) of surface of PA66 membranes in the form of films prepared in FA (a) and prepared in HCℓ (b) (2000x)

Shih et al.[28]and Lin et al.[9], also studied the mechanisms of phase separation on PA66 membranes in FA and, this polygonal characteristic with limits almost linear are similar to spherulites, that are obtained for phase separation solid-liquid (S-L) (crystallization).

The morphology of the PA66films in formic acid and hydrochloric acid is typical of polyamide films precipitate on considered aggressive bath (water), with dense layer and cellular and pore structure. The micrograph (Figure 6a) shows a PA66film in FA with a dense layer of approximately 4 µmthickness and pores cell small surrounded by a polymer matrix. The micrograph (Figure 6b) shows a PA66 film in HCℓ with dense layer of approximately 23 µm in thickness and

also has pores cell small surrounded by a polymer matrix.

Figure 6 shows the cross section of the PA66 films in FA and HCℓ which present similar morphologies.

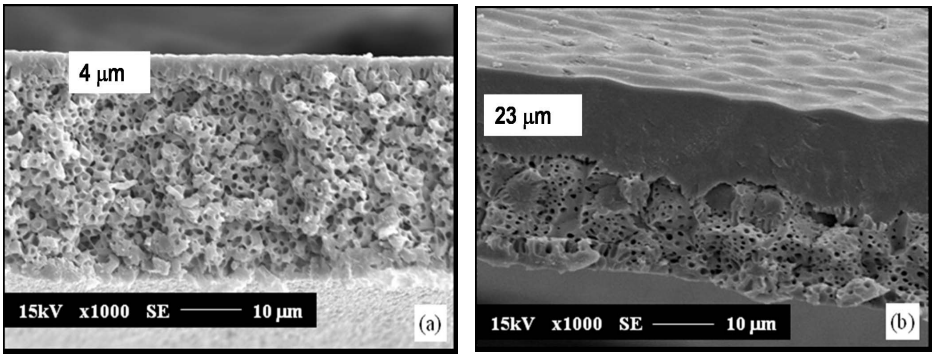

Figure 6: Micrograph(SEM) of the cross section of the PA66 films in: FA (a) and HCℓ (b) (1000X)

Looking at Figure 6 of the standpoint of Lin et al. [9], the non-porous dense layer formation is due to the increased concentration of polymer in the film interface with the bath (water). During precipitation a thick gel layer was formed on the surface excluding the possibility of nucleation liquid micelles in the region (pore formation). This process was more intense in PA66/HCℓ films, but these films were subjected to a solvent evaporation step, where according Mulder [2] is the beginning of precipitation, prior to the immersion bath. This probably favored the polymer concentration highest on the surface, resulting in increased thickness of the dense layer (23 μm) in relation to PA66/FA films (4 μm).

IV. Conclusions

The solution of PA66 in FA is considered a true solution in the literature, however, to solution PA66 in HCℓ was not found reference. The specific properties analyzed proved that the solution of PA66 in HCℓ behaves as a true solution, including the final morphology of the membranes that are similar membranes obtained by binary system PA66/FA.

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

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Abstract- The aim of this study was to characterize adult distribution with respect to light and analyze ovary contents in the four seasons of the year. The swimming behavior of *Ciona intestinalis*, *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia mammilata* larvae against certain abiotic factors were commented. For the field data on adult distributions, one-way analysis of variance (ANOVA) was applied to test for differences in adult orientation, with surface orientation as a fixed factor. Two adult species (*Ciona intestinalis* and *Molgula manhattensis*) showed no orientation with respect to light while in the other two species (*Ascidella aspersa* and *Phallusia mammilata*) light exerted a significant effects on the orientation and density of individuals. To evaluate among the different species the level of gregariousness found in the field, the number of individuals per clump for each species has been compared using one-way ANOVA, with species as a fixed factor. Artificial heterologous inseminations were carried out.

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

GJSFR-B Classification : FOR Code: 069999

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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]

Abstract- The aim of this study was to characterize adult distribution with respect to light and analyze ovary contents in the four seasons of the year. The swimming behavior of *Ciona intestinalis*, *Molgula manhattensis*, *Ascidella aspersa* and *Phallusia mammilata* larvae against certain abiotic factors were commented. For the field data on adult distributions, one-way analysis of variance (ANOVA) was applied to test for differences in adult orientation, with surface orientation as a fixed factor. Two adult species (*Ciona intestinalis* and *Molgula manhattensis*) showed no orientation with respect to light while in the other two species (*Ascidella aspersa* and *Phallusia mammilata*) light exerted a significant effects on the orientation and density of individuals. To evaluate among the different species the level of gregariousness found in the field, the number of individuals per clump for each species has been compared using one-way ANOVA, with species as a fixed factor. Artificial heterologous inseminations were carried out. Three experiments were investigated in the laboratory on metamorphosed larvae of the four species. The first experiment tested geotaxis with respect to phototaxis during larval settlement. One species (*Ciona intestinalis*) showed a clear preference for settlement on top surfaces Top > Lateral = Bottom whereas the three species (*Molgula manhattensis*, *Ascidella aspersa* and *Phallusia mammilata*) settled on the bottom than elsewhere Bottom > Lateral = Top. In the second experiment, larvae were placed in wells in which half of the top, bottom or lateral surfaces were covered by black tape while the other half of these surfaces were exposed to light. Few number of larvae in one species preferred the top regardless of light direction dark = light while three species showed a significant interaction between light/ darkness and position, showing a marked preference for dark surfaces and bottom orientation. Larvae of two species continued to prefer bottom surface in the light but selected both bottom and top in the dark. One species changed light preferences depending on the surface considered, The third experiment tested the effect of adult mantle extract on larval settlement. One species (*Ascidella aspersa*) showed little effect of mantle tissues extract while the other three species (*Ciona intestinalis*, *Molgula manhattensis* and *Phallusia mammilata*) showed a significant inhibition of settlement.

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

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.

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

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

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

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

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

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

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

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,

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

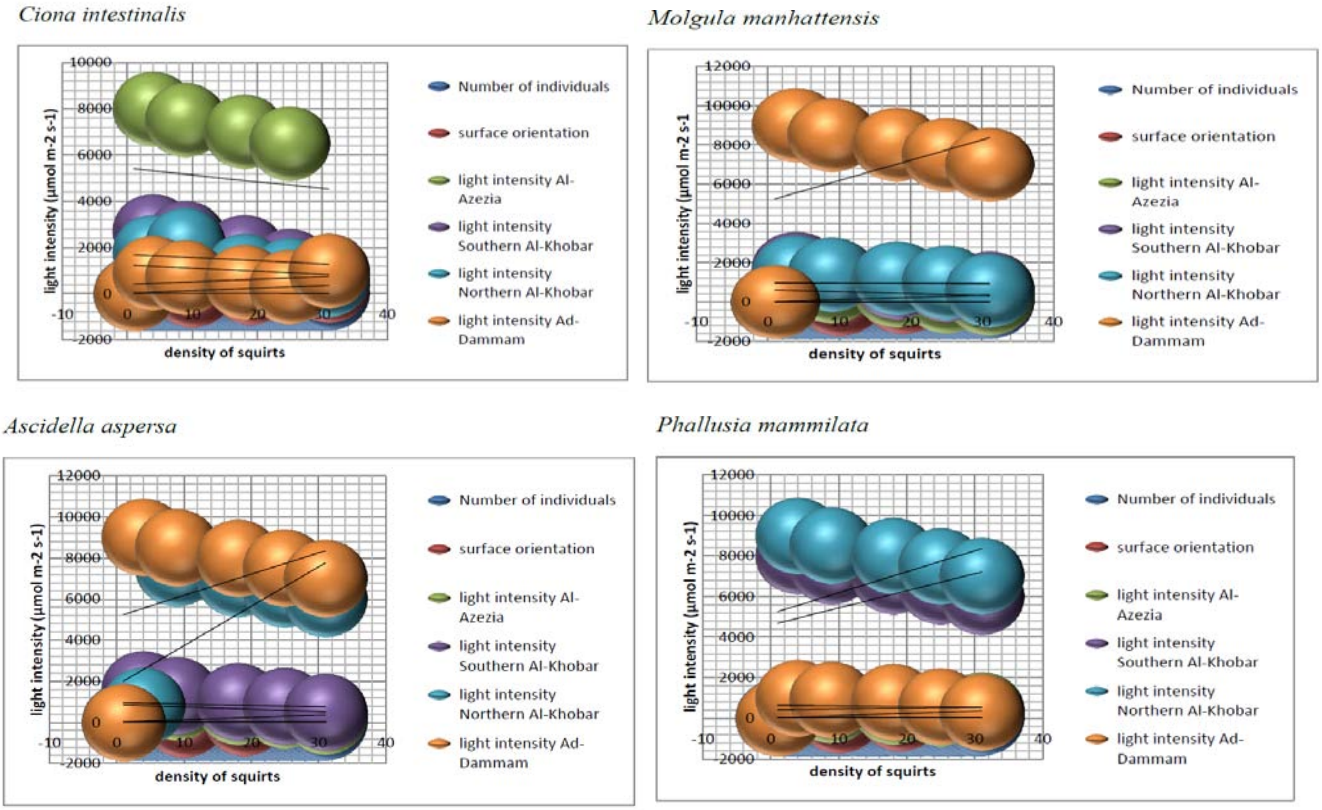
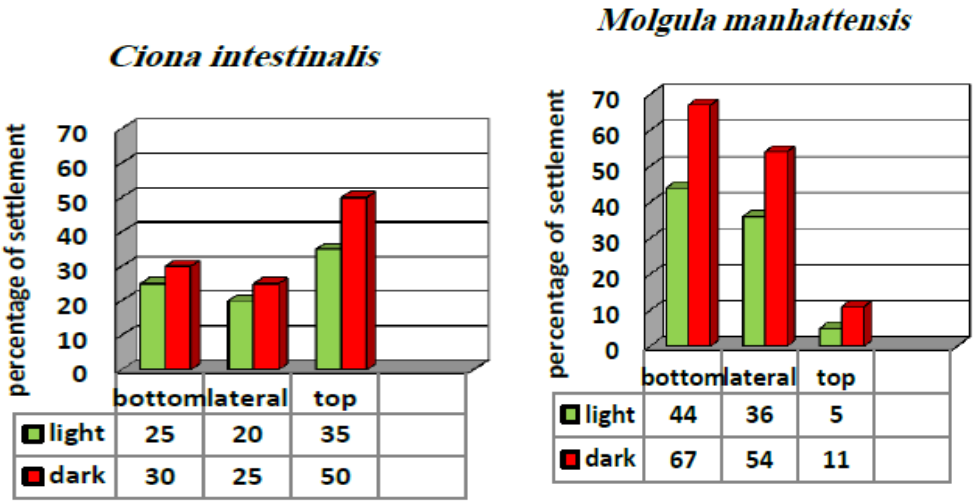


Fig. 1 : Phase contrast photomicrograph of a whole mount of a hatching larval stage of Ciona intestinalis (beginning of differentiation of the trunk and tail regions)

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



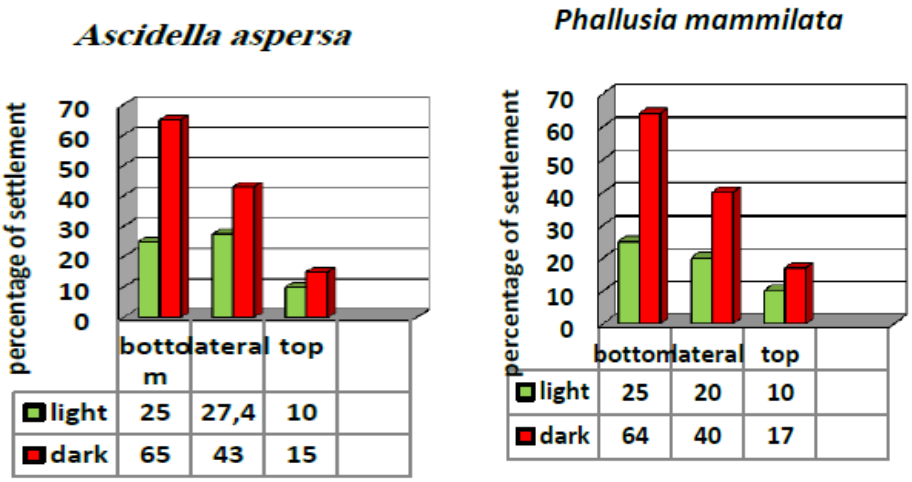


Fig. 2 : Phase contrast photomicrograph of a whole mount of a larval stage of Ciona intestinalis (Early tail resorption is indicated by an arrow)

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.

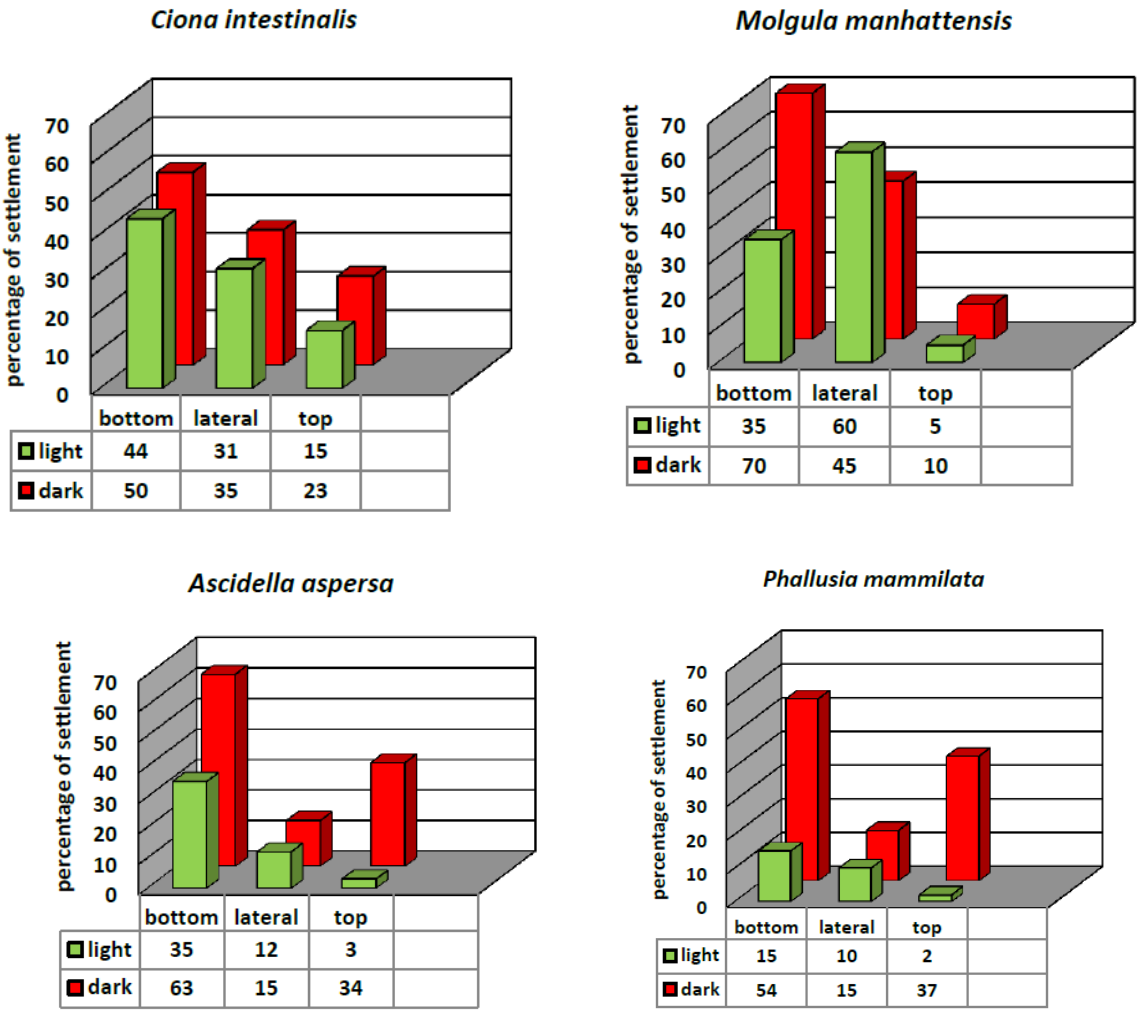


Fig. 3 : Phase contrast photomicrograph of a whole mount of a late metamorphosed larval stage of Ciona intestinalis

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.

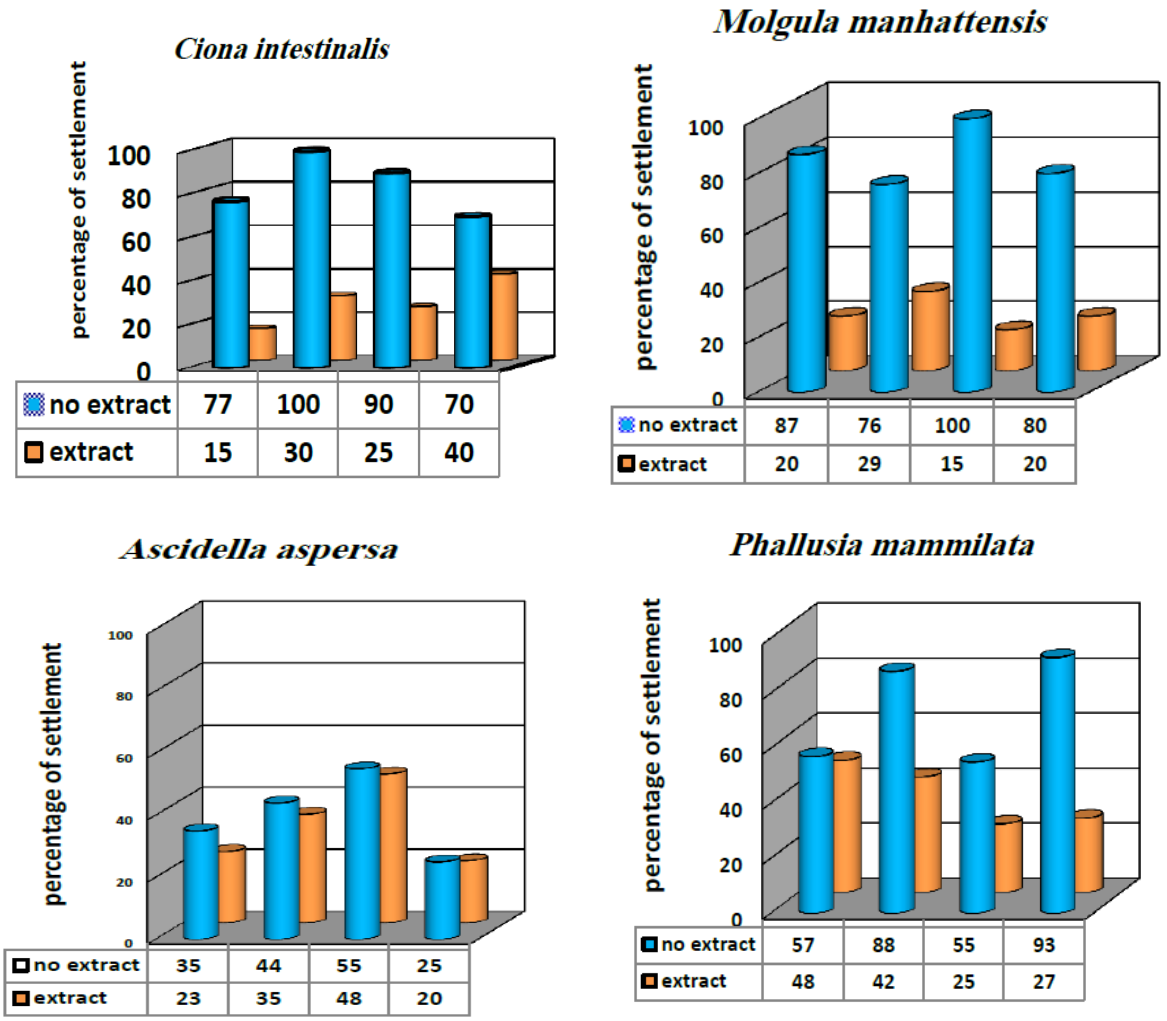
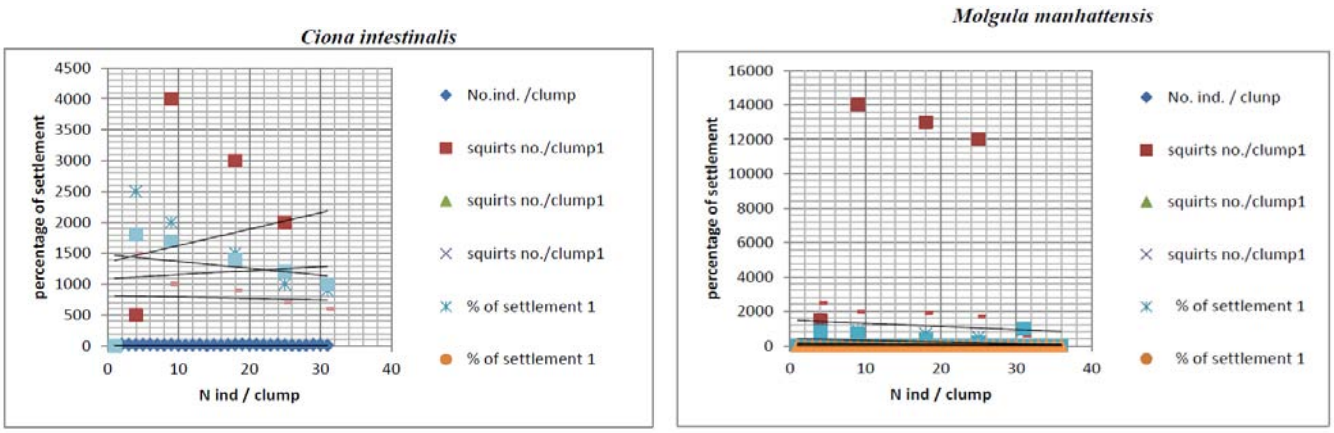
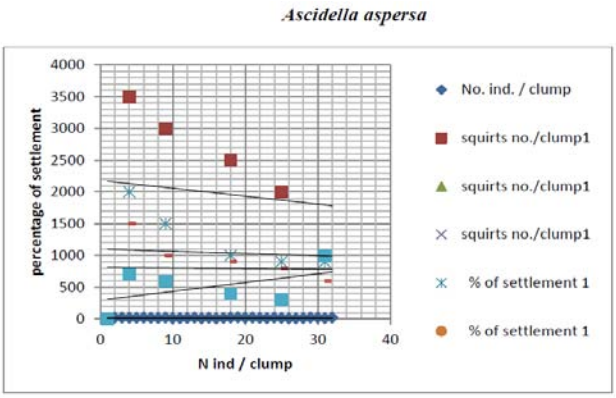
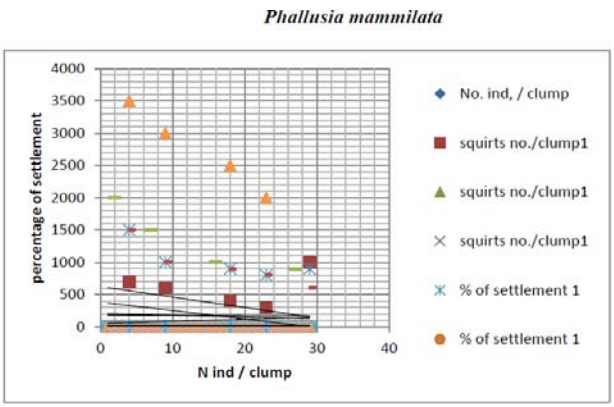


Fig. 4 : Photomicrograph of a transverse section of Phallusia mammilata showing the gonad. The squirt was collected during summer

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



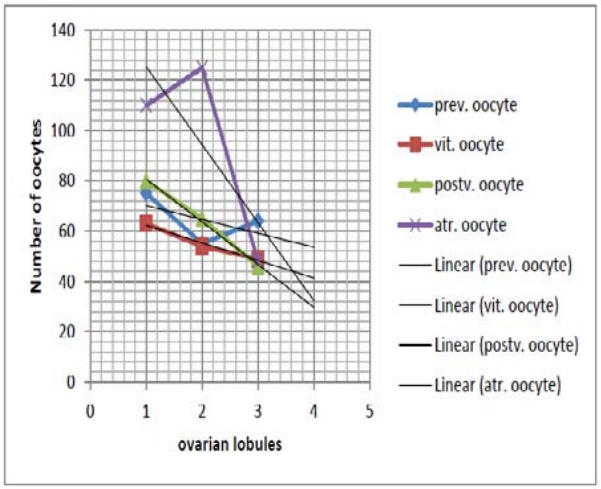




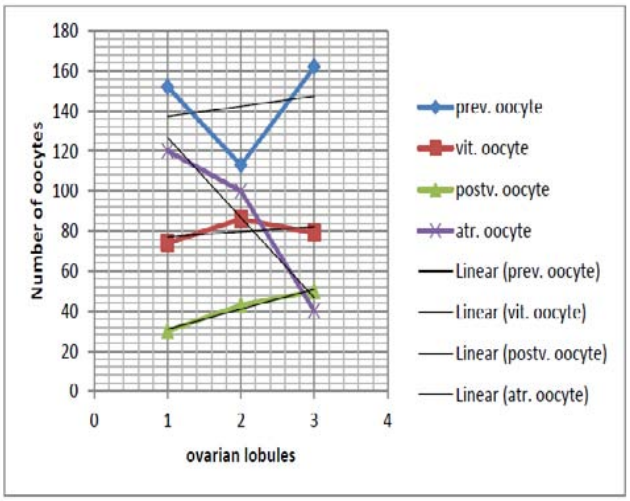
Figs. 5-6 : Photomicrograph of a transverse section of Phallusia mammilata ovary showing germinal epithelium and previtellogenic oocytes. The squirt was collected during spring

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

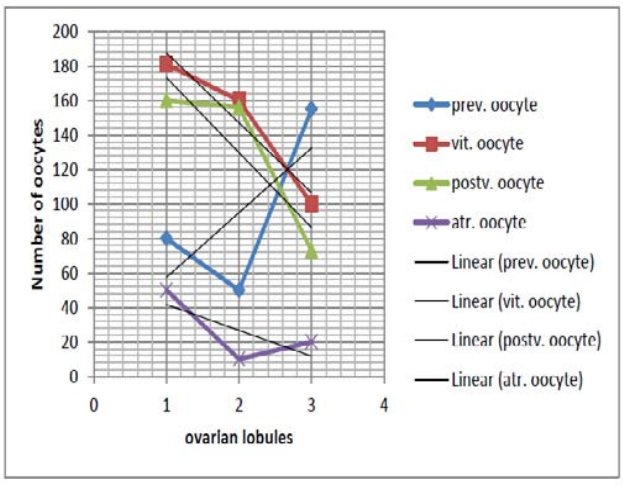
During spring



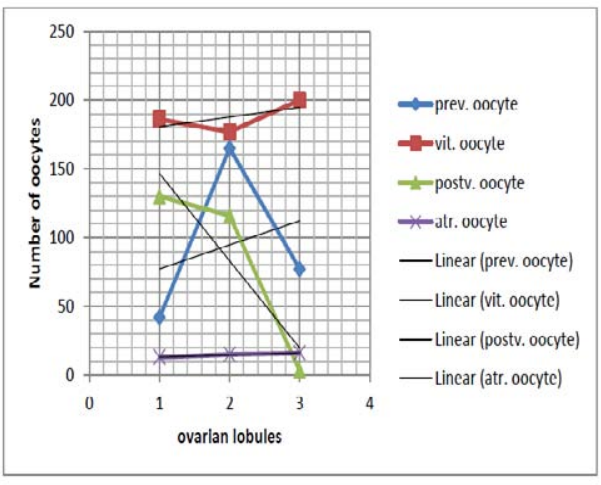
During summer



During autumn



During winter



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

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

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			

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			

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

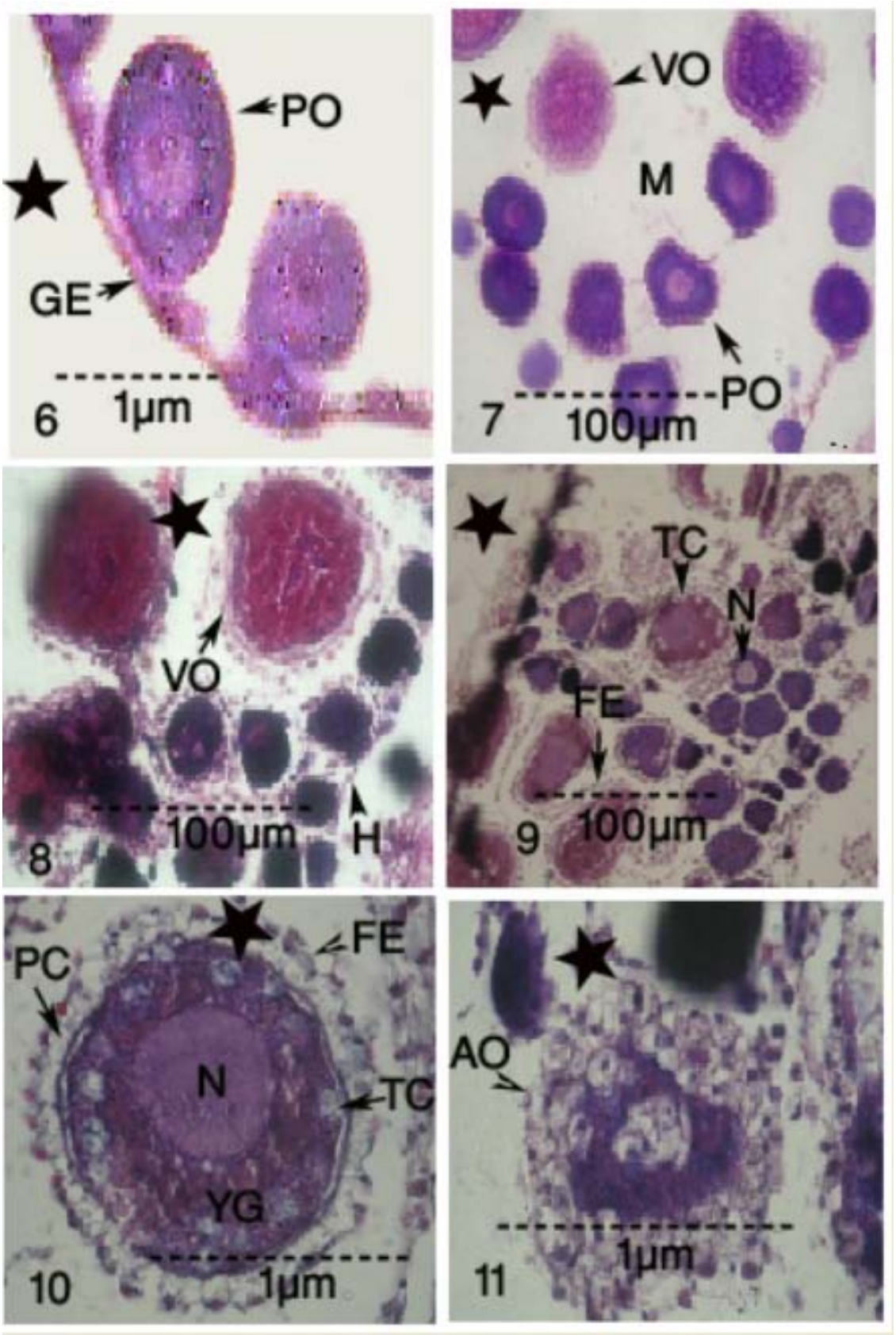
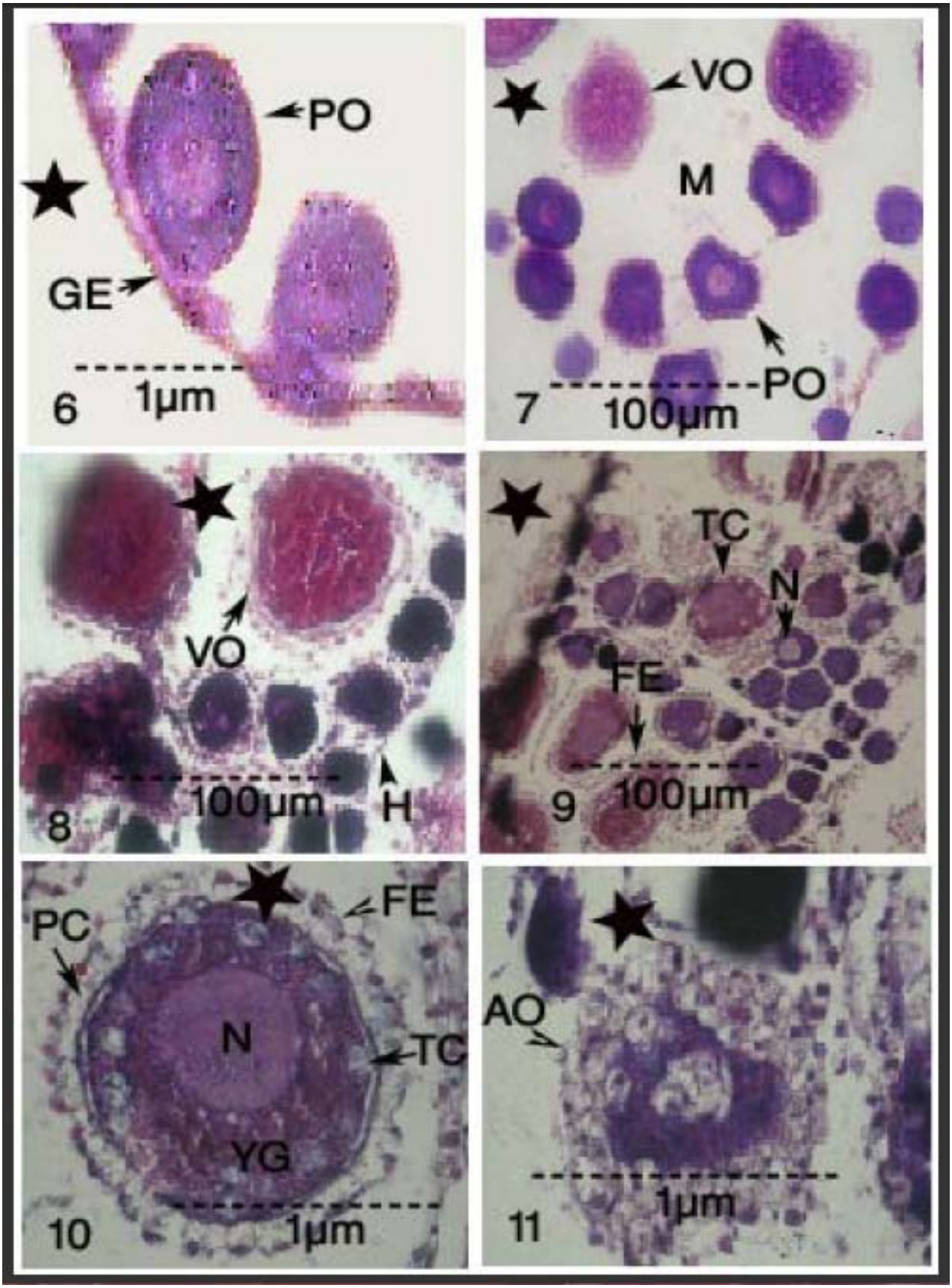


Fig. 7 : Photomicrograph of a transverse section of *Phallusia mammilata* ovary showing previtellogenic and early stage of vitellogenic oocytes. The squirt was collected during summer



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.

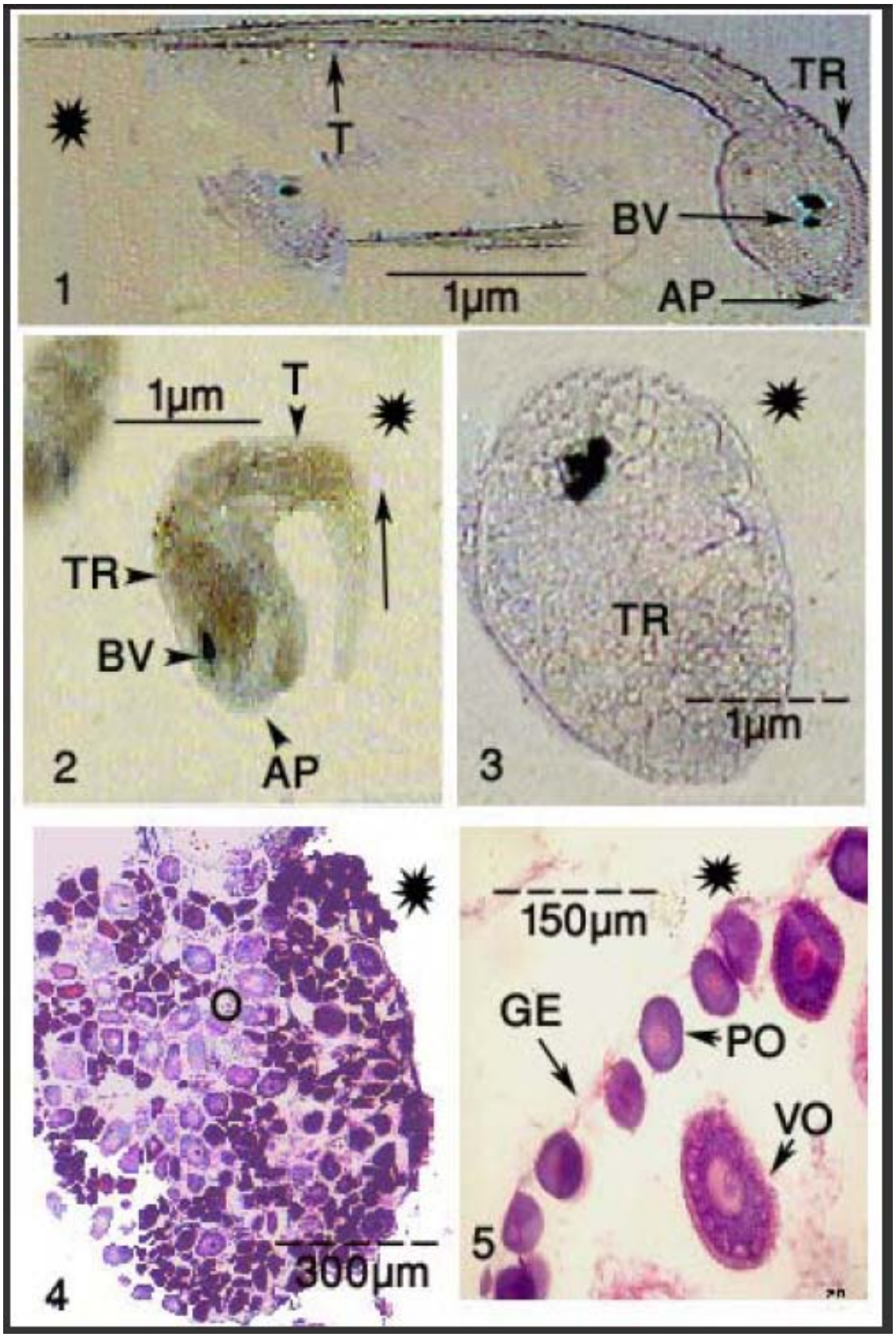


Fig. 10 : Photomicrograph of a transverse section of *Phallusia mammilata* ovary showing late stage of vitellogenic oocytes. Note, follicular epithelium, test cells and pervitelline space were developed. The squirt was collected during winter.

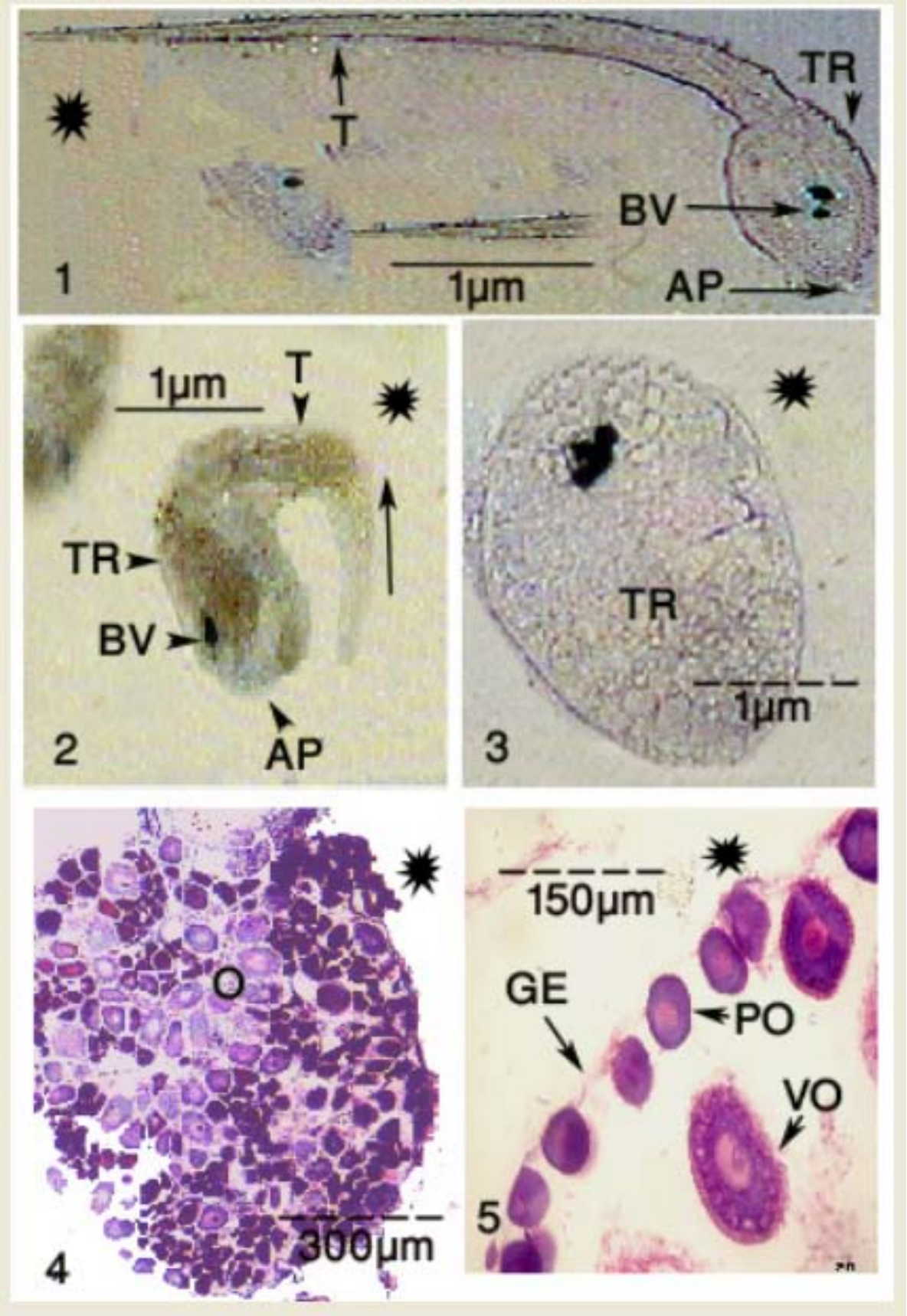


Fig. 11 : Photomicrograph of a transverse section of *Phallusia mammilata* ovary showing atretic oocytes. The squirt was collected during late winter.

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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FELLOWS

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Note :

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"

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The Author can submit the paper either online or offline. The authors should prefer online submission.Online Submission: There are three ways to submit your paper:

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(II) Choose corresponding Journal.

(III) Click 'Submit Manuscript'. Fill required information and Upload the paper.

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- Large Images must be in One Column
- Numbering of First Main Headings (Heading 1) must be in Roman Letters, Capital Letter, and Font Size of 10.
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2. Ethical Guidelines,

3. Submission of Manuscripts,

4. Manuscript's Category,

5. Structure and Format of Manuscript,

6. After Acceptance.

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- One should avoid outdated words.

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Numerical Methods: Numerical methods used should be clear and, where appropriate, supported by references.

Acknowledgements: Please make these as concise as possible.

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Acrobat Reader will be required in order to read this file. This software can be downloaded

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TECHNIQUES FOR WRITING A GOOD QUALITY RESEARCH PAPER:

1. Choosing the topic: In most cases, the topic is searched by the interest of author but it can be also suggested by the guides. You can have several topics and then you can judge that in which topic or subject you are finding yourself most comfortable. This can be done by asking several questions to yourself, like Will I be able to carry our search in this area? Will I find all necessary recourses to accomplish the search? Will I be able to find all information in this field area? If the answer of these types of questions will be "Yes" then you can choose that topic. In most of the cases, you may have to conduct the surveys and have to visit several places because this field is related to Computer Science and Information Technology. Also, you may have to do a lot of work to find all rise and falls regarding the various data of that subject. Sometimes, detailed information plays a vital role, instead of short information.

2. Evaluators are human: First thing to remember that evaluators are also human being. They are not only meant for rejecting a paper. They are here to evaluate your paper. So, present your Best.

3. Think Like Evaluators: If you are in a confusion or getting demotivated that your paper will be accepted by evaluators or not, then think and try to evaluate your paper like an Evaluator. Try to understand that what an evaluator wants in your research paper and automatically you will have your answer.

4. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

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6. Use of computer is recommended: As you are doing research in the field of Computer Science, then this point is quite obvious.

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9. Use and get big pictures: Always use encyclopedias, Wikipedia to get pictures so that you can go into the depth.

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11. Revise what you wrote: When you write anything, always read it, summarize it and then finalize it.

12. Make all efforts: Make all efforts to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in introduction, that what is the need of a particular research paper. Polish your work by good skill of writing and always give an evaluator, what he wants.

13. Have backups: When you are going to do any important thing like making research paper, you should always have backup copies of it either in your computer or in paper. This will help you to not to lose any of your important.

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17. Never use online paper: If you are getting any paper on Internet, then never use it as your research paper because it might be possible that evaluator has already seen it or maybe it is outdated version.

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19. Know what you know: Always try to know, what you know by making objectives. Else, you will be confused and cannot achieve your target.

20. Use good quality grammar: Always use a good quality grammar and use words that will throw positive impact on evaluator. Use of good quality grammar does not mean to use tough words, that for each word the evaluator has to go through dictionary. Do not start sentence with a conjunction. Do not fragment sentences. Eliminate one-word sentences. Ignore passive voice. Do not ever use a big word when a diminutive one would suffice. Verbs have to be in agreement with their subjects. Prepositions are not expressions to finish sentences with. It is incorrect to ever divide an infinitive. Avoid clichés like the disease. Also, always shun irritating alliteration. Use language that is simple and straight forward. put together a neat summary.

21. Arrangement of information: Each section of the main body should start with an opening sentence and there should be a changeover at the end of the section. Give only valid and powerful arguments to your topic. You may also maintain your arguments with records.

22. Never start in last minute: Always start at right time and give enough time to research work. Leaving everything to the last minute will degrade your paper and spoil your work.

23. Multitasking in research is not good: Doing several things at the same time proves bad habit in case of research activity. Research is an area, where everything has a particular time slot. Divide your research work in parts and do particular part in particular time slot.

24. Never copy others' work: Never copy others' work and give it your name because if evaluator has seen it anywhere you will be in trouble.

25. Take proper rest and food: No matter how many hours you spend for your research activity, if you are not taking care of your health then all your efforts will be in vain. For a quality research, study is must, and this can be done by taking proper rest and food.

26. Go for seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

28. Make colleagues: Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

29. Think technically: Always think technically. If anything happens, then search its reasons, its benefits, and demerits.

30. Think and then print: When you will go to print your paper, notice that tables are not be split, headings are not detached from their descriptions, and page sequence is maintained.

31. Adding unnecessary information: Do not add unnecessary information, like, I have used MS Excel to draw graph. Do not add irrelevant and inappropriate material. These all will create superfluous. Foreign terminology and phrases are not apropos. One should NEVER take a broad view. Analogy in script is like feathers on a snake. Not at all use a large word when a very small one would be sufficient. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Amplification is a billion times of inferior quality than sarcasm.

32. Never oversimplify everything: To add material in your research paper, never go for oversimplification. This will definitely irritate the evaluator. Be more or less specific. Also too, by no means, ever use rhythmic redundancies. Contractions aren't essential and shouldn't be there used. Comparisons are as terrible as clichés. Give up ampersands and abbreviations, and so on. Remove commas, that are, not necessary. Parenthetical words however should be together with this in commas. Understatement is all the time the complete best way to put onward earth-shaking thoughts. Give a detailed literary review.

33. Report concluded results: Use concluded results. From raw data, filter the results and then conclude your studies based on measurements and observations taken. Significant figures and appropriate number of decimal places should be used. Parenthetical remarks are prohibitive. Proofread carefully at final stage. In the end give outline to your arguments. Spot out perspectives of further study of this subject. Justify your conclusion by at the bottom of them with sufficient justifications and examples.

34. After conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print to the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects in your research.

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Key points to remember:

- Submit all work in its final form.
- Write your paper in the form, which is presented in the guidelines using the template.
- Please note the criterion for grading the final paper by peer-reviewers.

Final Points:

A purpose of organizing a research paper is to let people to interpret your effort selectively. The journal requires the following sections, submitted in the order listed, each section to start on a new page.

The introduction will be compiled from reference matter and will reflect the design processes or outline of basis that direct you to make study. As you will carry out the process of study, the method and process section will be constructed as like that. The result segment will show related statistics in nearly sequential order and will direct the reviewers next to the similar intellectual paths throughout the data that you took to carry out your study. The discussion section will provide understanding of the data and projections as to the implication of the results. The use of good quality references all through the paper will give the effort trustworthiness by representing an alertness of prior workings.

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To make a paper clear

· Adhere to recommended page limits

Mistakes to evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

· Shun familiar wording, don't address the reviewer directly, and don't use slang, slang language, or superlatives

· Shun use of extra pictures - include only those figures essential to presenting results

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The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing references at this point.

An abstract is a brief distinct paragraph summary of finished work or work in development. In a minute or less a reviewer can be taught the foundation behind the study, common approach to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study, with the subsequent elements in any summary. Try to maintain the initial two items to no more than one ruling each.

- Reason of the study - theory, overall issue, purpose
- Fundamental goal
- To the point depiction of the research
- Consequences, including definite statistics - if the consequences are quantitative in nature, account quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
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- A conceptual should situate on its own, and not submit to any other part of the paper such as a form or table
- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else

Introduction:

The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

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- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.

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This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

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- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
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- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

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The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.

Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
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- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

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- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

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The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a argument should be. Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly described. Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved with prospect, and let it drop at that.

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- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

- When you refer to information, differentiate data generated by your own studies from available information
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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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