

The eventful history of non-embryonic developments in tunicates

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3 **The eventful history of non-embryonic development in tunicates**
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ABSTRACT

Tunicates encompass a large group of marine filter-feeding animals and more than half of them are able to reproduce asexually by a particular form of non-embryonic development (NED) generally called budding. The phylogeny of tunicates suggests that asexual reproduction is an evolutionarily plastic trait, a view that is further reinforced by the fact that budding mechanisms differ from one species to another, involving non-homologous tissues and cells. In this review, we explore more than 150 years of literature in order to provide an overview of NED diversity and we present a comparative picture of budding tissues across tunicates. Based on the phylogenetic relationships between budding and non-budding species, we hypothesize that NED diversity is the result of seven independent acquisitions and subsequent diversifications in the course of tunicate evolution. While this scenario represents the state-of-the-art of our current knowledge, we point out grey areas that need to be further explored in order to refine our understanding of tunicate phylogeny and NED. Tunicates, with their plastic evolution and diversity of budding, represent an ideal playground for evolutionary developmental biologists to unravel the genetic and molecular mechanisms regulating non-embryonic development, as well as to better understand how such a profound innovation in life-history has evolved in numerous metazoans.

1 2 3 **1. INTRODUCTION**

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5 Tunicates (Phylum Chordata) encompass a large group of marine filter-feeding animals that
6 inhabit a wide variety of marine ecosystems around the world (Shenkar and Swalla 2011, Stolfi
7 and Brown 2015). They typically have a biphasic life cycle, with a planktonic larva and a post-
8 metamorphic phase that is benthic in ascidians and planktonic in thaliaceans (Brusca *et al.*
9 2016). While solitary tunicates reproduce strictly sexually, many can propagate both sexually
10 and asexually. During asexual reproduction, somatic tissues undergo a budding process that,
11 bypassing fertilization, embryonic development, a larval stage and metamorphosis, gives rise
12 to one or several adult individuals, called blastozooids. Individuals generated asexually
13 generally have a bauplan that is very similar to the individuals formed by embryonic
14 development (named oozoids). Budding has also been referred to as blastogenesis (Manni
15 *et al.* 2014), clonal replication (Hughes 1989), asexual propagation (Kürn *et al.* 2011), and more
16 recently non-embryonic development or NED (Alié *et al.* 2018). The latter includes also whole-
17 body regeneration, *i.e.* budding induced by exogenous inputs (Tiozzo and Copley 2015). In
18 some tunicates blastozooids remain connected to each other and form colonies, and then we
19 talk about colonial tunicates, as opposed to solitary species.
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22 Budding and non-budding species are scattered among the four main tunicate orders:
23 Thaliacea, Phlebobranchia, Aplousobranchia, and Stolidobranchia, and their phylogenetic
24 distribution unambiguously demonstrates that asexual reproduction arose convergently
25 several times (Tsagkogeorga *et al.* 2009, Pérez-Portela *et al.* 2009, Govindarajan *et al.* 2011,
26 Shenkar *et al.* 2016, Alié *et al.* 2018, Delsuc *et al.* 2018, Kocot *et al.* 2018). Convergent
27 acquisition of NED becomes even more evident by the variety of tissues and mechanisms
28 involved in bud formation across the different species (Berrill 1951, Godeaux 1957, Oka and
29 Watanabe 1957, Scelzo *et al.* 2019). In fact, in contrast to embryonic development, which
30 shows an extraordinary level of conservation amongst almost all the main tunicate orders, non-
31 embryonic developmental processes involve a variety of cells, tissues, and ontogenesis, all
32 displaying different degrees of interaction between epithelial and mesenchymal cells (even
33 within a single order).
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36 While in the last few decades the study of tunicate budding has focused on a few stolidobranch
37 model species (from Sabbadin 1958 to Kassmer *et al.* 2019, reviewed in Manni *et al.* 2018),
38 much knowledge about the diversity of NED is scattered across almost two centuries of
39 literature. This knowledge has been brilliantly reviewed by others (Berrill 1951, Nakauchi 1982,
40 Kawamura *et al.* 2008, Brown and Swalla 2012), but an explicit reconstruction of NED history
41 based on a modern understanding of the tunicate phylogeny is still needed. In this review, we
42 first introduce an up-to-date consensus phylogeny of Tunicata, which includes budding and
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non-budding species from the four tunicate orders (Figure 1). This phylogenetic framework provides a map for navigating through the following sections in which, for each of the main orders, we attempt to summarize the knowledge and the controversies about NED and its evolution.

2. Budding and non-budding species across the new tunicate phylogeny

A robust phylogenetic tree of tunicates is an indispensable prerequisite to portray the evolution of asexual development within an explicit cladistic framework. Authors of the first half of the twentieth century proposed various evolutionary scenarios regarding budding evolution, but in this pre-cladistic area asexual reproduction was alternatively viewed as an ancestral trait lost in solitary species (e.g. Van Name 1921), or as secondarily acquired with diverse speculations as to which budding modes would be more primitive or more derived than others (e.g. Garstang 1928). More recent phylogenetic studies of tunicates have started to specifically tackle the question of NED evolution (Zeng and Swalla 2005, Moreno and Rocha 2008, Pérez-Portela *et al.* 2009, Shenkar *et al.* 2016, Alié *et al.* 2018). Even if they sometimes led to conflicting results, especially between morphology-based and molecular-based reconstructions, they offered an explicit framework to discuss the evolution of asexual reproduction in this group.

In this review we based our discussion on a consensus phylogenetic tree of tunicates (Figure 1) that is a combination of several molecular phylogenies published in the last fifteen years (Turon and Lopez-Legentil 2004, Tsagkogeorga *et al.* 2008, Pérez-Portela *et al.* 2010, Tatián *et al.* 2011, Govindarajan *et al.* 2011, Shenkar *et al.* 2016, da Silva Oliveira *et al.* 2017, Delsuc *et al.* 2018, Kocot *et al.* 2018, Alié *et al.* 2018). From each study, we only retained the nodes that received maximal statistical support ($PP \geq 0.99$ or $BS \geq 0.95$) and we only included species for which detailed histological data on budding are well known from the literature (Figure 1, see legend for the details of the procedure). For instance, many thaliaceans and clavelinids included in phylogenies in Govindarajan *et al.* 2011 and da Silva Oliveira *et al.* 2017 are absent in our phylogeny, because their budding process is still unknown. On the other hand, several species in which the budding mode is well documented have not been included in molecular phylogenies (e.g. Diazonidae, Distomidae), and therefore these species will not be considered in this review. Finally, budding mechanisms have sometimes been carefully described in one particular species, but only a related species has been used in phylogenies. For instance, the phylogenetic position of *Aplidium conicum* and *A. tabarquensis* is known from Shenkar *et al.* (2016) but budding has been described in *A. pallidum* and *A. turbinatum* (Brien 1948). In such a situation we included the latter close to the former, based on the taxonomic

classification (Figure 1, dotted lines). We also included in the tree as many solitary species to accurately depict evolutionary relationships between solitary and colonial species.

According to the proposed phylogeny, budding species are separated into six distinct groups. (i) Thaliacea, that are all able to propagate by budding; (ii) all the Aplousobranchia with the exception of the solitary *Rhopalea idoneta*; (iii) the Stolidobranchia species *Polyandrocarpa zorritensis* and (iv) all the other colonial Stolidobranchia. For the species traditionally classified in the paraphyletic “Phlebobranchia”, budding species are (v) the species *Polyoctacnemus patagoniensis*; (vi) the three species belonging to the family Perophoridae (*E. turbinata*, *P. viridis*, *P. japonica*). Below, we describe and compare the structure and the nature of budding tissues in these different groups. Then we discuss the evolutionary scenario implied by this distribution of budding types across the phylogeny.

3. Budding in Stolidobranchia: the peribranchial, the vascular...and the vasal

Stolidobranchia form a monophyletic order of ascidians that comprises budding and non-budding species (Figure 2a-d), and among which Styelidae is the only family where NED has been reported (Figure 1). Peribranchial budding, sometimes referred to as atrial, palleal, or pallial budding, is the most common form of non-embryonic development adopted by Styelidae. Since the first description of peribranchial budding by Metschnikoff (1869) in *Botryllus* sp., it has been described in many styelids (Pizon 1893, Ritter 1896, Selys-Longchamps 1917, Berrill 1940, 1948a, Abbott 1953, Watanabe and Tokioka 1972, Kawamura and Watanabe 1981, Mukai and Watanabe 1976, Akhmadieva et al. 2007). Peribranchial buds always arise from a thickening zone of the mono-layered peribranchial epithelium, which progressively folds forming a hemisphere overlaid by the parental epidermis (Figure 2e). Then, the extremity of the young budlet continues to invaginate and then forms a double vesicle: the inner vesicle is derived from the peribranchial epithelium, the outer from the epidermis, and free hemocytes (also called blood cells) are trapped between these two layers (Figure 2e). As development proceeds, the outer vesicle gives rise to the epidermis and the inner vesicle differentiates into most of the adult organs, including the digestive tube, the endostyle, the central nervous system, the pharynx, and the peribranchial chambers (Selys-Longchamps 1917, Berrill 1941, 1948a, Watanabe and Tokioka 1972, Kawamura and Watanabe 1981, Mukai and Watanabe 1976). Germ cells are derived from circulating hemocytes (Izzard 1968, Sugimoto and Nakauchi 1974, Mukai and Watanabe 1976, Sabbadin and Zaniolo 1979, Brown et. al 2007, 2009a, Kawamura and Sunanaga 2010, Kassmer et al. 2015, Langenbacher and Tomaso 2016, reviewed in Rodriguez et al. 2017).

The mechanisms regulating cell sources and lineages that lead to a fully functional blastozoid are far from fully elucidated. In *Botryllus schlosseri*, transcription factors known to specify the three canonical germ layers during embryogenesis are co-opted and re-expressed in distinct and overlapping domains of the inner vesicle, suggesting early cell commitment in the different regions of the latter (Ricci *et al.* 2016a). Some authors suggested that in *B. schlosseri* the nervous system and musculature may differentiate from a transient anatomical structure during inner vesicle morphogenesis (Burighel *et al.* 1998; Prünster *et al.* 2018, 2019). In *Polyandrocarpa misakiensis* mesenchymal cells are integrated into the budding epithelium and may play a role in organogenesis (Kawamura *et al.* 1991, Tatzuke *et al.* 2012). Also, experiments of somatic chimerism, generated by the fusion of *Botryllus schlosseri* histocompatible colonies or after blood transplantation, suggested that circulating stem cells could participate in blastozoid organogenesis (Stoner and Weissman 1996, Laird *et al.* 2005, Voskoboinik *et al.* 2008, Rinkevich *et al.* 2013). However, in these studies it is hard to determine if the chimerism occurs at the tissue level or if it is due to contamination of donor hemocytes circulating in the intricate network of the hemocoel sinuses.

Another form of NED in Styelidae is named vascular budding (Figure 1 and Figure 2f). The existence of vascular budding was a controversial issue until Oka and Watanabe described this process in *Botryllus primigenus* (Oka and Watanabe 1957) and *Botrylloides violaceus* (Oka and Watanabe 1959). These studies showed the participation of undifferentiated hemocytes, referred to as hemoblasts (also named lymphocyte-like cells), which migrate to the epithelia of the blood vessels and trigger the vascular bud (reviewed in Kawamura and Sunanaga 2010) (Figure 2f). Similar processes have been later reported in *Botryllus schlosseri* (Watkins 1958, Milkman and Therrien 1965, Sabbadin *et al.* 1975, Voskoboinik *et al.* 2007, Ricci *et al.* 2016a), *Botrylloides leachi* (Milne-Edwards 1841, Burighel *et al.* 1976), *Botryllus delicatus* (Okuyama and Saito 2001), and most recently in *Symplegma brakenhielmi* (Gutierrez and Brown 2017). The cluster of homing hemocytes develops into a hollow vesicle that grows in size and becomes enclosed by the surrounding vasculature epithelia. The result is a double vesicle comparable to the one observed during peribranchial budding (Figure 2f). From this point, the inner vesicle will give rise to future zooid organs, and the outer vesicle to the epidermis. In *Botryllus schlosseri* the temporal and spatial expression pattern of “germ layer markers” in the inner vesicle of is comparable to the expression reported for peribranchial budding (Ricci *et al.* 2016a).

Despite efforts in the last decade to characterize the cells building vascular buds (Sunanaga *et al.* 2006, 2010, Voskoboinik *et al.* 2007, Brown *et al.* 2009b, Rinkevich *et al.* 2010), their nature has remained elusive. Only very recently, Kassmer *et al.* (2019) showed that injection of a single Integrin-alpha6-positive (IA6+) hemoblast in a colony of *Botrylloides leachi*

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3 previously treated with a mitosis inhibitor can rescue budding capacities. The number of IA6+
4 hemoblast rapidly increases at the onset of vascular budding, participating in the formation of
5 the bud tissues (Kassmer *et al.* 2019). Taken together, these results functionally show for the
6 first time the presence of blood-borne multipotent stem cells in *Botrylloides leachi*. Indeed, the
7 nature of these cells may differ from one species to the other. While in *Botrylloides leachi* the
8 primordial blast express the stem cell markers Piwi2 and Vasa (Kassmer *et al.* 2019), Vasa
9 and Piwi1 are not expressed in vascular buds of *Botryllus primigenus* (Sunanaga *et al.* 2006,
10 2010), while Piwi proteins are only detected in some peripheral cells of vascular bud in
11 *Botrylloides violaceus* (Brown *et al.* 2009b).

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13 The third form of NED reported in Stolidobranchia is vasal budding (Figure 1 and Figure 2g),
14 recently described only in *Polyandrocarpa zorritensis* (Brunetti and Mastrototaro 2004; Alié *et*
15 *al.* 2018; Scelzo *et al.* 2019). Vasal buds appear along a stolon, which consists of a blood
16 vessel up to several centimeters long, protruding from the epidermis of the parental zooid and
17 covered by a thin layer of tunic. The regions designated for budding, characterized by
18 accumulations of vascular ampullae, are called nests (Figure 2g). These nests can resist
19 adverse conditions after thickening the tunic and accumulating reserves, forming dormant
20 spherules able to germinate under favorable conditions (Figure 2b). Budding is triggered by
21 abscission of the stolon and isolation of the nest from its parent. Vasal buds start in the nests
22 from the thickening and invagination of a patch of cells on the vascular epidermis. The
23 invagination proceeds until a double vesicle is formed (Figure 2g). The aggregation of
24 undifferentiated hemoblasts around the forming inner vesicle suggests that circulating
25 mesenchymal cells also participate in organogenesis (Scelzo *et al.* 2019). Unlike peribranchial
26 and vascular budding, both the inner and the outer vesicle derive from the epidermis of the
27 parental zooid. Nevertheless, the expression of the transcription factor NK4 (Alié *et al.* 2018)
28 in vascular, peribranchial and vasal budding cells suggests that despite the different nature of
29 this budding tissues, they may share a common, independently co-opted budding-specific
30 molecular identity.

49 50 **4. Budding in “Phlebobranchia”: the stolonial and the unknown**

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52 Ascidians traditionally classified as Phlebobranchia (Figure 3) form a paraphyletic group with
53 respect to Aplousobranchia, and due to conflictual results between recent phylogenomic
54 studies, their deep relationships remain ambiguous (Figure 1). Budding “Phlebobranchia” are
55 distributed in three distinct families: Perophoridae that are all budding *Perophora* and
56 *Ecteinascidia*; Plurellidae, with solitary *Microgastra* and colonial *Plurella*; and Octacnemidae,
57 containing solitary species and the budding *Polyoctacnemus patagoniensis* (Kott 1972, 1985).

Octacnemidae is a very poorly known family of abyssal ascidians and to our knowledge, there is only one incomplete report on the species *P. patagoniensis* mentioning a stolon that connects the adult zooids with no description of the budding process (Metcalf, 1893). Plurellidae are not very well known either, their phylogenetic affinity is unclear and budding in *Plurella* remains completely undescribed (Kott, 1972, 1985). All the knowledge that we have about NED in "Phlebobranchia" comes from studies on the family Perophoridae (Figure 3), which adopts a form of stolonial budding.

Stolonial budding has been carefully described in *Perophora viridis*, *Perophora listeri*, and *Ecteinascidia turbinata*, and is apparently conserved across the whole family (Kowalewsky 1874, Van Beneden and Julin 1886 Ritter 1896, Lefèvre 1897, 1898, Dale Beers 1923, Brien and Brien-Gavage 1928, Deviney 1934, Freeman 1964, Fukumoto 1971, Mukai et al. 1983). Buds arise along stolons that grow from the base of each zooid and creep along the substrate (Figure 3e). While in some species zooids remain connected by their stolon and exchange blood cells (e.g. *Perophora viridis*), in others the stolonial connection is lost and zooids are independent of each other (e.g. *Ecteinascidia turbinata*) (Figure 3b-d). The Phlebobranchia stolon is an epidermally-protruded vessel surrounded by a thin layer of tunic. The stolonial lumen is separated into two longitudinal compartments by a central mesenchymal septum, on each side of which blood circulates in opposite directions (Lefèvre 1897, 1898, Brien and Brien-Gavage 1928, Deviney 1934, reviewed in Kawamura et al. 2008). The septum consists of an epithelial-like aggregation of mesenchymal cells that results from the contact of two blood sinuses in the zooid and that is in histological continuity with a mesh of mesenchymal cells underlying the epidermis (Brien and Brien-Gavage 1928, Deviney 1934). The first histological sign of budding in *Perophora* arises near the tip of the growing stolon (Figure 3f). Mesenchymal cells including hemoblasts gather at the extremity of the septum. Their number increases by the addition of new cells and by proliferation, until they form a hollow vesicle of more or less spherical shape that becomes the inner vesicle of the bud (Figure 3f) (Brien and Brien-Gavage 1928, Koguchi et al. 1993). In *Ecteinascidia turbinata* the septum, albeit mesenchymal as in *Perophora*, has a tubular shape and the inner vesicle grows by evagination of the septal wall (Lefèvre 1897, Dale Beers 1923). The outer vesicle, as in other ascidians, derives from the epidermis. While some specific variations have been reported, the general process of morphogenesis from the double vesicle stage seems to be shared by all Perophoridae. It has been carefully described in *Perophora viridis* and *Ecteinascidia turbinata*, where the outer epidermis provides the zooid epidermis and the inner vesicle gives rise to the branchial and peribranchial chambers, the endostyle, the digestive tract and the neural gland. The cerebral ganglion, pericardium, and gonads arise from circulating hemocytes with or without participation of cells wandering from the inner vesicle (Lefèvre 1897, 1898).

The role of mesenchymal cells and particularly of circulating hemoblasts in bud formation has been confirmed by experimental manipulation of budding in *Perophora viridis*. In his remarkable experiments, Freeman (1964) irradiated colonies of *P. viridis*, which consequently blocked stolon growth and bud formation. He subsequently rescued the budding process by reinjecting a non-irradiated blood fraction containing only hemoblasts. This elegant experiment showed that the sole hemoblast morphotype is necessary and sufficient to trigger bud formation, regardless of whether they are part of the septum or not. In the same line, when pieces of stolon are experimentally isolated in *Perophora viridis* (Deviney 1934) or *Perophora orientalis* (Fukumoto 1971), they spontaneously regenerate new zooids from the remnant pieces of the septum as in normal budding but, if the septum is absent or highly deteriorated, the bud arises from free mesenchymal cells.

5. Budding in Aplousobranchia: stolonial, larval, and the epicardial conundrum

Aplousobranchia encompasses around 1500 described species, almost all colonial (Figure 4) with the exception of some *Rhopalea* species, which secondarily returned to a solitary lifestyle (Shenkar 2013, Shenkar *et al.* 2016). Aplousobranchs are recognizable by their elongated body divided into an anterior thorax and the posterior abdomen containing the digestive tract, gonads, and heart. In some species, heart and gonads are located even more posteriorly into a post-abdomen. It is the abdominal and the post-abdominal region that are merely concerned with budding via different forms of strobilation (or stobilization), while one family, the Clavelinidae, also undergoes a divergent mode of stolonial budding (Figure 1).

Epicardial budding across Aplousobranchia

In most Aplousobranchia, budding takes place in the abdominal and/or post-abdominal regions, but the nature and behaviors of the budding tissues changes substantially from one species to the other (reviewed by Berrill 1935, 1951 and Nakauchi 1982). Still, a structure called epicardium always plays a prominent role in the budding process as a multipotent organogenetic tissue. The epicardium of Aplousobranchia is a tube-like sac that originates as two posterior invaginations of the pharynx (Kawamura *et al.* 2008). These two invaginations generally fuse to form a single vertical structure that runs parallel to the digestive tube and contact the pericardium in its posterior-most extremity. Budding arises by epidermal constriction that enclose a piece of epicardium and other entrapped tissues, and therefore this budding mode has often been referred as stobilization (or strobilation) (Figure 4g). Here we

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3 will use the term epicardial budding (Figure 1), owing to the conserved role of the epicardium
4 in this wide and complex array of non-embryonic developmental modes in Aplousobranchia.
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7 Abdominal and post-abdominal budding has been described in many *Aplidium* species (Figure
8 4a-b) (Nakauchi 1982). *Aplidium turbinatum* is characterized by a very long post-abdomen
9 containing the epicardium, the gonads and the heart at its posterior-most extremity. During
10 budding, the post-abdomen detaches from the abdomen and then divides into a dozen pieces
11 by strobilation, which begins in the most anterior part and proceed posteriorly (Brien 1948),
12 with each of the strobilae giving rise to a bud. While the epidermis of the parent becomes the
13 bud epidermis, each trapped fragment of the epicardium reshapes itself into a monolayered
14 spherical vesicle that eventually gives rise to the internal organs (Figure 4g). Trapped between
15 these two vesicles, the gonads become the future genital glands, while other mesenchymal
16 cells function as storage of nutrients for the developing bud (Brien 1948). A closely related
17 species, *Aplidium pallidum*, has a very short post-abdomen. Thus strobilation arises in the
18 abdomen and buds contain not only an epicardial fragment but also different sections of the
19 digestive tract, of gonads, and of a neuron-like strand of cells called the dorsal cord (Brien
20 1925, Brien 1948). The epicardium develops into the pharynx, the peribranchial chambers, and
21 the heart. The fragments of the old digestive tube regenerate a new digestive system and the
22 dorsal cord gives rise to the neural and germinal cells (Brien 1925, Brien 1948).
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25 Brien (1948) has speculated that the presence of additional tissues may limit the organogenetic
26 potentialities of the epicardium (Brien 1948). Indeed several *Aplidium* are able to strobilate
27 both in the abdominal and the post-abdominal regions (Nakauchi and Kawamura 1978,
28 Nakauchi 1981, 1982, 1986) but the relative role of the epicardium in those buds remains to
29 be studied. Berrill's hypothesis is nevertheless supported by experiments on *Aplidium*
30 *turbinatum* and on *Clavelina lepadiformis*. When abdominal sectioning is performed in these
31 species, the epicardium regenerates the thorax, while the digestive loop is formed by the rectal
32 and esophageal tissues. In contrast, if the animals are sectioned in the post-abdominal regions,
33 the epicardium ensures the regeneration of all the organs, including the digestive loop (Brien
34 1930, Brien 1948).

35 Budding in two other families, Didemnidae and Holozoidae, represents two illustrations of the
36 very diverse role that the epicardium plays in NED. In Didemnidae the epicardium is reduced
37 in size and remains as a pair of small abdominal sacs. The most detailed description of budding
38 in this family has been given by Pizon (1905) in *Diplosoma listerianum*, and despite some
39 specific variations, it arises similarly in other Didemnidae, e.g. *Polysyncraton lacazei* (Berrill
40 1948), *Trididemnum cereum*, and *Leptoclinum gelatinosum* (Salfi 1932). In these species there
41 is no sign of abdominal or post-abdominal strobilation, and the organs of the bud first remain
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physically associated with the parental organs. The anterior part of the epicardium forms diverticula that differentiate into a pharynx (including the branchial and peribranchial chambers and the nervous system) and an esophagus. The adult rectum forms diverticula that differentiate into another rectum. The posterior part of the epicardium form another heart, and the adult esophagus produces another digestive loop. The result is a double zooid possessing every organ in duplicate. Then, the new thorax (pharynx, rectum, and esophagus) gets associated with the old abdomen and the new abdomen (heart, intestine, stomach) with the old thorax (Pizon 1905). Budding also takes place at the larval stages in Didemnidae, by a process that, whereas likely similar to the above-described epicardial budding, remains largely unknown (Caullery 1895, Pizon 1905, Brien 1948).

In Holozoidae, the first budding event arises in the larva where the very well-developed epicardium is the main organogenic tissue (Della Valle 1881). This process has been described in detail in *Distaplia magnilarva* (Julin 1896, Brien 1948) and *Hypsistozoa fasmeriana* (Brewin 1959) and in several other Holozoidea (Caullery 1908, Berrill 1948b, Ivanova-Kazas 1967, Nishikawa 1990). In the larva that is still brooded, the left epicardium elongates until it reaches the neighbouring ectoderm (the right epicardium is much reduced). At that point of contact the epidermis evaginates and forms a stolonial outgrowth in which the epicardium penetrates (Brien 1948, Brewin 1959). In *D. magnilarva* this stolon is very short and soon separates from the adult to form a probud that subsequently divides and forms multiple buds. When the larva settles and metamorphoses, it already bears several mature blastozoids and several developing buds (Brien 1948). In *Hypsistozoa fasmeriana* in contrast, the stolon extends considerably and forms up to 14 buds by epidermal constrictions, which become mature blastozoids by the time the larvae is free-swimming. Thus the epicardial budding literally results in the formation of a swimming colony (Brewin 1959). Despite these variations, the bud comprises an epidermal outer vesicle and an inner vesicle derived from the epicardium that will give rise to all the internal organs, including the nervous system and the germ cells (Brien 1948). Budding in adult blastozoids after larval settlement was shown in *Distaplia magnilarva*, *D. bermudensis*, and *D. rosea* by simple epicardial budding highly reminiscent of seen in the larva (Berrill 1935, 1948b).

51 *Stolonial budding in Clavelinidae*

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53 In addition to epicardial budding, Clavelinidae undergo another form of NED called stolonial
54 budding (Figure 4e,f-h) Stolonial budding in Clavelinidae has been particularly well-studied in
55 *Clavelina lepadiformis* (Brien & Brien-Gavage 1927, Brien 1930) and is essentially the same
56 in *C. picta* (Berrill 1935), *C. oblonga* (Brien 1930), and *Pycnoclavella aurilucens* (Berrill 1947).
57 Clavelinidae possess hypertrophied ventral stolons consisting of an epidermally-derived
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3 circulatory vessel surrounded by a thin layer of tunic (Figure 4e,f). As in Perophoridea
4 described above, each stolon is divided into two longitudinal sinuses by a mesenchymal
5 septum, on each side of which blood flows in opposite directions. Brien and Brien-Gavage
6 (1927) showed the mesenchymal nature of the septum, which consists of a thin wall of
7 mesenchymal cells that is an extension of the adult hemocoel and is in physical continuity with
8 the mesenchymal network of cells underlying the stolonial epidermis (Brien and Brien-Gavage
9 1927). Along this stolon, opaque and lobulated regions become budding chambers (Figure
10 4h), which are congested with mesenchymal cells called trophocytes that likely play a role in
11 bud nutrition (Seeliger 1882, Kerb 1908, Brien & Brien-Gavage 1927, Brien 1930, Berrill 1947).
12 When the budding chamber is isolated from its parent, e.g. by necrosis or after zooid
13 regression, the mesenchymal septum forms ramifications that extend into the lobes of the
14 budding chamber. In one of these lobes, the tip of a septal ramification enlarges by
15 accumulation of free hemocytes. Then, this cluster of mesenchymal cells gets organized into
16 a monolayered inner vesicle surrounded by an epidermal outer vesicle (Figure 4h) (Giard and
17 Caullery 1896, Brien and Brien-Gavage 1927, Brien 1930). The outer vesicle gives rise to the
18 zooid epidermis and the inner vesicle to all the other organs, with the exception of the nervous
19 system and the germline that, according to Brien and Brien-Gavage (1927) derive from a
20 separate aggregate of mesenchymal cells that they call the “neuro-genital mass” (Brien 1948).
21 One dated report suggests that the inner vesicle derives from an invagination of the stolonial
22 epidermis (Kerb 1908) but there are no other publications that support this observation.
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25 Budding chambers produce zooids yearround, but they can also act as dormancy structures,
26 allowing the colony to bypass seasonal adverse conditions (Brien & Brien-Gavage 1927, Brien
27 1930, Berrill 1935, Berrill 1947, Mukai 1977, de Caralt 2002). During dormancy, the zooids
28 regress while the budding chambers, typically called “winter buds”, survive and go on to
29 repopulate the colonies when conditions improve. It is not clear if there are morphological
30 differences between normal budding chambers and winter buds, but it is possible that the
31 trophocytes may be allowed to further accumulate in the chambers prior to hibernation,
32 providing more reserve storage for the dormancy period (L. Hiebert, personal observations).
33 Finally, zooids of *C. lepadiformis* can also arise from isolated fragments of stolon, or from
34 budding chambers upon artificial section of the adult post-abdomen, probably resulting from
35 the arrest of blood circulation (Della Valle 1915, Brien 1930, Berrill and Cohen 1936). The
36 regeneration process is histologically identical to the stolonial budding described above, but
37 the ability of a stolon fragment to form a fully functional zooid is linked to its absolute size. For
38 instance, in fragments smaller than a half-millimeter the epidermal cells change shape and
39 begin to form the outer vesicle, but the inner cell mass never appears (Berrill and Cohen 1936),
40 likely because the number of septal cells is too low to initiate organogenesis.
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6. Budding in Thaliaceans: from bloom to bud

Thaliaceans are planktonic, transparent budding tunicates (Figure 5). Their fragility, difficult accessibility, and scarce availability explain why the 85 described species have been poorly studied (Piette & Lemaire 2005, WoRMS 2019). They are divided into three monophyletic groups: salps, pyrosomes, and doliolids (Figure 1). With the exception of doliolids, that retained a rudimentary free-living larvae, thaliaceans have lost tailed larvae and bear the developing embryos in the maternal body. Sexual and asexual phases coexist, and budding starts before oozooid development is completed. This budding process largely accounts for thaliacean seasonal blooming, a phenomenon that has an important effect on the oceanic trophic chain, through feeding upon phytoplankton and production of carbon-rich fecal pellets that may become trapped in deep sediments (Andersen 1998, Phillips *et al* 2009, Lebrato *et al* 2013, Conley *et al.* 2018). So far, NED mechanisms have only been studied in few thaliaceans, sometimes leading to conflicting results regarding the nature of tissues involved in bud constitution.

Salps

Colonies of salps are long chains of blastozoids, sometimes several meters long, attached to each other by the tunic (Figure 5a), until the chain breaks off and blastozoids live their own lives. Each blastozoid houses a single egg, which once fertilized develops within the maternal tunic until it starts to produce a budding stolon that will eventually give rise to several hundred blastozoids (Figure 5b-c). The stolon primarily arises as an invagination of the pharyngeal floor at the posterior extremity of the endostyle (Brooks 1893, Brien 1928, Berrill 1950) (Figure 5d). This inner-most growing tube is capped by overlying epidermis and these two epithelia trap mesenchymal cells in between. As the stolon elongates a process of epidermal constrictions defines the boundaries of the future blastozoids (Figure 5b). According to Brien (1928), who studied stolonal budding in *Thalia democratica*, *Salpa maxima*, and *Salpa fusiformis*, with the exception of the inner tube arising from the pharynx and the outer epidermis, mesenchymal cells play a prominent role as they give rise to the rudiment of the pericardium, the peribranchial tubes, the nervous system, and the genital mass (Figure 5g). Berrill's observations of *Salpa fusiformis* and *Pegea confoederata* led to a totally different interpretation: the neural tube arises by invagination from the stolonal ectoderm, the germline originates from the mesenchymal cells, while all the other organs develop from the inner tube of the stolon (Berrill 1950). Further investigations are therefore strongly needed to reconcile these discordant descriptions.

Pyrosomes

Colonies of pyrosomes have the shape of a tube, closed at one of its extremities and constructed of thousands of blastozooids (Figure 5e-f). Every zooid has its oral siphon facing outward and its atrial siphon oriented toward the lumen of the colony, where the exhalant current of each zooid converge and propel the colony forward. A fertilized oocyte produces the first individual, named the cyathozoid, a flat-bodied rudimentary larva that lays on the egg vitellus and never leaves the maternal cloaca. The budding process begins very early when the cyathozoid organs are not even fully-developed. The cyathozoid elongates posteriorly and rapidly strobilates by epidermal constrictions into four buds, in which each organ originates from its corresponding rudiment in the cyathozoid larvae (except the nervous system that is formed *de novo*) (Godeaux 1957, Salensky 1890, 1891, Julin 1912, Brien 1948). The four blastozooids (called tetrazooids) formed by this primary budding, in turn produce new buds by secondary budding. The secondary stolon is structurally similar to the stolon in salps, with an outer ectoderm and an inner tube that forms by invagination of the pharyngeal floor at the posterior extremity of the endostyle (Berrill 1950, Godeaux 1957). For Godeaux (1957) blastogenesis of the secondary bud involves multiple tissues: the inner pharyngeal tube gives the pharynx and the digestive system, while the peribranchial chambers, the heart, and the germ cells come from the corresponding parental tissues, and the neural complex has a mesenchymal origin (Figure 5h). In contrast, for Berrill (1950) the secondary budding in pyrosomes is similar to what he described for salps: the nervous system originates from the stolonial ectodermal while other organs derive from the inner tube.

Doliolids

Colonies of doliolids are composed of a barrel-shaped oozooid, called a nurse, that carries three rows of polymorphic blastozooids on a posterior dorsal appendix (the spur). Doliolids have a relatively complex life cycle with three different types of blastozooids (for a review, see Piette and Lemaire 2005, Bone 1998). Unlike pyrosomes and salps, some species of *Doliolum* possess a free swimming tadpole larva with a notochord, similar to the larva of ascidians. Budding starts in the larva, with the production of a stolon that, like in salps and pyrosomes, sprouts from the ventral floor of the pharynx, posterior to the endostyle, and strobilates by transversal epidermal constriction. The nature of the stolon has been debated for a long time (Grobben 1882, Uljanin 1884, Neumann 1906, 1935, Godeaux 1957) but Godeaux (1957) produced a convincing histological description. The stolon begins as two bilateral invaginations of the pharyngeal wall, capped by the overlying epidermis. During its elongation others structures get incorporated: one pericardial cord sprouting from the larval heart will give rise to the neural complex, three invaginations of the cloacal chamber, one median and two lateral,

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3 will form the digestive system and the peribranchial chambers, and one mass of mesenchymal
4 cells with unknown origin and fate (Figure 5i). According to this description, the budding stolon
5 of doliolids differs from the one of pyrosomes and salps but they all share the involvement of
6 the pharynx - in the same position - and therefore we named it pharyngeal budding and we
7 proposed it to be homologous in Thaliaceans (Figure 1).
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15 **7. Reconstructing gains and losses of NED: a phylogeny and homology issue**

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17 The combination of a reliable phylogeny and detailed anatomical descriptions allows for the
18 inference of gains and losses of NED across tunicates. A parsimonious reconstruction of
19 solitary *versus* colonial clearly favors a solitary tunicate ancestor, and therefore NED most
20 likely results from several convergent acquisitions during tunicate evolution. This view is
21 strongly supported by the diversity of budding processes that involve disparate and in many
22 occasions non-homologous tissues. In fact, tunicate buds always contain an outer layer
23 derived from the parental epidermis but their inner layer originates from a variety of tissues.
24 The different budding modes have been traditionally classified upon the nature of the budding
25 tissues (Berrill 1951) as well as ecological aspects (Nakauchi 1982, Turon 1992). Following
26 the descriptions of NED provided in the previous paragraphs, we distinguish -six different
27 budding modes based on the origin of the inner layer (see table 1).
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36 In Stolidobranchia, the position of the solitary Pyuridae, Molgulidae, and Styelidae strongly
37 supports the hypothesis of a non-budding ancestor. Thus, we can speculate that three
38 acquisitions of budding arose in the evolutionary history of Styelidae (Alié *et al.* 2018). (i) Vasal
39 budding has been acquired in the species *Polyandrocarpa zorritensis*, (ii) peribranchial
40 budding in the last common ancestor of all peribranchial budders and (iii) vascular budding
41 was acquired secondarily in an ancestor that already possessed peribranchial budding (Figure
42 1). The evolutionary scenario of NED evolution in Stolidobranchia could prove even more
43 complex, as NED in numerous budding species is undescribed (e.g. *Styela complexa* (Kott,
44 1995), *Syncarpa composita* (Hasegawa and Kajihara 2019)).
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51 In Aplousobranchia, the epicardial budding is shared by all colonial species and therefore it is
52 very likely ancestral to this order (Moreno and Rocha 2008, Shenkar *et al.* 2016). While
53 different families of Aplousobranchia have particular variations of the epicardial budding
54 (Moreno and Rocha 2008), the presence of a multipotent epicardium involved in NED is not
55 reported in other tunicates and likely represents a synapomorphy of this order (Figure 1).
56 Clavelinidae combine epicardial and stolonial budding, and even if some authors have initially
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considered the stolonial septum as derived from the epicardium or the pericardium (Van Beneden and Julin 1886, Giard and Caullery 1896) its mesenchymal nature has been firmly demonstrated (Brien and Brien-Gavage 1927). Therefore, we can hypothesize that stolonial budding has been secondarily acquired in the clavelinid ancestor in addition to the already present, ancestral epicardial budding (Moreno and Rocha 2008, Figure 1).

In the paraphyletic order “Phlebobranchia”, the position of Perophoridae close to the solitary Ascidiidae suggests that stolonial budding is a synapomorphy of the Perophoridae family, acquired independently from the Clavelinidae (Moreno and Rocha 2008) (Figure 1). Due to the paucity of budding phlebobranchs in the current phylogenies, the relationships between budding and non-budding species remain largely unknown, as well as their affinities to Aplousobranchia. This strongly limits our understanding of the evolution of NED in these groups, which would benefit from data on enigmatic species like *Polyoctacnemus patagoniensis* and *Plurella* (Kott 1972, 1985).

In pyrosomes, salps, and doliolids, stolonial buds arise from the pharyngeal floor immediately posterior to the endostyle. This type of budding is therefore ancestral to thaliaceans and may represent a synapomorphy for this clade. However, some authors suggested that the pharyngeal expansion in thaliaceans is homologous to the epicardial budding of Aplousobranchia (Bonnevie 1896, Brien 1928, Berrill 1935, Godeaux 1957, Ivanova-Kazas 1967). If true, it may imply that epicardial budding was either convergently acquired in these two groups, or inherited from a deeper common budding ancestor. Once again, further morphological descriptions of the budding tissues and clarification of the deepest nodes of the tunicate phylogeny are needed (Tsagkogeorga et al. 2009).

8. Tunicata: a playground for experimental Evo-Devo

The discipline of evolutionary developmental biology (evo-devo) is based on the comparisons of developmental mechanisms among species to explore the phenotypic changes during evolution. Tunicates provide different levels in which these comparisons can be explored. First, all budding tunicates retain sexual reproduction and embryonic development. Despite being extremely different, in many tunicate budding and sexual development lead to similar post-metamorphic body plans. Then, embryogenesis, metamorphoses and non-embryonic development can be analyzed side-by-side in the same species to explore how homologous features can be obtained following completely different developmental mechanisms. For instance, studies in *Botryllus schlosseri* have already shown how key developmental genes have been co-opted from embryogenesis and redeployed during peribranchial budding (Tiozzo et al. 2005, Ricci et al. 2016a, 2016b, Prünster et al. 2018, 2019). The redeployment of

embryonic developmental programs during asexual development and regeneration is not a novel idea in the literature. However, relatively few experimental studies compared gene expression and function between different developmental pathways. Comparisons between embryonic and NED, literally in the same organism, is indeed a powerful approach to highlight the rewiring of gene regulatory networks and describe the plasticity of regulatory modules.

The previous chapters show how budding modes are diverse in terms of triggering cells and tissues and early ontogenesis. Studies in a limited number of ascidian models suggested that the triggering mechanisms may also not be conserved. For example, peribranchial budding in *Polyandrocarpa misakiensis* involves a fully differentiated epithelium that undergoes transdifferentiation (Kawamura *et al.* 2008) whereas vascular budding in *Botrylloides leachii* and stolonial budding in *Perophora viridis* engages stem cells with different degrees of potency (Freeman 1964, Kassmer *et al.* 2019). The combination of transcriptomic (RNAseq, sc-RNAseq) and functional approaches can highlight conserved modules, such as genes, portions of gene regulatory networks, or particular cell or tissues “types” that control budding. Then, comparisons between similar and different NED modes in diverse species, or even different NED modes in the same species may illuminate deep homologies in budding mechanisms. Particularly interesting would be investigating conserved molecular modules in clusters of budding cells in non-homologous epithelia, like the peribranchial epithelia in Styelidae, the posterior ventral pharynx in thaliaceans and the epicardium in Aplousobranchia. The latter shows a disparate degree of potency across different species belonging to the same order and provides a framework to study plasticity of tissue potency and cell commitment. Possible mechanisms of heterochronic shifts could also be investigated, like in the case of larval budding in Holozoidae and Didemnidae: possibly a consequence of a predisplacement (McNamara 2012) of the respective post-metamorphic NED.

The multiple gains and losses of budding in tunicates provides an opportunity to explore the mechanisms underlying the acquisition of NED. For example, one can explore the function of undifferentiated mesenchymal cells (referred to as hemoblasts or lymphocyte-like cells), which have been reported in both solitary and colonial ascidians. In the solitary *Styela plicata* these cells show characteristics of stem cells, and they have been suggested to play a role during regeneration (Jimerez-Merino *et al.* 2019, *in press*). In *Botrylloides leachi*, also a stylelid, cells with similar characteristics have been shown to initiate vascular budding (Kassmer *et al.* 2019b). This scenario raises the hypothesis that hemoblasts may have acquired different degrees of potency underlying NED evolution.

Understanding the evolution of NED in tunicates allows for a deeper understanding of the source of the tremendous colony diversity within this subphylum. With the exception of some

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3 thaliaceans, the bauplan of a zooid is relatively conserved across the different tunicate groups.
4 On the other hand, the variations in shapes, colors, and sizes of colonies are remarkable, a
5 diversity deeply rooted in the variability of NED ontogenetic steps. For example, in Styelidae the
6 variation in the length of the bud peduncle defines the degree of connection between the
7 zooids, resulting in more or less integrated colonies. In Aplousobranchia, it is the buds
8 movements within the parental tunic that determine the shape of the colony. Therefore, the
9 regulation of one structure or behavior may not change the bauplan of the zooid but influences
10 the colony phenotype. Such changes can also affect the ecology of the colony, such as in the
11 species that have convergently evolved planktonic buds, allowing for somatic dispersion
12 (Fujimoto and Watanabe 1976, Mukai et al. 1983, Turon 2005).
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15 In the light of the techniques and genomic resources now available, the diversity of
16 developmental modes characterizing Tunicata and the relative richness of anatomical
17 knowledge, clearly supply a well-stocked toolbox to explore developmental mechanisms and
18 better understand their evolution.

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FIGURE LEGENDS

Figure 1. Phylogenetic relationships between budding and non-budding tunicates. Budding species are represented in red, non-budding species in black. For each branch, letters refer to the studies in which this clade was retrieved, including all the represented species. Letters between parentheses refer to studies that support the corresponding clade, *i.e.* with some of the represented species and when no contradicting group were found. Species with dotted lines are positioned not based on phylogenetic studies but on classification. When a new budding mode arise in a budding taxa, it's preceded by "+".

Figure 2. Stolidobranchia. (a) *Polycarpa spongiabilis* (courtesy of Rosana Rocha). (b) Colony of *Polyandrocarpa zorritensis* hatching from dormant spherules (courtesy of Alexandre Jan). (c) *Styela plicata*. (d) Small colony of *Botryllus schlosseri*. (e) Schematic representation of peribranchial budding at three successive stages, from peribranchial invagination to double vesicle. (f) Schematic representation of vascular budding at three successive stages, from

hemocyte clustering to double vesicle. (g) Schematic representation of vasal budding from nest formation to the double vesicle stage.

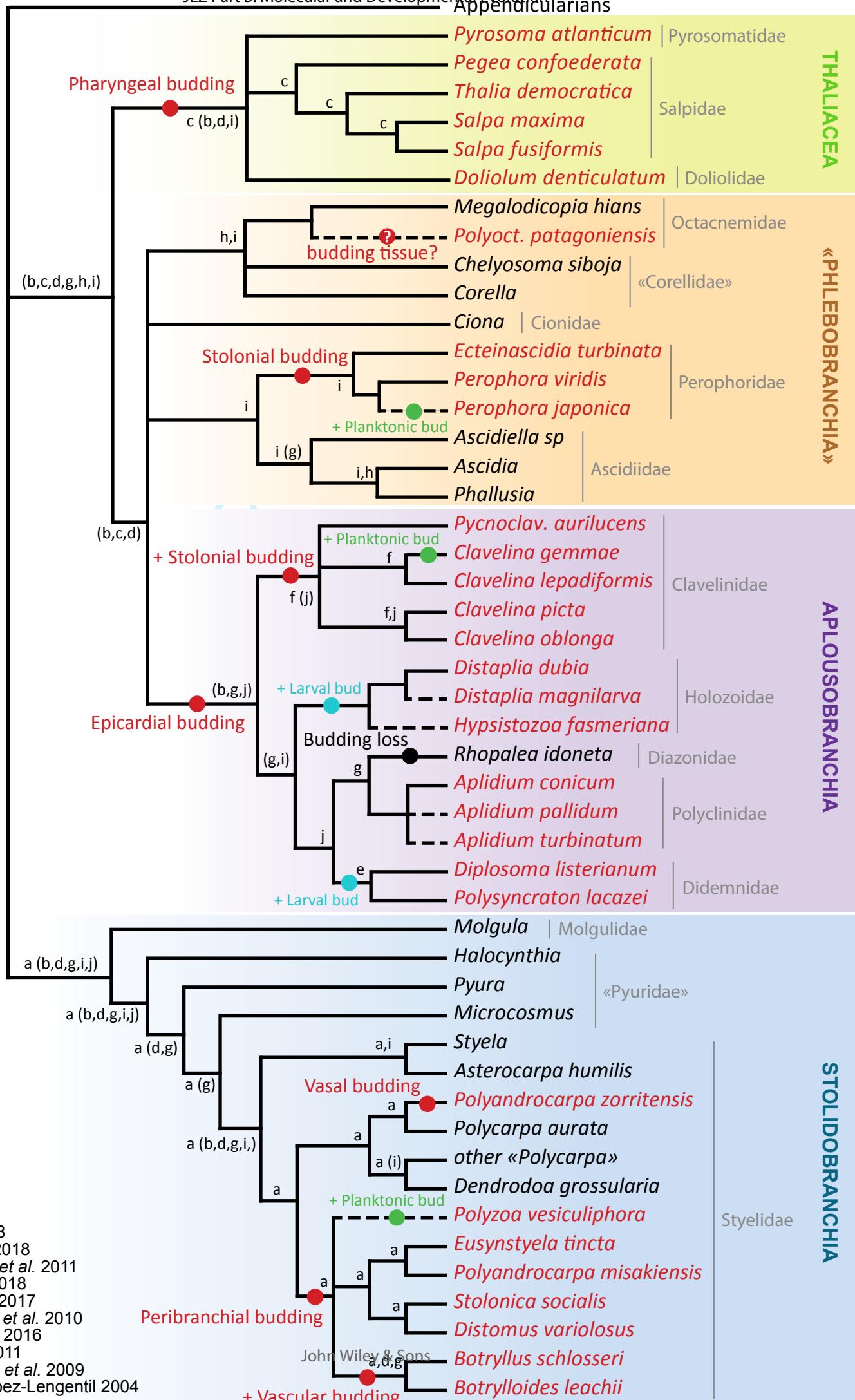
Figure 3. Phlebobranchia. (a) *Ciona robusta* (courtesy of Alexandre Jan). (b) *Ecteinascidia turbinata* (courtesy of Rosana Rocha). (c) *Perophora* sp. (Cifonauta image database. Available at: <http://cifonauta.cebimar.usp.br/media/10591/>). (d) Colony of *Perophora viridis* (courtesy of Rosana Rocha). (e-f) Schematic representation of stolonial budding from bud primordium to the double vesicle stage.

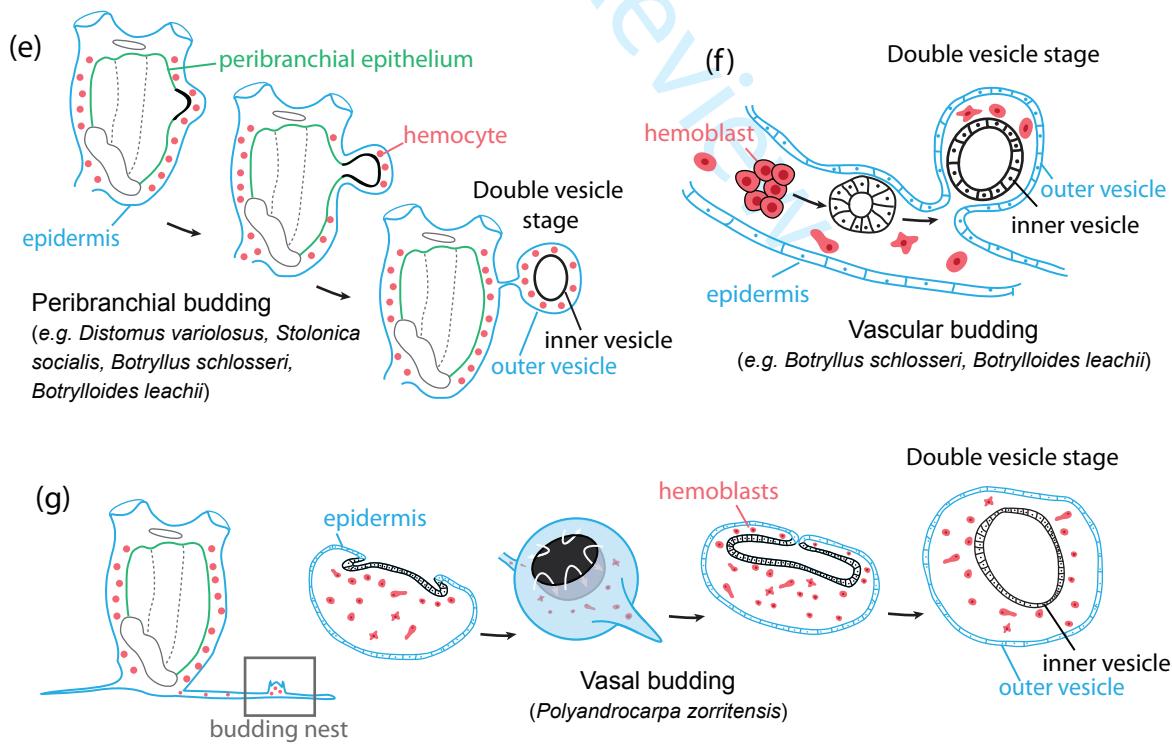
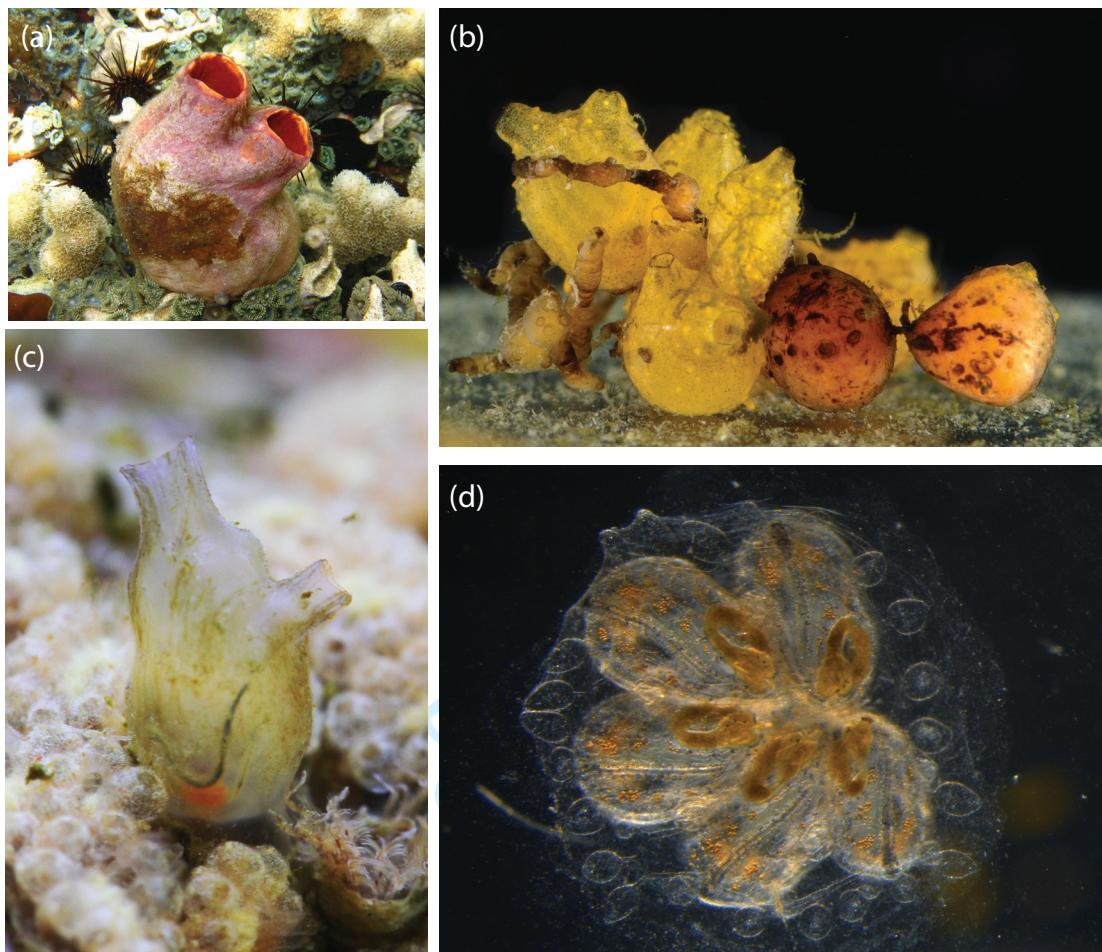
Figure 4. Aplousobranchia. (a) Colony of *Aplidium conicum* and (b) *Didemnum fulgens* (courtesy of Xavier Turon). (c) Planktonic bud of *Clavelina gemmae* (courtesy of Xavier Turon). (d) *Diplosoma listerianum* in the process of budding. (e) Colony of *Clavelina lepadiformis* and (f) detailed view of budding chambers showing a forming zooid (courtesy of Kazuo Kawamura). (g) Schematic representation of epicardial budding and detailed view of the post-abdominal budding, from strobilization to the double vesicle stage. (h) Schematic representation of stolonial budding in Clavelinidae.

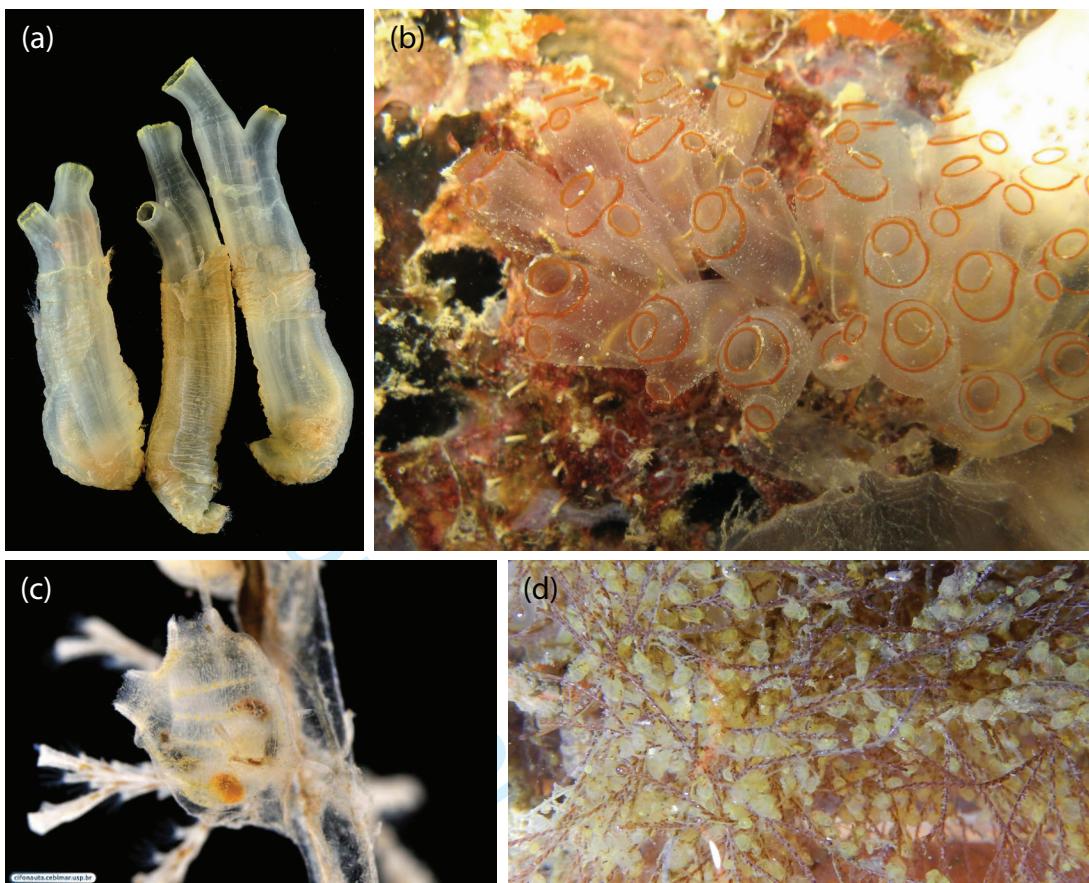
Figure 5. Thaliacea. (a) Chain of *Pegea confoederata*. (b) Close-up view of a stolon in the process of strobilization in *Salpa fusiformis*. (c) Oozoid of *Thalia democratica*, bearing a budding stolon. (d) Microscope view of the early bud initiation in *Salpa maxima*. (e) Colony of *Pyrosoma atlanticum* (courtesy of David Luquet) and (f) close-up view. (g) Schematic representation of pharyngeal budding in Salpidae. (h-i) Schematic transverse section of budding stolons in (i) *Doliolum denticulatum* and (h) *Pyrosoma atlanticum*.

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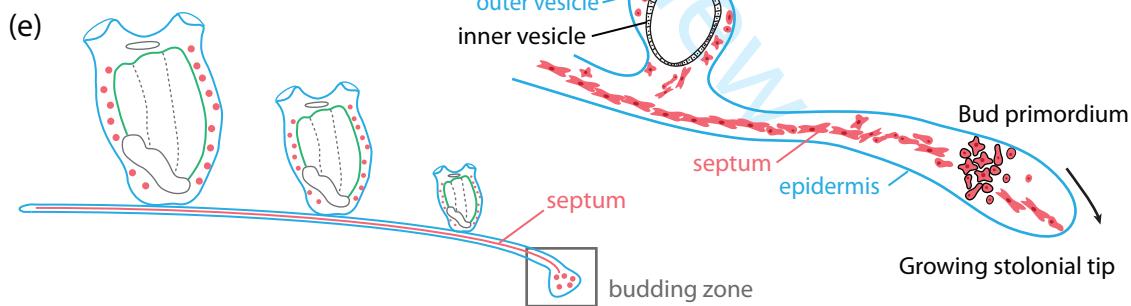
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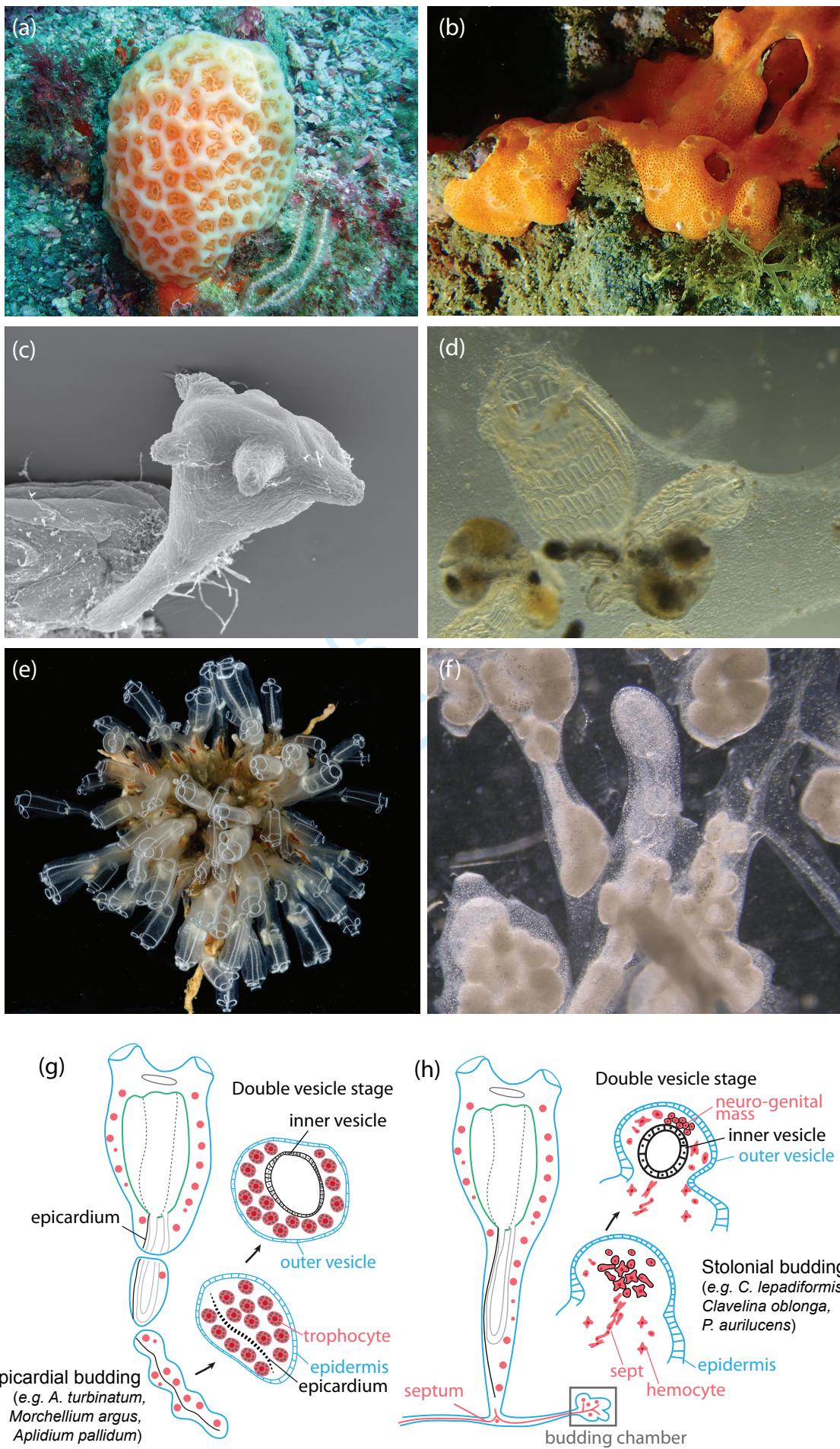






Stolonal budding (e.g. *Perophora listeri*,
Perophora japonica, *Ecteinascidia turbinata*)





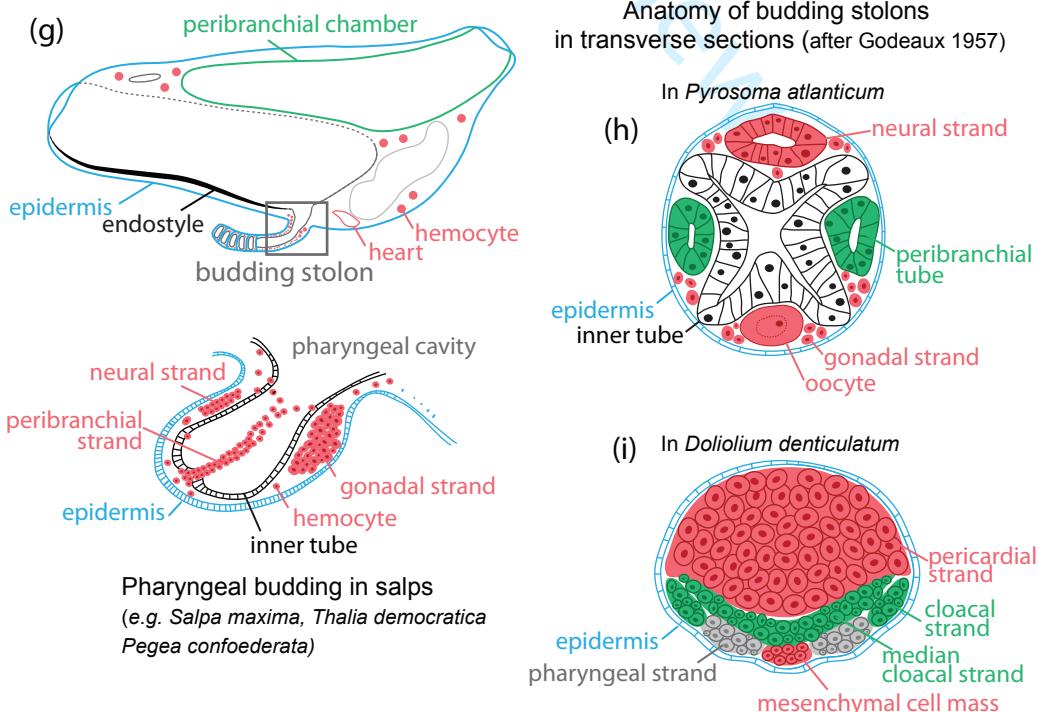
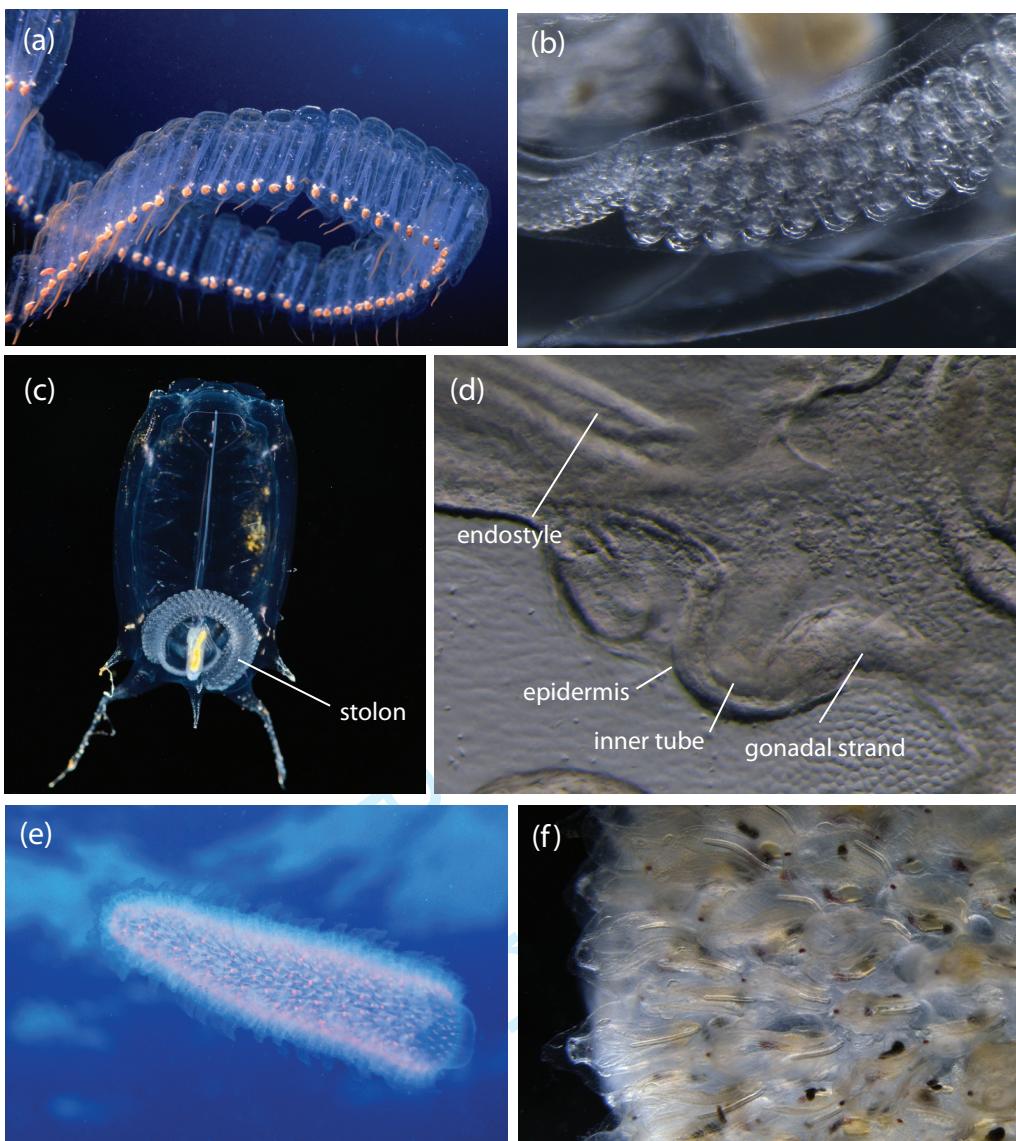


Table 1. Six budding modes in Tunicates

Budding mode	Tissues forming the bud inner vesicle	Example species	References
Peribranchial budding	Peribranchial epithelium	<i>Botryllus schlosseri</i> , <i>Polyandrocarpa misakiensis</i> , <i>Polyzoa vesiculiphora</i>	Pizon 1893, Selys-Longchamps 1917, Watanabe and Tokioka 1972
Vascular budding	Hemoblasts	<i>Botrylloides leachii</i> , <i>Botryllus schlosseri</i>	Burigel et al 1976, Ricci et al 2016a
Vasal budding	Vascular epidermis	<i>Polyandrocarpa zorritensis</i>	Alié et al. 2018, Scelzo et al 2019
Stolonial budding	Mesenchymal septum	<i>Perophora viridis</i> , <i>Ecteinascidia turbinata</i>	Dale Beers 1923, Brien and Brien-Gavage 1928, Freeman 1964
		<i>Clavelina lepadiformis</i> , <i>Pycnoclavella aurilucens</i>	Brien & Brien-Gavage 1927, Brien 1930
Epicardial budding	Epicardium	<i>Distaplia dubia</i> , <i>Aplidium turbinatum</i> , <i>Diplosoma listerianum</i>	Pizon 1905, Berrill 1948b, Nakauchi 1986
Pharyngeal budding	Pharyngeal floor (epicardium?)	<i>Pyrosoma atlanticum</i> , <i>Salpa fusiformis</i> , <i>Doliolum nationalis</i>	Berrill 1950, Godeaux 1957