FAUNA D'ITALIA
Vol. LI

ASCIDIACEA
of the European Waters

COMITATO SCIENTIFICO PER LA FAUNA D'ITALIA

Lucio Bonato, Ferdinando Boero, Marco A. Bologna,
Achille Casale, Marco Curini Galletti,
Bruno Massa, Alessandro Minelli, Marco Oliverio,
Augusto Vigna Taglianti, Marzio Zapparoli

FAUNA D'ITALIA

Sotto gli auspici dell'Accademia Nazionale Italiana di Entomologia
e dell'Unione Zoologica Italiana
con il patrocinio del Ministero dell'Ambiente e della Tutela
del Territorio e del Mare

ASCIDIACEA
of the European Waters

---

a cura di

RICCARDO BRUNETTI
e
FRANCESCO MASTROTOTARO

CALDERINI

1ª edizione: maggio 2017

Cover photos from left to right: *Ciona edwardsi* (photo M. Relini); *Clavelina lepadiformis*; *Distaplia bermudensis* (photos F. Vitale); zooid of *Didemnum commune*; larva of *D. coriaceum* (photos F. Grieco); *Polycitor cristallinus*; *Diplosoma spongiforme* (photos F. Mastrototaro); *Ascidiella scabra*; *Styela plicata* (photos F. Vitale); *Aplidium elegans* (photo F. Mastrototaro); *Ecteinascidia turbinata* (photo F. Vitale); *Didemnum maculosum* (photo F. Grieco); *Microcosmus polymorphus* (photo F. Mastrototaro); *Rhopalaea neapolitana* (photo E. Trainito); *Polyandrocarpa zorritensis*; *Botrylloides leachii* (photos F. Vitale); *Halocynthia papillosa* (photo E. Ricchitelli)

new Business Media
gruppo tecniche nuove

via Eritrea, 21 - 20157 Milano
Redazione: piazza G. Galilei, 6 - 40123 Bologna
Vendite: tel. 051/6575833; fax 051/6575999
e-mail: libri.edagricole@newbusinessmedia.it
http://www.edagricole.it

5529

Stampa: Andersen S.p.A., via Brughera IV - 28010 Boca (NO)
Finito di stampare nel maggio 2017

ISBN 978-88-506-5529-8

of subsequent reviewers. Only consolidated synonyms as well as papers where more extensive descriptions of the individual taxa are given have been included in the species accounts.

Two kinds of key are used: ordinary dichotomus keys leading to the identification of all known families and genera and tabular keys with diagnostic characters for the European species within each genus. In this case, tabular keys are preferred because they allow to take a large set of characters simultaneously into account. This permits the identification of a species even in the absence of some information, in which case the identification process is usually interrupted when using a dichotomus key (Monniot *et al.*, 1991). On the other hand dichotomus keys may have a didactic value and can be memorized unless the taxon is very large.

We hope that our work will help young researchers in environmental studies, and excite renewed interest in taxonomy, a field of zoological research too much and too long neglected.

This work is dedicated to Armando Sabbadin (21 Dec. 1920 – 19 Feb. 2016), *Emeritus* Professor of Comparative Anatomy at the University of Padua, that with his unflinching study of several aspects of the colonial ascidian biology greatly contributed to renew the Italian interest in ascidiology.

To Franca for her moral support.
Riccardo

Voici que le semeur est sorti pour semer
(Marseille 11/07/1999)
A Fiammetta
Francesco

PREFACE (Figures 1, 2)

Our aim with this volume is to provide a summary of the current knowledge of the ascidian fauna of the European waters delimited, as in Costello *et al.* (2001), by the Arctic Ocean to the latitude 25°N with the western boundary marked by the Mid-Atlantic Ridge (Figure 1).

Figure 1 - Geographic scope of this volume. The isotherms of February are shown. 1. Azores; 2. Madeira; 3. Canaries. AR. Arctic Ocean; BAS. Baltic Sea; BLS. Black Sea; CA. cold Atlantic Ocean; EM. Eastern Mediterranean Sea; NAS. Northern Adriatic Sea (it is distinguished from EM because its very cold winter temperature); TA. temperate Atlantic Ocean; WA. warm Atlantic Ocean; WM. Western Mediterranean Sea.

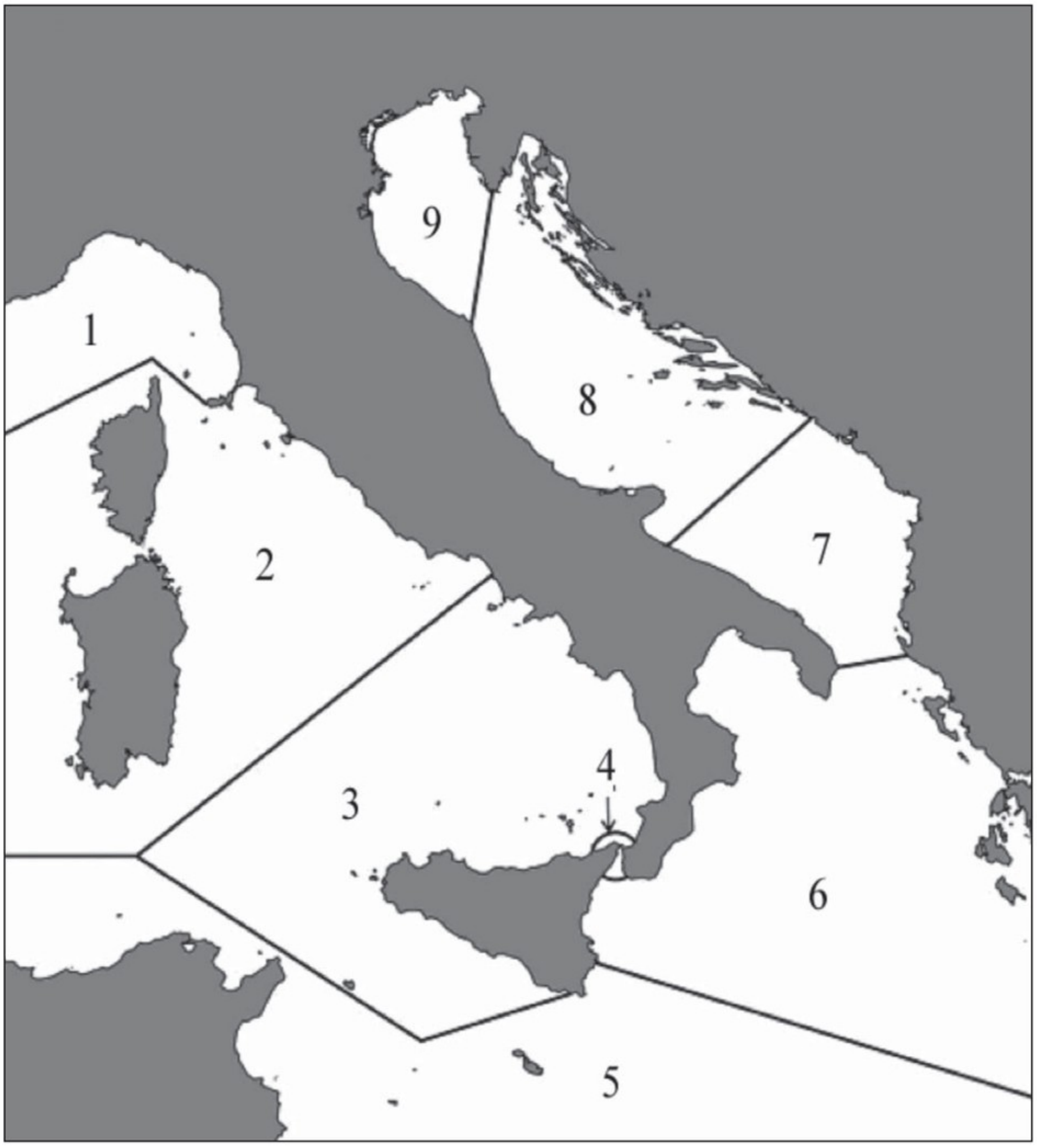

Figure 2 - Conventional partitioning of the Italian seas into nine faunistic areas (Bianchi, 2004). 1. Northern Tyrrhenian Sea; 2. Mid Tyrrhenian Sea; 3. Southern Tyrrhenian Sea; 4. Strait of Messina; 5. Strait of Sicily; 6. Ionian Sea; 7. Southern Adriatic Sea; 8. Mid Adriatic Sea; 9. Northern Adriatic Sea.

Particular attention was given to the finds of ascidian species along the Italian coasts Mastrototaro & Tursi, 2010, following the division of the Italian seas into 9 geographical areas as proposed by Bianchi (2004) (Figure 2).

Every species is briefly described, paying attention to the morphological characters useful for species identification.

The present work being addressed to a readership broader than the group specialists, the space devoted to the biology of the group is felt by us useful to a better understanding of the original descriptions and illustrations, when available, are always shown and have been integrated with the contributions

CONTENTS

THE AUTHORS

Riccardo Brunetti:
Professor of Marine Biology at the University of Padua until an early retirement (30/06/2000) later on voluntary collaborator of the Museo di Storia Naturale di Venezia and subject Editor (Tunicata) of Zootaxa
ric.brunetti@gmail.com

Francesco Mastrototaro:
Researcher in Zoology at University of Bari (Italy)
Via Orabona, 4 – 70125 – Bari – Italy
francesco.mastrototaro@uniba.it

Acknowledgments
The Authors wish to thanks **Vittoria Catalano** and **Giacomo Masato** for their help in old manuscripts searching.
Paolo Burighel and **Alessandro Minelli** for their exciting discussions about the studied taxa, **Renzo Mazzaro** for his help in the electronic drawing of figures and **Flavia Grieco**, **Federica Montesanto** and **Barbara Rotella** for their help in the rereading of the proofs.
.

PART ONE

INTRODUCTION

INTRODUCTION

(Tables 1-2; Plates 1-8)

Historical Outline

The earliest description of ascidians was given by Aristotle (350 B. C.) in his *History of Animals* (cf. Cresswell, 1883). He recognized the animal nature of the solitary forms, which he named *Tethya*, and described their test (or tunic) and body wall. Moreover he recognized the inhalant and exhalant function of the siphons. Subsequent authors interested in marine zoology (Pliny, ~A.D. 70; Aelianus, ~A.D. 200; Bellonius (or Belon), 1553; Rondeletius, 1555; Gesner, 1558; Aldrovandi, 1606; Avicenna, 1608; Jonstonus, 1650; Redi, 1684; Sloane, 1707) contributed very few new data to extending the knowledge on these animals. Rondeletius used the names *Uva marina* (marin grapes) and *Malum insanum* (mad fruit) for two compound ascidians, the first certainly a *Botryllus* sp., the second one probably a *Botrylloides* sp., and Redi identified two solitary forms which he named *Mentula marina* (little marine penis) and *Microcosmus marinus*. In 1757 Schlosser & Ellis, described *Botryllus* and in 1762 Baster introduced the name *Ascidium* for a species which he likened to oysters. Finally Linnaeus (1767) erected the genus *Ascidia* to replace both *Tethyum* and *Ascidium* for the solitary forms, maintaining the name *Alcyonium* for the compound species. From the end of the XVIII century the number of ascidian species, which in Linnaeus' *Systema Naturae* (12 ed.) was only 10, began to increase. A summary of ascidiological knowledge of the time is found in Bruguière's contribution to the *Encyclopédie Méthodique* (1789, 1791). [For old references see Hopkinson, 1913].

In 1815 an article on *Botryllus* was published by Desmarest & Lesueur, but the most important work of this time is Savigny's (1816) famous *mémoir*. In this splendid publication, in which several species are described and depicted, the author clarifies the structure of the colonial species, which are separated from the *Alcyonaria* (Cnidaria) with which they were previously united. The increased understanding of the anatomy of this group due to Cuvier (1815) and Savigny (1816) made it possible for Lamarck (1816) to accommodate both simple and colonial species into the new class *Tunicata*. The following years witnessed the publication of articles by Delle Chiaje (1827-30) and Audouin & Milne-Edwards (1828) in which the larval metamorphosis was described for the first time, while the list of species continued to increase (Risso, 1826). In 1834 the circulation of the blood was described by Lister in *Perophora,* but the most important work of these years is Milne-Edwards' *Observations sur les Ascidies Composées des côtes de la Manche* (1841). A few years later Schmidt (1845) discovered the presence of cellulose in the test, Van Beneden (1846) illustrated the embryology of a simple ascidian and the articles of Macdonald on *Chondrostachys* (1858) and *Diplosoma* (1859) were published. In the meantime new species were described by Forbes & Hanley (1848-52), Alder (1850), Stimpson (1852) and Grube (1861).

The year 1866, in which Kowalevsky published his *Entwickelungsgeschichte der einfachen Ascidien*, marks a milestone in the history of ascidiology: the structure of the ascidian larva was recognized as reflecting the basic body ar-

chitecture of the chordates. Kowalevsky's article stimulated the study of the embryology and budding of the colonial forms. The works of Metschnikoff (1868), Kupffer von (1869, 1870), de Lacaze-Duthiers (1870), Giard (1872b), Della Valle (1882a, b, c) and Pizon (1893) are also worthy of mention. New acquisitions in anatomy and physiology were due to Ussow (1875) and Julin (1881), who studied the nervous system and its relation with the dorsal tubercle, while Fol (1874) studied the structure and function of the endostyle. In the same years important studies contributed to increasing the list of species (Verril, 1871, 1872; Giard, 1873; Heller, 1874, 1875, 1877; de Lacaze-Duthiers, 1877; Della Valle, 1877, 1881; Traustedt, 1880, 1882, 1883a, b, 1885; Drasche von, 1883, 1884a, b; Roule, 1884a, b, c), but above all the three volumes of the *Challenger Reports* devoted to the Tunicata by Herdman (1882, 1886, 1888) in which 163 species, many of them previously unknown, were described and depicted. The extraordinary production of the XIX century is summarized in Seeliger's and Hartmeyer's contributions in Bronn's *Klassen und Ordnungen des Tierreichs* (Seelinger 1893-1911; Hartmeyer, 1909-11).

An overview of morphology and biology of Ascidians

Morphology
(Plate 1)

Ascidians that do not bud are called *simple* or *solitary*. Those that undergo budding, so increasing the zooid number to form a colony, are called *colonial* or *compound*.

This first classification was done by Savigny (1816) later Milne-Edwards (1841) split the colony-forming ascidians in two groups: compound and social ascidians, both are colony-forming, but in social ascidians the zooids remained free and not enclosed in a common test. The division suggested by Milne-Edward met with no success and already from the first part of Herdman's (1882) Challenger reports it was abandoned.

Simple ascidians have a sac-like structure, whereas the individuals of compound ascidians are usually elongate and their body can be divided into two or three segments: the *thorax* containing the pharynx, the *abdomen* containing the digestive organs and also the reproductive organs, unless a *posterior abdomen* is recognizable, in which case the latter hosts the gonads and the heart. The body of a simple ascidia or of a single element of a colony is called a *zooid* or *ascidiozooid*; if developed from a bud it is called a *blastozooid*. The zooid has two apertures: the *oral* or *branchial aperture* and the *atrial* one. Water is drawn into the pharynx through the oral aperture and moved out of it through the atrial one. These apertures are sometimes at the end of cylindrical extensions of the body called *siphons*. The axis passing through the oral aperture and the oesophagus is defined as anteroposterior, the neural complex marks the dorsal side.

The *test* (or tunic) (Plate 1, Figs 1, 2) is a protective layer externally covering the body. It consists of polysaccharides (*tunicin*) structurally similar to plant cellulose. Proteins, blood cells, pigments and sometimes calcareous *spicules* may be present within the test. The test's aspect can vary, from transparent as glass to completely opaque, from mucilaginous to as hard as leather, sometimes the

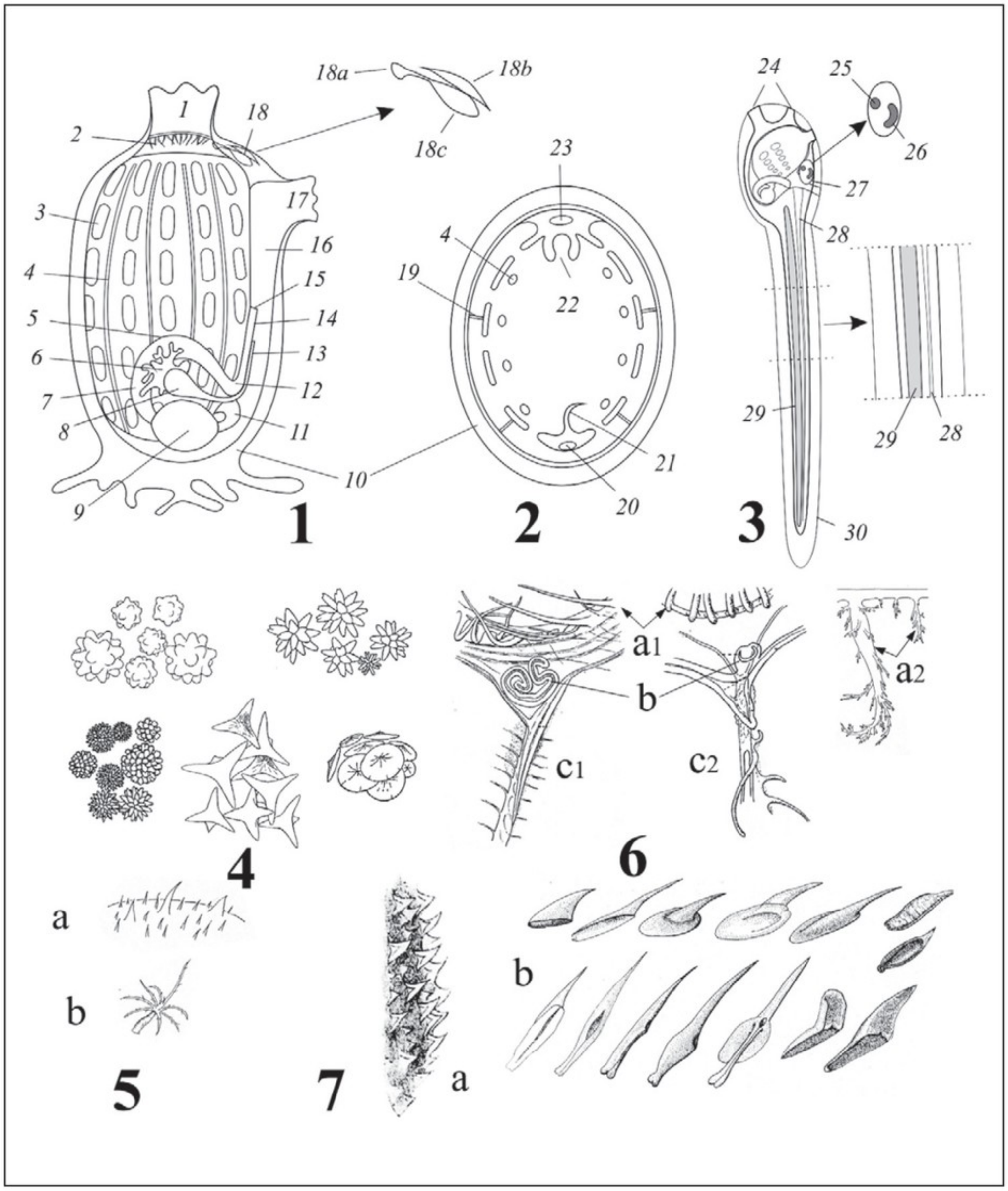

Plate 1 - Some aspects of ascidian morphology (I). **1** Diagram of ascidian body. **2** Cross-section of the same. **3** Tadpole larva. **4** Some types of spicules. **5** Spinules, *a*, simple; *b*, branched. **6** Dorsal anterior portion of branchial sac. Tentacles, a_1, simple; a_2, branched; b, dorsal tubercle, c_1, dorsal lamina with smooth edged; c_2, dorsal lamina divided in languets. **7** Siphonal armature, a, appearance of internal test of Pyurid's siphon; b, some type of siphonal scales. (4, 6, 7a from Hŭus, 1937. 5 from Millar, 1966. 7b from Kott, 1985).

1, oral siphon; *2*, tentacles; *3*, stigmata; *4*, internal longitudinal vessel; *5*, primary intestinal loop; *6*, testis; *7*, intestine; *8*, ovary; *9*, stomach; *10*, test; *11*, oesophagus; *12*, secondary intestinal loop; *13*, gonoduct; *14*, rectum; *15*, anus; *16*, peribranchial cavity; *17*, atrial siphon; *18*, neural complex; *18a,* dorsal tubercle; *18b*, neural gland; *18c*, ganglion; *19*, connection between body wall and branchial sac; *20*, dorsal vessel; *21*, dorsal lamina; *22*, endostyle; *23*, endostylar (or subendostylar) vessel; *24*, adhesive organ; *25*, otholith; *26*, photolith; *27*, sensory vesicle; *28*, neural tube; *29* notochord; *30,* fin.

surface of the test presents simple or branched *spinules* (Plate 1, Fig. 5). Sand or debris may adhere to its surface or be embedded within it. The test is secreted by the external epithelium delimiting the body wall.

The test invaginates into the siphons terminating in front of the tentacles. In the Pyuridae and in some genera of the Styelidae, in this invaginated part of the test there are *siphonal scales* oriented toward the aperture. These scales, varying between the species, are an important taxonomic character (Turon, 1987a).

The *body wall*, sometimes improperly called *mantle* [a term to be used only in Mollusca, where this body part is responsible for secretion of the shell], adheres to the test in proximity to the apertures. It consists of two epidermal sheets with mesenchimatic tissue between them (for the microscopic anatomy of ascidians see the review of Burighel & Cloney, 1997). The neural complex, blood lacunae and smooth muscle are contained within the body wall.

The *branchial sac* or *pharynx* is the largest internal organ (Plate 1, Fig. 2); it opens to the external environment by means of the oral aperture. Surrounding the internal base of the latter there are simple or ramified *tentacles* (Plate 1, Fig. 6) which prevent the entry of coarse particles.

Along its ventral edge the branchial sac is externally joined to the body wall and internally covered by a glandular groove: the *endostyle.* The branchial sac is perforated by *stigmata* normally arranged in transverse rows. The edge of the stigmata is generally ciliated and the movement of the cilia creates an incoming stream of water which flows through the stigmata. This flux of water permits gaseous exchanges as well as food uptake. In fact ascidians are filter-feeders which rely on water-suspended particles as a source of nutrition. However the stigmata are larger than the particles which the animals are normally able to capture: therefore the pharynx is not the true filtering structure. Instead this role is played by a *mucous microfilter* the meshes of which have a diameter of 0.1-0.2 μm (Monniot F., 1979 a, b) (on tunicate feeding see the review of Bone *et al.*, 2003). Thus the branchial sac serves as a water pump and as a support for the actual mucous filtering structure. The mucous filter is continuously produced by the glandular cells of the endostyle and transported by ciliary movements into the middle-dorsal line where the *dorsal lamina,* sometimes replaced by a row of *languets* (Plate 1, Fig. 6), arises in front of the endostyle. Once the mucous filter reaches the dorsal lamina it rolls up to form a chord which then descends toward the oesophageal opening.

The *gut loop* (Plate 1, Fig. 1) starts proximally in the *oesophagus*, followed by the *stomach*, and distally by the *intestine* ending with the *rectum*. The gut loop may form a simple arc or be curved in the form of an S, in the latter case the gut loop can be distinguished into a *primary* (proximal) and a *secondary* (distal) *loop*. The external stomach wall is smooth or folded or presents various kinds of reliefs. The wall of the intestine is enveloped by a *pyloric gland* formed by several vesicles. The latter are connected by a fine duct to the posterior region of the stomach. In some cases, in proximity of the duct opening, the stomach wall presents a blind diverticulum known as the *pyloric caecum* (see examples in Plates 114-115). The *anus*, which shows a smooth or lobed edge, opens into the *body cavity* (or *peribranchial* or *atrial*) *cavity* (or *chamber*). The latter communicates with the external environment by means of the *atrial* (or *cloacal*) *aperture*, which also can present tentacles internally (see examples in Plates 118-119).

The *neural complex* is located on the mid-dorsal line between the siphons

(Plate 1, Fig. 1). It is formed by the closely associated *neural gland* and *neural ganglion*. A duct runs from the neural gland to the prebranchial (imperforated) part of the pharynx where it opens on its inner surface, behind the ring of oral tentacles. The neural gland aperture (*dorsal tubercle*), which is ciliated, can be a simple opening or a variously curved slit (Plate 1, Fig. 6). None of the hypothesized functions ascribed to this complex has been fully proved.

The *blood system* is open (Plate 2). The *heart* consists of two concentric tubes: *pericardium* and *myocardium*. Only the latter is contractile due to the presence of striated muscle in its wall. A peculiarity of the ascidian heart is the periodical change in the direction of the contractions. The blood runs in *sinuses*, which are cavities devoid of an endothelial wall. *Vessels*, characterized by an epithelial wall, are only present near the heart and within the test. However there is a definite circulatory scheme (Brunetti & Burighel, 1969; Burighel & Brunetti, 1971) The blood does not contain respiratory pigments but various cells are present. These have been described by authors under different names; De Leo (1992) reduced the approximately 40 described categories to only six cellular types.

The *epicardium* and the *excretory organs*. The epicardium consists of paired sacs developing from the posterior end of the dorsal mid-line of the pharynx (Plate 2, Figs 7-9). Despite the name, it develops independently of the heart. It is an important organ from both an embryological and a phylogenetic point of view, but it is difficult to see, without an accurate histological analysis in adult zooids. In Cionidae, the left sac is larger than the right sac. Communication with the branchial cavity persists in some *Ciona* species as two small holes on the bottom of the branchial sac (*pharyngo-epicardiac openings*). In *Clavelina* and the majority of Aplousobranchia (see Classification) the left sac separates to form a single chamber isolated from the branchial sac. In Didemnidae the epicardia have lost their communication with the pharynx and persist as very small sacs in the oesophageal region. In Polyclinidae they are very developed, as a single long tube in the abdomen and posterior abdomen. A review about epicardium can be found in Berrill (1950: 26-29).

The function of this organ is problematic; in Cionidae it is probably related to the regeneration capacity, in Clavelinidae, Didemnidae and other Aplousobranchia it is involved with the budding function (Brien, 1948; Berrill, 1950). In Phlebo- and Stolidobranchia (see Classification) this organ is exclusively embryonic. However its vestiges remain as an excretory organ, as "vesicles" surrounding the gut loop in the Phlebobranchia and a "kidney" in Molgulidae (Stolidobranchia).

The *spicules* (Plate 1, Fig. 4) are small calcareous bodies (generally less than 1 mm in diameter) present in the test of some species belonging to Didemnidae, Polycitoridae and Pyuridae. Those of the first two families consist of aragonite crystals (Prenant, 1925), while in *Herdmania momus* (Pyuridae) they are made of vaterite a rare form of calcium carbonate (Lowenstam & Abbott, 1975) and are present in the blood vessels too. The spicules of the Didemnidae are globular or star-shaped. Among the Polycitoridae, *Cystodytes* has discoid spicules crowded around the zooid abdomen, while *Polycitorella* presents spicules similar to those of the Didemnidae (Monniot F., 1970). The spicules of the Pyuridae are formed by superimposed whorls of spines; they are surrounded by an organic sheath, grow in time and finally migrate into the test and are finally expelled externally (Lambert & Lambert, 1987).

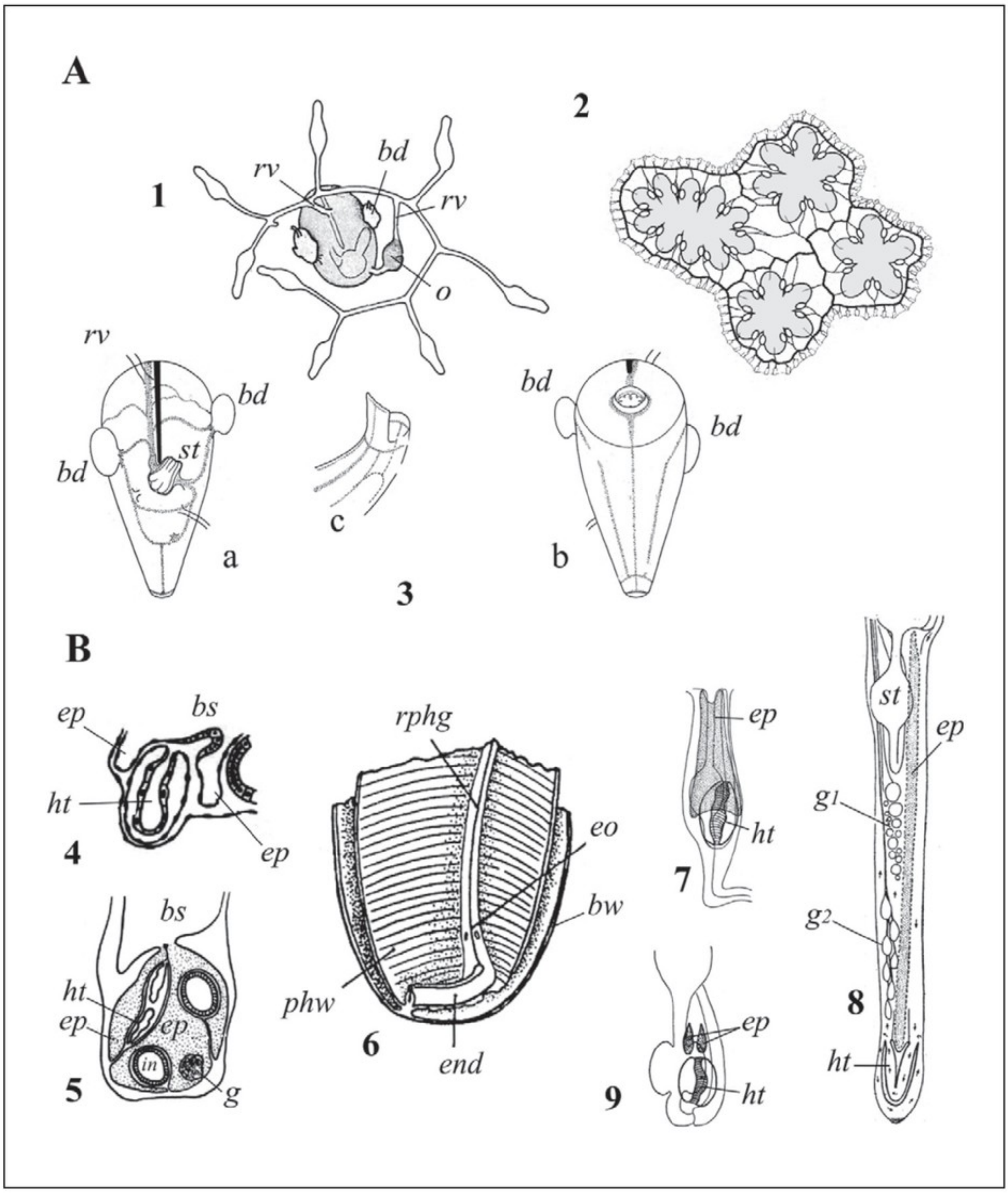

Plate 2 - Some aspects of ascidian morphology (II). **A. Blood circulatory system**. **1**, circulation in sinuses of *Botryllus* body wall; **2-3**, circulation in the vessels of the test colony of *Botryllus*. **B. Origin and development of epicardium. 4**, in *Ciona* origin of the two epicardia from the bottom of the branchial sac; **5**, the two epicardic cavities envelop heart, gonads and intestine; **6**, but they remain in communication with the branchial cavity; **7**, in *Clavelina* the epicardia fuse to form a single chamber isolated from the branchial sac; **8**, in *Aplidium* they fuse to form a single sac extending along the length of the abdomen and posterior abdomen; **9**, in the Didemnidae the epicardia are reduced to two small sacs.
bd, bud; *bs*, branchial sac; *bw*, body wall; *end*, endostyle; *eo*, epicardic openings; *ep*, epicardium; *g*, gonad; g_1, ovary; g_2, testis; *ht*, heart; *in*, intestine; *o*, regressed oozooid; *phw*, pharyngeal wall; *rphg*, retropharyngeal groove; *rv*, radial vessel; *st*, stomach. (1 from Burighel & Brunetti, 1971; 2,3 from Brunetti & Burighel, 1969; 4,5,7,8 from Berrill, 1950; 6 from Millar, 1953b; 9 from Brien, 1948).

For a long time it was thought that the spicules originated from mineralization exclusively due to chemico-physical factors. It is now known that they are the result of biomineralization under cellular control (Bellan-Dufrançaise *et al.*, 1995). In Didemnidae the spicules are formed in special organs, the *thoracic lateral organs* (TLO) (Lafargue & Kniprath, 1978; Kniprath & Lafargue, 1980).

The colours. Most of the solitary species are poorly coloured, covered in life by fine sediment or by epibionts, often only the siphons are pigmented. On the contrary, colonial species are usually rich in pigments which may be transported by blood cells to form delicate patterns (Sabbadin, 1962). Besides the true pigments (carotenoids, flavins, melanins) coloration can be due to products of nitrogenous metabolism (purines, pterins, urates) (Sabbadin & Tontodonati, 1967). These colours are probably under genetic control, as has been shown in *Botryllus schlosseri* by Sabbadin (1959, 1964). Some colours are due to the presence of bodies included in the test, as in the case of the calcareous spicules of Didemnidae, or even to the presence of pigments of endosymbiotic algae. There are also structural colours due to microcrystals, fibrils or other light diffracting structures (Monniot C. *et al.*, 1991). Pigments are usually not permanent, in fact after death they generally disappear within a few hours or days both in alcohol or formalin preserved samples. In many botryllid species the bright colours rapidly turn black, which may be confusing for those who have not seen the live specimen. Often the specific name refers to the colour observed on fixed material while the living animal has a very different coloration. That should advise against the use of specific names based upon the colour.

Sexual reproduction

Gonads. Ascidians are hermaphrodites with male and female gonads which generally ripen together; however, self-fertilization is normally prevented (Sabbadin *et al.*, 1992). Only in a few cases, in *Distaplia magnilarva* and, perhaps, in the genus *Sycozoa* (Kott, 1990), testes and ovaries may ripen at different times.

Testes and ovaries, either as separate organs or, more frequently, as a single structure, may be located in the body wall (Pleurogona) or in the intestinal loop or underneath it (Enterogona). In the Aplousobranchia (a suborder of the Enterogona) they are separated and located in the abdomen, below or beside the intestinal loop, or in the posterior abdomen. In the Phlebobranchia (a suborder of the Enterogona) and in the Stolidobranchia (a suborder of the Pleurogona) gonads are usually hermaphroditic; the male and female parts are separated only in a quite small number of species among the Botryllinae and Styelidae. In the Phlebobranchia the single gonad is usually enclosed within the intestinal loop. In the Stolidobranchia, gonads are in the lateral body wall, usually as several elongate units on both sides. Only some genera of the subfamily Styelinae (*Polycarpa, Dicarpa* and *Psammostyela)* have globose gonads (*polycarps*). Finally, in the Molgulidae there is only one gonad on each side. *Fertilization* can be external (*oviparity*), in which case a great number of isolecitic eggs are produced, or internal. In the latter case a small ovary produces few eggs which are rich in yolk and develop inside the parental body (*ovoviviparity*). In some species there are isolecitic eggs which are however associated with transfer of nutritional material from the parent to the embryos (*viviparity*) (Zaniolo *et al.*, 1998).

Spawning is probably induced by a photic stimulus (Lambert & Brandt, 1967; Whittingham, 1967; West & Lambert, 1976; Brunetti *et al.*, 1988) and is facilitated by the contraction of the whole zooid (*squirting*). In species with internal fertilization, the sperm is probably carried by the incoming water flux through the oral siphon and, as suggested by Kott (1992a, p. 386), its passage through the stigmata might be facilitated by a concurrent block in the secretion of mucus, as reported by Carlisle (1951) for *Ciona intestinalis*. (For more on the mechanism of fertilization in ascidians, see Satoh, 1994).

Larva
(Plate 1, Fig. 3)

The ascidian larva resembles the larval stage of the frogs (*tadpole*) (Plate 1, Fig. 3). It is free swimming for a short time, from a few minutes to some hours. In species with external fertilization, pelagic life may last some days, but in this case most of the time is spent floating as a developing egg. In some viviparous species of Styelidae and Molgulidae, development is direct and the pelagic stage is lost: the parental zooid emits young zooids which are ready to settle. (On the embryonic development of ascidians, see Brien, 1948 and Satoh, 1994).

The larval body consists of a *trunk* and a *tail*. It is covered by a transparent test, which along the tail extends into a dorsal and a ventral fin. At the anterior end of the trunk there are three, rarely two, *adhesive organs* which may be triradially arranged, or in a single vertical plane. Along the tail run, internally, the *notochord* and, dorsally to this, the *central nervous system* which consists of a cellular tube which anteriorly, into the trunk, expands to form a *cerebral vesicle* containing an *otolith* (by some authors called a "*statocyte*") and an *ocellus*. The otolith is formed by a single cell containing a granule of melanine and connected to the internal wall of the cerebral vesicle by a narrow peduncle (Whittaker, 1966; Torrence, 1986). The ocellus consists of a pigmented (melanine) cup-shaped cell with the concave side closed by three unicellular lenses and the convex side placed on a layer of pyramidal light-sensitive cells (Dilly, 1964). This description is valid for oviparous species: *Ciona* and the majority of Phlebobranchia and Stolidobranchia, however in the Styelidae there is a reduction of the ocellus which in *Polycarpa* is completely absent.

In colonial (ovoviviparous and viviparous) Styelidae the larva is slightly larger in size (usually with a trunk length over 0.5 mm) and shows more developed organs. The ocellus is also absent in these larvae but the light sensitive functions are carried out by the otolith which contains light-sensitive cells (Grave & Riley, 1935). The resulting organ, called *photolith* (Garstang S. & Garstang W., 1928), has both photo-tactical and geo-tactical functions. In the Perophoridae (the only ovoviviparous Phlebobranchia) and in the Aplousobranchia (all remaining ovoviviparous species), the larvae are large (usually with a trunk measuring over 1 mm), have both an ocellus and an otolith and show a high degree of structural complexity. The larvae of these groups are diverse both in shape and in the spatial arrangement of the adhesive organs. *Ectodermic papillae* and *epidermic vesicles* may also be present (Cloney, 1978; Kott, 1990; Turon, 1991).

The larvae are positively phototropic and swim actively upward. The *phototropism* is inverted immediately before settling (Crisp & Ghobashy, 1971), when

the larvae probably also have some ability to choose the substrate (on ascidian larval ecology, see also Millar, 1971; Svane & Young, 1989; Goodbody, 1995).

Metamorphosis

The transition from pelagic larva to benthonic young ascidian is called *metamorphosis*. This period of rapid changes (for a detailed description, see Cloney, 1978) begins when the larva adheres to a substrate by means of the larval adhesive organs which are soon replaced by other adhesive organs such as the *vascular ampullae* or the *stolons*. As the larva settles, the tail is retracted and reabsorbed, after which the trunk goes through a 90° rotation bringing the zooid into its final position (Plate 3, Fig. 2). Finally, the young zooid (*oozooid*) opens the apertures, extends the branchial sac and water pumping begins. The temporal extension of metamorphosis is related to the level of development of the larva. Thus, it is longer in oviparous species, where organogenesis must be completed. In Phlebobranchia the oozooid has two atrial apertures and the branchial sac has two horizontal stigmata (*protostigmata*) per side (Plate 3, Fig. 3a). Then the two atrial apertures unite and the protostigmata become more numerous and divide into pieces which will attain the final vertical position. There are also two atrial apertures in the Stolidobranchia but their fusion takes place within the larva before settling. In the Polyzoinae the oozooid usually has 4 rows of vertical stigmata and this number increases in time to attain the number characteristic of the adult zooid. In the Botryllinae the oozooid presents only protostigmata (Plate 3, Fig. 3b) while the definitive vertical stigmata are present in the zooids, which originate by budding.

Budding
(Plates 4-8)

In addition to sexual reproduction, several species perform *vegetative reproduction* (*budding*) by means of which every zooid produces one or more copies of itself, originating a clone. If several zooids of a clone remain connected (as, for example, in the Botryllinae) they create a *colony*, as defined by Rosen (1979). However, in some species the new zooids disconnect from the parental zooid, for example in *Clavelina*, *Seriocarpa*, and *Polyzoa* (Kawamura & Watanabe, 1981). As stressed by Kott (1982b), only in the latter case the origin of the new zooids deserves to be defined as *asexual reproduction*, while in the former case it is more correct to use the term *replication*. The origin of new zooids, in fact, has a different meaning in the two cases: sexual and asexual reproduction allow dispersion and an increase in population, while replication causes only colony growth (Harper, 1977). The possible adaptive role of *coloniality* was elucidated by Kott (1982b) and Sabbadin (1994). Ascidian budding has been accurately studied since the end of XIX century and has been extensively targeted in morphogenetic studies (Nakauchi & Kawamura, 1986).

The main budding types are:

1 – *Larval budding* (Plate 4). This takes place in *Distaplia*, *Sycozoa* and *Hypsistozoa*. In the embryo, inside the larva, a ventral stolon develops and narrows to form a spherical bud. This primordial bud (first described by Della Valle,

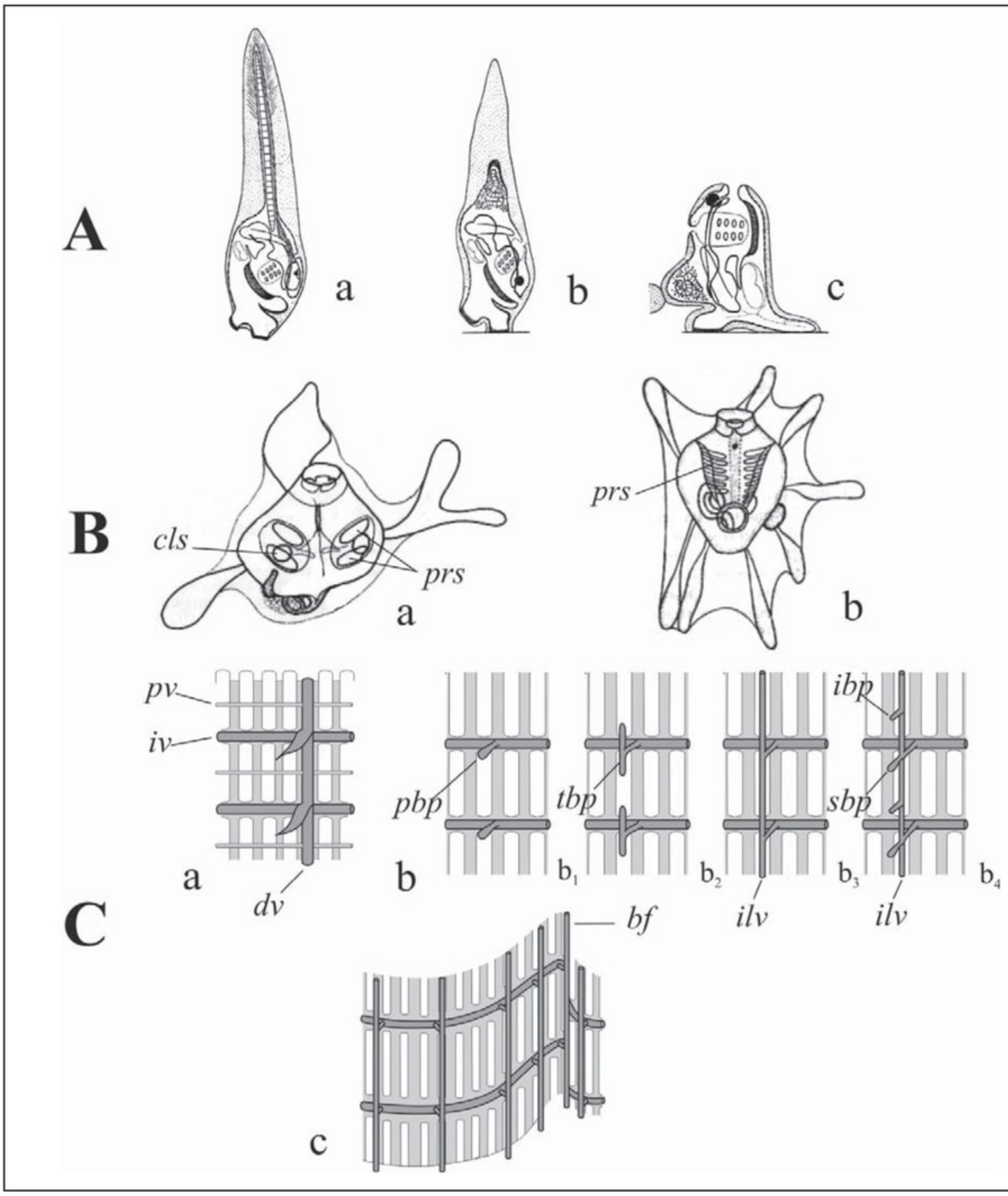

Plate 3 - Some aspects of ascidian morphology (III). **A. Metamorphosis:** the larva reach the substratum (a), settles with the adhering organs and the tail is reasorbed (b); oozooid opens apertures, after having rotated of 90° to the definitive position (c). **B. Oozooid** in a *Phallusia* (a) and in a Botryllinae (b). **C. Diagrams of the branchial structure:** Aplousobranchia (a); Phlebobranchia (b) (b1 to b4 subsequent steps in complexity); Stolidobranchia (c).
bf: branchial fold; *cls*: cloacal chamber siphon; *dv*: dorsal vessel; *ibp*: intermediate branchial papilla; *ilv*: internal longitudinal vessel; *iv*: interstigmatic vessel; *prs*: protostigmata; *pv*: parastigmatic vessel; *pbp*: primary (or simple) branchial papilla; *sbp*: secondary branchial papilla; *tbp*: T-shaped branchial papilla. (A and Bb from Brien, 1948; Ba from Brunetti & Zaniolo, 2009).

1881) divides into three parts, one of which will originate the *blastozooid* while the other two go on dividing, giving origin to a population of buds which will develop into zooids. This process was first described by Salfi (1928) who highlighted the fact that the zooids are devoid of budding capacity. On the opposite,

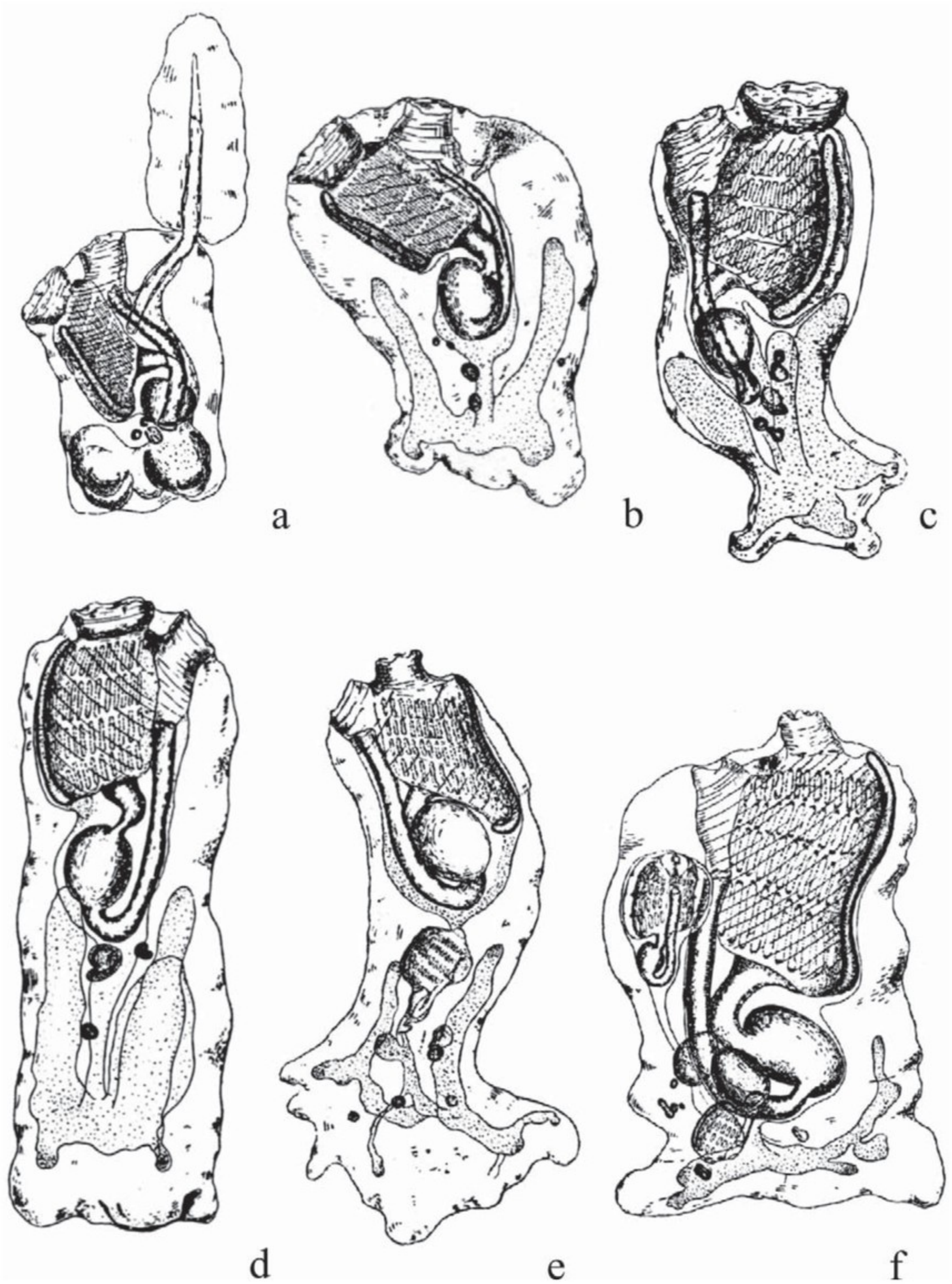

Plate 4 – Budding (I). **Larval budding in *Distaplia***: a, larva immediately after settlement: near the stomach there are three buds derived from the primordial bud, one of these will originate the blatozooid; b, c, new buds are produced from the previous ones; d, «cylindrical stage», few days after settlement the young colony undergoes to a stationary period lasting about one month; e, f, the activity is resumed in the young colony and all new zooids develop from the buds derived from the primordial larval bud (From Salfi, 1928).

Berrill (1948a) believed that every zooid at the end of its life cycle produced buds with the same modality as the larva but later on Ivanova-Kazas (1967) carefully repeated the study of the *Distaplia* cycle confirming Salfi's observations.

Some authors (Salfi, Van Name, Berrill, Kott and others) used the term *blastozooid* exclusively to indicate the replicated individuals in the larvae and the term *zooid* for the replicated individual in the colony, therefore they distinguish the *blastogenesis* in the larva from the *replication activity* in the colony. However, currently most authors agree with Brien's (1948, p. 708) definitions: *La formation et l'évolution des bourgeons constituent la blastogenèse. Les organismes qui en dérivent sont les blastozoides*.

2 – *Stolonic budding* of *Perophora* and *Clavelina* (Plate 5). A stolon bearing mesenchymatic and blood elements develops from the zooid body wall (Freeman, 1964; Fukumoto, 1971). Along the growing stolon the buds develop as swellings which contain a mesenchymatic septum. From this moment the development proceeds in two ways: "*Perophora*-type" and "*Clavelina*-type". In the first case the connection between zooid and bud persists, and along the stolon, zooids of different age develop and remain vascularly connected. In the *Clavelina*-type development the bud, after having accumulated reserve food cells, breaks the connection with the parent zooid, afterwards the new zooid starts to develop. These separated buds (also known as *budding chambers*) can give rise to new zooids even after long time, enabling the species to survive through periods of unfavourable environmental conditions. At medium latitudes these buds of *Clavelina lepadiformis* are quiescent during the winter and at the beginning of spring they originate zooids which appear as elegant bouquets. For the reasons explained above (cf. Brien, 1948, p. 713), these clusters are not considered as colonies. Actually they are frequently not even a clone (and this must be kept in mind by experimental biologists). In fact an oozooid of *Clavelina* may produce a population of buds but other oozooids close to it may produce other buds that might join with the first to form a mixed bud population. Therefore the cluster of zooids developed from such buds is not necessarily a clone.

3 – *Planctonic buds* (Plate 5, Fig. 3). These originate as an outgrowth of the body wall, covered by several ampullae, which separate and are scattered by currents (Watanabe & Tokioka, 1973). In some cases, *Polyzoa vesiculiphora*, *Perophora japonica*, *Clavelina gemmae*, planctonic buds are produced in addition to the usual budding (see Watanabe & Tokioka, 1972; Mukai *et al*., 1983; Turon, 2005, respectively).

4 – *Budding by strobilation* (Plate 5, Fig. 4). A resorption of the internal organs occurs in the zooid which then becomes constricted and each part generates a new zooid. This kind of budding is present in the Polyclinidae and Polycitoridae and may involve only the abdomen, the posterior abdomen or both (Nakauchi, 1982).

5 – *Recto-oesophageal budding* (Plate 6). The *recto-oesophageal budding* of the Didemnidae is probably the most complex budding process known in Ascidiacea. In these animals, the thorax is connected to the abdomen by a thin peduncle formed by the rectum and the oesophagus. An *abdominal bud* (*ab* in the plate) originates from the oesophagus and gives rise to an intestine while a *thoracic bud* (*tb* in the plate) arises from the rectum and originates a branchial sac. For many years ascidiologists, accepting the opinion of Ganin (1870), thought that the new zooid originated following the union of these two parts. However

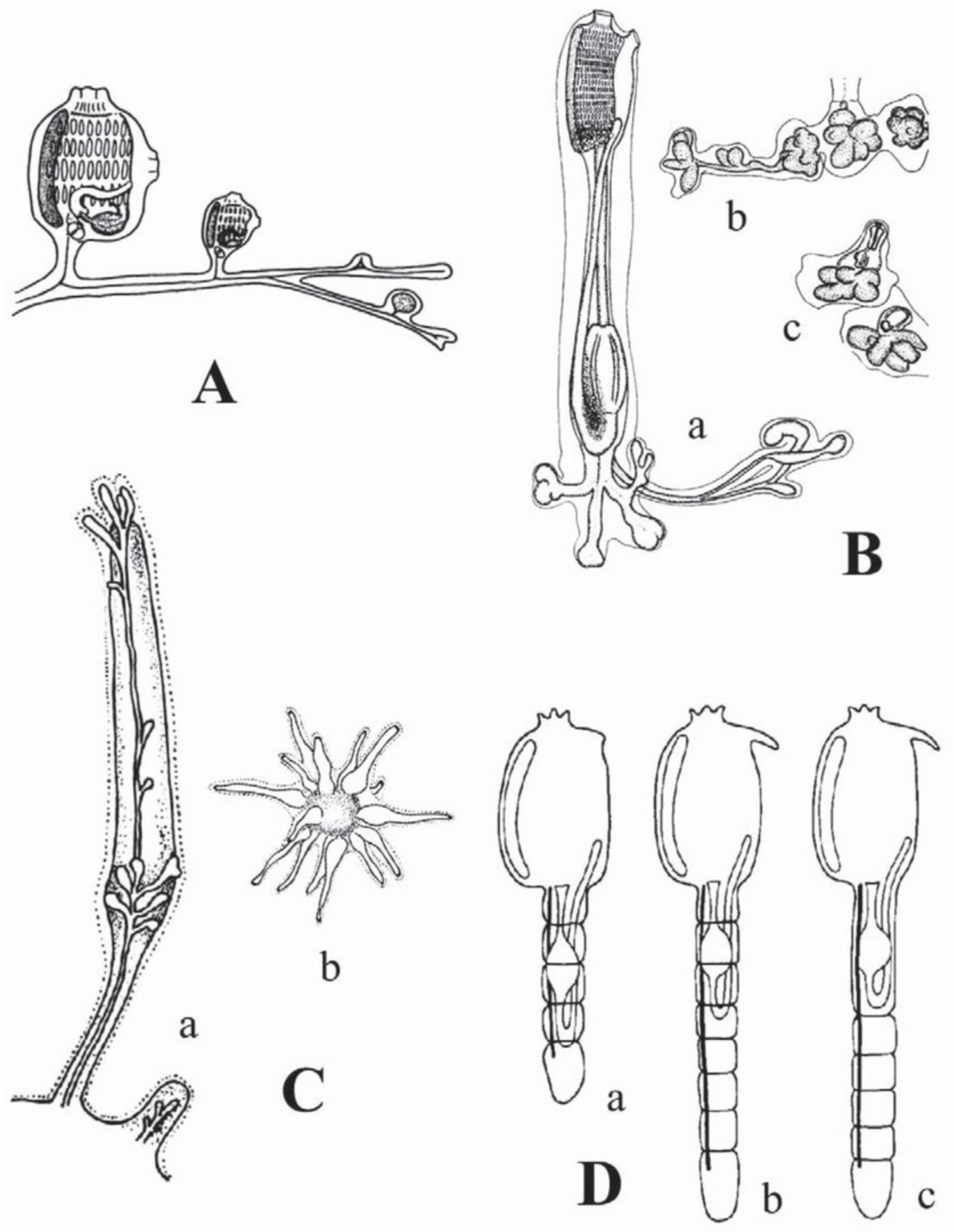

Plate 5 - Budding (II). **A. Stolonic budding in *Perophora*.** The bud develops from basal stolons connected to the parent zooid. **B. Stolonic budding in *Clavelina*.** a, adult zooid with stolonial vessel where reserve material is accumulated; b, constriction and isolation of terminal ampullae; c, regeneration of replicates from the isolated ampullae. **C. Planktonic bud formation in *Polyzoa*;** a, stolon outgrowing from the surface of the parental zooid; b, planktonic bud liberated from stolon. **D. Strobilation in polycitorines and polyclinids.** The fragmentation may involve the abdomen (a), the abdomen besides the posterior abdomen (b) or the posterior abdomen only (c) (A, B from Berrill, 1935; C from Watanabe & Tokioka, 1972; D from Nakauchi, 1982).

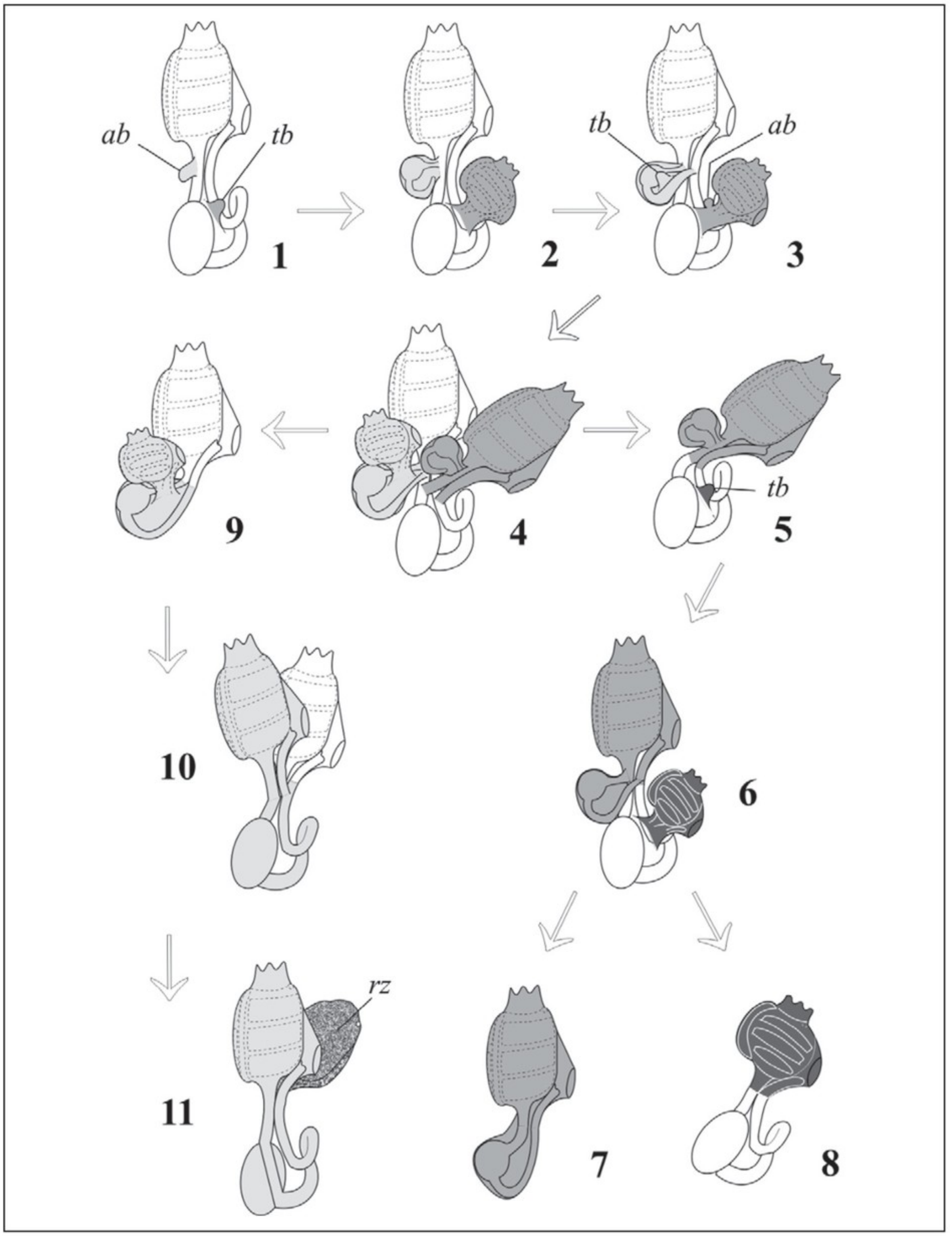

Plate 6 – Budding (III). ***Recto-oesophageal budding in*** **Didemnidae**. Explanation in the text section on budding. (Redrawn from Salfi, 1932b).
ab, abdominal bud; *rz*, regressed part of the zooid; *tb*, thoracic bud.

careful observations conducted by Salfi (1932b, 1933) on different species of *Didemnum*, *Tridemnum* and *Diplosoma* showed that both abdominal and thoracic buds are true buds, and both of them are able to generate a whole zooid. The difference between the two buds is that the abdominal bud gives rise firstly

to an intestine and only later to a thorax, while the contrary happens in the case of the thoracic bud (Figs 1 to 3). The result of this process is the formation of a zooid with two thoraces and two abdomens (Fig. 4) which soon separate into two heterogeneous zooids: one formed by the thorax of the parent and the newly generated abdomen; the other by the abdomen of the parent and the newly generated thorax (Fig. 5). In *Diplosoma* this division takes place already in the larva so that, after metamorphosis, the young colony is constituted by two heterogeneous zooids, but in the usual vegetative cycle the heterogenous zooids represent only a transient stage (Salfi, 1932b, 1933). Not always do the two buds develop: in periods of less active vegetative development the abdominal bud can be missing and the development of the thoracic bud generates a bi-thoracic zooid (Fig. 10). Generally, after some time, the new thorax replaces the parental one, which then regress (*rz* of Fig. 11).

Salfi also studied the origin of the two buds (Salfi, 1933). The abdominal bud originates from a mass of undifferentiated cells which accumulate against the parental oesophageal wall. An intestine develops from this rudimentary group of cells, whose cavity soon communicates with that of the parental oesophagus. (The new oesophagus does not develop as an outgrowth of the parental oesophagus, as thought by previous authors). The remaining undifferentiated material gives rise to a U-shaped tube, the central part of which leads to the formation of the heart, while the lateral ones produce the epicardic tubes. Therefore the parental epicardic tubes do not play any part in the origin of the abdominal bud. On the contrary, the thoracic bud originates from the parental epicardic tubes. In fact the primordium of the thoracic bud originates from a cellular mass due to the proliferation of the ends of the epicardic tubes which, once they have approached each other, lean against the ectodermal wall of the abdomen.

6 – *Palleal or peribranchial budding* (Plate 7). This budding modality is probably the most studied process although it is present in a limited number of styelid species. Three successive blastogenetic generations coexist in a Botryllinae colony: the filtering zooid, its bud and the bud of the latter, usually called buds of first and second generation respectively (a, b and c in the plate). The development of the bud can be described as a succession of stages, and since in the colony all buds of the same generation develop synchronously, the stages of the three successive blastogenetic generations show also the developmental stage of the colony, the knowledge of which is often useful to interpret the observed sample.

In plate 7 we present the sequence of stages suggested by Berrill (1941a). The first developmental stage (stage 1) consist in a disc-shaped thickening of the body wall of the parent (first generation bud); then the disc evaginates (stage 2) and closes to form a sphere (stage 3) which remains vascularly connected to the parent (Berrill, 1941a, b; Burighel & Brunetti, 1971). Then the organogenesis begins: peribranchial cavities and the primordial stomach characterise stage 4, and the primordium of the radial vessel stage 5. At stage 6, the heart is clearly visible, and at stage 7 a new bud primordium (second generation bud) distinctly appears. At stage 8 the heart is beating. Finally at stage 9 there is the opening of the oral siphon and the regression of the old parent begins by apoptosis (Burighel & Schiavinato, 1984; Lauzon *et al.*, 1992) and the filtering zooid is reabsorbed by macrophages; this last stage marks the *change of generation* (by some authors called *takeover*).

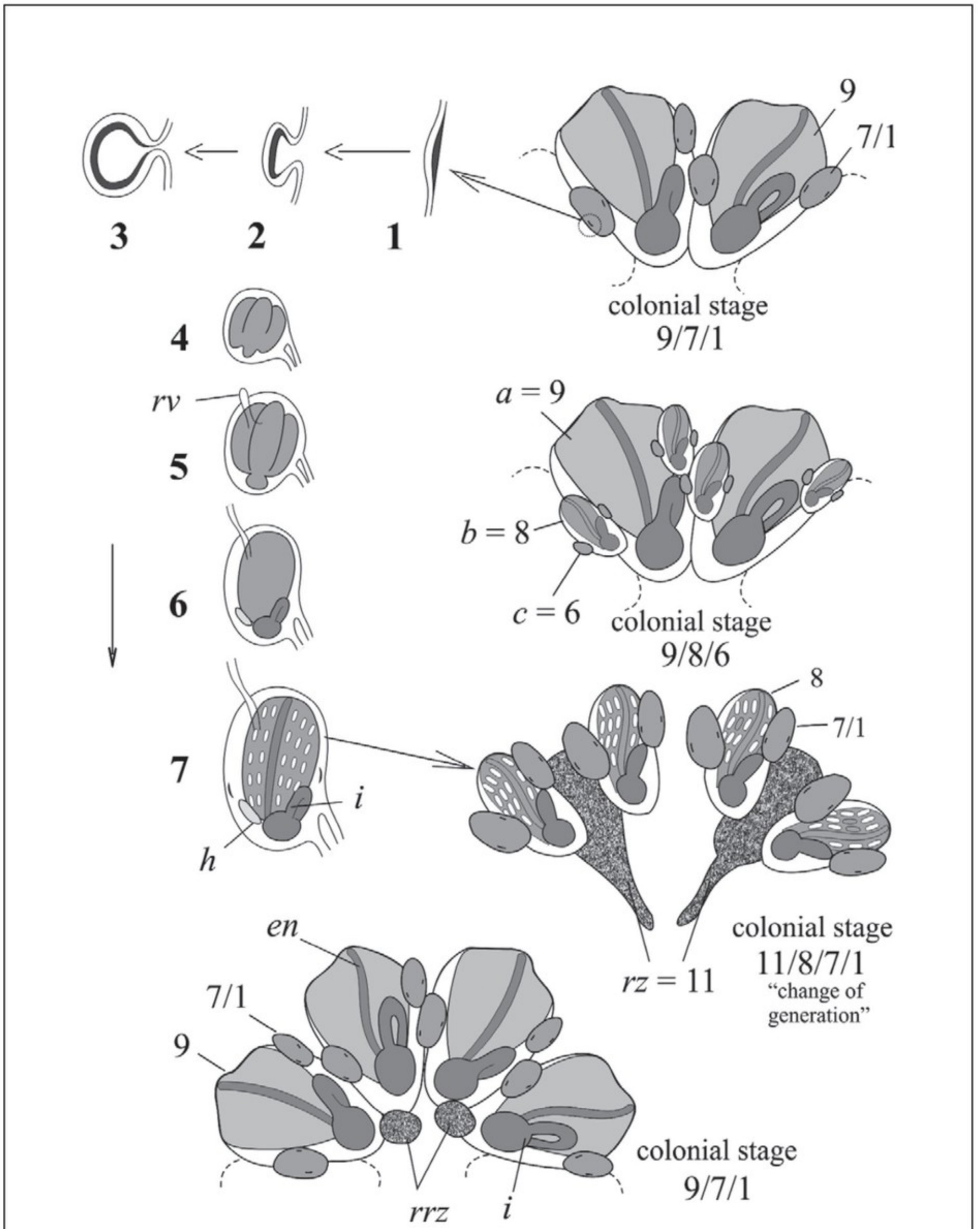

Plate 7 - Budding (IV). ***Palleal budding in Botryllinae.*** Diagram of a colony observed from the ventral side adhering to substrate. On the right, the developmental stages of the three generations of zooids in the colony are indicated with a number (Berrill, 1941a), and the developmental stage of the colony defined with a formula (for instance 9/7/1) in which the numbers indicate the stage of the zooids in each of the three generations (Sabbadin, 1955). On the left details of the development of the bud since stage 1.
a, filtering zooid (Stage 9); *b*, bud of first generation (stages 1-7); *c*, bud of second generation. *en*, endostyle; *i*, intestine; *rrz*, residuals of regressed zooid (stage 11).

This sequence of stages was framed on microscopical observations of living colonies growing in aquaria and is not easily applicable to fixed samples other than in an approximate way. Alternative sequences were thus suggested (Table 1).

TABLE 1 - COMPARISON AMONG SOME PROPOSED SEQUENCES OF COLONIAL STAGES.

Generation number	Watanabe, 1953	Berrill, 1941a*	Lauzon *et al.*, 2002
N	C	9/8/5	C - 2
N→N+1	A	change of generation → 9/8/6 → 11/9/71	A - 1 A - 2
N+1	B	9/8/2	B - 1
		9/8/3	B - 2
	C	9/8/4	C - 1
		9/8/5	C - 2
N+1→N+2	D	change of generation → 9/8/6 → 11/9/7/1	A - 1 A - 2
N+2	B	9/8/2	B - 1

* The formula indicates the developmental stage of the colony.

Watanabe (1953) described the development of the bud as a succession of three stages; his method, only slightly modified by Mukai & Watanabe (1976), consisting of a grouping of Berrill's stages, is not very useful, because of its oversimplification. More recently Lauzon *et al.* (2002) have proposed a sequence of seven steps which follow Berrill's description without adding any really new knowledge. In our opinion these developmental stages, which in any case require careful microscopical observation, do not bring any real advantage.

7 - *Vascular budding* (Plate 8). This kind of budding was first observed in *Botryllus primigenus* (Oka H. & Watanabe, 1957) in which buds develop at the base of the vascular ampullae in addition to the palleal modality (Plate 8, Fig. 1). The phenomenon also occurs in *Botrylloides violaceus* but has only been observed experimentally in isolated pieces of colony devoid of zooids (Oka H. & Watanabe, 1959) and in regenerating colonies of *Botrylloides leachii* (Burighel *et al.*, 1976) (Plate 8, Fig. 2). In this case the diversity in the budding mechanism is also related to the type of tissue involved in the process: the vessel wall (epidermis) and blood cells in the absence of the peribranchial wall (Miyamoto & Freeman, 1970).

8 - *Transverse constriction* of *Seriocarpa* (Plate 8, Fig. 3). In this genus, which belongs to the Styelidae, the zooid first closes its siphons while the internal organs undergo dedifferentiation, then it divides transversally into two separate zooids with regenerating organs (Diehl, 1972).

COLONY

Irrespective of the replication modality adopted, the colony (or clone) generated, can be very different. The simplest colonies are those of the Perophoridae, where zooids are separate and are connected only by a basal stolon. There are then colonies which look like the bouquets of *Clavelina* but in which the abdomens are embedded in a thick basal test (examples: *Pycnoclavella* and *Diazona*). Finally there are colonies in which zooids are completely embedded in the common test, either each one as an independent unit or organized in systems. In the first case the atrial siphons of the zooids open directly on the colony surface, in

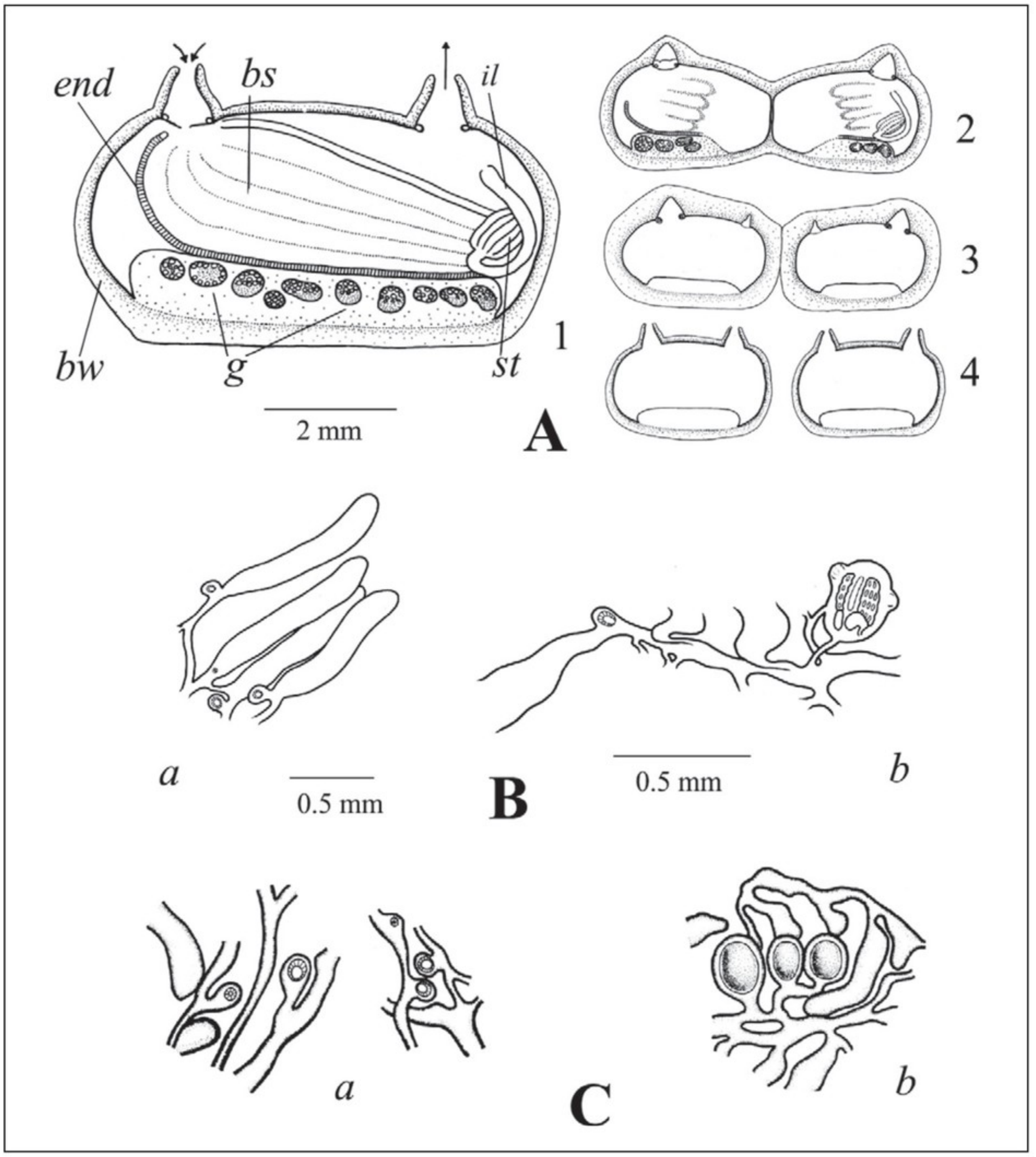

Plate 8 - Budding (V). **A. Transverse costriction of *Seriocarpa*** (1-4 succeeding steps of the process, see in the text)**. B. Vascular budding in *Botryllus primigenus*. C. Vascular budding in *Botrylloides violaceus*:** development of buds in an isolated small piece of colony devoid of zooids. *a*, young buds of different size at the base of ampullae; *b*, two buds formed consecutively on the same ampulla; *bs*, branchial sac; *bw*, body wall; *end*, endostyle; *g*, gonads; *il*, intestinal loop; *st*, stomach. (A from Diehl, 1972; B from Oka H. & Watanabe,1957; C from Oka H. & Watanabe, 1959).

the second case the atrial siphons open into a common cloacal chamber which communicates with the outside through one or more cloacal siphons. The simplest systems are circular in shape (*Aplidium*, *Botryllus*) but the common cloacal chamber can be more or less extensively branched with several cloacal siphons (*Didemnum*, *Botrylloides*). The progressive increase in colonial complexity probably reveals an increase in the level of integration.

The opinion that the greatest integration is present in the colonies with vascularly connected zooids (for example: Perophoridae and Botrillynae and *Symplegma*) is probably due to the influence of Berrill's authority who thought that only in such cases are we in the presence of a "true colonial organism" that is a strongly integrated structure (Berrill, 1950: 52). However, this hypothesis has still to be demonstrated. From a morphological point of view it is certain that the most complex colonial structure is present in the Aplousobranchia in which a colonial vascular system is lacking. If integration is due to the ability of zooids to communicate between one another, then one must assume that such communication occurs not only by means of the vascular system but also, or especially, by diffusion of substances into the test. In other words, as supposed by Nakauchi (1982, p. 760), "some information other than so-called *genetic information"* is involved in morphogenesis.

Histocompatibility and colonial fusion

In the Botryllinae, when two conspecific colonies come into contact, within a few hours the ampullae from each contacting organism undergo a process of vascular fusion or rejection; in other words the colonies are able to recognize each other ("self" or "non-self") (for a review see Taneda *et al.*, 1985). Fusion or rejection are genetically controlled by a multi-allelic genetic locus and fusion occurs when even a single allele is shared (Oka H. & Watanabe, 1960). Compatibility/incompatibility has also been shown experimentally in solitary ascidians by means of injection of cells or tissue grafts (Fuke, 1980; Raftos, 1996). However the phenomenon has been observed in the field only in colonial species. Of the two contacting colonies one is resorbed, though its blood cells continue to be present in the resulting chimera. The adaptative significance of fusion is not clear. In *Botryllus schlosseri* blood-related larvae settle close to each other and this increases the probability of successful fusions (Grosberg & Quinn, 1986), suggesting that chimeras may have a selective advantage, however this hypothesis is not supported by experimental results (Rinkevich & Weissman, 1992). This problem was carefully analyzed by Rinkevich (1996). However, multichimeras derived from the fusion of several colonies were subsequently found in the field and also obtained in the laboratory. In addition, an interesting paper by Rinkevich & Shapira (1999) showed that multichimeras are rarely reabsorbed, do not fragment, and grow more than bichimeras. These results suggest that in multichimeras the conflict resulting from intraspecific differences is reduced while the resulting entity is more adaptable to environmental changes.

Ecology

Ascidians are exclusively marine animals which are widely distributed at all latitudes and depths. They are usually sciaphilous organisms and present their greatest diversity in the deeper part of the subtidal zone. Ascidians are sessile, often investing, on hard substrates though they may also be present on sandy or muddy sediments. Some species are interstitial (Monniot F., 1965). Ascidians may give rise to dense populations in lagoons, harbours or semi-enclosed environments where the far from optimal range of the chemico-physical characters

are compensated by a high trophic capacity, due to the great quantity of suspended particles (Brunetti & Menin, 1977; Gabriele *et al.*, 1999; Mastrototaro *et al.*, 2008). In these eutrophic environments some widely distributed species such as *Ciona intestinalis*, *Styela plicata* and *Botryllus schlosseri* are usually present. However, ascidians are typically marine organisms and even the above cited species have scarce osmoregulatory capacity. Only *Molgula manhattensis* (which is a quite rare species) appears to prefer or tolerate low salinities (below 25‰) and can be defined as an 'estuarine' species (Millar, 1971; Kott, 1976; Nakauchi & Hajihara, 1981).

Life cycle. Metamorphosis, settling, growth, reproduction and death, are strongly controlled by water temperature (Grave, 1935; Sabbadin, 1958; Millar, 1971). This is well established for a limited number of littoral zone species, however the progress in diving technology is extending our knowledge to open sea species too. As expected, temperature influences generation time. At intermediate latitudes, during the warmer season, a succession of short generations is usually followed by one generation which survives the colder period and reproduces the following spring (Sabbadin, 1957; Brunetti, 1974, 1976; Brunetti *et al.*, 1988; Grosberg, 1988; Caralt *et al.*, 2002). Generally, for each species there is a thermal optimum for each function, so in *Botryllus schlosseri* the greatest vegetative reproduction activity (even if it is slow) takes place at temperatures below 10 °C at which gonadal ripening is prevented (Brunetti, 1974). When the temperature drops close to the species tolerance value, this usually produces a rise in the mortality rate which causes a strong reduction, or the total disappearance, of the population as often occurs to *Ciona intestinalis* in the Venice lagoon (Brunetti & Menin, 1977; Marin *et al.*, 1987). Nonetheless, some colonial species are able to survive a period of unfavourable environmental conditions by means of zooid regression and the establishment of a resting stage. In particular, in *Botrylloides leachii* low temperatures induce the regression of all zooids and the colony appears as a carpet of ampullae (Brunetti, 1976). In these cases colony rebuilding takes place by regeneration. Thus in *Clavelina phlegraea* hybernation consists of the regression of the thorax which regenerates during the following spring (Brien, 1931), while in the case of *Botrylloides leachii* regeneration occurs by vascular budding (Burighel *et al.*, 1976). High temperatures may also induce resting stages (Brunetti, 1976; Turon & Becerro, 1992). Turon (1992) described a regression and subsequent regeneration of the branchial sac in *Polysyncraton lacazei*. This phenomenon, though similar to that occurring in *Clavelina phlegraea*, according to the authors has the function of rejuvenating the colony by prolonging the life of zooids. The effects of temperature on metabolism are influenced by changes in salinity, however the interaction between these two factors has only been clearly shown in laboratory experiments (Brunetti *et al.*, 1980, 1984, 1985; Marin *et al.*, 1987; Vázquez & Young, 2000).

Lifespan and mortality. Apart from the mass mortality induced by catastrophic phenomena such as a sudden drop in salinity value (Goodbody, 1961) or the death and detachment of the biological substrate of which ascidians are epibionts (McDougall, 1943), very little is known concerning the extent of ascidians' lifespan (for a review, see Millar, 1971). Immediately after the settling of the larvae, the population is generally characterized by a high mortality rate which in *Ascidia nigra* has been evaluated between 67% and 100% (Goodbody

& Gibson, 1974). Similar values have been found in *Botrylloides leachii* and *Botryllus schlosseri* (Brunetti, unpublished data). The causes of this mortality are not clear, according to Goodbody & Gibson (1974) it might be due to predation and overall suffocation caused by benthic diatoms (the observed decrease in mortality with increasing depth appears to indirectly confirm this hypothesis). Similar results were observed in *Didemnum candidum* (Hurlbut, 1991) and *Diplosoma* spp. (Keough & Downes, 1986). In *Trididemnum opacum* the same authors found a juvenile mortality rate of up to 19% per day and ascribed it to predation by fishes and sea urchins. The lifespan is known only for a few species. Usually the solitary species live for 1-2 years (Sabbadin, 1957; Millar, 1971; Goodbody & Gibson, 1974) and the colonial ones less than one year (Brunetti, 1974, 1976; Brunetti *et al.*, 1988; Grosberg, 1988; Chadwick-Furman & Weissman, 1995). In the absence of external causes, death seems to be due to senescence. This has been found in *Botryllus schlosseri* (Brunetti & Copello, 1978; Chadwich-Furman & Weissman, 1995). Rinkevick *et al.* (1992) and Lauzon *et al.* (2000) experimentally demonstrated that the lifespan in this species probably has a hereditary basis, though the exact mechanism is not yet clear. In any case, death is thought to be mainly due to apoptosis (Lauzon *et al.*, 1993).

Symbiosis with unicellular algae. The presence of algae in colonial ascidians was first shown by Herdman (1906), later they were described as zooxanthellae or zoochlorellae because of their photosynthetic pigments. The presence of zooxanthellae has not been proved; while Newcomb & Pugh (1975) showed that the zoochlorellae of previous authors were blue-green algae which Lewin (1975) subsequently described as a new species *Synechocystis didemni*. Later, another species of the same genus, *S. trididemni*, was described in *Trididemnum cyanophorum* by Lafargue & Duclaux (1979). However, the majority of the algae which are present in the Didemnidae have characteristics which are intermediate between those of prokaryotes and those of eukaryotes. In particular, as in the case of prokaryotes, they lack a nuclear membrane and show the typical structure of cyanophytes, but like eukaryotes they have chlorophyll b, lack phycobilins and may have stacked thylakoids. Lewin (1977) named these microscopic algae *Prochloron* and introduced the new division Prochlorophyta for them. In the Didemnidae in which the symbiosis is obligate (Kott, 1980, 1982a, 1984a, b), there is a transfer of substances from the algal cells to the host (Griffiths & Thinh, 1983). Moreover, algal cells are carried from one generation to the next by means of a special organ, the *rastrum*, situated at the posterior end of the larval trunk (Kott, 1981). Non-obligate symbiosis can be present in some species of *Aplidium*, *Eudistoma* and *Botryllus* (Kott, 1981). Symbiosis, obligate or not, between algal cells and ascidians is usually known in tropical and sub-tropical regions. At other latitudes *Prochloron* has been detected in *Didemnum fulgens* in the Adriatic Sea (Müller W.E.G. *et al.*, 1984) while cells similar to *Synechocystis trididemni* were noted by Hernández-Mariné *et al.* (1990) in *Didemnum lahillei* and in *Didemnum granulosum* in the East Mediterranean.

Until now no case of symbiosis with algal cells has been reported among the solitary ascidians; however, De Leo & Patricolo (1980) noted the presence of algal material (tentatively identified as blue-green algae) in the test of *Ciona intestinalis*.

Classification
(Table 2)

Aristotle placed ascidians (as *Tethya*) among the bivalved molluscs. This classification remained almost unchanged until the end of the XVIII century. In Linnaeus' twelfth edition (1767) of *Systema Naturae* the ascidians are placed in the class Vermes: the simple ones (under *Ascidia*) in the ordo Mollusca and the compound ones (under *Alcyonium*) in the ordo Zoophyta. During the first decades of the XIX century new schemes of animal classification were suggested by Cuvier and Lamarck, in both cases ascidians remained in the Vermes group. Lamarck introduced the name Tunicata (during his Zoology course of 1816) to indicate the ascidians and the pelagic *Salpa* and *Pyrosoma* as a whole. Moreover, he distinguished the simple from the compound forms. Savigny (1816) maintained Lamarck's distinction (using the name Tethyes as a synonym of Tunicata) and placed them in the Mollusca Acephala following Cuvier's classification. In 1841 Milne-Edwards used the name Tunicata and separated the social ascidians from the compound ones, defining the former as species which reproduce by budding but do not give rise to colonies characterized by the presence of a common test. This point of view was also followed by Herdman in his account in the Challenger Report (1882, 1886). In 1843 Milne-Edwards gathered together Tunicata and Bryozoa in a class named Molluscoida to indicate a distinction of these groups from the Mollusca, while at the same time suggesting a certain affinity with the latter group.

In 1866 Kowalewsky, observing the early developmental stages of a simple ascidian, noted the affinity of these organisms to the Vertebrata. About 15 years after Kowalewsky's article everybody recognized ascidians as belonging to the phylum Chordata, within which they form the taxon (subphylum) Tunicata or Urochordata (Lankester, 1877; cf. Nielsen, 2012).

The present classification of ascidians originates from Lahille's work (1886, 1888 and 1890) who divided ascidians, according to the structure of the branchial sac, into Aplousobranchia (without folds and internal longitudinal vessels), Phlebobranchia (without folds but with internal longitudinal vessels) and Stolidobranchia (with folds and internal longitudinal vessels) (Plate 3). A few years later, Garstang (1896) removed the pelagic tunicate from the Aplousobranchia where Lahille had placed them. Finally, Perrier (1898) suggested an alternative system based on the arrangement of the gonads and erected the three groups Hemigona (or Enterogona), Hypogona and Pleurogona: with gonads in the intestinal loop, under the intestinal loop or in the body wall, respectively. In 1928 Garstang, taking into consideration the developement of epicardic cavities and the modalities of sexual reproduction, recognized only two groups which he named Endoblastica and Periblastica, the first one corresponding to the Enterogona plus the Hypogona, the second to the Pleurogona.

In 1937-1940 Hŭus combined the classification of Lahille with that of Perrier, as modified by Garstang, considering Enterogona and Pleurogona as orders and Aplousobranchia, Phlebobranchia and Stolidobranchia as sub-orders. Berrill (1936) however doubted the value of the branchial structure in the classification of the group and suggested a new classification, based essentially on the nature of the epicardium, which divided the ascidians into four orders: Nephrocoela, Acoela, Diplocoela and Epicardiocoela. However, this classification, be-

TABLE 2 - SOME STAGES IN THE PROGRESS OF THE CLASSIFICATION OF TUNICATA.

According **Savigny** (1816)
- Class **Ascidiae** (= Tunicata Lamarck)
- Order **Tethydes**
  - Family Tetyae
    - Simplices (*)
    - Compound (**)
  - Family Luciae (Pirosomida)
- Order **Thalides** (Doliolida and Salpida)

According **Herdman** (1882)
- Class **Tunicata**
- Order I. **Ascidiacea**
- Suborder Ascidiae Simplices
  - Family Molgulidae
  - Family Cynthiidae
  - Family Ascidiiadae
  - Family Clavelinidae
- Suborder Ascidiae Compositae
  - Family Botryllidae
  - Family Didemnidae
  - Family Distomidae
  - Family Polyclinidae
  - Family Diplosomidae
- Suborder Ascidiae Salpiformes
  - Family Pyrosomatidae
- Order II. **Thaliacea**
  - Family Doliolidae
  - Family Salpidae
- OrderIII. **Larvacea**
  - Family Appendiculariidae

According **Berrill** (1950)
- Class **Ascidiacea**
- Order 1. **Enterogona**
- Suborder **Aplousobranchiata**
  - Family Clavelinidae
    - Subfamily Polycitorinae
    - Subfamily Clavelininae
    - Subfamily Holozoinae
  - Family Polyclinidae
    - Subfamily Polyclininae
    - Subfamily Euherdmaninae
  - Family Didemnidae
- Suborder **Phlebobranchiata**
  - Family Cionidae
  - Family Diazonidae
  - Family Perophoridae
  - Family Corellidae
  - Family Ascidiidae
- Order 2. **Pleurogona**
- Suborder **Stolidobranchiata**
  - Family Styelidae
    - Subfamily Styelidae
    - Subfamily Botryllinae
  - Family Pyuridae
  - Family Molgulidae
- Class **Thaliacea**
- Order **Pyrosomida**
- Order **Doliolida**
- Order **Salpida**
- Class **Larvacea**
- Order **Copelata**

According **Kott** (1985, 90 92, 2001)
- Class **Ascidiacea**
- Order 1. **Enterogona**
- Suborder **Aplousobranchia**
  - Family Cionidae
  - Family Diazonidae
  - Family Clavelinidae
  - Family Pycnoclaveliidae
  - Family Holozoidae
  - Family Stomozoidae
  - Family Didemnidae
  - Family Polycitoridae
  - Family Placentelidae
  - Family Protopolyclinidae
  - Family Ritterellidae
  - Family Euherdmaniidae
  - Family Distomidae
  - Family Polyclinidae
- Suborder **Phlebobranchia**
  - Family Ascidiidae
  - Family Plurellidae
  - Family Agnesiidae
    - Subfamily Agnesiidae
    - Subfamily Ciallusiinae
  - Family Corellidae
    - Subfamily Corellidae
    - Subfamily Rhodosomatinae
  - Family Perophoridae
- Order 2. **Pleurogona**
- Suborder **Stolidobranchia**
  - Family Styelidae
    - Subfamily Styelidae
    - Subfamily Botryllinae
  - Family Pyuridae
  - Family Molgulidae
  - Family Hexacrobylidae
  - Family Octanemidae

(*) *Boltenia, Cynthia, Phallusia, Clavelia*
(**) *Diazona, Distoma, Sigillina, Synoicum, Aplidium, Polyclinum, Didemnum, Eucoelium, Botryllus*

ing based on an organ which is always difficult to observe, soon went out of fashion. Eventually, the study of vanadium biochemistry (Webb, 1939) confirmed the validity of Perrier's two groups, the Enterogona and the Pleurogona, as natural subdivisions. Van Name (1945) only recognized as valid Lahille's three divisions, which he accepted at the order level. Moreover, he criticized, without quoting it, Berrill's article (1936), if his words are correctly interpreted: *Attempts to classify ascidians on the basis of any single character or organ, such as, for instance, that on the basis of the epicardium, are less satisfactory, as they either group together very unlike forms or break up evidently natural assemblages recognized in generally adopted classifications* (Van Name, 1945, pag. 3). Finally, Van Name re-introduced Lahille's terms Aplousobranchia, Phlebobranchia and Stolidobranchia that Seeliger (1893-1906) had replaced, without valid reasons, with Krikobranchia, Diktyobranchia and Ptychobranchia respectively. In *The Tunicata* (1950) Berrill dropped his classification based on the epicardium and followed that suggested by Hŭus bringing only few modifications (i.e. raising Perophoridae and Diazonidae to family rank). Hŭus' classification was also followed by subsequent authors (Millar, 1960a; Nishikawa, 1990, 1991; Monniot *et al.*, 1991b).

A substantial change was introduced by Kott (1969) who placed the Cionidae and the Diazonidae within the Aplousobranchia. Starting from some of Berrill's ideas (1936, 1950), *Ciona intestinalis* was recognized as the most primitive ascidian species comparable to the common ancestor from which the evolution of the class started off. From the ancestral cionid forms two trends followed, each characterized by different functions of the epicardium. In the Aplousobranchia the epicardium is always involved in the vegetative reproductive process while in the Phlebobranchia and the Stolidobranchia it is implicated in excretion processes. However more recent research (Stach & Turbeville, 2002) has confirmed the position of these two families within the Phlebobranchia.

Collection and Preservation

Ascidians are exclusively marine organisms, very common in shallow waters but also present in abyssal environments. They are all sedentary, mostly growing on hard substrates but also on soft bottoms or in sediment. The collecting methods therefore change according the habitat studied. The external appearance of living animals is quite different from that of preserved material.

First of all the whole specimens or colonies must be observed, noting the nature of the test, its shape and the eventual presence of inclusions, the shape of openings, the colours and, in colonial species, the arrangement of the zooids in systems.

Colours usually change or completely disappear in the fixative and the soft body undergoes strong modification (contraction), so any attempt at specific identification on the basis of the appearance of the living animals is generally problematic and the result uncertain. Identification at the species level is therefore based on the morphology of the internal organs, which require the dissection of the fixed animal. To avoid the contraction of the body, that dramatically alters the shape and appearance of the species, the living specimens should be narcotized. Narcotization of ascidians is not easy, especially in Aplousobranchia, and several methods using different chemicals have been suggested.

Unfortunately, an experimental study on which chemical, what concentrations and time are best for the chief ascidian groups is missing. Menthol crystals seem to be an appropriate agent. However narcotization with menthol requires long time, five or more hours for large solitary specimens and up to three hours for colonies (after narcotization the menthol crystals, which do not dissolve very well in water, can be saved). In some cases these times are much too long, for example colonial species may regress and botryllid colonies may undergo a premature change of generation. In these cases it is necessary to reduce the time necessary for relaxation. The effectiveness of menthol can be increased by keeping the crystals in seawater and adding some of this water to the specimen with further crystals, or pulverizing the crystals of menthol to hasten their solution in sea water. Kott (1990, pag. 6) suggested: *More rapid relaxation is achieved by using a very weak solution of MS222 (Sandoz Ltd.). A few grains of powder in 5 ml seawater was made up as a stock solution. About 2 drops of this solution per 100 ml of sea water was found to relax most species rapidly and completely*.

The specimen is narcotized when siphons do not close when stimulated. The narcotized specimens are fixed by adding formaldehyde (40%) to obtain a 5-10% solution in sea water.

Dissection is easy in most Phlebobranchia and Stolidobranchia, but requires some experience for the small zooids of the Aplousobranchia. In the first two cases the specimens should be opened with a cut along the ventral mid line, then the body (zooid) can be removed from the test and likewise opened to show the structure of the branchial sac. The latter can be removed to observe the gut, the gonads and other structures located on the body wall.

The small zooids of the colonial Aplousobranchia must be removed from the colonial test and observed in a drop of glycerol on a slide. However, the fine structures of these small bodies, just like the larvae, often require staining (for example with Mayer's hemalum solution), clearing and mounting for light microscopy according to the usual histological techniques. The extraction of the zooids from didemnid colonies may be difficult, in these cases a portion of the colony can be decalcified in a HCl solution (about 3%).

Identification

The absence of rigid structures, the different degree of relaxation, the possibility that environmental factors affect the habitus of individuals and colonies, and often a wide range of intraspecific variation, all contribute to making ascidian identification very difficult.

The main characters to take into consideration when identifying ascidians are:

Solitary ascidians

– *Test* (=external tunic): characters of the external surface including colour, presence of epibionts; thickness, inclusions, blood vessels.

– *Apertures*: position, shape, siphonal armatures.

– Oral and atrial *tentacles*.

– Body *muscles*.

– *Dorsal tubercle*: position, shape of slit.

– *Dorsal lamina*: entire (surface appearance); divided (languets).

– *Branchial sac wall*: flat, folded, branchial formula, shape of stigmata and meshes.

– *Intestine*: course, subdivisions, stomach morphology, liver, anus.

– *Gonads*: number, shape, arrangement, gonoducts, relationship of testis to ovary.

Colonial ascidians

– In addition to the characters listed for solitary ascidians, carefully observe the following:

– Arrangement (or not) of the zooids in *systems*.

– *Zooids*: their arrangement in the systems; structure of common cloaca; presence of retractor muscles, lateral organs and vascular appendices.

– *Larvae*: size, form, number and arrangement of adhesive organs, ampullae.

– Morphology of *test spicules*.