Vasal budding : characterization of a new form of non-embryonic development in the colonial ascidian Polyandrocarpa zorritensis

Marta Scelzo

▶ **To cite this version:**

Marta Scelzo. Vasal budding : characterization of a new form of non-embryonic development in the colonial ascidian Polyandrocarpa zorritensis. Development Biology. Sorbonne Université, 2020. English. NNT : 2020SORUS467 . tel-04002082

HAL Id: tel-04002082
https://theses.hal.science/tel-04002082

Submitted on 23 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

ED515 - Ecole doctorale Complexité du Vivant

Vasal budding: characterization of a new form of non-embryonic development in the colonial ascidian *Polyandrocarpa zorritensis*

Thèse de doctorat en Biologie du Développement

Par Marta Scelzo
Sous la direction du Dr Stefano Tiozzo

Présentée et soutenue publiquement le 16 Septembre 2020
Au Laboratoire de Biologie du Développement de Villefranche-sur-Mer
Devant un jury composé de:

Dr Eric Röttinger	Rapporteur
Pr Xavier Turon	Rapporteur
Dr Aldine Amiel	Examinatrice
Dr Sebastien Darras	Examinateur
Dr Eve Gazave	Examinatrice
Pr Elisabeth Christians	Examinatrice
Dr Stefano Tiozzo	Directeur de thèse
Pr Federico Brown	Co-Directeur de thèse

This work is licensed under a Creative Commons
Attribution - Non Commercial - No Derivatives 4.0 International license

A chi ho amato e c'è sempre stato,

perché i traguardi raggiunti contano di più se puoi condividerli.

A chi ho amato e se n'è andato troppo presto,

perché cercando un senso alla morte ho capito come vivere davvero.

Alla Marta di domani:

anche questo è stato fatto per te, ma stavolta fanne l'uso che vuoi. Sii felice.

Acknowledgments

The last four years went by so fast and so slowly at the same time. One day, thinking back to this period, I know that I will think about the smell of a fig tree, the song of cicadas and the colors of the sunset on the city of Nice. And for sure, I will think about all the people that walked by my side during these four years. To them, to all the "Villefranche people", I want to say thank you.

First of all, I would like to thank my supervisor, Stefano Tiozzo, for giving me the opportunity to work in his team and for guiding me during my entire PhD. I am also very grateful to all the other members of the "Regeneration and pluripotency" lab. Thanks to: Alex, for introducing me to the *Polyandrocarpa* world; Federico, for your precious advices; Sonia, for your kindness and the technical (and emotional) support; Irene, to let us observe the Nature through the beauty of Art; all the students that worked in lab, in particular Maxence and Rita, for creating a nice working environment.

A very special gratitude goes to my favorite labmates: Mandela and Laurel for our stimulating, judgment-free, (often) crazy scientific discussion, and my petite Marie for her help and her incredible determination. You have been an inspiration to me, and I am very grateful to have worked with you.

I would like to thank all the members of my thesis jury, for accepting to read my manuscript and to participate to my defense. I also want to thank all the people who gave me scientific and technical support during my PhD: the members of my thesis committee, Stefania Castagnetti and Francesc Cebrià, for their advices; the members of the de Tommaso lab, in particular Tony and Susannah, for the precious help with the Iα6; Sophie Pagnotta for the amazing TEM images; Ludovic Cervera for the cell sorting; Cathrine Pons for teaching me how to use the cryostat; Martine Fioroni for providing me all the fascinating old papers available at the biblioteque. A special mention goes to Lolo, AlexJ, Loann, Sophie and Regis for taking care of my babies *Polyandrocarpa*.

I wish to thank all the occupants of the JM student office, Geraldine, Julie, Cat, Marion and Manon, for all the laughs, the teardrops and the crazy moments I shared with you. I also wish to thank also the occupants of the mezzanine, in particular Angelica, Anna, Julia, Anne, David, AlexJ for the great moments spent together.

I am also grateful to the all the JM people. Clare, Evelyn, Sonia, Cathy, Sandra, Carine, Yas, Tsuyo and all others, thanks for the funny lunch and coffee breaks, the rides and all the interesting discussions.

A big thanks goes to the entire Villefranche family of the Institut de la mer. Thanks to: Christelle and Frédéric for taking care of all the administrative procedures; the service I4, Sameh, Faisal, Mébarek and Philippe for taking care of our microscopes and our safety; Jocelyne, Didier and Thierry for feeding me with good food; Guy, François, Lucas and all the others (sorry if I forget someone) for these incredible 4 years spent with you.

Ringraziamenti

Per le mie tesi precedenti ho intenzionalmente evitato di mettere nero su bianco dei ringraziamenti, un po' per pudore, un po' perche' penso che sia piu' importante mostrare riconoscenza anziche' scriverla su un foglio. Tuttavia, per questo che spero sara' il mio ultimo manoscritto, ho deciso di ringraziare pubblicamente con tutto il mio cuore le persone a me care che mi hanno accompagnato (anche se da lontano) in questi quattro anni.

Ringrazio la mia famiglia, per avermi sostenuta sempre. Questi quattro anni non sono stati semplici, molte volte si è fatta sentire la mancanza un abbraccio, ma anche se solo per 5 minuti e con uno schermo che ci separava mi avete fatto sentire la vostra presenza ogni giorno. Grazie ai miei genitori, per avermi sempre spinta a seguire i miei sogni e le mie ambizioni e per aver fatto tutto il possibile per permettermi di realizzarli. Potete essere ben orgogliosi di chiamarmi dottoressa da oggi, perché questo titolo che mi avete concesso da almeno un ventennio adesso è ufficialmente riconosciuto! Grazie a mia sorella, alla parte più bella di me. Abbiamo sempre condiviso tutto, e il pensiero di averti al mio fianco anche in questo giorno mi riempie il cuore di gioia. Grazie a mia nonna, per tutte le preghiere e i rosari fatti in questi anni di studio. Apparentemente hanno funzionato! Grazie anche a chi per quattro anni sarei mancata alle cene del venerdì e a chi avrebbe adorato parlare con me in francese, perché il mio cuore vi ha sempre sentiti al mio fianco.

Grazie al mio Lyam per avermi sempre incoraggiata e confortata. Grazie per i tuoi consigli, i tuoi "è una pigna nel culo" e "sei troppo fortissima". Per tutte le volte che hai saputo calmarmi durante le mie crisi e hai saputo darmi un altro punto di vista (meno amaro e più razionale) sulle situazioni insormontabili che credevo di trovarmi davanti. Senza di te non credo avrei finito questo percorso! Merci beucoup mon tresor!

Grazie ai miei amici. Alla mia Vittoria, perché con i nostri buongiorno e i tuoi calendari hai scandito ogni singolo giorno di questi quattro anni, odiando i lunedì e agognando i weekend. Alle mie ciofeche Chiara e Maria, perché credo fermamente che la ciofecaggine sia un punto di forza che ci porterà lontano. D'altronde, saremo le prime ciofeche PhD al mondo, mica è roba da poco? Al mio Ughetto, per aver sempre creduto alle mie capacità scientifiche. Senza di te non sarei dove sono ora (letteralmente, perché non avrei mai applicato per la posizione 4 anni fa), e spero davvero un giorno di lavorare ancora con (o per) te. A Marina, per aver continuato a ricordarmi negli ultimi 11 anni la bellezza della storia, dell'arte e del "classicismo". A Pasquale per tutti i gelati e le interessanti discussioni dei fine settimana nizzardi.

A tutti voi va il mio piu' sincero grazie!

Abstract

Title: Vasal budding, a new form of non-embryonic development in the colonial ascidian *Polyandrocarpa zorritensis:* histological and ultrastructural description.

Colonial organisms can generate a new adult body by asexual reproduction and whole body regeneration, two forms of non-embryonic development (NED). Colonial tunicates are the closest relatives of vertebrates able to regenerate by NED, starting from a portion of adult epithelia and/or mesenchymal cells. In tunicates different modes on NED are defined depending on the nature of the organogenetic tissues. Interestingly, this capacity is scattered across the sub-phylum, with species able of NED (colonial) closely related to species where regenerative capabilities are absent or reduced (solitary) (Alie et al 2020). This suggests that NED has been acquired or lost several times among the group. In recent phylogeny of family Styelidae, the colonial species *Polyandrocarpa zorritensis* seems to have acquired independently the capability of NED (Alie et al 2018).

During my PhD I characterized the NED in this species, identifying the stages of NED under laboratory conditions and the tissues/cells involved. By histological and ultrastructural analyses I highlighted the participation to NED of vascular epithelium and mesenchymal cells. This type of NED was undescribed before in colonial ascidians, and we decided to call it "vasal budding" (Scelzo et al. 2019). During the early stages of vasal budding I observed undifferentiated mesenchymal cells cluster and proliferate at the regenerative point; their distribution varies during vasal budding, increasing in the developing areas. I characterized histologically and ultrastructurally the mesenchymal cells, identifying in the proliferating cells an undifferentiated morphotype, the hemoblasts. These cells are known as putative stem cells responsible for NED in other colonial ascidian. Preliminary data suggest the expression of Integrin alpha 6, a stem cell marker, in hemoblasts at the onset of organogenesis during vasal budding. In addition, I also described the presence of a dormant stage, the spherule, in the life cycle of *P. zorritensis*, and I characterized the environmental variable involved in dormancy in this species and in a distantly related species, *Clavelina lepadiformis*. In order to elucidate the molecular mechanisms underlying the dormancy process and identify dormancy-specific elements, I am currently participating to the molecular characterization of dormant and non-dormant tissues by transcriptomic analyses.

Overall my work has helped to generate basic biological understandings of a new organism, *Polyandrocarpa zorritensis,* suitable for comparative studies on the cellular and molecular mechanisms of NED in tunicates.

Chapter 1 - Introduction

1.1 Asexual reproduction in metazoans **2**

1.1.1 Some definitions 2

1.1.2 Asexual reproduction and its relationship with sexual reproduction 5

1.1.3 Asexual reproduction and coloniality 5

1.2 Regeneration in metazoans **6**

1.2.1 Definition of regeneration 6

1.2.2 Evolution of regeneration and its distribution 8

1.2.3 Regeneration and embryonic development 11

1.3 Relationship between asexual reproduction and regeneration: the Non-Embryonic Development (NED) **11**

1.3.1 Cellular and molecular mechanisms of NED 12

1.3.2 Diversity of NED modes within metazoans – some examples 14

Cnidaria 14

Annelids 16

Platyhelmonthes 17

1.3.3 Approaches to study evolution and mechanisms of NED 18

1.4 Tunicates: suitable models to study NED **19**

1.4.1 Tunicates: some history 19

1.4.2 Taxonomy and phylogeny 21

1.4.3 Lyfe cycle: solitary vs. colonial 21

1.4.4 The ascidian body plan 24

Body wall 24

Water flow and nutrition 24

Circulatory system 26

1.4.5 A higher level of organization: formation of colonies 27

1.5 NED in tunicates: generalities and distribution **29**

1.5.1 Pharyngeal budding in Thaliaceans 32

1.5.2 NED in Phlebobranchia 34

Stolonial budding 34

Dormancy in *Perophora spp* 34

Whole body regeneration in *Perophora* 34

1.5.2 NED in Aplousobranchia 37

Epicardial budding 37

Stolonial budding in *Clavelina* 40
Dormancy and rejuvenation 41
WBR in *Clavelina lepadiformis* (stolonial and epicardial regeneration) 42
1.5.2 NED in Stolidobranchia 43
Peribranchial budding 45
Vascular budding 51
Dormancy in Styelidae 53
1.6 Stolidobranchia as models to study NED evolution 54
1.6.1 Cellular and molecular signatures of NED in Styelidae: state of the art 55
Molecular signature odf peribranchial budding 55
Role of mesenchymal cells during perobranchial budding 56
Mesenchymal cells as progenitors of vascular budding 58
1.7 Thesis objectives 60

Chapter 2

2.1 Novel budding mode in *Polyandrocarpa zorritensis*: a model for comparative studies on asexual development and whole body regeneration 62

Chapter 3

Introduction 65
3.1 Immunocytes 67
3.1.1 Phagocytes 67
3.1.2 Cytotoxic cells 68
3.2 Storage cells 68
3.3 Undifferentiated cells: hemoblasts 69
3.4 The role of hemoblasts during NED 71
Results 73
3.5 Identification of eight hemocyte morphotypes in *Polyandrocarpa zorritensis* 73
3.5.1 Hyaline amoebocytes 73
3.5.2 Macrophages-like cells 73
3.5.3 Granular amoebocytes 74
3.5.4 Morula cells 75
3.5.5 Granulocytes cells 76

3.5.6 Storage cells (nephrocytes and pigment cells) 77
3.5.7 Hemoblasts 77
3.6 Relative distribution of hemoblastsin localization in budding tissues 78
3.7 Patterns of epithelial and hemoctes proliferation during vasal budding 80
3.8 Hemocytes dynamic during vasal budding 86
3.9 Integrin-alpha6 positive cells are present in the developing buds 88
3.10 Flow cytometric characterization of hemoblasts 89
Discussion 92
3.11 Conserved (and not conserved) role of hemocyte morphotypes in *Polyandrocarpa zorritensis* 92
3.12 Interplay between vascular epithelia and hemoblasts during vasal budding 93
3.13 Proliferating activity leads to the double vesicle formation 94
3.14 Iα6 as a potential marker for stem cells in vasal budding 96
3.15 Towards a functional characterization of hemoblasts in *P. zorritensis* NED 96
3.15.1 Control of the EdU chase efficiency 97
3.15.2 Identification of hemoblast markers with immunostaining, flow cytometry and *in situ* hybridization 97
3.15.3 In vivo hemocyte tracking 98
3.15.4 NED inhibition and rescue experiments 99
Material and methods 100
3.16 Hemocyte extraction and characterization 100
3.17 Cell counting 100
3.18 Cell proliferation assays 101
3.19 Localization of Integrin-alpha6 positive cells by immunohistochemistry on cryosections 102
3.20 Flow cytometry 103

Chapter 4

Introduction 106
4.1 Dormancy : definitions and generalities 106
4.1.1 Dormancy : evolution and ecology 106
4.2 Physiological and morphological changes of dormancy in invertebrates 108

4.3 Dormancy and development in invertebrates **110**
4.3.1 Dormancy and sexual development 110
4.3.2 Dormancy as a form of NED in colonial invertebrates 111
4.4 Dormancy in ascidians **112**
4.5 Aim of the study **114**
Results **115**
4.6 Identification of field conditions regulating dormancy in *P. zorritensis* **115**
4.7 Effecr of salinity and temperature on dormancy in *P. zorritensis* in laboratory conditions **116**
4.7.1 Production of *Polyandrocarpa zorritensis* spherules induced by temperature 116
4.7.2 Temperature dynamics of spherule production and reactivation 118
4.7.3 Viability of spherules during exposure to long cold periods 120
4.7.4 Range of resistance to environmental cues of adult zooids and spherules in *P. zorritensis*.. 120
4.8 Morphological description of laboratory-produced *P. zorritensis* spherules **122**
4.9 Production of dormant forms in *Clavelina lepadiformis* **123**
4.9.1 Observationd on winter bud production in specimens of *C. lepadiformis* bred in the laboratory 123
4.9.2 Range of resistance to environmental cues of adult zooids and spherules in *C. lepadiformis* 125
4.10 Ultrastructural description of dormant and non-dormant tissues of *Clavelina lepadiformis* . **127**
4.11 Dinamic of gene expression between dormant and non-dormant stages **130**
4.11.1 Sample quality controls 132
4.11.2 Differential expression analysis and production of co-expression clusters 134
4.11.3 Tissue/sample enrichment analysis 138
Discussion **140**
4.12 production of spherules as a strategy for colony survival and dispersion in *P. zorritensis* **140**
4.13 Production of winter buds in *Clavelina lepadiformis* and comparisons with *P. zorritensis* **141**
4.14 Dealing with temperatures: does dormancy represent the seasonality of NED? **142**
4.15 Dormant forms of *P. zorritensis* and *C. lepadiformis* are specialized buds **143**
4.16 Potential insights from the differential gene expression **144**
Material and methods **145**
4.17 Sample collection and specimen hysbandry **145**
4.18 Light and transmission microscopy (TEM) **145**
4.19 Effects of different environmental cues on colonies of *P. zorritensis* and *C. lepadiformis* **145**
4.20 Differential expression analysis **146**

Chapter 5 – General remarks and discussions

5.1 Exerimental models for wvo-devo : tunicate clades and tunicate species **150**

5.2 Can the hemoblasts plasticity lead to NED in colonial ascidians? **151**

5.3 Dormancy, budding and WBR: the "NED triad" **152**

5.4: A star is born: *Polyandrocarpa zorritensis* as key species for investigating NED mechanisms and evolution **150**

Annex 1

The eventful history of non-embryonic development in tunicates **155**

Annex 2

Convergent acquisition of non-embryonic development in Styelid ascidians **157**

Annex 3

Curriculum vitae **159**

References

References **161**

Glossary

Asexual reproduction: developmental process that generates individuals (clones) genetically identical to the parent by mitosis.

Dormancy: state of metabolic depression. In this manuscript, I refer to all the hypometabolic states (diapause: endogenous induced by physiological cues; quiescence: regulated by variation in environmental conditions; cryptobiosis: severe hypometabolic state) with the general term of "dormancy".

Hemocytes: circulating mesenchymal cells of the tunicates. Different morphotypes are described.

Hemoblasts: undifferentiated hemocytes

NED non-embryonic development: developmental process in which an adult body is formed starting from somatic cells/tissues instead of gamets. Asexual reproduction, whole-body regeneration and dormancy can be considered forms of NED.

Propagative budding: budding process that allows to the colony growth and propagation (Nakauchi, 1982). It includes asexual reproduction.

Survival budding: budding process that allows the survival of the colony under adverse environmental conditions (Nakauchi, 1982). It includes WBR and dormancy.

WBR whole-body regeneration: regeneration of the entire body starting from few cells of small fragments.

List of abbreviations

CPSCs: Circulating putative stem cells

FSC: Forward scatter

GA: Granular amoebocytes

HA: Hyaline amoebocytes

Iα6: Integrin-alpha6

MC: Morula cells

MET: Mesenchymal-to-epithelial transition

MLC: Macrophage-like cells

NED: Non-embryonic development

PO: Phenoloxidase

PSCs: Primordial stem cells

SSC: Side scatter

Chapter 1 - Introduction

1.1 Asexual reproduction in metazoans

1.1.1 Some definitions

Sexual reproduction - that involves the production of gametes and implicates genetic recombination and crossing over - is the way of reproduction ubiquitously distributed among metazoans. Yet, it is not the only one and many animal species are also able to reproduce by asexual reproduction (Otto and Lenormand, 2002; Subramoniam, 2018). Asexual reproduction involves only mitosis and the newly generated individuals (clones) are genetically identical to the parent. We can identify two general groups of asexual reproduction: parthenogenesis and agametic reproduction. During parthenogenesis, referred to as amictic reproduction, an egg develops into a new organism without being fertilized (de Meeûs et al., 2007; Normark, 2013; Suomalainen, 1950). This type of asexual reproduction is present in Rotifers (Carmona et al., 2009; Simion et al., 2020) or many species of arthropods such as Hymenoptera (Rabeling and Kronauer, 2013) and crustaceans (*Daphnia spp.*, Decaestecker et al., 2009). On the other hand, agametic asexual reproduction does not require reproductive organs and gametes, because new clonal individuals originate from somatic cells and/or tissues (Sibly and Calow, 1982). From now on, I will use the term "asexual reproduction" to refer to agametic reproduction.

Asexual reproduction is often referred to as self-division or propagation (Morgan, 1901) if the clones are originated from detached pieces of the parental animal body and are freely released in the surrounding environment (as in some annelids, Giese, 2012). More specifically, the phenomenon of propagation is called fragmentation if the new clones generate from pieces cut off by external forces, while it is called fission if it involves endogenous processes (Brockes and Kumar, 2008; Hughes, 1989). If the new clone is formed by sprouting of a small area of the parental body and then increases in size, the asexual process is called budding (Hughes, 1989). The new clones can stay connected with the parent or be detached, and often, their body axis are oriented differently (i.e. about 180 degrees in *Hydra*) (Otto and Campbell, 1977).

Asexual reproduction has been described in a wide range of animals, in particular soft-bodied invertebrates (Subramoniam, 2018) (Table 1.1, Figure 1.1). At first glance, its distribution across the metazoans' trees already suggests many independent gains and losses.

	Phylum	Class	Order	Species
1. Agametic cloning				
1a. Budding	Placozoa	Trichoplacoidea	Trichoplacida	Trichoplax adhaerens
	Cnidaria	Anthozoa	Scleractinia	Acropora palmata
	Ctenophora	Tentaculata	Lobata	Bolinopsis infundibulum
	Xenacoelomorpha	Acoela		Convolutriloba macropyga
				Convolutriloba retrogemma
	Bryozoa	Phylactolaemata		Lophopus crystallinus
1b. Fission	Placozoa	Trichoplacoidea	Trichoplacida	Trichoplax adhaerens
1b. i. Binary fission (Simple)	Echinodermata	Asteroidea	Forcipulatida	Allostichaster polyplex
	Cnidaria	Anthozoa	Actiniaria	Haliplanella lineata
1b. ii. Transverse fission	Xenacoelomorpha	Acoela		Convolutriloba hastifera
				Convolutriloba longifissura
	Platyhelminthes	Rhabditophora		Girardia tigrina
1b. iii. Longitudinal fission	Annelida	Oligochaeta	Tubificida	Paranais litoralis
	Acoelomorpha	Acoela		Paratomella unichaeta
	Platyhelminthes	Rhabditophora	Tubificida	Planaria fissipara
1c. Fragmentation	Ctenophora	Tentaculata	Lobata	Mnemiopsis leidyi
	Cnidaria	Anthozoa	Actiniaria	Metridium senile
	Nemertea	Anopla	Heteronemertea	Lineus longissimus
	Xenacoelomorpha	Acoela		Convolutriloba retrogemma
	Hemichordata	Enteropneusta	Enteropneusta	Balanoglossus capensi
2. Parthenogenesis	Platyhelminthes	Trematoda	Echinostomia	Philophthalmus megalurus
	Rotifera	Monogononta	Ploimida	Brachionus calyciflorus
	Arthropoda	Crustacea	Cladocera	Daphnia pulex
	Arthropoda	Insecta	Hemiptera	Myzus persicae
			Hemiptera (Aphididea)	Rhopalosiphum padi

Table 1.1: Taxonomic distribution of different types of asexual reproduction within invertebrates (from Subramoniam 2018).

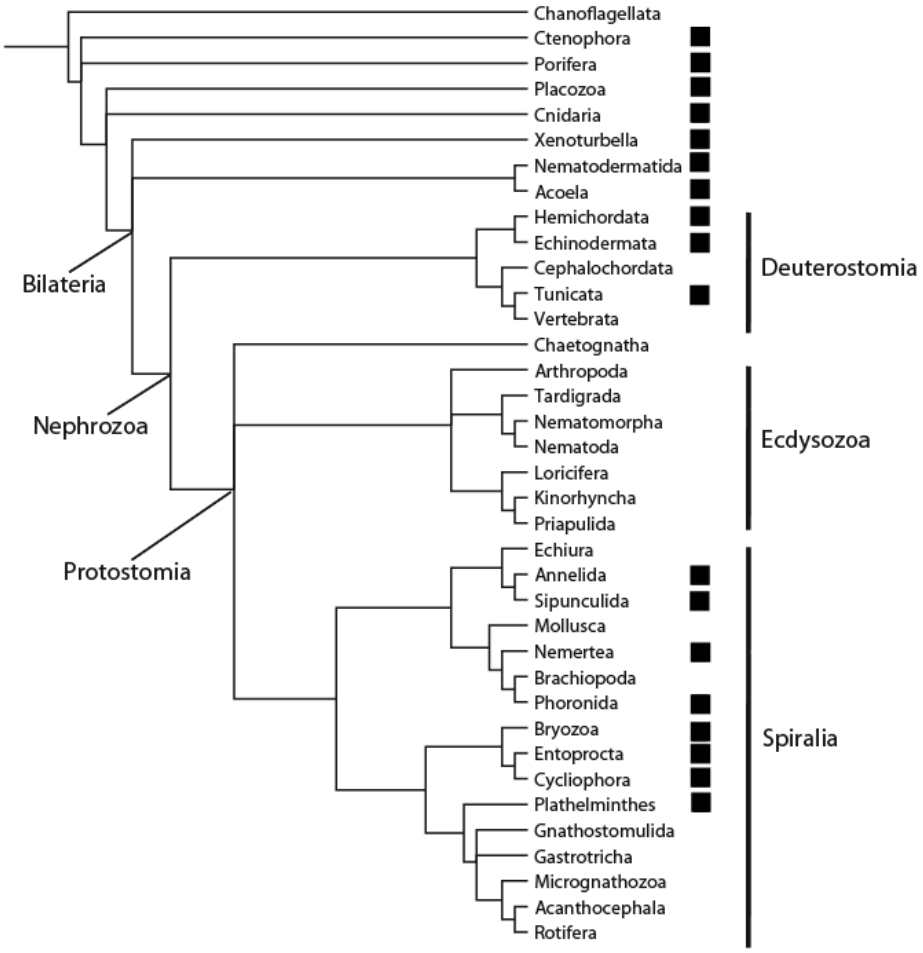


Figure 1.1: Distribution of asexual reproduction among the major metazoan phyla. Black squares indicate the taxa in which asexual reproduction has been reported. (Modified from Nilsson Sköld and Obst, 2011).

1.1.2 Asexual reproduction and its relationship with sexual reproduction

Unlike sexual reproduction, recombination and crossing over do not occur during asexual reproduction, excluding the generation of any genetic variation and the probability to eliminate deleterious mutations (Barton and Charlesworth, 1998; Crow, 1992). Nevertheless, species able to propagate asexually are often also able to reproduce sexually. In this way, they can maintain the advantages of both reproductive strategies (Bell, 1982; Williams, 1975, reviewed in Trivers, 1983).

In the beginning of the 20th century, some authors considered asexual reproduction as an ancestral trait, for example in ascidians (van Name et al., 1921; Selys-Longchamps, 1917): because asexual reproduction is a very "complex character", it was considered less likely that different groups could have acquired this character independently. The absence of asexual reproduction in some organisms (as in many vertebrates) was thought to be the result of an increase in morphological complexity, where the capacity to generate *de novo* asexual clones would have been less likely to evolve. More recently, comparative studies consider sexual reproduction ancestral, while asexual propagation would have evolved independently multiple times in different groups, such as in annelids (Zattara and Bely, 2016) or tunicates (Alié et al., 2020).

1.1.3 Asexual reproduction and coloniality

In some species, individuals that develop by asexual reproduction detach from the parent and disperse. This strategy permits colonizing microhabitats, as occurs in many species of corals (as *Acropora spp.*) and sponges (as *Amphimedon spp.*) living in reefs (Smith and Hughes, 1999; Wulff, 1991). In other species, the clones remain attached to the parent, maintaining physical connection for an exchange of metabolites and other chemical signals, therefore forming colonies (Hughes, 1989; Mackie et al., 1986). When the physical or physiological contact is not maintained despite the spatial proximity, we speak of clonal aggregates (Hiebert et al. 2020). The modular units of a colony or a clonal aggregate are called zooids (e.g., in tunicates), ramets (i.e. and bryozoans), or polyps (e.g., in cnidarians). In a colony, all the zooids participate in the colony homeostasis. In some species, such as ascidians, the zooids are also phenotypically identical, while in other marine invertebrates, we observe polymorphisms, namely the division of labor among morphologically different modules (Harvell, 1991, 1994). Polymorphisms have been frequently reported in some cnidarians (e.g. the siphonophores *Physalia physalis*, Munro et al., 2019), in bryozoans (Schack et al., 2019) or pelagic tunicates such as doliolids (Braconnot, 1970).

1.2 Regeneration in metazoans

1.2.1 Definition of regeneration

Regeneration can be defined as the ability to restore lost or injured parts of the body, starting from pre-existing cells or tissues (Carlson, 2007). A regenerative process can be classified in different modes based on the nature of the stimuli that trigger it, on the structure that can be regenerated or on the mechanisms employed in response to damage (Carlson, 2007). Physiological regeneration includes the natural cell turnover responsible for the "*natural replacement of extruded or worn-out body parts*" (Carlson, 2007) - as in the case of epithelial regeneration (intestinal epithelium: Barker, 2014), the erythropoiesis (Alberts et al., 2002a) and the seasonal loss and regrowth of structures as feathers in birds (Voitkevich, 1966) or antlers in cervids (Goss, 2012) - or the reparative or post-traumatic regeneration, where the tissues are rebuilt after been removed by self-induced amputation or external injury (Kostyuchenko et al., 2016; Morgan, 1901; Poss, 2010). Regeneration can occur at different levels of biological organization (Bely and Nyberg, 2010; Slack, 2017) (Figure 1.2): cellular level (e.g. nerve axons, Huebner and Strittmatter, 2009), tissue level (e.g. epidermis, Odland and Ross, 1968), organ level (e.g. heart, Laflamme and Murry, 2011) or structural level (e.g. limb, Bryant et al., 2004). In some cases, the "entire" body can be rebuilt starting from few cells or tiny fragments, and some authors use the term whole-body regeneration (WBR) (i.e. planarians Gehrke and Srivastava, 2016).

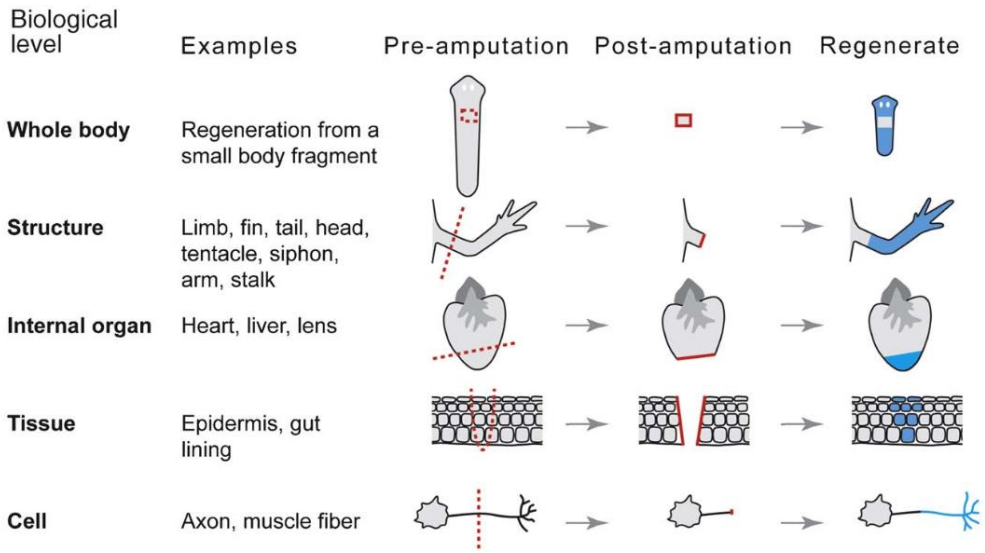


Figure 1.2: Regeneration at different levels of biological organization. It remains unclear which aspects of regeneration are homologous across successive levels. Colony-level 'regeneration', as seen in colonial animals such as corals and ascidians, occurs through asexual reproduction rather than through regeneration of individuals and thus is not included here. Dashed red lines indicate amputation planes; solid red lines indicate wound surfaces; blue fill indicates regenerated body parts (From Bely and Nyberg, 2010).

Thomas H. Morgan (1901) divided the regenerative mechanisms into two big categories, based on the cellular events occurring after an injury, and he coined the terms epimorphosis and morphallaxis. During the epimorphic regeneration, a structure called blastema is formed at the wound surface. The blastema is made of undifferentiated or dedifferentiated cells that migrate to the wound site and proliferate. A typical example of epimorphosis is the limb regeneration in amphibians (Suzuki et al., 2006). In contrast, during morphallaxis, a blastema is not formed and pre-existing cells participate in regeneration by remodeling; an example occurs in Hydra head regeneration (Bosch, 2007) (Figure 1.3).

However, these two mechanisms can overlap, and their distinction is not so strict (Pellettieri, 2019; Tiozzo and Copley, 2015 Agata et al 2007). For example, in the annelid *Sabella spp.*, when abdominal fragments are isolated, they can regenerate both anterior and posterior ends (Carlson 2007, Berrill, 1931). The head regeneration occurs through epimorphosis with blastema formation; however, at the same time, the abdominal fragments close to the future head are completely remodeled by the reorganization of the pre-existing tissues through morphallaxis, to form thoracic segments (Hill, 1970). *Hydra* has been often used as an example for morphallactic regeneration(Bosch, 2007), in which the parental epidermal cells are recruited and reorganized for the formation of the new tissues without blastema formation (Cummings and Bode, 1984). However, although a blastema is not formed, cell proliferation occurs during all the processes in the remodeling tissues, and multipotent interstitial cells migrate toward the regenerating area and participate in morphogenesis (Buzgariu et al., 2018) (for details, paragraph 1.3.2).

The regenerative process can be dissected in multiple chronological steps: wound healing, mobilization of cell precursors, and morphogenesis (Tiozzo and Copley 2015). Wound healing represents a process of re-epithelialization, which leads to the regeneration of the epithelium damaged after injury (Carlson 2007). It permits to restore the tissue homeostasis preventing infection and water loss and it is usually a conserved process, present also in species without extensive regenerative abilities, such as in mammals (Borena et al., 2015). Wound healing seems to be necessary for the initiation of the regenerative cascade in some species: for example, during limb or tail regeneration in amphibians wound healing is responsible for the correct organization of blastema (Murawala et al., 2012). The features of re-epithelialization are similar in species that can regenerate and in species that do not regenerate; however, the final result (a functional tissue in the former, a scar in the latter) is not the same (Bielefeld et al., 2013). Wound healing and scar formation have been proposed to influence the regenerative ability of animals, but how it occurs it is still not clear (Carlson 2007).

The cellular source of regeneration activated for restoring the lost structure can vary in different organisms. Carlson (2007) reported three main cellular origins: "*dedifferentiation of mature cells*

in the remaining tissue", "*proliferation of remaining cells without dedifferentiation*", and "*proliferation of stem cells*" (Jopling et al., 2011). These progenitor cells can be already present in the damaged tissues or migrate from other sites. Yet, these mechanisms can co-exist, like during heart regeneration in zebrafish (Jopling et al., 2010).

The morphogenetic event leads to the rebuilding of the lost part. As result of this process, a new structure - functional identical or similar to the one present before - is reconstructed. At this step developmental signals like *Wnt*, *BMP*, *Nodal* can be re-deployed during regeneration in different organisms, such as cnidarians, planarians and mammals (Adell et al., 2010; Clevers et al., 2014; Holstein et al., 2003) (see paragraph 1.2.3).

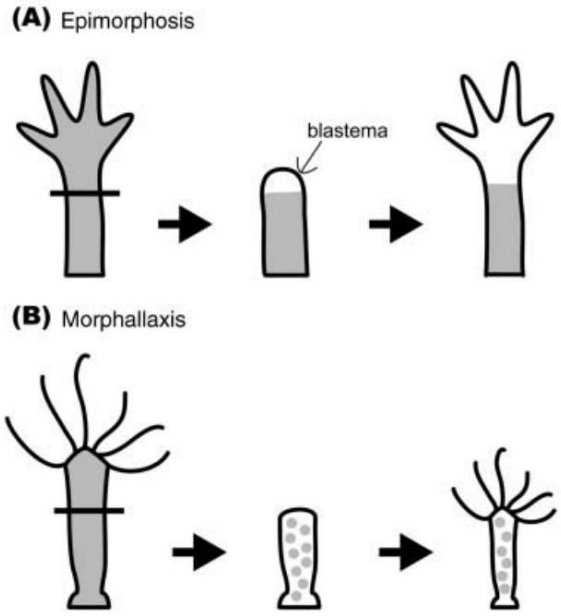


Figure 1.3: Two examples of epimorphic and morphallactic regeneration. (A) Limb regeneration in amphibians is a representative example of epimorphosis. In this type of regeneration, a mass of undifferentiated cells referred to as the 'blastema' is initially formed after wound healing and then blastema cells actively proliferate to restore the lost part of the amputated organ; (B) Hydra regeneration is categorized as morphallaxis. A blastema is not formed. Direct rearrangement of pre-existing cells in the stump contributes to regeneration (from Agata et al 2007).

1.2.2 Evolution of regeneration and its distribution.

Regeneration is a widespread process across metazoans. However, it is not uniformly distributed. Species that can regenerate extensive body parts and species with limited regenerative capabilities can coexist even in the same clade, (Figure 1.4). In addition, the regenerative potential can also vary depending on intrinsic (e.g. age or size) and environmental (e.g. temperature or food availability) cues (Henry and Hart, 2005; Morgan, 1901). The identification of "regeneration" as a trait, and the debate about the evolution of regeneration can

be dated back to the 1890s, between two important evolutionary biologists: August Weismann and Thomas Hunt Morgan (reviewed in Esposito, 2013). Weismann considered regeneration as an adaptive character, secondary evolved in response to injury (Weismann 1893). Morgan strongly opposed Weissman's idea, considering regeneration as an epiphenomenon of development and not necessarily connected to injury (Morgan, 1898). Since then, many scientists got fascinated by this topic (Alvarado, 2000; Barr, 1964; Bely and Nyberg, 2010; Birnbaum and Alvarado, 2008; Brockes and Kumar, 2008; Brockes et al., 2001; Giangrande and Licciano, 2014; Goss, 1969, 1992; Lai and Aboobaker, 2018; Reichman, 1984; Tiozzo and Copley, 2015). According to Korschelt, that defined regeneration as "Ursprungs Erecheinung", a primordial trait of life (Korchelt, 1928), regeneration is a an ancestral character in metazoans, lost or modified multiple times (reviewed in Goss, 1992).

The ancestrality of regeneration has been critically evaluated, considering that some aspects may arise independently in different lineages (Goss, 1969, 1992; Tiozzo and Copley, 2015). Indeed, regeneration is a complex trait, characterized by multiple cellular and molecular mechanisms, often preceded by wound healing; it begins with the mobilization of cellular precursors and continues with morphogenetic events. Despite this, it is often still considered as a single trait and plotted into phylogenetic trees. Nevertheless, plotting in the tree the diverse mechanisms of regeneration - like the cellular origin of the regenerating part or the molecular mechanisms driving the development - represents a more complex task. Furthermore, the efforts to fit "regeneration" into a phylogenetic tree lead to more general challenges linked to evolutionary biology, for instance, the definition of homology. Experimental approaches that compare the "regenerating capability" in closely related species – i.e. belonging to the same family and sharing the same bauplan– may help to understand the evolution of cellular and molecular events during regeneration. The evolution of "regeneration" as a character and the dissection and comparison of its mechanisms is still a proliferating field of investigation, which need further investigation in many species and metazoans clades (like for example in mollusk, Imperadore et al., 2018)

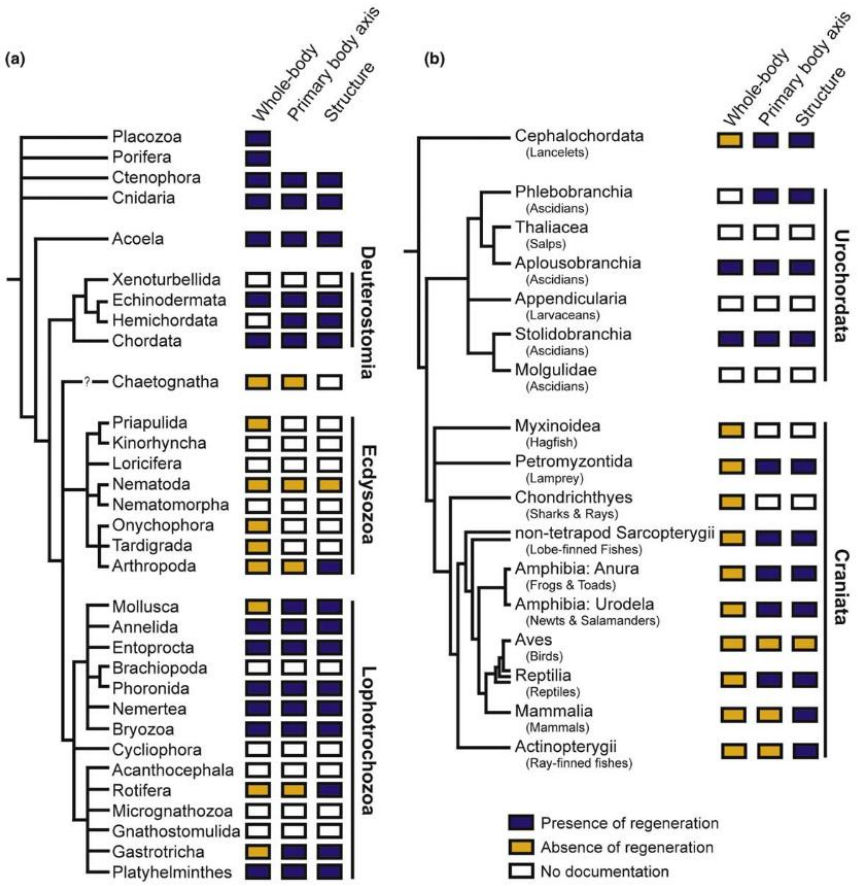


Figure 1.4: Phylogenetic distribution of regeneration across (a) the Metazoa and (b) the Chordata. 'Presence of regeneration' indicates that at least one well-substantiated report exists for regeneration in that taxon and does not imply that all species in that taxon can regenerate. 'Absence of regeneration' indicates that there is at least one well-substantiated report for the lack of regeneration in that taxon (and none indicating the presence of regeneration). We define 'whole-body regeneration' as the potential to regenerate every part of the body (although not necessarily simultaneously or from a tiny fragment). The ability to regenerate the primary body axis is scored independently for each taxon and does not assume homology of body axes across or within phyla (from Bely and Nyberg, 2010)

1.2.3 Regeneration and embryonic development

More than one century ago, Morgan introduced the idea that regeneration is a characteristic of the growth process and suggested to be an epiphenomenon of development (Morgan 1901). The major evidence supporting the connection between development and regeneration is the fact that, when the equilibrium of the organism is altered by injury, the developmental patterns responsible for embryonic development are recruited for regeneration (Alvarado and Tsonis, 2006; Barr, 1964). An example of an embryonic molecular signal re-deployed during regeneration is given by *Wnt* signaling. During embryonic development, the canonical *Wnt/β catenin* pathway is involved in primary axis polarity, controlling particularly the posterior specification in different bilaterians (Petersen and Reddien, 2009). In various species this signaling is redeployed during regeneration, as in the case of head regeneration in *Hydra* (Lengfeld et al., 2009), head and tail regeneration in *Schmidtea mediterranea* (Gurley et al., 2010), fin regeneration in *Danio rerio* (Wehner and Weidinger, 2015) and tadpole tail regeneration in *Xenopus* (Lin and Slack, 2008). However, it is important to remember that regeneration requires various mechanisms that are typical of this process and are not involved in the development, as a response to injury or cell mobilization (Brockes and Kumar, 2008; Vervoort, 2011).

1.3 Relationship between asexual reproduction and regeneration: the Non-Embryonic Development (NED)

Asexual reproduction and regeneration are often considered as strictly connected. Generally, many species that are able to reproduce asexually show also high regenerative potential (Sanchez Alvarado 2000, Martinez et al 2005). Many types of propagation such as fission, strobilation, or fragmentation, can be seen as whole-body regeneration (WBR). An example is the case of segmentation in annelids: this process is considered a form of asexual reproduction because new clones are produced, but when the isolate fragment regenerate its entire body we can refer to this process as WBR (Berrill, 1952). One difference proposed to distinguish WBR from asexual reproduction is that WBR can be triggered by external stimuli or injury (Sanchez Alvarado 2000). From this perspective, the causality is the discriminant event: if the fragmentation is part of the life cycle we would consider it as a propagation/reproduction, on the other hand, if the fragment is generated by an exogenous event (i.e. the knife of a biologist), then we could talk of WBR. Nevertheless, the relationship between asexual reproduction and WBR has been poorly studied, both from a mechanistic and from a philosophical point of view. Both processes lead to the formation of functional clones and some molecular mechanisms may be shared (Martinez et al., 2005). Whole-body regeneration and asexual reproduction can be

both considered as processes of post-embryonic development, in which the entire body is newly formed agametically (Kostyuchenko et al., 2016) and, to emphasize both the mechanistic aspects (the ontogenesis) and the lack of a zygotic origin, the term Non-Embryonic Development (NED) has been proposed to unify these two developmental processes (Alié et al., 2018).

1.3.1 Cellular and molecular mechanisms of NED

One key question on regeneration and asexual reproduction concerns which cells and tissues are at the origin of the regenerating body, and which molecular signals drive their development. In invertebrates, this problem has been explored in detail only in a limited number of model organisms and restricted phylogenetic groups, especially at the molecular level (Lai and Aboobaker, 2018). As cellular sources of NED, two main mechanisms have been described: dedifferentiation or transdifferentiation of somatic cells and proliferation of resident progenitors (Poss, 2010). During dedifferentiation, somatic differentiated cells can revert to a non-differentiated pluripotent or multipotent status; these cells differentiate again in the developing area, forming the same cell type than before or a different one (transdifferentiation). The mechanisms underlying dedifferentiation and transdifferentiation have been mostly studied for regeneration in mammals (Jopling et al., 2011). In invertebrates, examples of dedifferentiation and transdifferentiation occur, for instance, during regeneration in sponges (Ereskovsky et al., 2020), gut regeneration of holothurians (Mashanov et al., 2005, 2015) and budding in some colonial ascidians (Fujiwara and Kawamura, 1992a; Kawamura and Fujiwara, 1995).

Resident progenitor cells are undifferentiated cells, which are able to proliferate, and self-renew (Carlson, 2007). Progenitor cells can be already present at the regenerating site or can be far from it but able to migrate to reach it. These cells have been described in different groups of invertebrates, such as in sponges (called archaeocytes, Müller, 2006), cnidarians (interstitial cells or i-cells, Frank et al., 2009), planarians (neoblasts, Baguñà, 2012) or tunicates (hemoblasts, Kassmer et al., 2019; Kawamura and Sunanaga, 2010; Laird et al., 2005). Some authors hypothesized the existence of pluripotent somatic stem cells, called primordial stem cells (PriSCs) (Solana, 2013), conserved in different groups of metazoans (Alié et al., 2015; Fierro-Constaín et al., 2017; Juliano et al., 2010) and able to express the germline multipotency programm GMP (that includes *Piwi*, *vasa*, *Nanos*, *Tudor*) (Juliano et al 2010) (Figure 1.5). According to this hypothesis, the GMP is expressed not only in the germline but also in the cells and tissue that represent the precursor of regeneration and asexual reproduction. The neoblasts in planarians are a good example of PriSCs (Rink, 2013).

Figure 1.5: Summary of available data on each gene expression/function in different cell types and/or cell lineages across metazoans. White cases indicate “not found/not expressed”, questions marks indicate “not yet studied”. Asterisks indicate that several paralogs are concerned. Data gathered from Alie´ et al. (2011) and Gazave, Behague, et al. (2013) and Fierro-Constaın et al. (2016). (Modified from Fierro-Constaın et al. 2016).

1.3.2 Diversity of NED modes within metazoans - some examples

A large number of modes of NED have been observed in metazoans. However, only in a few species these processes have been deeply investigated, while for a wide range of animals the morphological description of postembryonic development (when present) lacks deeper cellular or molecular characterization. In this section, I will give a few examples of the processes of asexual propagation and whole-body regeneration in three metazoans taxa: cnidarians, annelids and platyhelminthes. These groups encompass some well-established laboratory models able to undergo NED, and for some of them, the cellular source of NED and the molecular mechanisms are reported.

- ***Cnidaria***

NED is widespread within cnidarians. It has been reported to occur in a high variety of strategies, as transverse and longitudinal fission, tentacle autotomy, pedal laceration, or stolon formation (Bocharova, 2016; Fautin, 2002). However, only in a few species the cellular and molecular events have been fully identified. Two cnidarian model organisms developed for studying NED, *Hydra* and *Nematostella*, show differences in the cellular mechanisms of NED.

In *Hydra*, the molecular and morphological events as the environmental conditions that trigger the NED are well-described (Otto and Campbell, 1977; Shostak et al., 1968). The cellular events occurring during NED are very well described during bidirectional regeneration, where the epithelia are responsible for wound healing and the regeneration of a new head and/or foot (Wittlieb et al., 2006). Two populations of unipotent adult cells are mainly responsible for NED: the gastrovascular endodermal and muscular ectodermal epithelial cells (Bode, 1996; Hobmayer et al., 2012). When the animal is regularly fed, both epithelial cell lineages continuously divide by mitosis (David and Campbell, 1972; Holstein et al., 1991): the new cells are displaced at the extremities of the body column, differentiating in the cells of the tentacles in the oral region and in the cells of the foot in the aboral area (Bode, 2003; Holstein et al., 1991). The mitotic division of epithelial cells is also responsible for bud formation. Buds arise as ectodermal protrusions of the body wall that contain part of the gastrovascular endoderm: the distal portion of the bud forms the oral region with tentacles, while the aboral region can remain attached to the parental polyp, or detach at the end of morphogenesis after 1-2 days of budding (Otto and Campbell 1977, Holstein et al 1991). *Hydra* undergoes an extreme mode of WBR: isolated pieces of the polyp can regenerate the body and appendages (Bode and Bode, 1984; Shimizu et al., 1993), and mechanically isolated cells of *Hydra* can spontaneously reaggregate and reform a new polyp (Gierer et al., 1972; Graf and Gierer, 1980). A cluster of 5-15 isolated epithelial cells is sufficient

for the formation of a new head, and developmental patterns (as *Wnt* and *Brachyury*) are re-deployed for the axis polarity (Technau et al., 2000). However, isolated head, tentacles, and pedal disc do not regenerate, probably because of the lack of actively dividing epithelial cells (Bode, 2003). *Hydra* shows also a population of stem cells, the i-cells (Bode, 1996; David and Plotnick, 1980), located in the interstitial space between epithelial cells. These cells are multipotent undifferentiated cells, participating to the morphogenetic events as progenitors of different cells types (as nematocytes, nerve cells, and germ cells) (Hobmayer et al., 2012; Watanabe et al., 2009); they also express some genes of the GMP (as *vasa-like* genes, Mochizuki et al., 2001). These cells proliferate and migrate towards the regenerating site where they differentiate (Hager and David, 1997). When i-cells are depleted with colchicine (Campbell, 1976; Marcum and Campbell, 1978), irradiation (Brien and Reniers-Decoen 1955), nitrogen mustard (Diehl and Burnett, 1964), or hydroxyurea (Cummings and Bode, 1984), (reviewed in Frank et al 2009) the resulting "epithelial polyps" are able to regenerate, but they are unable to catch prey and feed (Sacks and Davis, 1979). This phenotype is due to the fact that the cell lineages derived from i-cell cannot be produced anymore (Bosch and David, 1987).
These results show that epithelial reorganization, epithelial proliferation, i-cells proliferation, and i-cell migration are necessary for NED in *Hydra,* and that the line distinguishing morphallaxis and epimorphosis is not well defined.

Another well establish cnidarian model is *Nematostella vectensis,* in which two types of fission were described (Darling et al., 2005; Reitzel et al., 2007): physal pinching and polarity reversal. During physal pinching the body column starts to constrict at the aboral region until the detachment of a small fragment, the physal end (Darling et al., 2005). After separation, the physal regenerates the tentacles and all the others missing structures. Polarity reversal was initially reported as abnormal development by different authors (Frank and Bleakney, 1978; Hand and Uhlinger, 1995), but is considered as second type of transversal fission by Reitzer and colleagues (2007). First, the aboral pole undergoes to drastic modification, with the formation of new tentacles, a new mouth and pharynx. Then, the mesenteries expand in the new formed pharynx and the second oral region becomes able to feed. When the two oral potions can feed independently, a new physa is formed in the middle of the body column, and the transverse fission separates the two new adults.
N. vectensis is also able of bi-directional regeneration (Bossert et al., 2013; DuBuc et al., 2014), a process that requires both tissue remodeling (particularly of the mesenteries) and proliferation of ectodermal and endodermal epithelium and mesenteries (Amiel et al., 2015; Passamaneck and Martindale, 2012). The mesenteries seem to trigger the cell proliferation and the regeneration of the body wall epithelia (Amiel et al., 2019), presenting quiescent stem cells that

start to proliferate and migrate after amputation. Notably, differently from *Hydra,* the presence and participation of pluripotent interstitial stem cells have not been described yet. The expression of developmental genes during asexual reproduction and regeneration have been investigated (Burton and Finnerty, 2009; Schaffer et al., 2016; Warner et al., 2018, 2019), identifying genes differentially expressed between oral and aboral regeneration (Schaffer et al., 2016) or genes partially co-opted from embryonic development during WB (Warner et al., 2019).

- ***Annelids***

NED has been reported in many annelid species (Özpolat and Bely, 2016).

Annelids reproduce asexually by fission in 2 ways, by architomy (when the worm separates in different fragments that subsequently regenerate completely the head and the tail, as *Lumbriculus variegatus*) and by paratomy (a new tail and a new head are generated before the two are separated), as in *Pristina leidyi* (Kostyuchenko et al., 2016); in the different groups, variations of these two modalities have been called with different names. Environmental factors, such as high temperature or food availability, induce rapid growth and a more intense fission activity (van Cleave, 1937). The correlation between asexual reproduction and regeneration has been studied in few species, mostly describing the morphological events, while the molecular mechanisms remain less investigated

Architomy is common in oligochaete worms. A well-studied example is *Lumbriculus variegatus*: the worm divides in two and each fragment regenerate the missing parts (Berrill, 1952). After mechanical fragmentation, small isolated segments can also regenerate the anterior and/or posterior part forming a new worm by WBR. Regeneration following both archiromy and mechanical fragmentation combines epimorphic and morphallactic mechanisms: cells proliferate and migrate to the fission site forming a blastemal (Tweeten and Anderson, 2008), but some body parts as the nervous system originate by the reorganization of pre-existing tissues (Martinez et al., 2005, 2006). The formation of blastemal by migration of progenitor cells has been reported also during regeneration in other species, such as for posterior regeneration in *Platynereis dumerilii* (Planques et al., 2019). In *Lumbriculus* and other oligochaetes, undifferentiates cells called neoblasts have been identified (Bilello and Potswald, 1974; Kostyuchenko et al., 2016; Randolph, 1892). Compared to neoblasts in planarians (see next section), neoblasts in annelids lack stemness characteristics of stem cells, such as expression of GMP genes (Myohara, 2004; Tadokoro et al., 2006). Neoblasts are not commonly distributed within species and their role during NED is far to be completely elucidated. However, studies

that compared the occurrence of neoblasts in closely related species suggested that these cells may be necessary for asexual reproduction (Myohara, 2012).

- ***Platyhelminthes***

Asexual reproduction by transverse fission is well described in different species of free-living flatworms (Egger et al., 2007; Reuter and Kreshchenko, 2004). Like in annelids, wo principal types of fission are shown, by architomy and paratomy.

When fission occurs by paratomy, the differentiation of new structures starts before separation. Catenulid species, such as *Stenostomum leucops* or *Catenula spp.*, and macrostomorphans, such as *Macrostomum lineare*, show this type of fission: one or more new heads are formed along the trunk before the physical detachment, forming a chain of individuals at different grades of development (Moraczewski, 1977; Palmberg and Reuter, 1983; Sonneborn, 1930)

In the case of architomy, the animal undergoes fission first, and then the missing parts are reformed: the posterior end of the animal adheres to the substratum while the rest of the body becomes longer and thinner, and pulls away. In WBR, the two fragments regenerate the missing parts, forming two new animals. This happens in the triclad group (e.g. *Dugesia*). The process of regeneration that follows fission is the result of a mix between morphallaxis and epimorphosis, with the participation of stem cells (Reuter and Kreshchenko, 2004).

Platyhelminthes are well known for their extensive regenerative abilities. Freshwater planarians can undergo WBR, regenerating the entire body from very small pieces (Morgan, 1898; Randolph, 1892; Sánchez Alvarado, 2006) and some species, as *Schmidtea mediterranea* or *Macrostomum lignano* are well-established models to study regeneration (Cebrià et al., 2018; Egger et al., 2007; Oviedo et al., 2008).

Planarians can regenerate and reproduce asexually thanks to the presence of adult somatic stem cells, the neoblasts (Gehrke and Srivastava, 2016). These cells are the only mitotically active cells in the adult body (Newmark and Sánchez Alvarado, 2000) and in not injured animals they are responsible of the physiological somatic cell turnover. Differently from annelid neoblasts, the participation of planarian neoblasts to NED has been well investigated. In amputated animals the neoblasts proliferate and occur beneath the wounding epithelium, forming the blastema (Wenemoser and Reddien, 2010). To test the neoblasts' role during WBR, experiments of irradiation and re-injection have been done (Baguna et al., 1989; Wolff and Lender, 1962): when neoblasts purified from a non-irradiated donor are transplanted in non-regenerating irradiated

animals, cell renewal and regeneration are restored, while irradiated animals do not regenerate or survive if injected with other cell types (Baguna et al 1989).

In *Schmidtea mediterranea* a single non-irradiated neoblast transplanted in a lethally irradiated animal can restore the regenerative ability (Wagner et al., 2011). These experiments suggest that neoblasts are pluripotent cells. Other evidence for the pluripotency of neoblasts is the presence of chromatoid bodies and the expression of GMP genes (Krishna et al., 2019; Rink, 2013; Shibata et al., 1999). However, neoblasts show a molecular and functional heterogeneity: for example, in single-cell transplantation assays, only one type of neoblasts named clonogenic neoblasts (cNeoblasts) was able to completely rescue the irradiated animals (Wagner et al., 2011). The single-cell gene expression and single-cell RNAseq analysis supported this heterogeneity, as well as identifying different populations of neoblasts and new potential markers of neoblast pluripotency, such as the cell-surface protein Tetraspanin-1 (Fincher et al., 2018; Plass et al., 2018; van Wolfswinkel et al., 2014; Zeng et al., 2018).

1.3.3 Approach to study evolution and mechanisms of NED

While the common result of NED is the (re)generation of a completely functional body, the previous examples clearly show that invertebrates possess a high diversity of NED modes. The heterogeneity of NED mechanisms can significantly vary not only across species belonging of distant clades but even among species that belong to hierarchically lower taxonomical groups, e.g. in the same phylum, order, family or even between species of belonging of the same genus (as in the case of some annelids, Özpolat and Bely, 2016). Indeed, this diversity suggests multiple acquisitions and high plasticity of NED. Yet, the cellular and molecular bases of NED are well investigated only in a handful of model organisms, often distantly related. In addition, the comparison between these organisms with different morphologies and evolutionary histories makes difficult to reconstruct the gains or losses of NED. Therefore, to better understand the cellular and molecular mechanisms underlying NED and at the same time to attempt to track their evolution, one strategy is to first compare NED in more restricted taxonomical groups e.g. order or family levels. This approach permits first to focus on a restricted number of species that share common morphological features and a relatively common evolutionary history. A second step is to include in the comparison species with different NED strategies and also include species that lack the capacity of undergoing NED. This comparative approach has been already successfully used in planarians (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013) and annelids (Bely 2010). For example, in some species of planarians, the limited regenerative capabilities have been ”reversed” by knocking down the components of the Wnt/βcatenin pathway (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013). Therefore,

interfering with a single signaling pathway can reverse the evolutionary loss of regeneration. These studies in closely related regenerating and non-regenerating species represent a milestone for understanding molecular mechanisms of NED. A key animal taxon to apply this approach and therefore to study mechanisms and evolution of NED are Tunicates.

1.4 Tunicates: suitable models to study NED

Tunicates represent the closest relatives to vertebrates in which different modes of NED have been acquired and lost multiple times during evolution (Aliè et al 2020). This subphylum encompasses solitary species, which do not undergo NED, and colonial species, which show a variety of NEDs (Figure 1.6). Solitary and colonial species can be closely related and in the same order or even family, colonial species can show different NED types (Alié et al., 2020). Analyzing the phylogenetic distribution of colonial species and compared the cellular and molecular mechanisms of different NED modes in closely related species, it may be possible to understand how NED evolved within this group.

1.4.1 Tunicates: some history

The first reported description of a tunicate was made in 350 BC by Aristotle, who named them "Thalia" and related them to the actual mollusks because of their soft body. For a long time, tunicates were classified as lamellibranch mollusks or as holothurian echinoderms. The name tunicates have been coined by Lamarck in 1816 from the external tunic (or test) that covers their entire external surface of the larval and post-metamorphic body, which represent a synapomorphy of this taxon. It has been only in the second half of the 19th century that tunicates have been included in the phylum Chordata thanks to the observations on the tadpole larva made by the Russian scientist Alexander Kovalensky (1866). In fact, during the larval stage, tunicates possess chordate characteristics, such as a dorsal hollow nerve cord, a notochord ventral to the dorsal tube, a post-anal tail in the larva and pharyngeal gill slits -the stigmata- in the adult. Then, tunicates have also been commonly called Urochordata, a word composed by the Greek word οὐρά ("tail") and the Latin word *chorda* ("cord"). At the beginning of the 21st century, thanks to phylogenetic analysis tunicates have been placed as the sister group of vertebrates, where they still stand (Delsuc et al., 2006).

Because of their soft body, it is extremely rare to find well-preserved tunicate fossils. However, some sites, as the Maotianshan Shale in the south of China (mudstone deposits dated at the Lower Cambrian, around 540 Ma) and the Vendian site on the White Sea (sandstone deposits dated at around 555 Ma), are particularly rich of soft-bodied chordates due to the nature of the fossiliferous layers. Several specimens as *Shankouclava* (Chen et al., 2003), *Ausia* and *Burykhya*

(Fedonkin et al., 2012) were proposed to be tunicate-like fossils, but the affinity with current tunicate is controversial (Aldridge et al., 2007; Lacalli, 2002; Shu et al., 2001). The only hard elements well conserved in fossil records are the small aragonitic spicules (less than 0.1 mm) contained in the tunic of some species (Brookfield, 1988). Because of a lack of fossil records, it is extremely difficult to infer the divergence time of Tunicates. Yet, the divergence time among different tunicate clades have been recently inferred trough phylogenomic analyses (Delsuc et al., 2018).

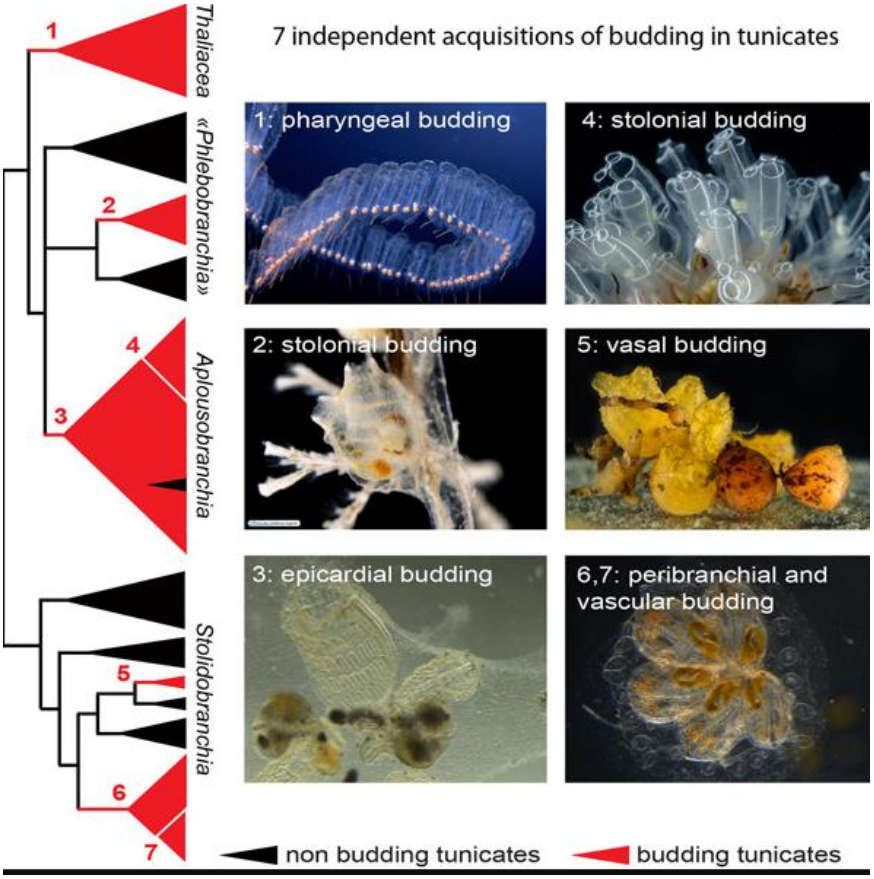


Figure 1.6: A) Phylogenetic distribution of solitary species not able to bud (black) and colonial species capable of NED (red) within tunicates. 1-6) Examples of different ways of NED, placed on the phylogenetic tree thanks to the corresponding number. 1) Chain of *Pegea confoederata* produced by pharyngeal budding. 2) A specimens *Perophora viridis*, able of stolonial budding 3) *Diplosoma listerianum* during the process of epicardial budding 4) Colony of *Clavelina lepadiformis* produced by stolonial budding 5) Colony of *Polyandrocarpa zorritensis* generated by vasal budding 6) Colony of *Botryllus schlosseri*. This species can undergo both peribranchial and vascular budding (Modified from Alie' et al 2020).

1.4.2 Taxonomy and phylogeny

The subphylum Tunicata encompasses more than 3000 strictly marine described species. It has been subdivided into three classes: Appendicularia, Thaliacea, and Ascidiacea. A fourth class, the Sorberacea, has been proposed for the predatory deep-sea species (Monniot and Monniot, 1990; Monniot et al., 1976). However, morphological and phylogenetic evidence group sorbeaceans within ascidians (Tatián et al., 2011).

Recent phylogenies (Braun et al., 2020; Delsuc et al., 2018; Kocot et al., 2018) placed the Appendicularia as a sister group of the other tunicates. Thaliaceans are grouped with the monophyletic clade composed of two ascidian orders, Phlebobranchia and Aplousobranchia. This position can suggest the evolution of Thaliaceans from a sessile ancestor. On the other hand, Phlebobranchia and Aplousobranchia are closer to Thaliacea than to the other ascidian order of Stolidobranchia, questioning the classification of the Ascidiacea as a real clade.

Tunicates show a variety of organisms that differ in size, color, distribution, and lifestyle (Holland, 2016; Shenkar and Swalla, 2011). The members of the classes Ascidiacea are sessile, attached to soft or most frequently hard substrata, while Thaliacea and Appendicularia are pelagic.

Despite the high diversity and the time of divergence between families (for example, the two solitary genera *Ciona* and *Phallusia* diverged about 280 million years ago, Delsuc et al., 2018), in almost all the species the characteristic of the post-metamorphic body plan is conserved. Since Ascidians are worldwide distributed and contain the majority of the described tunicate species, they represent the most studied tunicates, in which many aspects of their life cycle – such as seasonality of reproduction or larval behavior - have been characterized in detail. When not differently specified, from now on the following descriptions will be focused on ascidians.

1.4.3 Life Cycle: solitary vs. colonial

Depending on reproductive strategies, tunicates can be divided into solitary and colonial. Solitary or simple forms originate from a single metamorphosed larva and they are able to reproduce strictly sexually. On the other hand, the colonial or compound forms, in addition to sexual reproduction, are able to originate clones by NED, forming colonies (Figure 1.7).

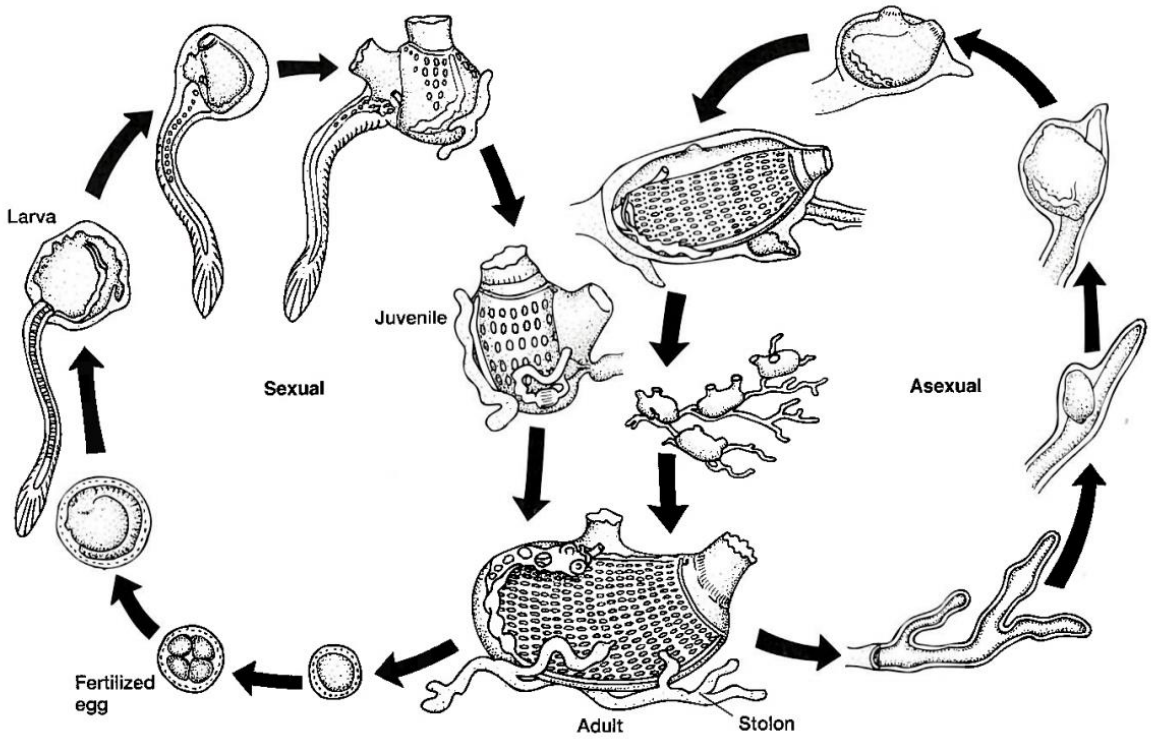


Figure 1.7: Prototypical ascidian life cycle. The life cycle of colonial ascidians includes a sexual (left) and an asexual (right) phase. In the sexual phase, the tunicate larva develops from a fertilized egg. This larva is planktonic and persists for a few hours or a few days at most. It soon settles on a solid substrate and undergoes metamorphosis into a sessile juvenile that grows into adulthood. The asexual phase begins with external buds from the root-like stolon or internal buds from organs within the body, depending on the species. These buds grow and differentiate into adults, often forming a colony of tunicates (from Kardong, 2002).

Both solitary and colonial forms reproduce sexually. Almost all tunicates are hermaphrodites, and they show very diverse reproductive strategies. The majority of solitary species release periodically the gametes and fertilization is external. In colonial species, fertilization is mostly internal and the embryos develop inside the maternal body, in a brooding chamber in the cloacal atrium or in the branchial sac (Mukai et al., 1987). At the beginning of the 20th century, Conklin carried different studies on ascidian embryos (Conklin, 1905, 1905, 1906) describing a highly determinative (or "mosaic") development, where the cell lineages are invariant and determined very early in development. Presumptive cell fate maps of embryos in many ascidians have been described (some examples: Nicol and Meinertzhagen, 1988a, 1988b; Nishida, 2005; Ortolani, 1955), showing that embryonic patterns are conserved in many species across families and orders.

A free-swimming tadpole larva is generally produced at the end of embryonic development, but some variations between groups exist. For example, almost all Thaliacea (salps and pyrosomes) have a direct development but some species (doliolids) have a larva that does not swim actively (Goudeaux et al 1998). In some species of the genus *Molgula*, the larva is tail-less, or anural: the notochord and the tail muscles do not develop, hampering swimming of the larva and inducing rapid settlement on the substrate (Bates and Mallett, 1991; Huber et al., 2000; Swalla and Jeffery, 1990).

After that, the larva undergoes a drastic metamorphosis (Figure 1.8): the tail and the larval nervous system are reabsorbed, the visceral organs and the siphons rotate of about 90° to reach their adult horizontal positions, and the attachment is increased thanks to the expansion of the tunic and the production of attachment organs, the epidermal ampullae (Cloney, 1982; Nakayama-Ishimura et al., 2009). In Appendicularia, the tail is not reabsorbed and it remains active for all the adult life cycle.

In colonial ascidians, the pattern of embryonic development seems conserved and comparable to solitary species (Prünster et al., 2019a, 2019b; Ricci et al., 2016) On the contrary, diverse types of NED are adopted in asexual reproduction: different in terms of tissue and cell of origin, as well as on early ontogenesis. The first zooid generated after metamorphosis of the larva is commonly called oozoid, while the zooids produced by non-embryonic development are called blastozooids. In some species, the formation of blastoozoids starts already at the larval stage, as in members of the ascidian family Holozoidae (Berrill, 1948a; Brewin, 1959) or doliolids (Godeaux 1957). NED modes will be described in section 1.5 and in Appendix 1.

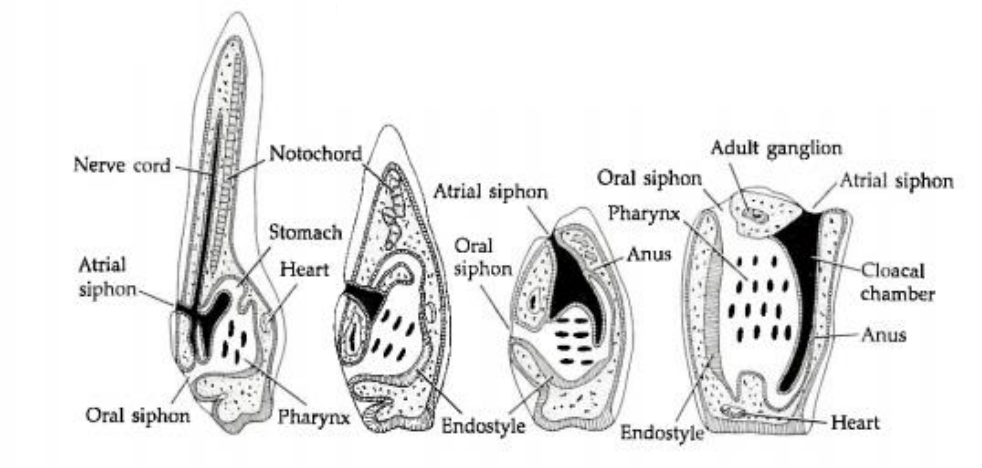


Figure 1.8: Metamorphosis of a settled tadpole larva. Tail resorption is followed by a reorientation of the body to bring the siphons to the adult position (from Brusca and Brusca, 2003).

1.4.4 The ascidian body plan

The post-metamorphic ascidian body is completely different from the larva and it has lost most of the chordate characteristics as the dorsal nerve cord, the notochord, and the post-anal tail. The body can show a variety of colors and shapes, but it can be generally divided into three regions: an anterior thoracic region that contain the pharynx, a central abdominal region with the digestive tract and a posterior post-abdominal region, which contains the heart and the reproductive organs (Figure 1.9).

- *Body wall*

Externally, the epidermis is completely covered by an extracellular matrix called tunic. The tunic is composed of water, proteins, and carbohydrates, but most of the fiber of cellulose-like polymer called tunicin (Hirose, 2009). The tunic provides support to the substrate and protection and can embed cells with unknown function, and in some species (i.e. Diddemnids and some Pyurids) it also contains calcareous spicules (Hirose et al., 2010). Spicules of various species differ in form and shape, and they were often used for taxonomic characterization (Kott 2011). The tunic grows with the zooid and it is produced by epidermal cells and tunic cells (Deck et al., 1966; Hirose et al., 1999). The ability to produce tunicin has been acquired via horizontal gene transfer from bacteria (Nakashima et al., 2004).

- ***Water flow and nutrition***

Almost the totality of the ascidian body is specialized in filter-feeding, except for the carnivore sorberaceans. The water enters through an opening called oral (or branchial or buccal or inhalant) siphon into the branchial basket (pharynx) (figure 1.9). The branchial basket is a sac-

like structure fissured by multiple gill openings, called stigmata; the stigmata are encircled by ciliated cells that are responsible for the movement of water flow in the pharynx (Martinucci et al., 1987). The branchial basket can be folded to increase the surface, as in stolidobranch ascidians (Shenkar and Swalla, 2011). Through the stigmata the water passes from the pharyngeal cavity into the cloacal atrium before being expelled through the atrial (or aboral or exhalant) siphon (Figure 1.9 A) (Petersen, 2007; Petersen and Svane, 2002).

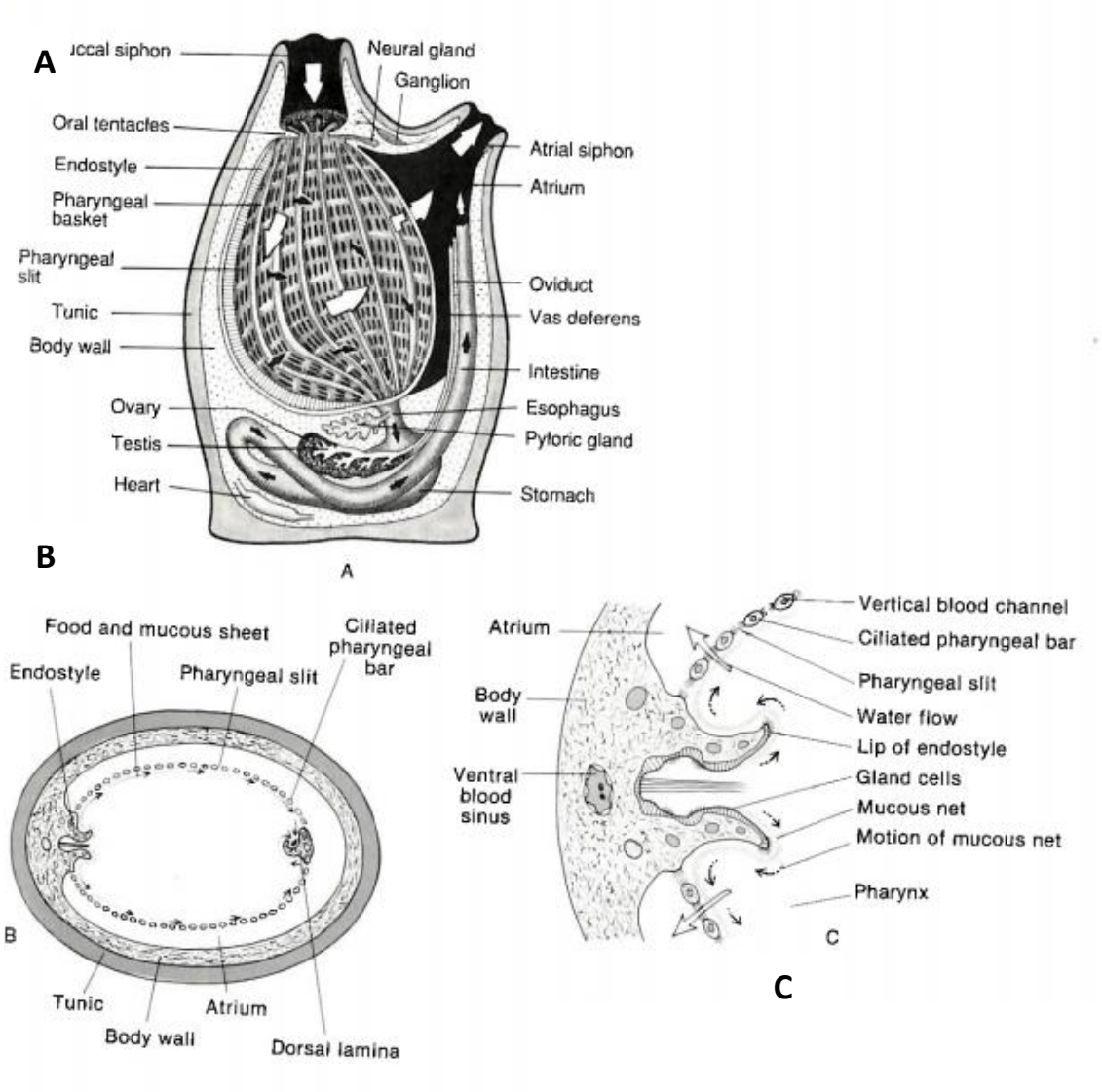


Figure 1.9: A) Diagrammatic lateral view of a solitary ascidian showing internal organs. Large arrows show the path of water flow, small arrows that of the mucus net; B) Cross section of the pharynx in A; C) Enlargement of the endostyle region of B to show the secretion of the mucus net and the water flow through gill slits. (from Ruppert and Barnes, 1994).

Inside the pharynx, the food particles suspended in the water flow are retained by the mucus produced by a ciliated groove, the endostyle (Fujita and Nanba, 1971; Goodbody, 1975); this structure runs along the ventral side of the pharynx until the esophagus and it is considered homologous of the vertebrate thyroid gland (Ogasawara et al., 1999).

The mucus film containing the food is then enrolled in a compact string and transported posteriorly by the dorsal lamina to the short esophagus and then the stomach (Flood and Fiala-Medioni, 1981); the dorsal lamina, located in the dorsal portion of the pharynx, is characterized by cilia and it can be organized as a continuous membrane or in a row of languets. The food is digested and absorbed in the stomach thanks to the presence of different types of granulated cells that compose the gastric epithelium (Burighel and Milanesi, 1973, 1975; Thorndyke, 1977). Also, one or more digestive glands (in particular the pyloric gland, present in all the species) are associated with the stomach and release digestive enzymes in the lumen (Goodbody, 1975). Not digested food particles pass through a U-shaped intestine, and are released through the anus intothe cloacal atrium. Here the remaining particles are released with the outgoing water flow through the atrial siphon.

- ***Circulatory system***

Ascidians present an open circulatory system. The heart is located posteroventrally, near the stomach. It is formed by a V-shaped tube that originated as an evagination of the pericardial wall. It consists of a tubular pericardial epithelium that invaginates to form a second channel, called myocardium. The myocardial wall presents contractile muscular cells that cause heart contraction. The heartbeat is assured by peristaltic waves, thanks to the presence of pacemaker regions at both heart's ends. The pacemakers alternate their activity making possible the peculiar periodic reversal of the blood flow (Goodbody, 1975; Hellbach et al., 2011; Kriebel, 1968). Different factors as salinity, temperature, and drugs have been reported to affect heart-rate reversals (Dijkstra et al., 2008; Hellbach et al., 2011; Pope and Rowley, 2002; Shumway, 1978; Waterman, 1943). At the two hearts ends two big vessels open, one called sub-endostyle vessel that runs ventrally to the endostyle, while the other called visceral vessel runs dorsally. These two vessels are connected to smaller vessels, which release the hemolymph in sinuses that serve all the organs. The hemolymph is characterized by blood cells (called in tunicates hemocytes) of different nature (see Chapter 3). The hemocytes do not contain respiratory pigments and the gasses are transported in solution in the hemolymph. In some colonial species like *Botryllus schlosseri*, the zooids that form the colony are embedded in a common extracorporeal vasculature, also called colonial circulatory system (Gasparini et al., 2007). This external network of vessels is connected to the open system of each zooid by the main vessel. The main vessel opens in a radial vessel, which connects all the zooids and buds. In the

periphery of the colony, these vessels end in sac-like structures, called ampullae, responsible for adhesion and contractility of the colony (Rodriguez et al., 2019).

1.4.5 A higher level of organization: formation of colonies

Solitary ascidians by definition do not form colonies, but many species often grow in clusters or aggregates as a consequence of gregarious settlement of larvae. Yet, these clusters do not constitute real colonies. Colonies are produced by the thaliaceans and colonial ascidians. The single modules (also called zooids) of a colony are usually smaller than the adult of solitary species, but the colony itself can reach the size of several square meters. The degree of fusion of the zooids in a colony can vary between species, forming sheet-like thin encrusting colonies or massive pedunculated ones (Hiebert et al. 2020); for Berrill (1935), this is influenced by the distance between zooids, their number and the distribution of extracorporeal vessels. According to Berrill, ascidian colonies can be subdivided into 4 major types depending on the grade of fusion (Berrill, 1951): zooids loosely connected at the base by body prolongations called stolons, as in *Clavelina lepadiformis* and *Perophora viridis*. Each zooid has its siphons and is surrounded by a separate tunic, looking like miniaturized solitary ascidians. Some authors call them social forms more than colonial (Figure 1.10 A); zooids originated by asexual reproduction and not connected anymore, probably because stolons degenerated after clonal propagation. However, the clones are densely compact and form gregarious colonies, as in *Distomus variolosus* (Figure 1.10 B); zooids moderately connected to each other, sharing a common tunic (usually in the abdomen) but still presenting separate siphons, as *Diazona violacea* and *Nephtheis fascicularis (*Figure 1.10 C);zooids strictly connected each other's, completely embedded in a common tunic; their atrial siphons open in common cloacal openings, and often share an external vascular system. *Botryllus schlosseri, Aplidium pallidum* and *Diplosoma listerianum* are some examples, forming very well organized zooidal systems (Figure 1.10 D).

The variability of zooid organization and therefore of the shape of the colony is the result of the mechanism adopted by their particular NED mode (see paragraph 1.5).

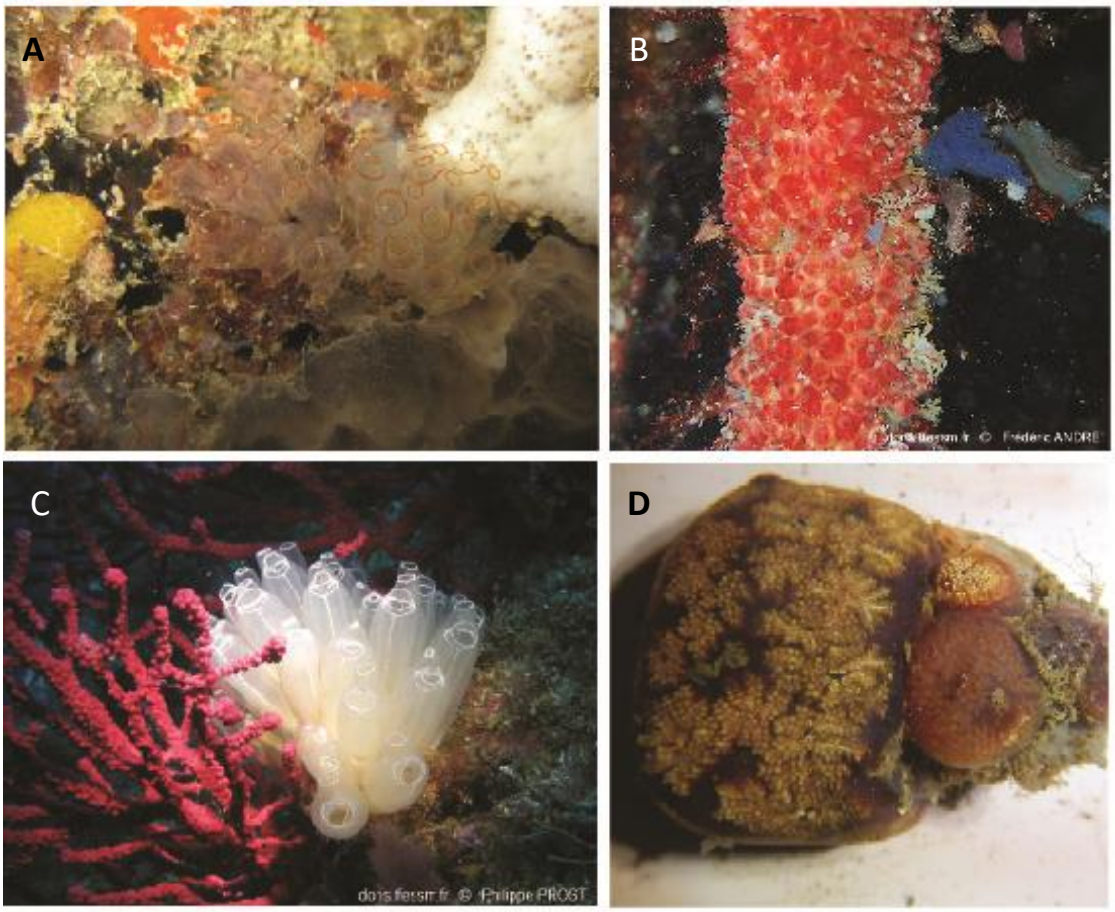


Figure 1.10: Four types of colonies formed by NED. A) Colony of *Ecteinascidia turbinata* (courtesy of Rosana Rocha); B) Colony of *Distomus variolosus* (© Frederic ANDRE, retrieved from https://doris.ffessm.fr/Especes/Distomus-variolosus-Ascidie-varioleuse-888); C) Colony of *Diazona violacea* (© Philoppe PROST, retrieved from https://doris.ffessm.fr/Especes/Diazona-violacea-Diazone-519); D) Colony of *Aplidium constellatum* (courtesy of Rosana Rocha).

1.5. NED in tunicates: generalities and distribution

Non-embryonic development occurs in tunicates by different forms of budding. Nakauchi (Nakauchi, 1982) divided budding into two functional categories: propagative budding and survival budding. Propagative budding occurs in all colonial forms as part of their life cycle and allows the colony growth and propagation, while survival budding is present only in some colonial species and allow the colony to get through adverse environmental conditions. Survival budding includes WBR, which occurs as a response of injury (regeneration *in sensu stricto*) but also dormancy, a state characterized by the formation of morphologically modified "buds" with reduced metabolic activity (Cáceres, 1997) (see Chapter 4). NEDs in tunicates are also categorized based on the tissues and cells that initiate the budding process (Berrill, 1951). In a recent review paper, we combined the latest phylogenic analyses and produced a consensus phylogenetic tree of the whole subphylum. Then, for each clade, we mapped the species undergoing NED and reported their description (Alič et al 2020, see Appendix 1). Colonial species that undergo NED are scatteredly distributed within the Tunicata (Figure 1.11). Based on the different tissues involved, the different ontogenies, and the phylogenetic relationship between solitary and colonial species, NED seems to have evolved independently several times in each of the three orders (Alié et al. 2020) (Figure 1.11).

Despite the differences in the ontogenic tissues and mechanisms, all NED modes lead to a conserved early stage, often referred to as triploblastic stage and characterized by the formation of a monolayered double vesicle (Tiozzo et al., 2008). The outer vesicle is always produced by the epidermis of the parental zooid, while the origin of the inner vesicle varies depending on the type of NED. Between these two vesicles, a mesenchymal space containing circulating mesenchymal cells, the hemocytes, is present. Usually, once the bud reaches the double vesicle stage, oganogenesis begins following a pattern that is similar in the different NED modes. The inner vesicle will generate almost all the tissues and organs of the new zooid by a series of folding and evagination processes (described in detail in *Botryllus schlosseri* by Berrill, 1941) while the outer vesicle usually gives rise to the epidermis of the bud (Figure 1.12).

In the following sections, I will present an overview of the different NED modes described in tunicates, including cases of dormancy. For a more detailed description see Appendix 1 (Alič et al., 2020).

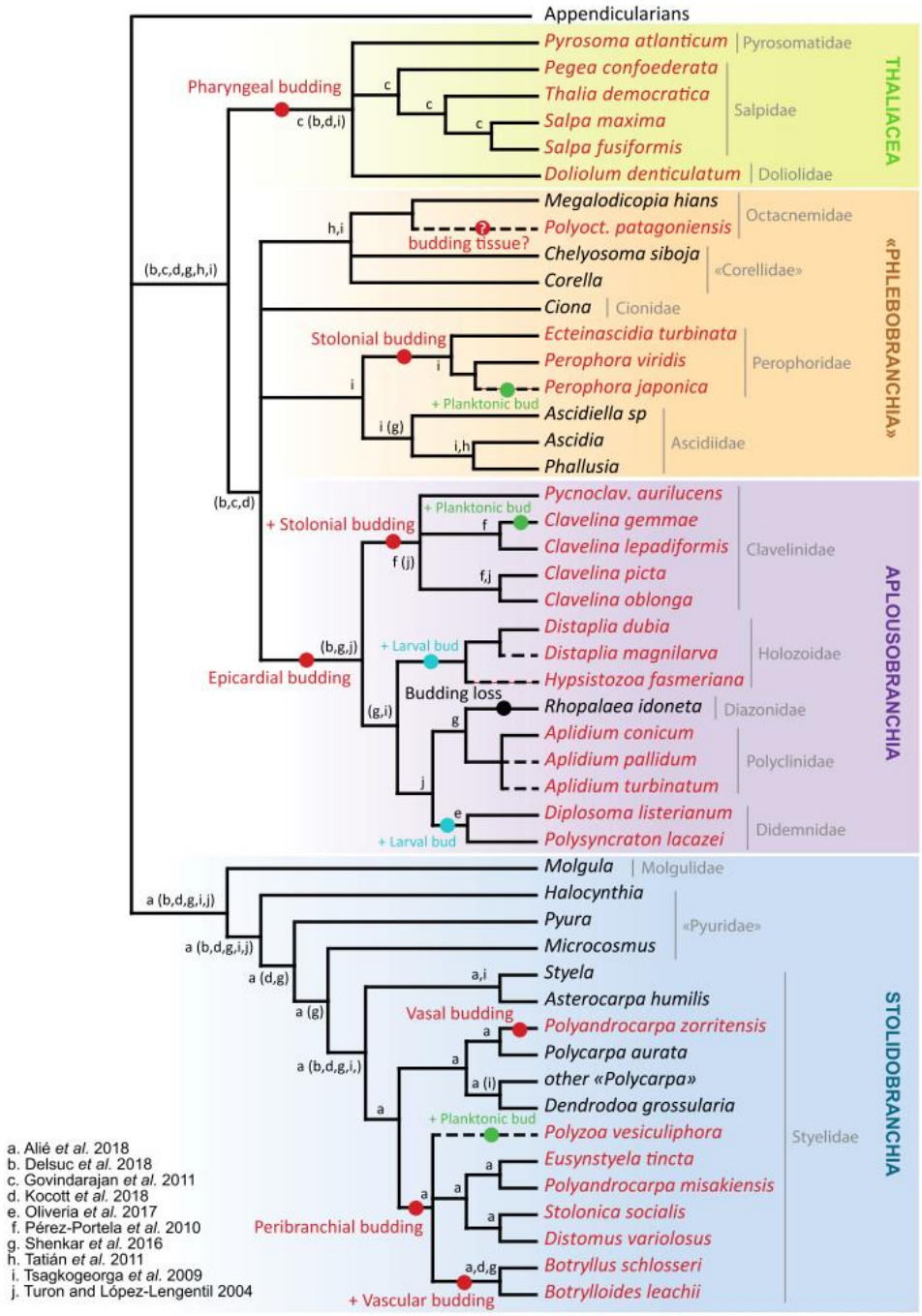


Figure 1.11: Phylogenetic relationships between budding and nonbudding tunicates. Budding species are represented in red, nonbudding species in black. For each branch, letters refer to the studies in which this clade was retrieved, including all the represented species. Letters between parentheses refer to studies that support the corresponding clade, that is with some of the represented species and when no contradicting group was found. Species with dotted lines are positioned not based on phylogenetic studies but classification. When a new budding mode arised in budding taxa, it's preceded by "+" (from Alié et al. 2020).

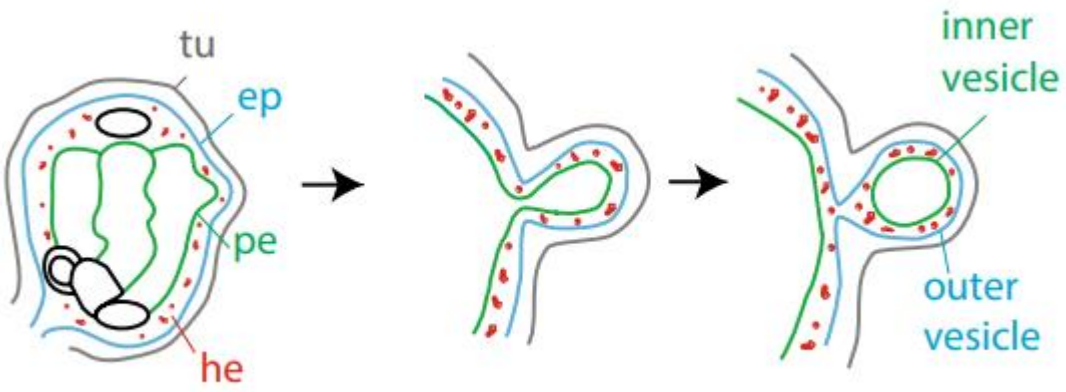


Figure 1.12: Schematic representation of peribranchial budding in *Botryllus schlosseri* from peribranchial evagination to double vesicle stage. In this species, the inner vesicle is formed from the peribranchial epithelium, while the outer vesicle originates from the zooidal epidermis (tu tunic, ep epidermis, he haemocytes, pe peribranchial epithelium) (from Scelzo et al., 2019)

1.5.1 Pharyngeal budding in Thaliaceans

Thaliaceans are all holoplanctonic colonial tunicates. The name comes from the Greek θαλλώ (blooming), reflecting the fact that the population rapidly expands by blooms when the environmental conditions are favorable (Loeb and Santora, 2012; Lucas and Dawson, 2014; Takahashi et al., 2015). Thaliaceans are very important under an ecological point of view (Henschke et al 2016): they are main players in marine food nets (Sutherland et al., 2010) and in biogeochemical cycles recycling a great quantity of organic materials by defecation (Phillips et al., 2009) and by sinking after death (Lebrato et al., 2013) (for a review, Anderson, 1998).

Thaliaceans are divided into three orders: Salpida, Doliolida and Pyrosomida. They represent a monophyletic group nested within ascidians (Delsuc et al., 2018; Govindarajan et al., 2011; Kocot et al., 2018), suggesting that they evolved from a sessile ancestor. Because the transparent bodies of thaliaceans are very delicate, it is quite difficult to collect intact specimens. This lack of specimens represents the main problem to study this interesting group, ideas expressed already in 1893 by Brook and Metcalf: "*The home of the Salpae is so remote from common observation that few persons have a true conception of their scientific interest or their importance in the economy of the sea*" (Brooks and Metcalf, 1893). These conditions make it particularly difficult to study NED in this group. New buds arise from an elongation of the pharyngeal floor, which forms a proliferating stolon; for these characteristics, budding in thaliaceans has been referred to as pharyngeal or stolonial budding (Figure 1.13) (Alié et al., 2020; Brown and Swalla, 2012; Piette and Lemaire, 2015). However, the nature of the tissues involved in stolon formation and bud organogenesis is not completely understood.

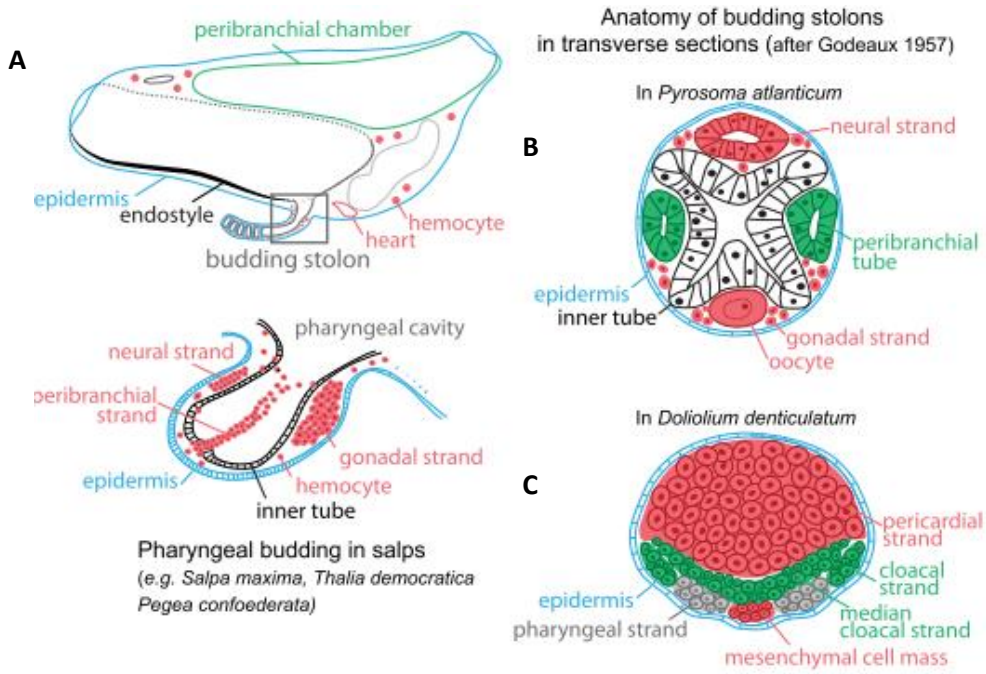


Figure 1.13: A) Schematic representation of pharyngeal budding in Salpidae. Schematic transverse section of budding stolons in B) *Doliolum denticulatum* and C) *Pyrosoma atlanticum* (from Alié et al. 2020)

1.5.2 NED in Phlebobranchia

The order Phlebobranchia encompasses both colonial and solitary species. The phylogenetic relationship between members of this group remains uncertain (Delsuc et al., 2018; Kocot et al., 2018). NED has been well characterized only in the family Perophoridae; however other phlebobranchs as *Polyoctanemus patagoniensis* and *Plurella spp.* have been described as colonial, but accounts on the way of budding lack in these species. In this order, the main NED described is stolonial budding.

- ***Stolonial budding***

In Perophoridae the buds arise along a stolon, a vascular protrusion growing at the base of the zooid (in *Ecteinascidia turbinata* (Beers, 1923), *Perophora viridis* (Freeman, 1964; Lefevre, 1898), *Perophora listeri* (Kowalevski 1874, Brien and Brien-Gavage 1928)). In a non-budding stolon a monolayered partition of mesenchymal origin, the septum, divides the stolonial lumen into two sinuses, in which the hemocytes circulate in opposite directions (Giard 1874, Brien and Brien-Gavage 1928).

Buds can be found along the stolon at unequal intervals, with younger buds closer to the distal end. At the stolonial tip the septum forms the hollow vesicle that will constitute the inner vesicle of the bud (Figure 1.14). Septal cells actively proliferate during this process (Koguchi, 1993).

Freeman (1964) demonstrated the role of undifferentiated hemocytes in stolonial budding. He irradiated small colonies of *P. viridis*, blocking the stolonial growth and the asexual reproduction. These irradiated colonies were then injected with hemocytes extracted from unirradiated specimens, and this was sufficient to rescue the growth and the budding. In order to identify which morphotype was responsible for the budding, Freeman manually sorted the different types of hemocytes cells and injected them separately in irradiated colonies. Only specimens reinjected with "lymphocytes", the small spherical undifferentiated cells, rescued the capability to bud. In addition, histology on irradiated colonies shows that the lymphocytes appeared highly damaged and their percentage was drastically reduced compared to unirradiated colonies. These results suggest that lymphocytes are the progenitor cells responsible for stolonial growth and stolonial budding in *P. viridis*.

During organogenesis in *Perophora viridis* - especially during the formation of epicardium, neural tube, neural ganglion, and sexual organs - mesenchymal cells lay on the inner vesicle and seem to be integrated into the forming organs. The fine structure of each of the blood cell types of *Perophora viridis* has been characterized (Overton, 1966). Lefevre hypothesized that these cells do not have already some "determinants" but they can develop in different tissues according to the location where they attach. In this way, the "destiny" will be given by the part of inner vesicle to which they enter in contact (hypothesis supported by the fact that during the

early stages of budding, before any appearance of organogenesis, some amoeboid cells are in contact with the inner vesicle but do not develop new organs) (Lefevre, 1898).

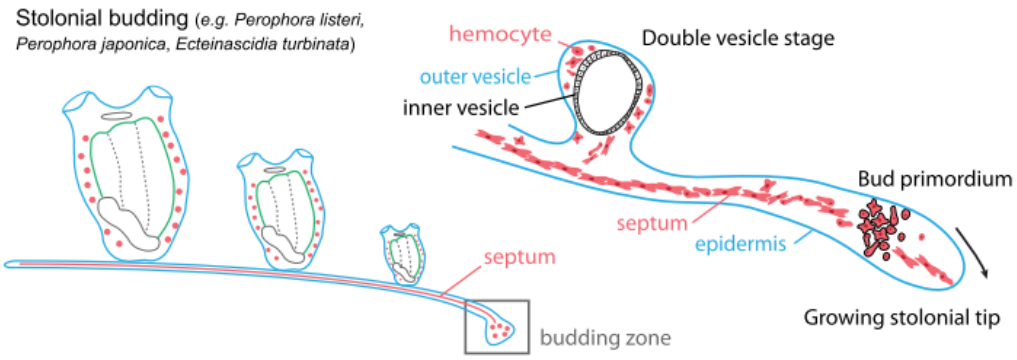


Figure 1.14: Schematic representation of stolonial budding In Perophora virids from bud primordium to the double vesicle stage (from Alie' et al 2020).

- ***Dormancy in* Perophora spp.**

At the end of the breeding season or under unfavorable conditions (as lack of food, lack of water circulation, presence of toxicants, changes in temperature, etc) the zooids can degenerate and be resorbed by the stolon (Huxley, 1921). The stolon looks opaque and yellowish, filled by mesenchymal cells. Mukai and colleagues (1983) identified these cells as granular amoebocytes based on the description from George (George, 1926), Freeman (1964) and Overton (Overton, 1966), and they renamed them trophocytes because of their nutritive functions: the components of the zooid are transported by trophocytes to the stolon, where they are accumulated as nutritive material ("pseudo-vitellus", Bien and Brien-Gavage 1928). When the environmental conditions are favorable, new buds are produced from the stolon, following the same budding patterns of normal colonies. The stolons can remain in this state for at least one year and after this period are still able to bud (Barth and Barth, 1966); because of this capability, they are called dormant or survival stolons (Mukai et al., 1983a).

Different species produce dormant stolons: *Perophora viridis* (Huxley 1921), *P. listeri* (Brien and Brien-gavage 1928) and *P. sagamiensis* (Mukai et al., 1983) hibernate, forming dormant stolons at the end of summer, when the temperature decrease, while other species as *P. japonica* tend to undergo to estivation to survive high temperatures (Mukai et al., 1983). However, under laboratory conditions, *P. viridis* zooids regress and dormant stolons are formed also at high temperatures (Barth and Barth, 1966), probably because these conditions are not optimal.

- ***Whole body regeneration in* Perophora**

In some Perophora species such as *P. viridis* (Goldin, 1948) and *P. listeri* (Brien and Brien-Gavage 1928) mechanically isolated pieces of stolons regenerate into new zooids. Both pieces extracted between two zooids and pieces excised at the tip have this capacity (Fukumoto, 1971; Goldin, 1948). The growth of the buds from stolonial fragments is similar to the development of normal stolonial buds (Deviney, 1934). Effects of salinity, light exposure, oxygen tension and temperature on the development of isolated stolons have been studied (Barth and Barth 1966), as well as the minimal stolon length that allows regeneration. Stolons of less than 1 mm in length were still able to bud (Barth and Barth, 1966). In very small pieces of stolon where the mesenchymal septum was too small, lymphocytes aggregated around the septum and participated in bud formation, supporting the idea that lymphocytes are necessary for NED in Perophoridae (Deviney, 1934, Freeman, 1964). Some mechanisms of humoral control have been proposed to regulate stolonial growth and inner vesicle formation during WBR in *P. orientalis* (Fukumoto, 1971). The thyroid hormones thyroxine and triiodothyronine stimulate stolon elongation but inhibit the formation of inner vesicles, while the inhibitor of thyroid peroxidase

thiourea blocks the propagation of the stolon but accelerates the formation of the double vesicle (Fukumoto, 1971).

1.5.3 NED in Aplousobranchia

All the five main Aplousobranchia families (Clavelinidae, Holozoidae, Diazonidae, Polyclinidae and Didemnidae) are colonial and therefore undergo NED. The only reported exceptions are some species of the genus Rhopalea, in the family Diazonidae (Moreno and Rocha, 2008; Shenkar et al., 2016). Aplousobranchia body is divided in an anterior thorax and a posterior abdomen. The abdomen can be more or less elongated, and in some species - for example in the family Polyclinidae- it is extended in a post-abdomen (Brien 1958, Berrill, 1950). Vascular stolons run from the posterior region in some species as Clavelina (Brien and Brien-Gavage 1927). Two main types of budding are known in this group: epicardial budding and stolonial budding.

- ***Epicardial budding***

The bud occurs in the post thoracic regions, involving either the abdomen, the post-abdomen or both and, in some instances, the vascular stolon (Nakauchi, 1970). Despite the differences in the region where budding takes place, in all the species the epicardium represents the multipotent organogenetic tissue (Kawamura et al., 2008). For this reason, this mode of NED has been called epicardial budding (Alié et al., 2020), but it has been referred also as abdominal or post-abdominal strobilization based on the body region where the buds are formed (Brown and Swalla, 2012).

Nutritive mesenchymal cells called trophocytes containing granules of nutritive substances (called pseudo-vitellus by Brien, 1948) participate often to epicardial budding: these cells migrate to the posterior end and accumulate in the future buds furnishing nutritive substances during organogenesis.

Polyclinidae - In the family Polyclinidae buds are produced by strobilization: the abdominal regions are subdivided in buds or strobilae by a series of transverse constrictions driven by the epidermis. The first constriction occurs at the level of the esophagus, while the consecutives are progressively more close to the posterior end, following an anteroposterior wave. The number of buds depends on the length of the post-thoracic region. The main organogenetic tissue is the epicardium; however, Brien (1925, 1948) suggested that other tissues may participate in organogenesis. In *Aplidium zostericola*, he described the formation of the digestive tube as regeneration of the remaining fragments of stomach and intestine, while the other tissues are

formed by the epicardium (Brien 1948). Brien speculated that the presence of parts of the digestive tract may reduce the regenerative capabilities of the epicardium, and he referred to budding in *A. zostericola* as entero-epicardial.

In some species of the family Polyclinidae as *Aplidium spp.* and *Polyclinum spp.* the events that characterize the budding are well described. In *Aplidium yamazii* (Nakauchi, 1970) budding occurs at the level of the abdomen throughout the year. The first signs of budding are the thickening of epicardial wall and the migration of trophocytes to the abdomen. Then the epidermal constrictions start, and the stomach begins to grow and elongate posteriorly; in this way, each strobila will contain part of the parental stomach. As result of the first constriction, the abdomen is separated from the parental thorax; only at this point all the other constrictions become complete and the buds are singularly released, remaining embedded in the same tunic. During strobilization, the parental thorax slightly shrinks and it stops to feed; when the abdomen separates, the internal organs expand and they return to the normal size. After 15-30 days these organisms will be ready to start another budding cycle.

In other species of the genus *Aplidium*, as *A. turbinatum* or *A. multiplicatum*, buds arise from both abdominal and post-abdominal regions (Nakauchi, 1986; Nakauchi and Kawamura, 1978) (Figure 1.15). Not all Polyclinidae species show regeneration of the parental thorax after strobilization. In *Aplidium petrense* and *Polyclinum planum* the thorax and abdomen completely regress and disappear at the end of strobilation (Holyoak, 1992; Millar, 1962). For Nakauchi (1982) regeneration of the thorax is related to the environmental conditions. If circumstances are favorable, the parental thorax can regenerate and reform another zooid, while if the conditions are adverse the thorax may degenerate. However, there is no evidence that species that regenerate the thorax stop to do it under adverse conditions; so this phenomenon may be species-dependent.

In the Polyclinidae, zooids are usually embedded in the same tunic and share a common cloacal opening. After strobilization, buds undergo to self-assembling movement to form a new organized colony (also called “cloister” by Brien 1948 and “cohort” by Holyoak 1992): the developing strobilae change position and migrate close to the parental zooid when it does not regress (i.e. *Aplidium multiplicatum,* Nakauchi and Kawamura 1978) or migrate in the region where the remnant thorax is degenerating when the parental thorax regress (i.e. *Polyclinum planum*, Holyoak 1992). It is not clear what induces this migration, but Nakauchi and Kawamura (1978) speculated that the parental thorax may release attractive substances in the tunic.

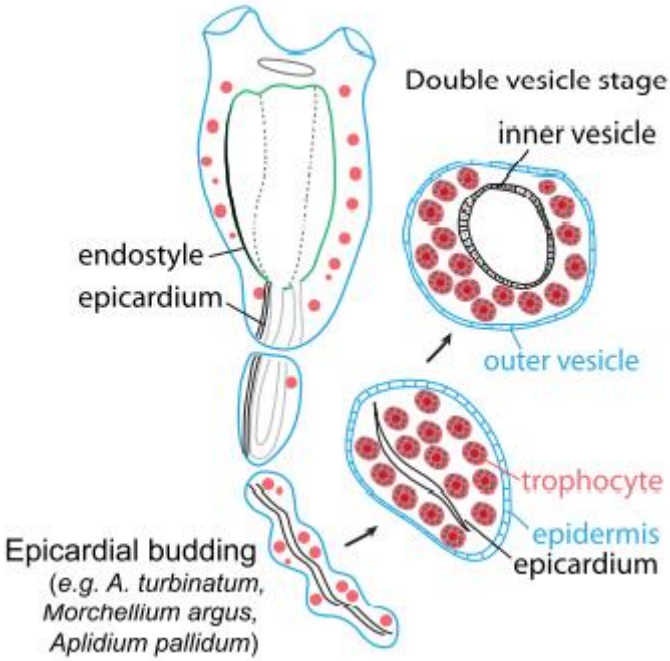


Figure 1.15: Schematic representation of epicardial budding and detailed view of the postabdominal budding, from strobilization to the double vesicle stage (from Alie' et al 2020).

Holozoidae – One interesting characteristic of Holozoidae such as *Distaplia rosea*, *D. bermudiensis* and *Hypsistoma fasmeriana* is that they start to bud already at the larval stage (Berrill, 1948b; Brewin, 1959).

In the ventral region of the *Distaplia* tadpole larva, the epicardial epithelium thickens and proliferates, bulging out with the overlying epidermis and forming an elongated probud or proliferative stolon. Soon from this probud 4-5 buds constrict off one at the time by epidermal constriction. As a result of this constriction, a double vesicle is produced, characterized by an outer epidermal vesicle and an internal one originated by epicardium, separated by mesenchymal space in which are present embryonic mesenchymal cells. The buds remain in the larval tunic during larval metamorphosis and oozoid formation.

Morphogenesis starts in all the buds, but only the biggest one will complete it and take the place of the oozoid becoming the first blastozooid, while the other buds will degenerate (Berrill, 1948). In *Hypsistozoa*, the proliferative stolon curves describing a hairpin bend on the larval left side. When it reaches its maximal length, the most distal part of the stolon extends to the right side of the larva (Brewin, 1959). The buds, separated by epidermal constrictions, are organized forming arcs of a spiral, and differently from *Distaplia,* all constrictions undergo morphogenesis to form blastozooids.

Buds are similarly produced by blastozooids (Berrill, 1948). When the zooid starts to regress, only the thorax and most of the abdomen degenerate. The most posterior region of the abdomen containing the epicardium progressively extends and becomes filled with trophocytes. Then, the epicardial tip enlarges and forms as in the larva a cylindrical protrusion, the proliferative stolon, which will produce new buds.

Didemnidae - Budding in Didemnidae as *Didemnus spp.*, *Diplosoma spp.* or *Polysyncraton spp.* has been called pyloric budding (Brown and Swalla, 2012). At the base of the oesophagus, the epicardium proliferates and enlarges forming two diverticula. The anterior diverticulum called abdominal bud regenerates a new abdomen comprising of heart, gonads and digestive tract, and it maintains connections with the parental thorax. The posterior diverticulum called thoracic bud regenerates the branchial basket and the nervous system and remains attached to the parental abdomen (Sköld et al., 2011)(Pizon 1905, Berrill 1935, Brien 1948). At the end of the regenerative process, the parental esophagus that connected the two buds degenerates and the two new zooids remain separated in the common tunic. This budding process required cell proliferation of different tissues, in particular, the epicardium and the oesophagus (Sköld et al., 2011).

- ***Stolonial budding in Clavelinids***

The members of the family Clavelinidae as *Clavelina lepadiformis* (Brien and Brien-Gavage 1927) or *Pycnocalvella aurilucens* (Berrill, 1947) acquired a way of budding completely different from other Aplousobranchia, the stolonial budding. The main actor of this type of budding is the post abdominal stolon, containing a mesenchymal septum that divides the lumen in two sinuses (Brien and Brien-Gavage 1927). This stolon can form multiple ramifications: the terminal ampullae become inflated and look opaque and whitish for the accumulation of trophocytes (called orange cells in *Clavelina miniata)* (Watanabe and Tokioka, 1973). These regions where the budding takes place are called budding chambers (Brien 1948, or nutritive chambers by Selinger, 1882). When the mesenchymal circulation is interrupted, the budding chamber starts to develop: the cells of the mesenchymal septum proliferate and form the inner vesicle, from which all the bud tissues except the epidermis will be generated (Figure 1.16).

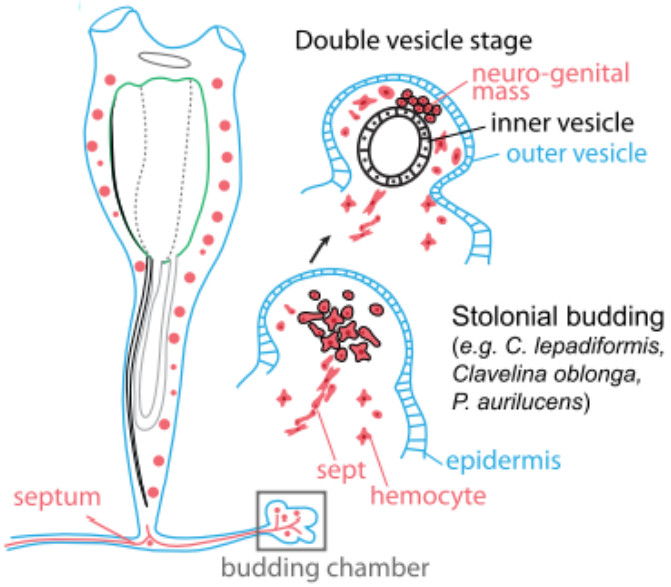


Fig 1.16: Schematic representation of stolonial budding in Clavelinidae (from Alie' et al 2020).

The new zooids are produced through the year and are completely detached from the parental zooid or remain connected by a reminiscence of stolon composed only by tunic. The mesenchymal septum is originated by aggregation of undifferentiated mesenchymal cells, described by Brien (1930) as lymphocytes. For Brien these lymphocytes and the mesenchymal septum are "two reversible aspects of the same element" (Brien 1930).

- ***Dormancy and rejuvenation***

Some Aplousobranchia can cope with environmental variations undergo dormancy or thorax rejuvenalization.

In Diazonidae the phenomenon of hibernation is well described in *Diazona violacea* (Berrill, 1950; Berrill and Watson, 1935). In early winter at the end of the sexual reproductive season, the thorax regresses, the tunic becomes thicker and smooth. Trophocytes engulf nutritive material from the degenerating area and migrate to the abdominal region, which appears congested of these cells. After trophocyte migration, strobilation by epidermal constriction takes place forming a series of 3 to 8 spherical buds. These buds remain aggregate in the tunic until the end of winter when they regenerate the missing organs from the epicardium (Salfi 1929, Berrill, 1948a). During this process, the nutrients from trophocytes are used by the developing bud and the number of these cells decreases (Berrill and Watson, 1935).

Clavelina lepadiformis undertakes hibernation. During the summer trophocytes start to be accumulated at the stolonial tips. In late summer the zooid regresses and the budding chambers separate from each other's, forming the dormant forms called winter buds able to survive to colonial regression and to resist to the winter cold temperature (Giard and Caullery 1896, Della Valle 1915, Huxley 1926). The winter buds can develop into new zooids in warmer conditions following the same pattern as normal stolonial budding (Berrill, 1951; Berrill and Cohen, 1936). The reserve material furnished by the trophocytes is gradually digested. Interestingly, Huxley (1926) reported the presence of winter buds during summer in the Gulf of Naples and he hypothesized that some environmental cues different than temperature may lead to formation of the winter buds. In *Clavelina flegrea* the process of hibernation is different, more similar to survival budding in Diazonidae (Stalfi 1929, Brien 1930. 1948). In winter the thorax degenerates and the abdomen survives covered by a thick tunic. In spring, these dormant abdomens regenerate the missing thorax from the epicardium. As in the colonies of Polyclinidae and Polycitoridae, the developing buds migrate in the tunic to reorganize the colony.

Species of the family Didemnidae undergo a phenomenon of thorax "rejuvenalization" (Turon, 1992). During summer colonies of *Polysyncraton lacazei* have been observed in a non-feeding state (Turon, 1992). The peribranchial basket shrinks and the siphons are closed and not in contact with the external environment. The spicules in the tunic are disorganized and the colony seems covered of a thick cuticle (Wahl and Lafargue, 1990). The abdomen maintains its integrity and mature gonads are still present in it. At the level of the oesophagus, a new thorax can be regenerated to replace the old ones in the new zooid. Other species of the genus *Didemnum*, such as *Didemnum immundum*, show a similar non-feeding state (Romanov 1989). This phenomenon is not reported as a survival budding because only the thorax is renewed and only part of the entire population is interested at the same time. Turon (1992) suggested that this phenomenon can be related to the increase of metabolic rates induced by higher temperatures during summer: for the animal it may be more convenient stop feeding than be subjected to an intense metabolic activity and an increase of production of thoracic waste.

- ***WBR in* Clavelina lepadiformis *(stolonial and epicardial regeneration)***

In *Clavelina lepadiformis*, mechanically isolated fragments of stolon can regenerate by WBR a new zooid from mesenchymal cells (Della Valle 1914, 1915, Brien 1930, 1948, Berrill and Cohen 1935). After isolation, the mesenchymal septum contracts and the lymphocytes that constitute it are separated. These cells clump at the region where the cut occurred and form the inner vesicle.

However, *C. lepadiformis* is also able to regenerate the whole body from the epicardium (Driesch 1902a,b, Brien 1930). When the body column is separated into four fragments - postabdomen, abdomen, esophagus and thorax - each of them can regenerate the missing part from the epicardium. Also if the main organogenetic tissue during budding in Clavelinidae is the mesenchymal septum, the epicardium maintains its multipotency, which is completely revealed during WBR.

1.5.4 NED in Stolidobranchia

Stolidobranchia forms a monophyletic order of ascidians that comprises budding and non-budding species, and among which Styelidae is the only family where NED has been well studied. Peribranchial budding, sometimes referred to as atrial, palleal, or pallial budding, is the most common form of NED adopted by Styelidae (Figure 1.17 A). Another form of NED in Styelidae is named vascular budding and in some species can occur as part of the asexual life-cycle (propagative budding) and in other only as whole-body regeneration in response to injury (survival budding) (Figure 1.17 B). The third form of NED reported in Stolidobranchia is vasal budding, which represent the main subject of this thesis. Different forms of dormancy (survival budding) have been reported in Stolidobranchia (see Chapter 4).

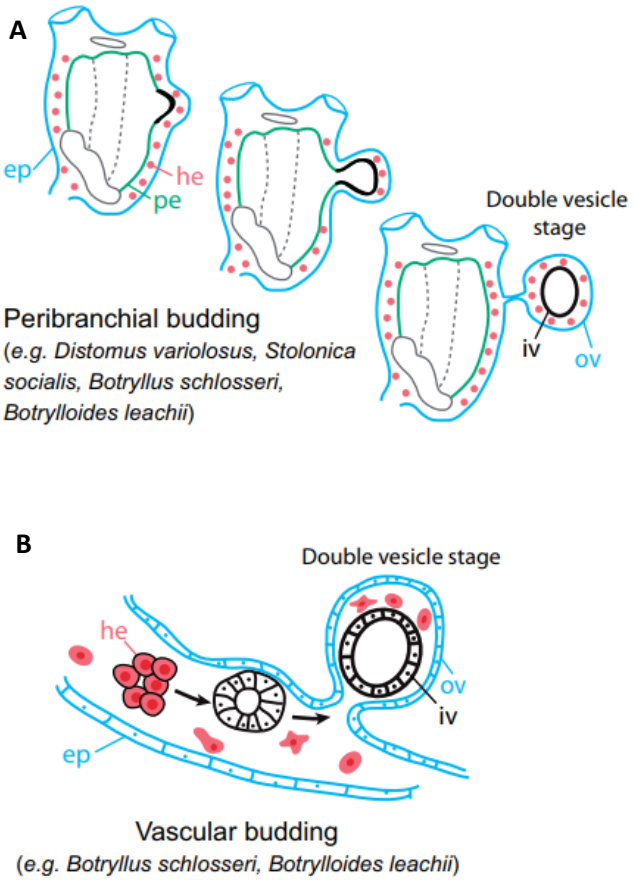


Figure 1.17: A) Schematic representation of peribranchial budding at three successive stages, from peribranchial invagination to double vesicle; B) Schematic representation of vascular budding at three successive stages, from hemocyte clustering to double vesicle. ep: epidermis, he: hemocytes, iv: inner vesicle, ov: outer vesicle, pe: peribranchial epithelium (from Alie' et al 2018).

- ***Peribranchial budding***

Peribranchial budding has been characterized in different species as *Botryllus* (Gasparini et al., 2015; Sabbadin, 1955), *Polyandrocarpa misakiensis* (Watanabe and Tokioka, 1973), *Polyzoa vesiculiphora* (Fujimoto and Watanabe, 1976a), *Symplegma spp.* (Berrill, 1940; Kawamura and Nakauchi, 1986).

The first sign of peribranchial budding is visible as a thickening of the peribranchial epithelium (called bud primordium by Izzard, 1973). This thickening bulges, carrying in its protrusion the overlying epidermis and tunic. The peribranchial epithelium will form the inner vesicle, which represents the organogenetic tissue (Figure 17 A).

One of the most studied Styelidae species is *Botryllus schlosseri* and peribranchial budding has been investigated in detail in this species. The colonies, organized in a star-shape system, contain three generations enclosed in the same tunic, which are all connected by the external vasculature. The adult blastozooid carries one to four primary buds, which in turn hold one to four secondary buds or budlets (Manni et al., 2007) (Figure 1.18). The budding cycle is characterized by periodic waves of zoodal resorption: during the takeover stage, all the zooids are resorbed and replaced by the primary buds, while the secondary buds become primary buds generating new secondary buds (Burighel and Schiavinato, 1984; Lauzon et al., 1992; Sabbadin, 1955). This cycle is accompanied by intense apoptotic activity (Lauzon et al., 2007; Tiozzo et al., 2006) and all the buds are perfectly synchronized and at the same developmental stage across the all colony.

Botryllus bud formation is restricted to specific regions of the peribranchial epithelium (Kawamura et al., 2008). After the thickening of the atrial wall, the bud primordium expands and arches forming a hemisphere. This hemisphere soon encloses on itself forming the inner vesicle, which remains close to the primary bud (Manni et al., 2007). The buds remain connected to the zooid with a thin epidermal stalk that permits also the connection with the colonial circulatory system. Other species of the subfamily Botrillidae as *Botrylloides spp.* show a similar mode of budding (Kawamura and Fujiwara, 1995, 1995; Kawamura and Nakauchi, 1986)..

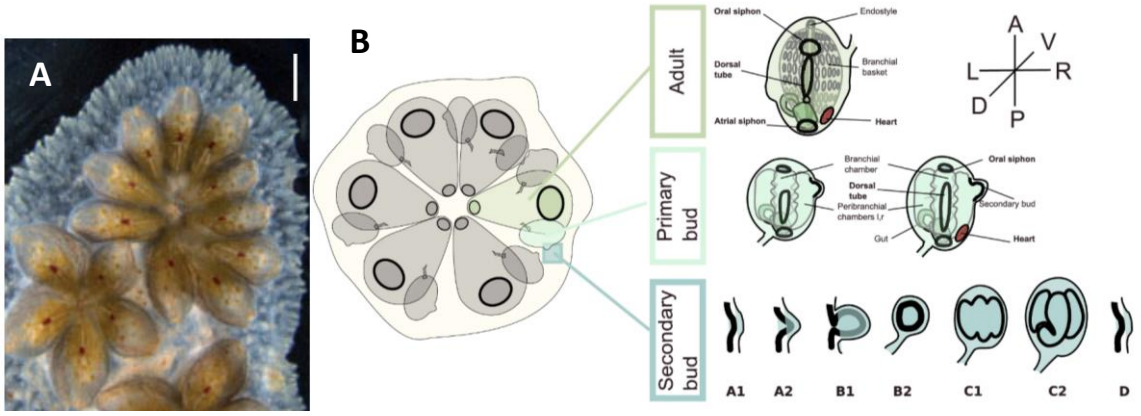


Figure 1.18: A) Colony of *Botryllus schlosseri* (scale 1mm) (from Alie' et al 2018); B) Schematic representation of development and staging of a *Botryllus schlosseri* colony. The secondary bud develops as thickening of the peribranchial epithelium and the epidermis (stages A1, A2), which evaginates and closes forming a double monolayered vesicle (stages B1, B2). The inner vesicle undergoes morphogenesis and is subdivided into three chambers (stages C1, C2). During "takeover" (stage D), the adult degenerate and get resorbed, the primary buds become adult, the secondary buds become the primary buds and start to bud again a new secondary bud, so a new blastogenetic cycle begins for the next secondary bud (A1) (from Prünster et al., 2019b).

A well-studied Styelid not belonging to the Botryllinae family is *Polyandrocarpa misakiensis.* The morphological and physiological changes of peribranchial epithelial cells have been well investigated during peribranchial budding (Fujiwara and Kawamura, 1992b; Kawamura and Fujiwara, 1995; Kawamura et al., 2008). Buds can be produced all along the peribranchial wall (KAWAMURA, 1982; Kawamura et al., 2008) (Figure 1.19). After the formation of the double vesicle, the inner vesicle undergoes transdifferentiation (Fujiwara and Kawamura 1992, Kawamura et al 2010). Cells acquire a cuboidal shape with an enlarged nucleus and a prominent nucleolus (Kawamura and Fujiwara 1994). Also, during organogenesis, they lose APL expression, a marker of epithelial cells in the peribranchial wall (Kawamura and Fujiwara 1994). Proliferating activity has been tested. During the earliest stages, when the bud is still attached to the parental zooid, mitotic dividing cells are randomly distributed in the protruding peribranchial epithelium and epidermis (Kawamura and Nakauchi 1991). When the inner vesicle is formed and the bud detaches, the organogenesis starts and the cells in the inner vesicle actively divide, particularly in the regions where the organ rudiments (also called organ placodes) form; cells in the outer vesicle divide too, but their percentage is less important (Kawamura and Nakauchi 1986).

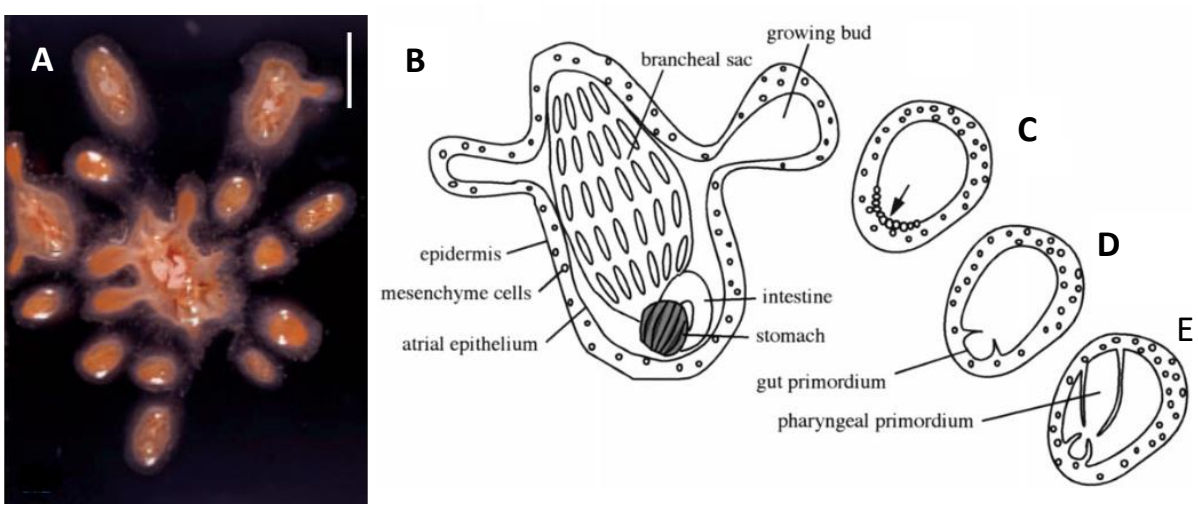


Figure 1.19: A) Colony of *Polyandrocarpa misakiensis* (scale bar: 1cm) (from Alie' et al 2018). B-E) Schematic representation of bud development in *Polyandrocarpa misakiensis* (from Ohashi et al 1999) B) The growing bud is an outgrowth of the parental body wall. The bud separates from the parent and starts cell differentiation and morphogenesis. C) The atrial epithelium in the most proximal region dedifferentiates and enters the cell cycle (arrow). D) The atrial epithelium of this region invaginates into the mesenchymal space andredifferentiates into the gut. E) The pharyngeal primordium is also formed as a folding of the atrial epithelium.

In *Polyzoa vesciculiphora*, *Distomus variolosus* and *Stolonica socialis* peribranchial budding occurs through the formation of a stolon and it is also called stolonial budding (Figure 1.20). However, this term is misleading. In fact, differently from the vascular stolon of *Perophora* or *Clavelina*, in *P. vesiculiphora*, *D. variolosus* and *S. socialis* the stolon originates from a protrusion of the peribranchial wall, surrounded by epidermis and tunic. Hemocytes flow in the stolon but in the mesenchymal space between the atrial epithelium and the epidermis.

In *P. vesiculiphora* the stolon can reach 10-15 mm in length and several buds (up to 7) are formed along it (Fujimoto and Watanabe 1976), while in *Stolonica* only one bud develops at the tip of the stolon (terminal budding, Selys-Longchamps 1916, Berrill 1948). Nutritive mesenchymal cells (called trophocytes by Watanabe and Tokioka (1976) in *Polyzoa* and pseudo-vitelline cells by Berrill (1948) in *Stolonica*) are accumulated in the region of the stolon where the bud will form before the circulation is interrupted. The new zooids are completely isolated from the parent in *Polyzoa*, while in *Stolonica* and *Distomus* they are loosely connected by a fragment of stolon.

In *Polyzoa vesiculiphora* a modified type of peribranchial budding called planktonic budding permits the propagation of buds (Fujimoto and Watanabe, 1976a). As the stolonial peribranchial budding, the peribranchial epithelium protrudes as if a stolon would be produced. However, it does not elongate too much, just 1 mm of length. The test vessel starts to ramify forming from 11 to 21 ampullae that surround the bud surface. Then, the bud constricts off from the parental zooid and it is freely released in the currents. The bud can attach to the substrate thanks to ampullar tips. Both stolonial and planktonic buds can be produced at the same time, but it remains unclear which mechanisms regulate the choice between stolonial and planktonic budding (Watanabe and Tokioka 1972, Fujimoto and Watanabe., 1976).

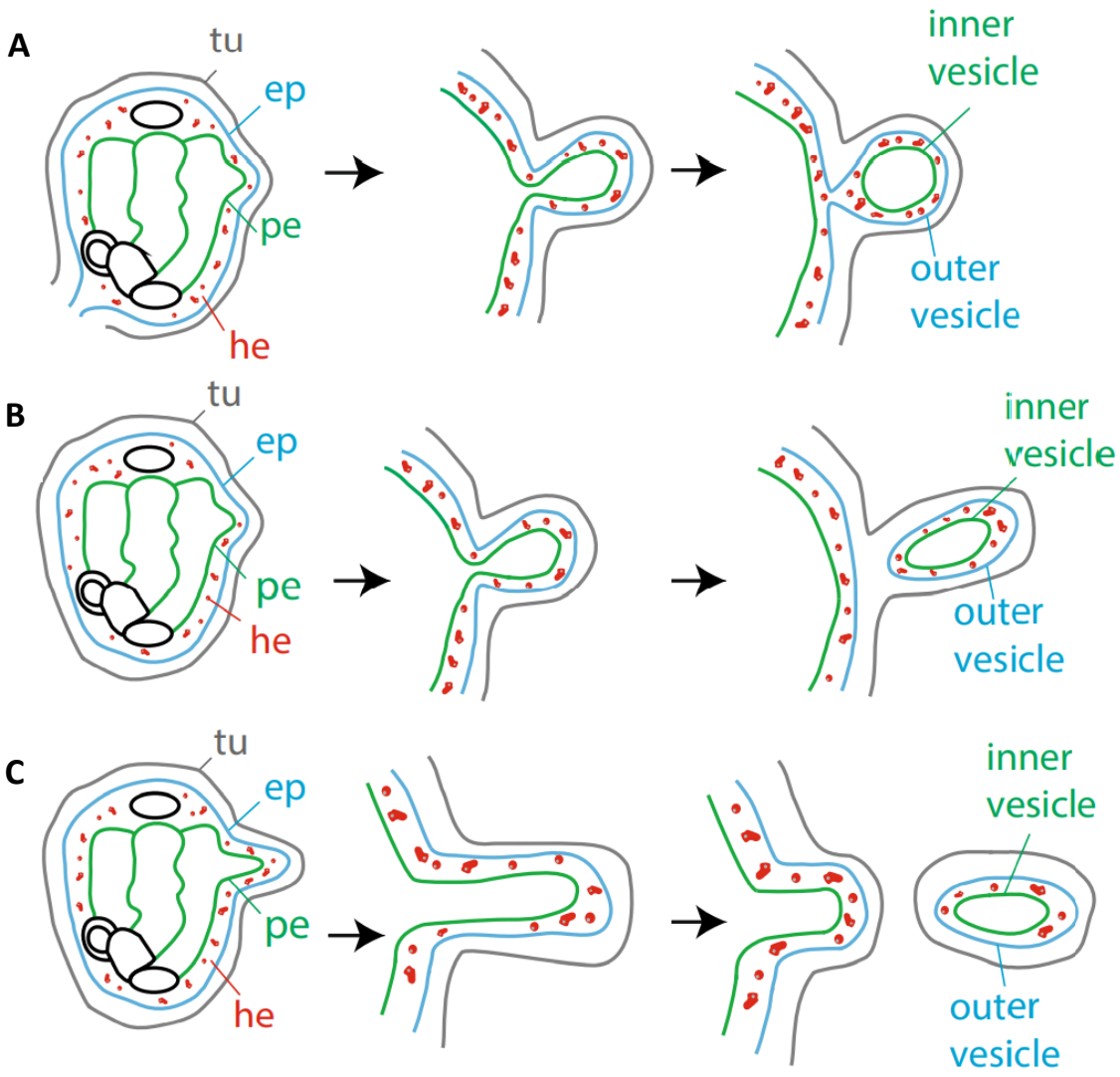


Figure 1.20: Schematic representation of peribranchial budding in *Botryllus schlosseri* (A), *Polyandrocarpa misakiensis* (B), and *Stolonica socialis* (C), from peribranchial evagination to double vesicle stage (from Scelzo et al., 2019).

- ***Vascular budding***

In Botrillidae species as *Botryllus primigenus* (Oka and Watanabe, 1957), *Botryllus tuberatus* (Akhmadieva et al., 2007) and *Symplegma brakenhielmi* (Gutierrez and Brown, 2017) the propagative buds originated from clusters of undifferentiated mesenchymal cells, possibly hemoblasts (see Chapter 3), nested within an evagination of the vascular epithelium (Figure 1.17 B). This aggregate of cells form a single-layered hollow vesicle encircled by epidermis; in this way, a double vesicle similar to the one of peribranchial budding is formed. This type of budding has been called vascular budding.

Interestingly, in some members of the same subfamily, such as *Botrylloides spp.* (Blanchoud et al., 2017; Brown et al., 2009; Kassmer et al., 2019a; Zondag et al., 2016) and *Botryllus schlosseri* (Ricci et al., 2016; Voskoboynik et al., 2007), can regenerate a new colony starting from isolated pieces of external vasculature (reviewed in Kassmer et al., 2019b). The early stages of this regenerative process are similar to the vascular budding: after the reorganization of the remaining vasculature and restoring of hemocytes circulation by ampullar contraction, undifferentiated mesenchymal cells cluster under the epidermis and eventually start to proliferate (in *Botryllus schlosseri*, Ricci et al., 2016) (Figure 1.21), forming a hollow vesicle visible as an opaque mass within the vasculature. In some cases, more double vesicles can be formed, but only one will successfully develop (Brown et al., 2009).
In these species, vasal budding represents an example of survival budding (i.e. it ensures the colony survival after a traumatic event) and the the term whole-body regeneration is used. WBR and vascular budding show the same patterns with the difference that WBR is triggered by injury.

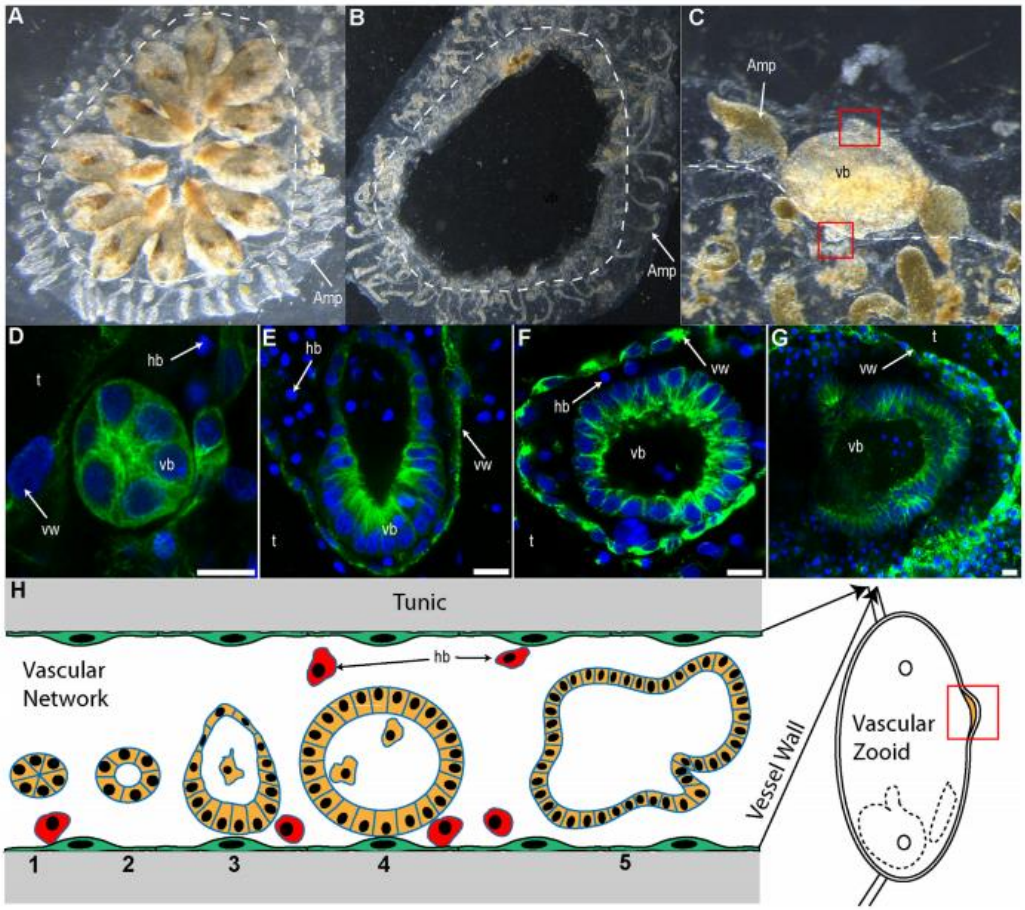


Figure 1.21: Induction and characterization of vascular budding (VB). (A): colony prior to dissection; (B): the same colony 48 h after ablation of all zooids and buds; (C): details of the regenerated zooid bearing palleal buds (red squares), 10 days after surgery. Dashed lines underline the peripheral vasculature. (D–H): details of the onset of VB. (D), (E), (F) and (G): regenerative vascular buds at stage 1, 3, 4 and 5, labelled with Tyrosinated tubulin (green), nuclei are counterstained with Hoescht (blue). Scale bars¼10 mm. H: schematic representation of the vascular bud morphology. Black numbers indicate arbitrary chosen stages of vascular bud development. Stage 1: compact cell cluster lining on the vessel wall. Stage 2: lumen formation, the vascular bud becomes a hollow vesicle. Stage 3: polarized vascular bud, exhibiting different cell shapes at two opposite sides of the vesicle (cuboidal versus flattened cells). Stage 4: double vesicle stage, where the vascular bud appears as an inner hollow sphere, and the vessel wall forming the outer vesicle. Stage 5: first epithelial folds, and beginning of the non-stereotyped morphogenesis. The last sketch indicates the adult regenerated body (vascular zooid), reinitiating PB (palleal bud in the red square). Amp: ampulla; vb: vascular bud; vw: vessel wall; hb: haemoblast; t: tunic (from Ricci et al., 2016).

- ***Dormancy in Styelidae***

In different species of Styelidae dormancy has been described. In *Botrylloides diegensis*, a kind of WBR is also triggered by low temperatures. During winter the colony enter in a dormant stage, called hibernation or torpor (Burighel et al., 1976; Hyams et al., 2017)[1], in which all the zooids are resorbed and the tunic is filled by hemocytes. When temperature increases during spring, new zooids are regenerated by clusters of hemoblasts similarly to vascular budding and WBR (Hyams et al., 2017).

In *Stolonica socialis* and *Distomus variolosus*, at the end of the breeding season the zooids regress and dormant buds can be observed, also called winter buds (Berrill, 1948c) or statoblasts (Berrill, 1950; Selys-Longchamps, 1917). These are characterized by an accumulation of nutritive trophocytes The following spring a new colony is reformed from the winter bud (Berrill, 1948c).

[1] Recently, Mediterranean populations of *Botrylloides spp.* reported as *B. leachii* have been identified as *B. diegensis* (Viard et al., 2019). It is likely that the specimens studied in the cited papers belong to the species *B. diegensis*.

1.6 Stolidobranchia as models to study NED evolution

As mentioned in Section 1.3 non-embryonic development is widespread across animal phyla. With its 38 genera and 551 accepted species, Stolidobranchia, and in particular the Styelidae, show a good diversity of NED modes and containrepresentative species that have gained or lost NED (Alié et al., 2018).

Indeed, a robust phylogenetic tree of Styelidae is an indispensable prerequisite to portray the evolution of asexual development within a comparative framework. In a recent phylogeny, we clarified the phylogenetic relationships between solitary and colonial species, identifying two independent acquisitions of NED (Figure 1.22) (Alié et al., 2018) (see Appendix 2). Acquisition of peribranchial budding occurred in the clade comprising Polyzoinae (e.g. *P. misakiensis*) and Botryllinae (e.g. *B. schlosseri*), which all share the same type of asexual reproduction. Within this clade, vascular budding arose in a Botryllinae ancestor, and therefore species of these sub-family posses both type of NED. Finally, NED also arose convergently in the species *Polyandrocarpa zorritensis*, which is phylogenetically distant from the other colonial Styelidae.

The molecural and cellular echnanisms of NED in *Botryllus schlosseri*, *Polyandrocarpa misakiensis* and *Botrylloides diegensis* (i.e. peribranchial and vascular budding) have been deeply investigated, and I will summarize this knowledge in the last paragraph of this introduction. In contrast, budding in *Polyandrocarpa zorritensis* was largely unknown. During my thesis I characterized this budding mode, a work that I will explain in more detail in the Chapter 2 and 3 of the present manuscript.

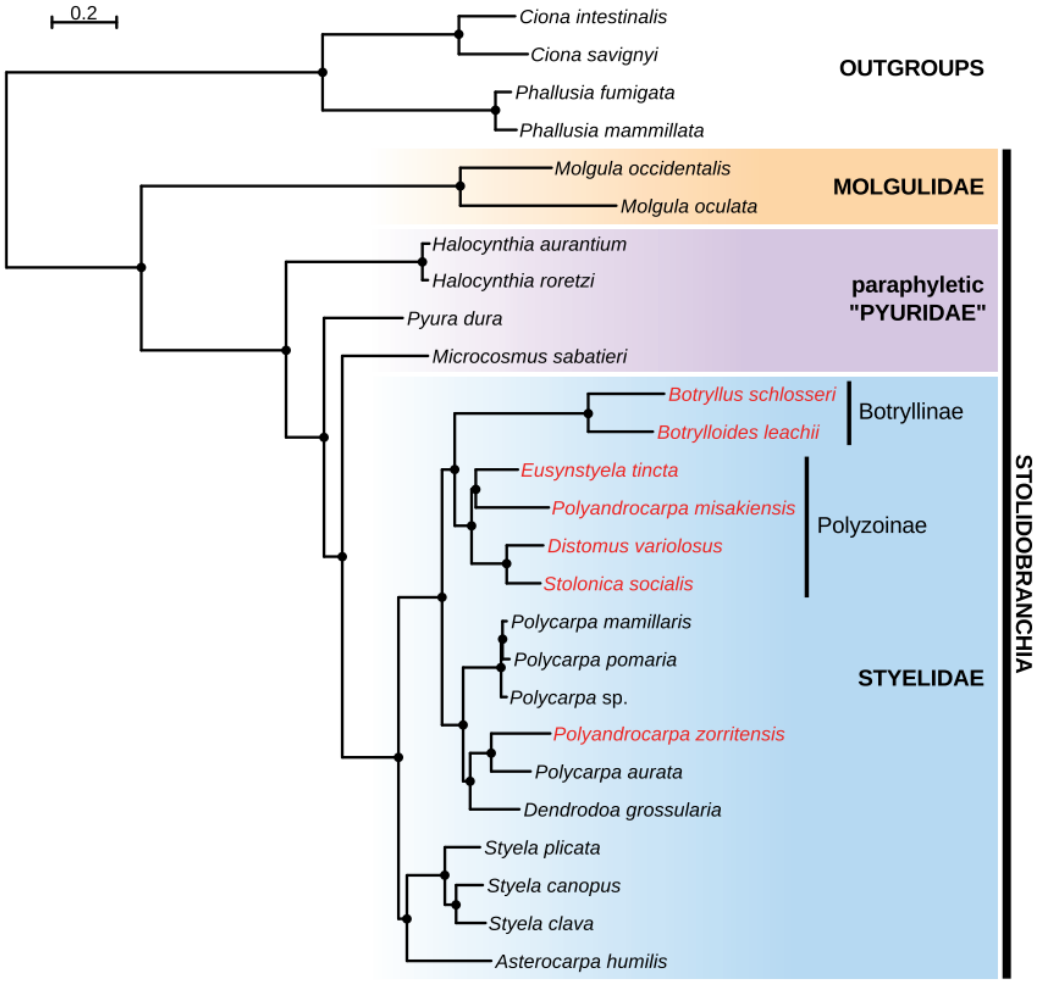


Figure 1.22: Phylogenetic relationships between Stolidobranchia, with a focus on Styelidae. Colonial species (capable of asexual reproduction via NED) are depicted in red, solitary species in black. (from Alie' et al 2018).

1.6.1Cellular and Molecular signatures of NED in Styelidae: state of the art

- ***Molecular signature of peribranchial budding***

The molecular regulation of peribranchial budding has been investigated in *Botryllus schlosseri*. To study the redeployment of embryonic developmental genes during budding, the expression of transcription factors implicated in germ layer specification has been delineated, showing a partial redeployment of these genes from the double vesicle stage onward (Ricci et al., 2016, 2016). siRNA knockdown of one of these genes, GATAa, resulted in budding arrest at early stages, before the double vesicle formation (Ricci et al., 2016). Embryonic genetic modules are also redeployed during *B.schlosseri*myogenesis (Prünster et al., 2019b) and neurogenesis (Prünster et al., 2019a).

Retinoic acid (RA) signals have been suggested to induce organogenesis in *Polyandrocarpa misakiensis*. Treatments with RA allows the formation of aberrant buds, with duplication of anteroposterior axis and supernumerary organs in the distal region, where normally morphogenesis does not occur (Kawamura et al., 1993). Homologous RA receptors, RAR and RXR, are expressed in the developing area, at the level of the epidermis and in a particular morphotype of mesenchymal cells, the glomerulocytes. Glomerulocytes are large cells that originate from epidermal cells (Hirose and Mukai, 1992; Mukai et al., 1990), and they were suggested to be the target of RA signaling in the bud (Kamimura et al., 2000). Kamimura and colleagues suggested the following model: the RA produced by the epidermis is incorporated in the glomerulocytes, which in response secrete transdifferentiation factors inducing organogenesis in the inner vesicle (Kamimura et al., 2000). One of these factors is the protein tunicate retinoic inducible modular protease TRAMP, expressed in glomerulocytes and upregulated in treatment with RA (Ohashi et al., 1999). TRAMP has been suggested to promote the transdifferentiation and proliferation of the atrial epithelium (Kamimura et al., 2000).
Interestingly, when a *P. misakiensis* bud is cut in two halves, each piece can regenerate the missing part (Kawamura et al., 2008; Taneda, 1985). In particular, the anterior fragment is able to completely regenerate the digestive tract (Kaneko et al., 2010). As during normal peribranchial budding, RA inhibitors suppress the gut regeneration, while RA treatments rescue it. TRAMP seems to mediate this process: the protein is expressed in peribranchial epithelium and mesenchymal cells at the wound site. This suggests the role of retinoic acid signaling in peribranchial epithelium transdifferentiation during WBR in *P. misakiensis* (Kaneko et al., 2010).

- ***Role of mesenchymal cells during peribranchial budding***

Different authors suggested the participation of hemoblasts, a population of undifferentiated haemocytes (blood cells) in organogenesis during the peribranchial budding. In *Symplegma reptans*, hemoblasts accumulate close to the forming oral siphon and differentiate in muscular cells forming the oral sphincter (Sugino et al., 2007). Epidermal cells make contact with the differentiating hemoblasts thanks to cellular projections, suggesting an inductive role of the epidermis. In *Botryllus schlosseri*, the heart primordium seems to form from an aggregate of hemoblasts that soon reorganizes in a hollow tube (Nunzi et al., 1979). In this same species, hemoblasts showing aldehyde dehydrogenase (ALDH) activity transplanted in a colony are integrated into the budding tissues (Laird et al., 2005).
Hemoblasts have also been proposed as germline precursors during the budding cycle. In *Botryllus spp.*, hemoblasts migrate to the secondary bud and adhere to the atrial epithelium; here they proliferate and differentiate into testis and ovarium (Mukai and Watanabe, 1976; Sabbadin

and Zaniolo, 1979; Sunanaga et al., 2006).These germline precursors express *vasa* and *piwi* (Brown et al., 2009; Sunanaga et al., 2010) and show high ALDH activity (Laird et al., 2005).

In *Polyandrocarpa misakiensis*, hemoblasts interact with the inner epithelium at the level of the organ rudiments (Kawamura et al., 1995). In chimera experiments, when an irradiated bud fragment is fused with a non-irradiated one, mesenchymal cells from the unirradiated piece aggregate on the irradiated epithelium and proliferate. Subsequently, from this cellular mass the gut rudiment forms (Kawamura et al., 1995). Kawamura and Nakauchi (1991) suggested that hemoblasts may be integrated into the developing organs through a mesenchymal-to-epithelial transition (KAWAMURA and NAKAUCHI, 1991). A key molecule in this process is the receptor for activated protein kinase C1, RACK1 (known in other species to induce the molecular cascades that promote cell-cell adhesion and lead to mesenchymal-to-epithelial transition - MET) (Swaminathan and Cartwright, 2012). RACK1 is expressed in the organogenetic epithelial cells and hemoblast aggregations (Tatzuke et al., 2012). RACK1 RNAi on developing buds causes the formation of the monolayered peribranchial epithelium, lacking hemoblast aggregates (Tatzuke et al., 2012).

In *P. misakiensis*, interaction between the inner vesicle and hemoblasts is assured by a calcium-dependent lectin of 14kDa, the tunicate-derived C-type lectin TC14 (Kawamura and Sugino, 1999; Suzuki et al., 1990). The epithelial cells of the inner vesicle contain lectin granules; during budding development, TC14 lectin is released by these granules in the mesenchymal space, forming an external matrix ECM (Kawamura et al., 1991). Single small hemoblasts (4-5um) were heavily positive to anti-TC14 antibody and were associated with the ECM. When buds were allowed to develop in the presence of a high concentration of anti-TC14 antibody to deplete the lectin, the buds did not undergo organogenesis. In these specimens, the hemoblast aggregates looked smaller and they were not able to link to the inner vesicle, remaining scatteredly distributed in the mesenchymal space; this suggests that the lectin TC14 is necessary to establish the contact between the ECM (and consequently the inner vesicle) and the hemoblast aggregates, and that this contact is required for the onset of organogenesis (Kawamura et al., 1991).

In some species, mesenchymal trophocytes have been described. In *Polyzoa vesiculiphora*, Fujimoto and Watanabe (1976) identified a morphotype of mesenchymal cells called granular amoebocytes containing glycogen granules and lipid droplets in their cytoplasm (Fujimoto and Watanabe, 1976a). In *P. misakiensis* when the double vesicle was separated from the parental zooid, the distal region appeared engulfed of mesenchymal cells containing pigment granules. These cells were similar to the granular amoebocytes of *Polyzoa vesciculiphora* (Kawamura and Watanabe, 1982) and have been renamed macrogranular leukocytes by Ballarin and Kawamura

(Ballarin and Kawamura, 2009). These cells are probably acting as energy supplies for the bud, which is not able to feed during the developing process until siphonal opening.

- ***Mesenchymal cells as progenitors of vascular budding***

WBR in *Botrylloides digenesis* is possible thanks to a population of pluripotent undifferentiated hemocytes, the hemoblasts. These cells express genes of the GMP as *pou3*, *vasa, Piwi 2*, (Kassmer et al., 2019a; Rinkevich et al., 2010) and si-RNA experiments against *Piwi* block the regenerative process (Rinkevich et al., 2010). The progenitors of WBR also express a marker of mammalian stem cells (Krebsbach and Villa-Diaz, 2017), integrin-alpha-6 (Iα6). Iα6+ hemoblasts actively proliferate and increase in number during early stages of WBR (Kassmer et al., 2019a; Rinkevich et al., 2007). The hemocyte aggregates (also called regeneration foci in Kassmer et al., 2019a)) are originated by clusters of Iα6+ cells, which form the hollow vesicle by proliferation. Treatments with Mitomycin C (MMC) stopped the regenerative process at initial stages by blocking cell proliferation. However, WBR was rescued injecting Iα6+ cells from non-treated donors, and a single Iα6 positive hemoblast was capable of rescuing WBR in 20% of treated specimens (Kassmer et al., 2019). To prove the involvement of these cells in the formation of the regenerating foci, Iα6 positive cells labeled with EdU were injected in treated samples; EdU positive cells were found in the hemocyte aggregates and in cells of the inner vesicle in later stages (Kassmer et al., 2019).

In *Botrylloides violaceus*, at the onset of organogenesis Piwi-positive undifferentiated mesenchymal cells were observed associated with the budding epithelium (Brown et al., 2009). These positive cells were also proliferating, as shown by immunostaining with an anti-PCNA antibody (Brown et al., 2009). Some epithelial cells in the developing bud were Piwi-positive too, suggesting that the Piwi-positive hemocytes that cluster next to the bud may be integrated into the inner vesicle and participate in organogenesis (Brown et al., 2009).

Retinoic acid (RA) signaling has been proposed to regulate vascular budding in *B*. *diegensis* (Rinkevich et al., 2007). A homolog of RA receptor is expressed in hemocyte aggregates and in the inner vesicle during WBR; the regenerative process was blocked when an inhibitor of RA synthesis was used, while treatments with RA resulted in the formation of multiple vascular buds (Rinkevich et al., 2007). In *B. diegensis* molecular signals involved in embryonic development have been proposed to induce activation of hemoblasts at the onset of WBR. Developmental signaling pathway genes, such as *Wnt*, *TGFb*, *Hedgehog* and *Notch*, were upregulated in the early stages of WBR, 24h post-injury, as well as genes of cell adhesion and apoptosis (Zondag et al., 2016). Inhibition of *Wn*t and *Notch* signaling with drug treatments blocked the regenerative process at early stages, but when the inhibitors were removed the

vascular buds continued to regenerate (Kassmer et al., 2019a). During drug treatments, the mesenchymal progenitors stopped to proliferate and their numbers decreased, while after the inhibitor was removed the proliferation activity recovered. This suggests that *Wnt* and *Notch* signaling are responsible for progenitor proliferation and activation, and when these pathways are blocked these cells are still present but in a quiescent state (Kassmer et al., 2019a).

In *Botryllus schlosseri* some transcription factors that specify germ layers during embryogenesis, such as *GATAa*, *GATAb*, Otx, *Tbx2/3* are expressed in the regenerating buds (Ricci et al., 2016). From the double vesicle stage, the expression profiles of these genes are comparable to the ones in peribranchial budding, suggesting a common developmental program for these two forms of non-embryonic development.

1.7 Thesis objectives

While the mechanisms of NED have been investigated in the monophyletic clade of peribranchial budders - i.e. *Botryllus schlosseri*, *Polyandrocarpa misakiensis,* and *Botrylloides diegensis*- the independent evolution of NED in *Polyandrocarpa zorritensis* was almost unknown. Different authors (Brunetti, 1978; Brunetti and Mastrototaro, 2004; Van Name, 1931) observed that buds formed along a vascular stolon and referred to the NED in this species as a form of vascular budding. Yet, these descriptions were based only on external observations and were not exhaustive on the nature of cells and/or tissues that triggered the budding. A more detailed description of the process in *Polyandrocarpa zorritensis* was required in order to infer the evolution of NED in the family Styelidae. In Chapter 2, I described the life cycle and the NED in *Polyandrocarpa zorritensis*, discovering a previously undescribed type of NED that I named "vasal budding". During vasal budding, the new zooids originate from a portion of the vascular epidermis in concert with undifferentiated mesenchymal cells. To explore the role of the mesenchymal cells, in Chapter 3 I characterized the different cell morphotypes present in *P.zorritensis* and I analyzed their distribution and proliferation activity at different stages of budding. These results together with preliminary experiments suggest that hemoblasts are involved in vasal budding, perhaps as putative stem cells. Chapter 4 is dedicated to the description of dormancy in *P.zorritensis.* I describe the abiotic environmental paramethers that drive *P.zorritensis* into and ouf of a dormant stage (spherule), and describe anatomically the different phases of these processes. The same approach has been repeated in another colonial species, *Clavelina lepadiformis*, which independently evolved NED and dormancy strategies. In collaboration with other members of the laboratory, we have started to search for dormancy-specific genes, by performing a differential gene expression analyses in dormant and non-dormant stages in both species. The preliminary data of these analyses is briefly presented. Finally, Chapter 5 contains a general discussion that intersects the different questions and results obtained in the previous chapters and present it in a broader perspective.

Chapter 2

Novel budding mode in Polyandrocarpa zorritensis: a model for comparative studies on asexual development and whole-body regeneration

Chapter 2 of my thesis is constituted of one manuscript that describes the life cycle and the morphological events occurring during early stages of NED in *Polyandrocarpa zorritensis*. At the beginning of my PhD, little informations were available on the budding process in *P. zorritensis*. Van Name described for the first time this species in Peru and named it *Stolonica zorritensis* (Van Name, 1931). Only later the author renamed the species with the actual name (Van Name, 1945). Van Name documented the new species as colonial and reported the presence of a stolon connecting all the zooids. Afterward, Brunetti described the buds produced along the stolons as spherical "*evaginations of the vascular wall*" (Brunetti, 1978). Based on external observations, Brunetti and Mastrototaro speculated that budding in *P. zorritensis* is a form of vascular budding (Brunetti and Mastrototaro, 2004). However, a detailed description of the cells and tissues responsible for NED in this species was lacking. The phylogenetic analysis of the family Styelidae produced by the laboratory exposed an independent acquisition of NED in *P. zorritensis* (Alié et al., 2018) (see Appendix 2), and made it necessary a further characterization of the process.

In this Chapter, I described the life cycle of *Polyandrocarpa zorritensis* under laboratory conditions. I reported the presence of a dormant stage, the spherule, which will be the object of a deepened study in Chapter 4. Then, I described a new type of NED, the vasal budding. Vasal budding is so far reported only in *Polyandrocarpa zorritensis*, in which the inner vesicle is originated from an invagination of the vascular epithelium and undifferentiated mesenchymal cells. The morphology and the dynamic of these cells will be the object of Chapter 3.

Scelzo *et al. EvoDevo* (2019) 10:7
https://doi.org/10.1186/s13227-019-0121-x

EvoDevo

RESEARCH Open Access

Novel budding mode in *Polyandrocarpa zorritensis*: a model for comparative studies on asexual development and whole body regeneration

Marta Scelzo[1†], Alexandre Alié[1†], Sophie Pagnotta[2], Camille Lejeune[1], Pauline Henry[1], Laurent Gilletta[1], Laurel S. Hiebert[1,3], Francesco Mastrototaro[4] and Stefano Tiozzo[1*]

Abstract

Background: In tunicates, the capacity to build an adult body via non-embryonic development (NED), i.e., asexual budding and whole body regeneration, has been gained or lost several times across the whole subphylum. A recent phylogeny of the family Styelidae revealed an independent acquisition of NED in the colonial species *Polyandrocarpa zorritensis* and highlighted a novel budding mode. In this paper, we provide the first detailed characterization of the asexual life cycle of *P. zorritensis*.

Results: Bud formation occurs along a tubular protrusion of the adult epidermis, the stolon, in a vascularized area defined as budding nest. The bud arises through a folding of the epithelia of the stolon with the contribution of undifferentiated mesenchymal cells. This previously unreported mode of bud onset leads to the formation of a double vesicle, which starts to develop into a zooid through morphogenetic mechanisms common to other Styelidae. The budding nest can also continue to accumulate nutrients and develop into a round-shaped structure, designated as spherule, which represents a dormant form able to survive low temperatures.

Conclusions: To understand the mechanisms of NED and their evolution, it is fundamental to start from a robust phylogenetic framework in order to select relevant species to compare. The anatomical description of *P. zorritensis* NED provides the foundation for future comparative studies on plasticity of budding and regeneration in tunicates.

Keywords: Ascidian, Evolution, Non-embryonic development, Tunicate, Vasal budding

Introduction

In addition to sexual reproduction and embryogenesis, a significant number of metazoan species are able to propagate asexually or regenerate completely and form new bodies through non-embryonic development (NED) [1–3]. Many species of tunicates, the sister group of vertebrates [4], are capable of asexual development and whole body regeneration [5]. Tunicates include solitary species, which can only reproduce sexually, and colonial species that, in addition to sexual reproduction, are also able to propagate asexually and have extensive regenerative capabilities [5–7]. In tunicates, asexual reproduction and whole body regeneration occur by clonal replication whereby somatic tissues undergo a budding process that leads to the development of clonally related zooids. Current hypotheses for the phylogeny of the tunicates suggest many convergent acquisitions of NED across the whole subphylum [8–10], a view further supported by the diversity of tunicate budding modes. In fact, mechanisms of asexual development differ substantially among colonial species and involve differences in the body region where new buds appear, and differences in the types of

*Correspondence: tiozzo@obs-vlfr.fr
†Marta Scelzo and Alexandre Alié have contributed equally to this work
[1] CNRS, Laboratoire de Biologie du Développement de Villefranche-sur-Mer (LBDV), Sorbonne Université, 06230 Villefranche-sur-Mer, France
Full list of author information is available at the end of the article

© The Author(s) 2019. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

cells and tissues that contribute to the development of new zooids [11–15]. Diversity in budding mode likely contributes to the wide variation in colony organization and integration among zooids found across the subphylum [16].

In order to dissect the mechanisms of budding in ascidians and to understand the acquisition and loss of complex characters such as asexual propagation and whole body regeneration, it is necessary to broaden our knowledge of budding modes among different species, and to compare them in a phylogenetic perspective relying on robust trees. Most of our current knowledge of tunicate NED comes from a limited range of species. One of the most well-studied colonial ascidians belongs to the family Styelidae, *Botryllus schlosseri* (Pallas 1776). In this species, the bud originates from an evagination and thickening of two epithelia, the peribranchial epithelium and the surrounding epidermis, with the possible participation of mesenchymal cells [17]. The evagination arches progressively to form a double vesicle (Fig. 1): the outer vesicle derives from the adult epidermis and gives rise to the epidermis of the new zooid, while the inner vesicle derives from the peribranchial epithelium and undergoes morphogenetic changes that eventually give rise to the internal organs of the functional new

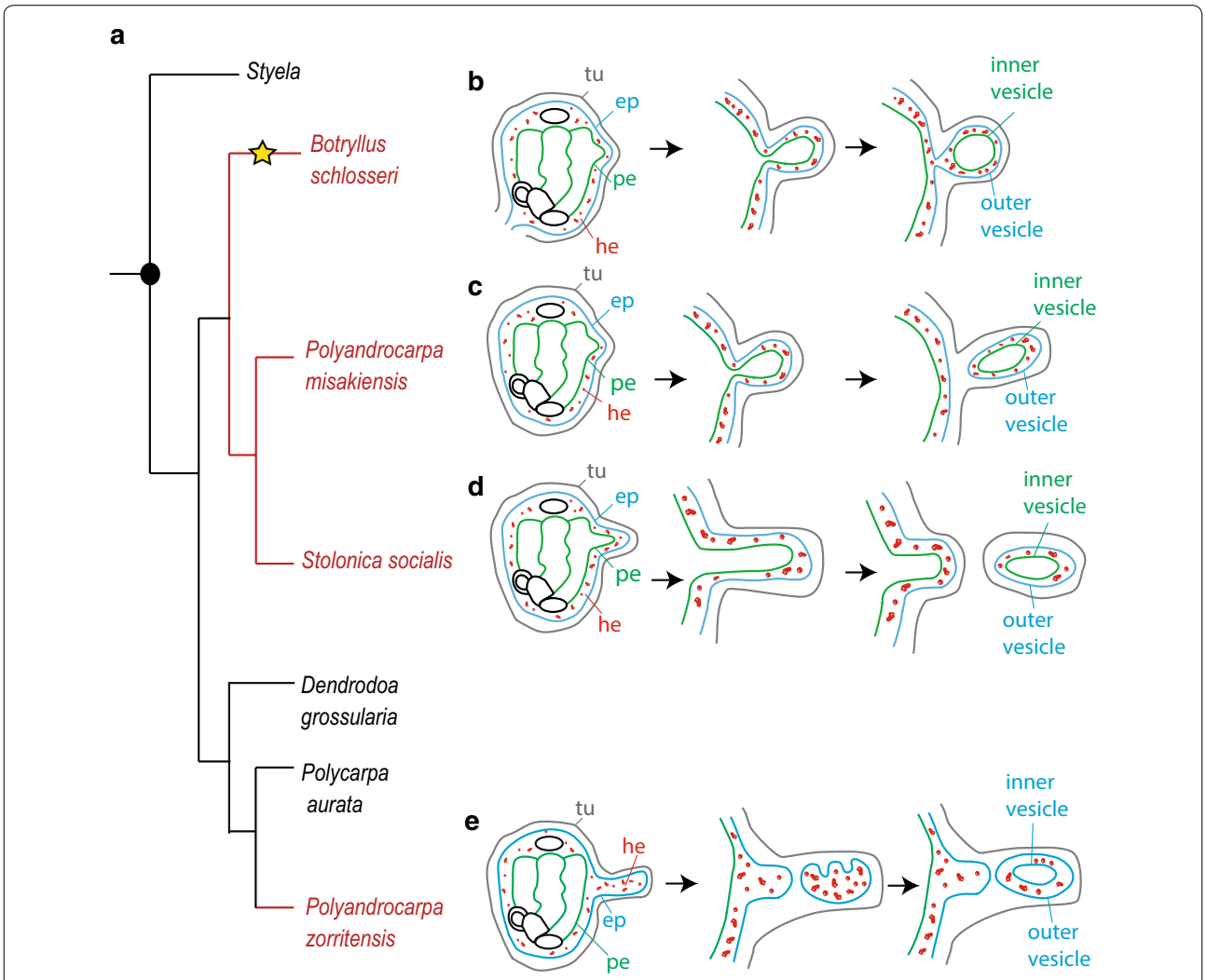


Fig. 1 Phylogeny of the family Styelidae and diversity of budding modes. **a** Simplified phylogenetic tree of Styelidae family (modify from Alié et al. 2018), showing the distribution of solitary species (shown in black) and colonial species (in red). The yellow star indicates the branches in which vascular budding evolved. **b**–**d** Schematic representation of peribranchial budding in *Botryllus schlosseri* (**b**), *Polyandrocarpa misakiensis* (**c**), and *Stolonica socialis* (**d**), from peribranchial evagination to double vesicle stage. **e** Schematic representation of the budding process in *Polyandrocarpa zorritensis* from the formation of vascular stolon to double vesicle stage. (*tu* tunic, *ep* epidermis, *he* haemocytes, *pe* peribranchial epithelium)

zooid. Circulating mesenchymal cells aggregate within the folding epithelium and proliferate, participating in the formation of the organs [18–20]. The resulting bud remains attached to the colony by a short peduncle. This mode of reproduction, called peribranchial budding or blastogenesis, permits colonial growth and periodic zooidal renewal. In the family Styelidae, peribranchial budding is the main form of asexual development, with some minor variations between species (Fig. 1). For instance, in *Polyandrocarpa misakiensis* [21], mesenchymal cells are thought to transdifferentiate and integrate into the inner vesicle and the evaginated bud separates from the parent [22, 23]. In *Stolonica socialis* (Hartmeyer 1903), the atrial epithelium and epidermis protrude and extend considerably from the zooid body forming a process called a stolon [24]. When the tip of the stolon reaches a certain mass, it is constricted off forming a bud disconnected from the maternal zooid [25].

In a recent phylogenetic reconstruction of the family Styelidae, we revealed that all the species undergoing peribranchial budding belong to a single clade (Fig. 1, from [10]). Within this clade, the genera *Botryllus*, *Botrylloides,* and *Symplegma* are also able to regenerate via a second budding mode, vascular budding, in which the bud originates from the aggregation of circulating mesenchymal cells interacting with the vascular epithelium [26, 27]. The above-cited phylogeny of Styelidae also revealed that the colonial species *Polyandrocarpa zorritensis* (Van Name 1931) does not group with the clade of "peribranchial budders" but is rather more closely related to solitary styelid species (Fig. 1). These results suggest a convergent acquisition of NED in *P. zorritensis*, and prompted us to further describe this process [10, 28]. Preliminary histological analyses revealed that, as in other colonial styelids, a double-layered vesicle is formed prior to bud organogenesis [10]. However, in *P. zorritensis*, the inner vesicle does not derive from peribranchial epithelium, but it originates from the epidermis of the circulatory system, which has ectodermal embryonic origin [7]. This undescribed mode of budding not only supports the independent acquisition of coloniality and makes *P. zorritensis* a key species for comparative studies aiming to understand the distribution of coloniality in the group, but also opens up new territory to explore the developmental mechanisms behind the capacity for budding.

In this paper, we provide the first detailed characterization of the asexual life cycle of *Polyandrocarpa zorritensis* and we describe the ontogenesis of this unreported form of ascidian budding that we name "vasal budding."

Materials and methods

Sample collection and specimen husbandry

Colonies of *Polyandrocarpa zorritensis* were collected in the harbors of Taranto (Mare Piccolo, 40°28′48.29″N 17°16′4.33″E) and La Spezia (Assonautica Benedetti, 44°06′10.7″N 9°49′34.5″E) (Italy). Colonies in the field are composed of feeding zooids and often a layer of smaller orange globular non-feeding structures that we have termed *spherules.* The spherules were gently separated from the colony and stored in circulating sea water system at 11 °C (at salinity of 39–40‰). New zooids were grown by transferring the spherules to glass microscope slides at 24 °C. We use the term "hatching" for the emerging of a new zooid from a spherule. The animals were maintained at 24 °C in open sea water systems and fed with a mix of living algae (*Tisochrysis lutea*) and concentrated algae (Isochrysis 100 and Shellfish Diet 1800, Red Maricolture Inc).

Light and transmission electron microscopy (TEM)

Inclusion of *Polyandrocarpa zorritensis* specimens for paraffin sectioning and hematoxylin/eosin staining were performed as described in Alié et al. [10]. Samples for semi-thin and ultra-thin sectioning were fixed with a 3% solution of glutaraldehyde in sodium cacodylate buffer (pH 7.3), post-fixed with osmium tetroxide (OsO_4) 1% cacodylate buffer, dehydrated using acetone, and embedded in epoxy resin. An UltracutE Reichert ultramicrotome was used for the sectioning. Semi-thin sections (1–2 µm) were stained with a solution of methylene blue 1% in sodium tetraborate + azur II 1% in water (v/v), and images were analyzed on a ZEISS microscope Axio ImagerA2. Ultra-thin sections (60–80 nm) were contrasted with uranyl acetate and lead citrate and observed under a transmission electron microscope TEM JEM 1400 JEOL, and imaged using a MORADA SIS camera (Olympus).

Effect of low-temperature storage and spherule size on hatching and growth

To test whether storing the spherules at 11 °C had any negative effect on the hatching rate, two different batches of spherules were used. The first corresponds to spherules collected in Spring 2016 (April) and directly transferred to glass slides at 24 °C in the laboratory. The second batch corresponds to spherules that were kept at 11 °C in circulating sea water for 7 months before being transferred to 24 °C. After 7 days, the number of the zooids that were generated in relation to the number of spherules sowed was counted. After 13 days, the number of zooids bearing stolons was counted.

In the following experiments, the size of zooids and budding nests (a term we use for newly formed pre-buds made of vascular ampullae aggregations) was estimated

photometrically, by delineating their periphery manually and measuring the area using ImageJ software [29]. To test the effect of spherule size, we arbitrarily split 45 spherules into three categories of size ("small" from 1 to 1.5 mm in diameter; "medium" from 1.5 to 2 mm in diameter; "big" 2 mm in diameter and larger) and then monitored the size of zooids derived from these spherules, considering also the number of stolons born by each zooid. Statistical significance was assessed using a Mann–Whitney test. To test the effect of budding nest size, 32 budding nests were followed during 6 days post-abscission (i.e., separation of the bud from its parental zooid by cutting the stolonial vessel with a razor blade) and the size of zooids was then measured. A linear regression curve was drawn using Excel software.

Results

Asexual life cycle of *P. zorritensis* in captivity

The budding process in *Polyandrocarpa zorritensis* has been briefly described by Brunetti and Mastrototaro [28] and by Alié et al. [10]; however, detailed descriptions of the colonial life cycle and bud histology are still missing. Wild colonies are composed of a few hundred zooids clustered together and connected by a dense network of orange-colored stolons covered by a thick tunic (Fig. 2a, b). Most of these stolons have the shape of a pearl necklace, with spherical structures of 1 to 2.5 mm in diameter connected to each other by thin segments of stolon (Fig. 2c). Each of these spherules can be easily separated from the colony—their stiffness permits their manipulation without damage—and stored at 11 °C for up to several months without any detectable morphological alteration.

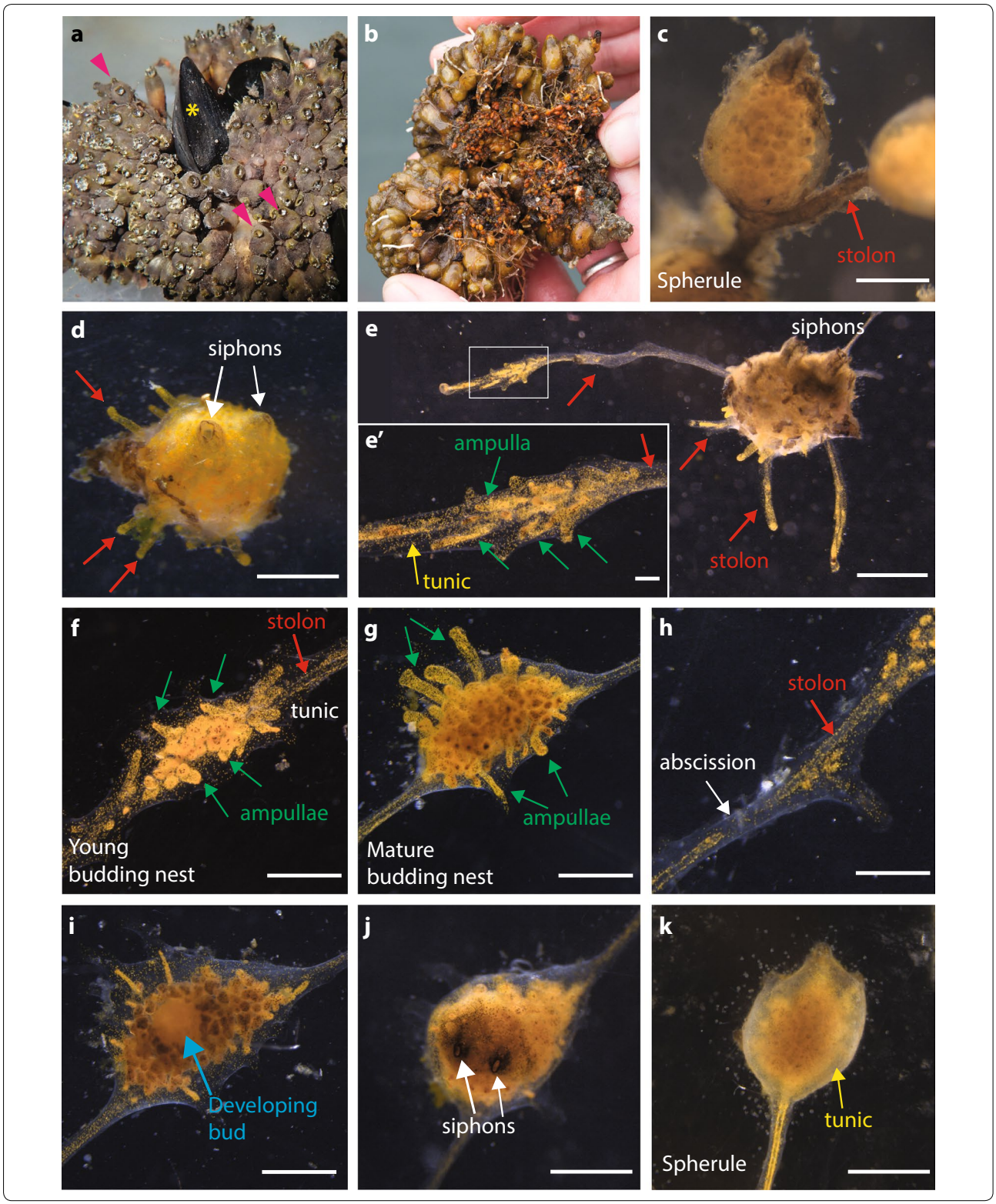
When transferred to glass slides at 24 °C, the spherules show morphological changes and eventually give rise to zooids. After 1 to 5 days, the spherule loses its round shape, becomes more elongated, and shows lighter orange coloration (Additional file 1: Figure S1); after 6 to 10 days two open siphons become visible (Fig. 2d) indicating that filter feeding activity has initiated. Up to 12 months at 11 °C, spherules are still able to "hatch" and develop into filtering zooids, although this process is slightly slower than freshly collected ones (Additional file 2: Figure S2). When a colony is placed at 11 °C, most of the zooids regress and die, while only the spherules remain unaffected.

During the development from spherule to zooid, the body surface sprouts several stolons along which buds will appear. The stolons begin as small protrusions that allow the attachment of the zooid to the substrate (Fig. 2d). Usually, one or several of these body protrusions keep growing producing elongated stolons running along the substrate (Fig. 2e). The size of a zooid as well as the number of the emitted stolons is correlated to the size of the spherule from which it develops (Additional file 3: Figure S3). The stolon consists of a monolayered epidermal vessel covered with a thin layer of tunic. Circulating mesenchymal cells, hemocytes, flow through the stolonal lumen switching direction periodically (Additional file 4: Movie 1). Unlike the stolons of other ascidian species [30, 31], no central septum is present in the lumen of the *P. zorritensis* stolon. Along the whole stolon, anastomosed oval structures named ampullae grow parallel to the central vessel (Fig. 2e–e'). In some points along the stolon, the number of ampullae increases and their orientation changes, forming a tridimensional highly pigmented dome-like cluster, here named "budding nest" (Fig. 2f, g). Bud development begins at the center of these structures. Along a single stolon, it is possible to observe several budding nests, which emerge without any clear temporal or spatial order, but rather form upon substrate irregularities such as the angle of a Petri dish or scratches on a glass slide (personal observation).

Bud development is triggered upon complete isolation of the budding nest from the colony, which occurs spontaneously by abscission of the stolonial epidermis and consequent interruption of hemocyte circulation between the colony and the nest (Fig. 2h). Under laboratory conditions, budding can be triggered by cutting the stolon with a razor blade. About 24 h after abscission, the first signs of budding become externally visible: the number of ampullae decreases and a new zooid grows in the center of the nest (Fig. 2i). Within 4–5 days after the abscission, all the ampullae have regressed and

(See figure on next page.)

Fig. 2 Asexual cycle of *Polyandrocarpa zorritensis* in captivity. **a** *Polyandrocarpa zorritensis* colony. Adult zooids can be found on other animals, such as mussels (yellow asterisk). Pink arrowheads show examples of individual zooids. **b** Bottom side of the same colony as in (**a**), showing the dense network of stolons and spherules. **c** Detail of a spherule and the stolon (red arrow) connecting the spherule to the rest of the colony. **d** One spherule one week after being transferred at 24 °C. The two siphons (white arrows) are open and the protrusions (red arrows) that will attach to the substrate are recognizable. **e** One completely developed zooid with several stolons (red arrows). **e'** Close-up view of one stolon. It is possible to recognize the main blood vessel (red arrow) and the ramified ampullae connected to it (green arrows), oriented in the same direction as the main vessel. The tunic (yellow arrow) covers the whole structure. **f** Young budding nest. The ampullae (green arrows) increased in number along the vessel (red arrow). **g** Mature budding nest, composed of more compact ampullae (green arrows) that form a dome, highly pigmented on the top. **h** Detail of a vessel at the abscission site (white arrow). **i** Bud (blue arrow) developing inside the nest after abscission. **j** Newly budded zooid with open siphons (white arrows). **k** Spherule obtained by transferring a budding nest from 24 °C to 11 °C. (Scale bars **c**–**e**: 1 mm; **e'**: 100 µm; **f**–**k**: 1 mm)


a
b
c
stolon
Spherule
d
siphons
e
siphons
e′
ampulla
tunic
stolon
f
stolon
tunic
ampullae
Young
budding nest
g
ampullae
Mature
budding nest
h
stolon
abscission
i
Developing
bud
j
siphons
k
tunic
Spherule

two siphons open at the top of the newly formed zooid (Fig. 2j), while the axis defined by the endostyle is always perpendicular to the substrate, the axis along the two siphons is established randomly in respect to the orientation of the stolon. Just as we observed with spherules, the size of a zooid is proportional to the size of the nest from which it comes (Additional file 5: Figure S4). If abscission does not occur, the budding nest keeps growing, and if transferred at 11 °C, it eventually transforms into a dormant spherule (Fig. 2k).

The origin of budding: formation of the budding nest and the epidermal accumulation of reserve nutrients

During formation of a budding nest, the epidermis of the stolonial vessel and its surrounding ampullae undergo drastic histological and cellular changes. Histological sections confirmed the absence of a mesenchymal septum in the stolon. In the growing stolon, before nest formation, the stolonial wall is constituted by a monolayered epidermis composed of flat cells of 6–7 µm, with the highly electron-dense basal lamina facing the vascular lumen, while the apical side is covered with a layer of tunic 5–10 µm thick (Fig. 3a–c). Cells contain extensive RER (rough endoplasmic reticulum) and an abundant Golgi apparatus (Fig. 3c). At the basal pole, elaborated intercellular contacts connect the nearby cells (Fig. 3b, black arrows). The continuity with the zooid epidermis and the orientation of the epithelial cells confirm the epidermal nature of the stolon rather than peribranchial expansion as in other styelids (e.g., *Stolonica socialis*, [25]) (Fig. 3a).

When a budding nest starts forming, the tunic thickens (to around 100 µm) and ramifying ampullae accumulate around the central vessel (Figs. 2f, 3d). Epithelial cells of both the central vessel and the ampullae become roundish, 10 µm thick and connected to neighbor cells by tight junctions at their basal halves (Fig. 3e, f, black arrows). RER becomes more prominent, mitochondria are abundant, and the cytoplasm is enriched in spherical electron-dense bodies of different dimensions (0.5–2 µm) (Fig. 3e, f, red arrows) in the vicinity of which very numerous smaller granules (around 0.5 nm) are also visible (Fig. 3e, f, green arrows).

When the budding nest reaches a dome shape of 1–2 mm in diameter, the number and density of ampullae clearly increase (Figs. 2g, 3g). At this stage, the central vessel and the ramifying ampullae are indistinguishable from each other in histological sections. The epidermal cells change their original round shape and become rather cuboidal, 5–10 µm thick, and in close contact with their neighbors (Fig. 3h). RER, mitochondria, and Golgi become indistinguishable, while numerous of the above-mentioned spherical electron-dense bodies were observed (0.5–2 µm) (Fig. 3h, red arrows) in addition to membrane-free inclusions with heterogeneous content (Fig. 3h, yellow stars) in which remnants of the Golgi apparatus are sometimes visible (Fig. 3i).

Budding nests and dormant spherules have very similar histological and cytological properties (Fig. 3j–l), though the level of differentiation observed during nest formation is even more remarkable in spherules: their cytoplasm is completely filled with dark granules of 2–5 µm and with very amorphous and heterogeneous inclusions (Fig. 3k, l, red arrows and yellow stars, respectively), while smaller granules observed in budding nests (0.5–2 µm) are not visible anymore. The tunic fibers are more compacted (Fig. 3k), potentially conferring the characteristic spherule stiffness.

Vascular epidermis and mesenchymal cells participate in vasal bud formation

After abscission, the stolonial epidermis and mesenchymal cells contained in the budding nest undergo cellular modifications and morphogenetic movements that eventually lead to the formation of a new individual. We called this process "vasal budding," and we described the main ontological steps.

Step1: swelling

From 0 to 20 h post-abscission (hpa), budding starts by a phenomenon that we called "swelling" (Fig. 4a, b). A more or less spherical cavity about 300 µm in diameter (Fig. 4b) appears at the center of the nest, resulting from inflation of the stolonial vessel. This cavity, which is delimited by a monolayered epithelium, becomes polarized: cells at the top side (i.e., opposite to the substrate) become columnar (approximately 15 µm high × 5 µm wide), while cells at the bottom side (i.e., facing the substrate) are cuboidal (6–8 µm) (Fig. 4c, d, respectively). In comparison with the epithelia of the surrounding ampullae, the cells of the swelling cavity contain few inclusions and a higher nucleo-cytoplasmic ratio (compare *ov* and *a* in Fig. 4d). Heterogeneous populations of mesenchymal hemocytes are visible inside the cavity, inside the connections with ampullae, and inside the ampullae (Fig. 4b,d; Additional file 6: Figure S5a).

Step 2: invagination

From 20 to 22 hpa, an epithelial invagination process starts in the swelling vesicle (Fig. 4a, e). At the thicker pole of the cavity (top side), a circular region of the epidermis begins to unfold, which appears as two invaginations on histological sections (Fig. 4e, black arrows). Cells located at hinge points have a typical bottle shape, with their basal surface wider than the apical side (Fig. 4f), while cells at the invaginating edges (Fig. 4g) are wedge-shaped with their basal surface narrower than the apical

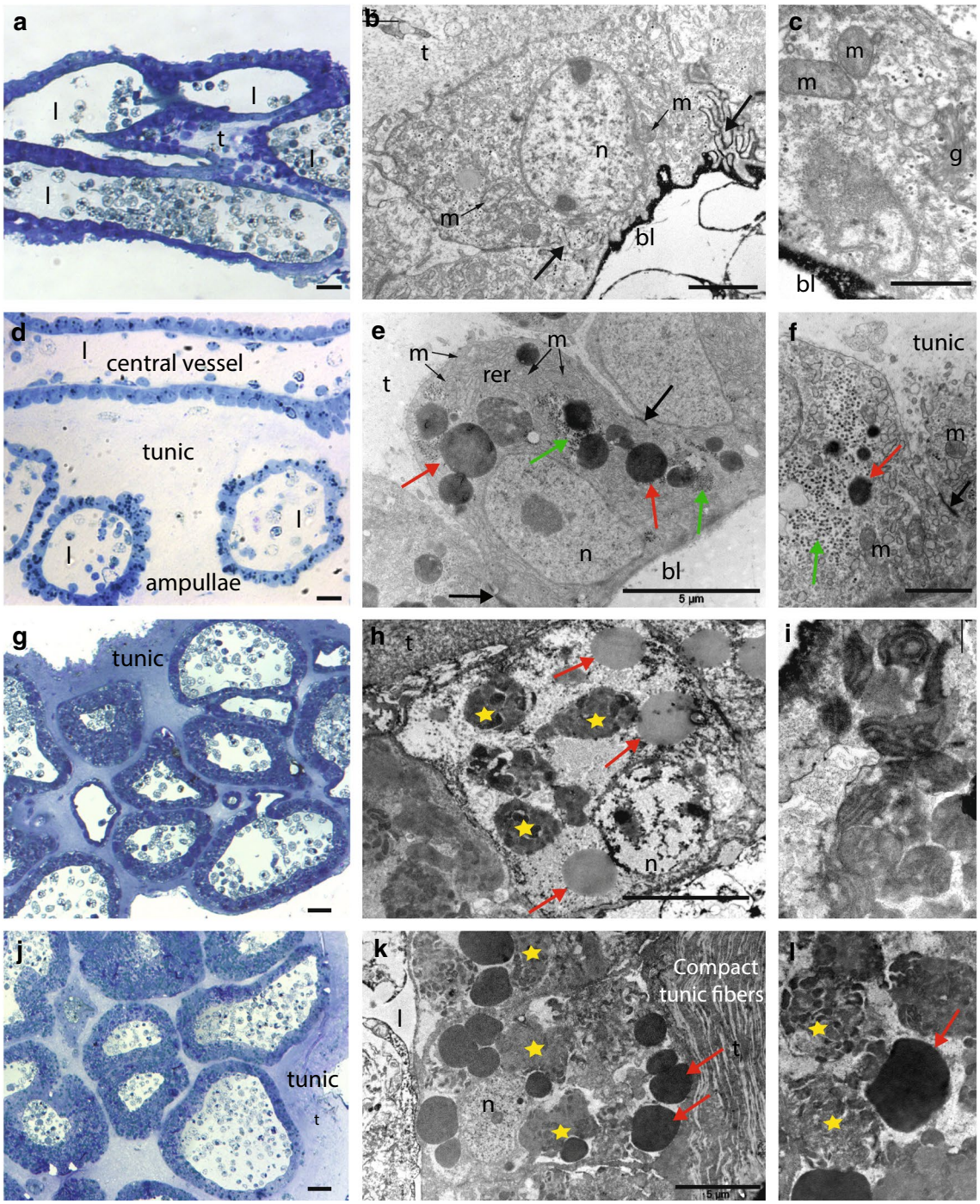


Fig. 3 Histological and ultra-structural characterization of the stolon, budding nest, and spherule. **a** Transversal semi-thin section of a stolon. **b** A stolonial epithelial cell, with strongly electron-dense basal lamina (bl) and several mitochondria (m). The cells are connected by elaborated intercellular contacts (black arrows). **c** Detail of a stolonial epithelial cell, showing Golgi apparatus (g), basal lamina (bl), and mitochondria (m). **d** Longitudinal semi-thin section of a young nest. **e** Epithelial cells of the stolonial vessel, containing extensive rough endoplasmic reticulum (RER), large electron-dense granules (red arrows), and glycogen granules (green arrows). Tight junctions (black arrows) connect the neighboring cells. **f** Detail showing the intercellular junctions (black arrow), some small electron-dense granules (red arrow) and glycogen granules (green arrow). **g** Semi-thin sections of a mature nest. **h** Epithelial cell from a budding nest, containing spherical electron-dense granules (red arrow) and heterogeneous inclusions (yellow star). **i** Detail of the inclusions' content. **j** Semi-thin section of a spherule. **k** An epithelial cell from a spherule, completely filled by heterogeneous inclusions (yellow stars) and electron-dense granules (red arrows). **l** Detail showing the two different types of inclusions present in the epithelial cells. (Scale bars. Semi-thin sections: 10 µm; **b**: 2 µm; **c**: 1 µm; **e**: um; **f**: 2 µm; **h**: 5 µm; **i**: 1 µm; **k**: 5 µm; **l**; 5 µm) (*bl* basal lamina, *g* Golgi, *l* vessel lumen, *m* mitochondria, *n* nucleus, *rer* rough endoplasmic reticulum, *t* tunic)

(See figure on next page.)

Fig. 4 Early stages of budding in *Polyandrocarpa zorritensis*. **a** Schematic summary of vasal budding, depicting the four main steps: swelling, invagination, fusion and double vesicle. **b** Semi-thin section of a vasal bud at the swelling stage. **c** Detail of the vascular epidermis on the top side (squared in **b**). **d** Detail of the vascular epidermis on the bottom side (squared in **b**). **e** Semi-thin section of a vasal bud at the invagination step, showing the invaginating epidermis (black arrows), the movement of the invaginating edges (pink arrows) and the part of the epidermis that goes on to form the floor of the inner vesicle (orange star). **f** Detail of the bottle-shaped cells at the invaginating hinge points (as squared in **e**). **g** Detail of a wedged-shaped cells at the invaginating edge (as squared in **e**). **h** Semi-thin section of a vasal bud at the fusion step, showing the inner vesicle floor (orange star), the inner vesicle lumen (green asterisk) and the movement of the fusing borders (pink arrows). **i** Detailed view of the inner vesicle wall. **i'** Close-up of the cilia apex of the inner vesicle cells (as squared in **i**). **j** Detail of hemocyte aggregate at the fusion area (as squared in **h**), showing undifferentiated hemoblasts (green arrows) and granules-containing cells (blue arrows). **k** Semi-thin section of a vasal bud at the double vesicle stage. **l** Detailed view of hemocytes located between the inner and the outer vesicle. **m** Ampullar wall showing absence of cell membrane and cytoplasmic continuity between cells (pink asterisks). **n–n'** Contact between an ampullar epidermal cell and a morula cell. Blue arrow shows cytoplasmic continuity (Scale bars. **b**, **e**, **h**, **k**: 50 µm; **c**, **f**, **g**, **m**: 10 µm; **d**, **j**, **l** 20 µm; **i**: 5 µm, **i'** 2 µm, **n** 2 µm, **n'** 1 µm) (*a* ampulla, *h* hemocytes, *iv* inner vesicle, *l* vessel lumen, *m* mitochondria, *n* nucleus, *ov* outer vesicle, *t* tunic)

side. In the course of invagination, the invaginating edges move toward each other (Fig. 4e, pink arrows), thus covering the portion of epithelium located between them (Fig. 4e, orange star).

Step 3: fusion

Around 22 hpa, the invaginating edges eventually come into contact and fuse to each other (Fig. 4a, h). This consequently delineates a thin cavity that is the future lumen of the forming inner vesicle (Fig. 4h, i, green asterisk). Cells lining this forming lumen are elongated (20–25 µm high × 3–7 µm wide) and polarized, with clear apical tight junctions as well as an apical cilium (Fig. 4i, i').

At the point of fusion, the epithelial organization of the invaginating epidermis is lost. A cluster of hemocytes appears around the closing aperture, between the prospective inner and outer vesicle (Fig. 4h, j). The vast majority of the clustering hemocytes show morphological features of undifferentiated hemoblasts, i.e., high nucleo-cytoplasmic ratio, a prominent nucleolus, poorly developed RER and mitochondria (Fig. 4j, green arrowhead), whereas other cells contain few electron-dense granules (Fig. 4J, blue arrowhead). While the basal lamina is present on every epithelial cell of the inner and the outer vesicle (Additional file 6: Figure S5b), it is indiscernible at the fusion point, so that the hemoblast cluster and the invaginating tissue constitute a morphologically indivisible unit without prominent cell junctions.

Step 4: double vesicle and initiation of organogenesis

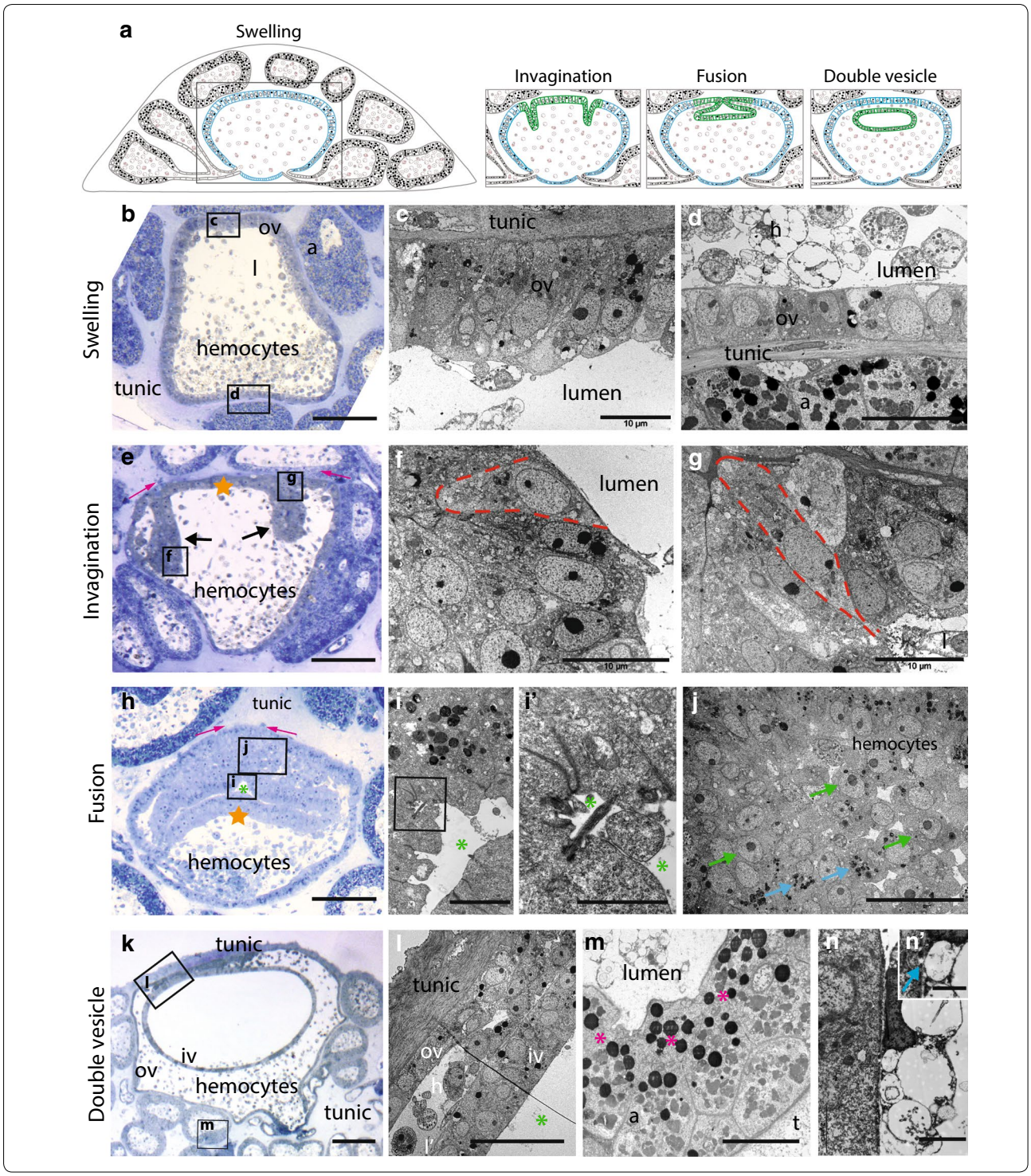
Fusion finally results in the formation of a monolayered inner vesicle, where the apical side of the epithelia faces the lumen of the vesicle, and a monolayered outer vesicle, with the apical side of the epithelium facing the tunic (Fig. 4a, k). Mesenchymal cells remain visible between them, in the region where the fusion occurred (Fig. 4l). While the outer vesicle will give rise to the external epidermis of the new zooid, the inner vesicle folds and undergoes organogenesis to give rise to the different organs of the zooid (Additional file 6: Figure S5c).

From this stage, regression of the surrounding ampullae begins. We have observed significant ultra-structural modifications in the ampullar epidermis, in which nutrients had accumulated during nest formation. At their basal side—facing the lumen—membranes of adjacent epithelial cells lose their integrity and therefore the cytoplasm of all the cells that constitute the ampullar wall is in continuity (Fig. 4m, pink asterisks). In addition, the cytoplasmic electron-dense bodies are displaced toward the basal side of the cells (Fig. 4m). Finally, many mesenchymal cells are in close contact with the ampullar wall (Fig. 4n). These cells contain large and empty vesicles, with a relatively small nucleus and sometimes a nucleolus, corresponding to the classical morphology of morula cells known in other ascidians. At some contact point between these cells and the epidermis, cell membranes are inconspicuous, so that cytoplasmic content of both partners seems to be in continuity (Fig. 4n', blue arrow).

Discussion

Among tunicates, solely sexually reproducing species and species that adopt different forms of NED are scattered across the whole subphylum, suggesting that budding and therefore coloniality have been acquired or lost several times [7, 10]. One colonial species in the family Styelidae, *Polyandrocarpa zorritensis,* shows a mode of budding that differs from other family members, which, along with its phylogenetic position [10], reinforces the hypothesis that NED has been acquired convergently in this species.

This paper describes the role of the vascular epidermis and circulating hemocytes in this novel form of NED. We also show that despite the original nature of the budding tissue, bud of *P. zorritensis* convergently acquired some similarities with other tunicate buds, such as the double vesicle stage, the possible



participation of hemoblasts, the nutritive role of the epidermis and the existence of a dormant stage. The terms "stolonial budding" and "vascular budding," which already designate budding modes in other tunicate species such as *Stolonica socialis* and *Botryllus primigenus* [24, 27], are inappropriate for *P. zorritensis*. Both of these budding modes involve cellular sources and early ontogenesis that differ from those we found in *P. zorritensis*. Therefore, given the peculiarities of *P. zorritensis* NED, we propose the term vasal budding.

Vasal buds originate from a novel combination of tissues

While tunicate budding modes are diverse in terms of tissues involved and early morphogenetic movements [7], most tunicate NED strategies converge morphologically toward a common ontogenetic stage: a triploblastic vesicle formed by two monolayered polarized epithelia that sandwich a mesenchyme of circulating hemocytes [11, 15]. While the outer vesicle always originates from the parental epidermis, the anatomical source of the inner vesicle is highly variable across NED modes. Consequently, budding modes in tunicates have been categorized according to the tissues that give rise to the inner vesicle [16]. For instance, the inner vesicle can originate from: (1) the peribranchial epithelium (e.g., *Botryllus*, *Stolonica*); (2) the epicardial tissue, with or without the participation of the intestinal epithelium (e.g., *Diazona, Aplidium,* and *Polyclinum*); (3) an endostyle outgrowth together with other mesenchymal cells (e.g., *Salpa*, *Pyrosoma,* and *Doliolum*); (4) the stolonial mesenchymal septum (e.g., *Clavelina* and *Perophora*) and (5) the circulating hemoblasts, as in the case of vascular budding (e.g., Subfamily *Botryllinae*) [26, 27, 32]. Here, we describe an additional, yet unreported budding mode that challenges the paradigm that "mature epidermis never gives rise to any tissue other than epidermis itself" [16]. Indeed, in *P. zorritensis*, both the outer and the inner vesicle derive from the parental epidermis through an invagination process. The peculiar onset of *P. zorritensis* vasal budding supports the idea that among ascidians the budding capacity of a tissue may be uncoupled from its embryonic origin.

Studies of two different NED modes in *Botryllus schlosseri*, peribranchial and vascular budding, show that the double vesicle stage exhibits the first spatial segregation of cell fate domains, notably for ectodermal and endodermal identities [33–35]. During vasal budding, the tissue differentiation and the organogenesis also start from the double vesicle stage, wherein organ primordia appears as foldings of the inner vesicle [10]. Therefore, it is possible that, despite the different onset, developmental modules responsible of the zooid morphogenesis are conserved in a convergently evolved NED.

The role of hemocytes in vasal bud ontogenesis

Formation of vasal buds in *P. zorritensis* involves a coordinated interplay between epithelial and mesenchymal (hemocyte) cells. In several tunicate species, hemocytes are known to participate in bud formation. In particular, undifferentiated cells referred to as hemoblasts (or lymphocyte-like cells), which have been shown to possess multi- or pluripotent stemness capacity, directly contribute to the formation of the inner vesicle [30, 36]. For instance, during stolonial budding of *Perophora*, *Ecteinascidia,* and *Clavelina* the inner vesicle originates from hemoblasts arising from the mesenchymal septum [30, 37, 38], and during Botryllinae vascular budding the circulating hemoblasts give rise to the inner vesicle [27, 32, 39]. Moreover, during peribranchial budding of *Polyandrocarpa misakiensis,* hemocytes appear to integrate into the growing bud by mesenchymal–epithelial transition [40, 41]. In *P. zorritensis* vasal budding, undifferentiated hemoblasts accumulate in the ridge formed by the invaginating epidermis during inner vesicle formation. In this area, the basal lamina is disrupted, the epithelial organization is lost and replaced by a mass of undifferentiated cells. Although based only on morphological data, we hypothesize that hemoblasts integrate into the forming inner vesicle, contributing to its growth and potentially to its differentiation. Studies combining cell proliferation dynamics with molecular markers specific for hemoblasts will be necessary to assay their role in the onset and/or differentiation of the vasal bud. It is unknown so far whether colonial tunicates possess particular population(s) of hemocytes involved in asexual reproduction, which are absent in solitary species. Alternatively, one can speculate that every tunicate possess homologous hemocyte populations, some of them being co-opted for a new function in NED. Morphological and molecular comparative studies on hemocytes at the single cell level would certainly help to solve this intriguing question.

The role of vascular epidermis and hemocytes in bud nutrition

Among tunicate NED, various populations of differentiated hemocytes function as reserve cells, i.e., they supply nutrients at the onset of budding and during bud growth [25, 42, 43]. Although not functionally tested, this metabolic role of nourishing a bud physically and physiologically separated from the parental zooid has been documented in many species across the three major ascidian taxa, e.g., in trophocyte accumulation in winter stolons of *Clavelina* [44], in strobilated buds of Polyclinidae [16] and in winter buds of *Stolonica socialis* and *Distomus variolosus* [24]. In *P. zorritensis*, formation of the budding nest starts by a thickening of the ampullar epidermis that accumulates lipid and glycogen granules, together with amorphous membrane-free inclusions similar to lysosomal bodies described in other species [45–48]. The lipid nature of the granules is confirmed by their gray color after methylene blue staining (Fig. 4 b) [49] as well as their electron density following osmium fixation (Fig. 3e, f) [50]. Later during bud development, the membranes of these ampullar cells disintegrate, while they are in close contact with numerous hemocytes resembling morula cells [26, 51, 52]. Morula cells have been proposed

as cytotoxic cells based on their phenoloxidase activity in different styelid species [53]. Therefore, we suggest that during vasal budding, the energy may be stored in epidermal cells of the ampullae rather than in trophocytes or reserve hemocytes. This is accomplished by glycogen and lipid accumulation and by self-digestion of these epidermal cells that release nutrients to the hemocoel and eventually to the growing buds. Such a nutritive function of the bud epidermis and/or hemocytes has been convergently acquired in species whose buds are physically detached from their parental zooids [24, 25, 31, 45]. Molecular comparison of anabolic and catabolic pathways involved in nutrients accumulation and consumption may shed light on the mechanisms underlying this evolutionary convergence.

Co-existence of propagating and dormant forms in *P. zorritensis* asexual life cycle

Previous authors have attempted to classify different tunicate NED modes according to their function within the asexual life cycle of the colony. Mukai et al. [31] distinguished between "buds which are concerned with the growth of the colony," "buds which are destined to found new colonies," and "buds whose function is primarily to survive adverse conditions." The more popular classification by Nakauchi [54] recognized only two categories: "propagative budding"—used for colony growth—and "survival budding"—allowing colonies to survive adverse conditions or damages. To these two categories Turon [45, 55] added "rejuvenative budding" and "multiplicative budding," respectively, for synchronous regression/renewal cycles and for production of new colonies. In the asexual life cycle of *P. zorritensis*, new zooid can arise either directly from the budding nest or from spherules (Fig. 5). Vasal buds of *P. zorritensis* seem to transcend all of the previously described functional categories, since they play (1) a survival role, in the form of the spherule, which allow for resistance to cold temperatures over winter [56], (2) a propagative role for colonial growth and expansion in the form of budding nest, and potentially

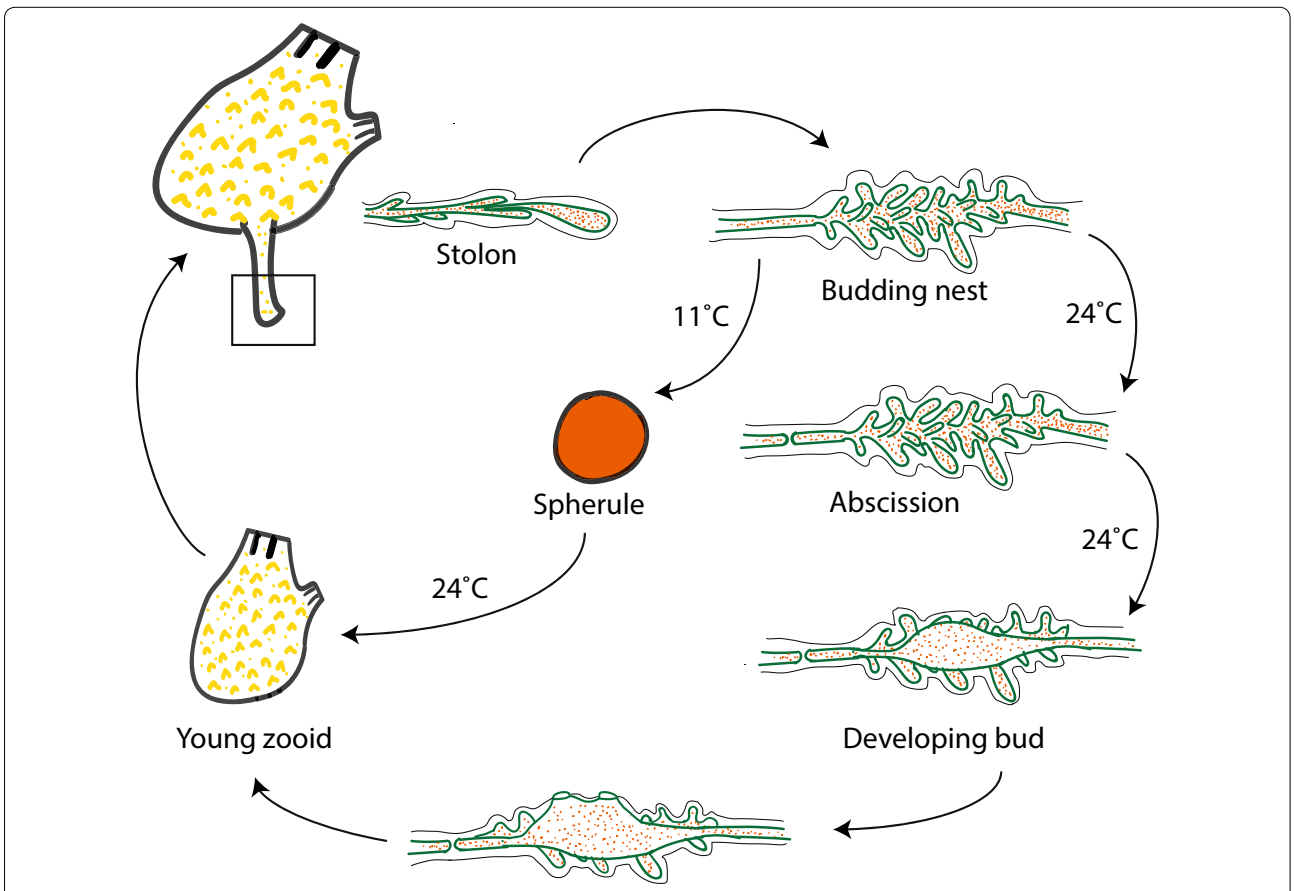


Fig. 5 Scheme of the life cycle of *Polyandrocarpa zorritensis*. The adult zooid emits a vascular stolon, along which the ampullae accumulate to form the budding nest. The nest can endure to two different fates: if transferred to low temperature (e.g., 11 °C), it forms a dormant spherule able to develop into a new zooid once transferred to higher temperature; if kept at 24 °C, abscission occurs along the stolon and the nest goes through vasal budding forming a new zooid. In both cases a new zooid able to produce stolons is formed, repeating the cycle and expanding the colony

(3) a colony-founding role through the dispersal of spherules, which are easily detachable from their mother colony by mechanical action, can be transported by water flow, and eventually allow colony multiplication.

Their behavior and ultrastructure suggests that *P. zorritensis* spherules represent a dormant stage. Dormancy, in general, is a change in physiological state during the life cycle in terms of morphology and metabolism that allows an organism to survive in hostile environments [57], and it appears to have evolved in almost all the groups of colonial animals. Dormancy has not been reported in any tunicate solitary species but it is present in different lineages of colonial ascidians. It is intriguing to speculate that the cellular and molecular mechanisms underlying ascidian NED, such as the stem cells involved in asexual reproduction and regeneration, were shared with dormancy. To our knowledge, *P. zorritensis* is the only known ascidian species where propagative buds and dormant stages coexist at the same time in the same colony. Therefore, *P. zorritensis* represents a striking model to study the relationship between the evolution of NED and the evolution of dormancy.

Conclusion

In order to understand the evolution of complex traits like non-embryonic development and coloniality, it is necessary to identify suitable, closely related species in which to trace the gains, losses, and modifications of such characters [58]. Subsequently, one must provide detailed descriptions of their NED in order to uncover the cellular and molecular mechanisms that lie behind the budding processes. Due to the heterogeneity and the scattered phylogenetic distribution of NED within the Tunicata, this clade provides an excellent model to take such a comparative approach to understand the origins of NED. A recent phylogenetic analysis of the ascidian family Styelidae [10] highlighted *P. zorritensis* as an amenable model to compare budding mechanisms with NED modes in other more well-studied, closely related, models like in *B. schlosseri* [17, 32] and *P. misakiensis* [27]. The morphological description here forms a foundation for future molecular and cellular studies on the convergent origins on NED within the ascidians.

Additional files

Additional file 1: Fig. S1. Spherules at different degrees of transformation.

Additional file 2: Fig. S2. Effect of low-temperature storage on rate of budding and stolon production.

Additional file 3: Fig. S3. Relationship between the size of zooids and the size of spherules.

Additional file 4: Movie 1. Tip of a stolon with mesenchymal circulating cells.

Additional file 5: Fig. S4. Relationship between the size of zooids and the size of budding nests.

Additional file 6: Fig. S5. Details of a bud at stage 1, stage 2 and early bud organogenesis.

Authors' contributions

M.S, A.A., and S.T. conceived and designed the study. M.S., A.A., S.P., C.L., P.H., and L.H. performed the experiments. L.G and F.M. provided *P. zorritensis* breeding support. M.S, A.A., and S.T analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Author details

[1] CNRS, Laboratoire de Biologie du Développement de Villefranche-sur-Mer (LBDV), Sorbonne Université, 06230 Villefranche-sur-Mer, France. [2] Centre Commun de Microscopie Appliquée, UFR Sciences, Faculté des Sciences del'Université de Nice - Sophia Antipolis, 06108 Nice, France. [3] Departamento de Zoologia, Instituto Biociências, Universidade de São Paulo, São Paulo 05508-090, Brazil. [4] Department of Biology and CoNISMa LRU, University of Bari Aldo Moro, 70125 Bari, Italy.

Acknowledgements

We would like to thank S. Lotito, S. Collet and L. Gissat for technical assistance, the EMBRC France for the support with the aqua-culture infrastructure, the team of Assonautica (La Spezia) and Giovanni Chimienti (Taranto) for help collecting colonies of *P. zorritensis*.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Funding

This work was supported by ANR (ANR-14-CE02-0019-01) and CNRS (PICS 07679) to S.T., and FAPESP-BEPE fellowship (2018/05923-3) to L.H.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 6 February 2019 Accepted: 21 March 2019
Published online: 03 April 2019

References

1. Bell G. The masterpiece of nature: the evolution and genetics of sexuality. Berkeley: University of California Press; 1982.
2. Sköld HN, Obst M, Sköld M, Åkesson B. Stem cells in asexual reproduction of marine invertebrates. In: Rinkevich B, Matranga V, editors. Stem cells in marine organisms. New York: Springer; 2009. pp. 105–137.
3. Brusca RC, Moore W, Shuster SM. Invertebrates, 3rd edition. Sunderland, Massachusetts, USA: Sinauer Associates, Inc., Publishers; 2016
4. Delsuc F, Brinkmann H, Chourrout D, Philippe H. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature. 2006;439:965–8.
5. Brown FD, Swalla BJ. Evolution and development of budding by stem cells: ascidian coloniality as a case study. Dev Biol. 2012;369:151–62.

6. Satoh N. Developmental biology of ascidians. Cambridge University Press, New York; 1994.
7. Stolfi A, Brown FD. Tunicata. In: Wanninger A, editor. Evolutionary developmental biology of invertebrates 6: deuterostomia. Vienna: Springer. 2015; p. 135–204.
8. Delsuc F, Philippe H, Tsagkogeorga G, Simion P, Tilak M-K, Turon X, et al. A phylogenomic framework and timescale for comparative studies of tunicates. BMC Biol. 2018;16:39.
9. Kocot KM, Tassia MG, Halanych KM, Swalla BJ. Phylogenomics offers resolution of major tunicate relationships. Mol Phylogenet Evol. 2018;121:166–73.
10. Alié A, Hiebert LS, Simion P, Scelzo M, Prünster MM, Lotito S, et al. Convergent acquisition of nonembryonic development in styelid ascidians. Mol Biol Evol. 2018;35:1728–43.
11. Tiozzo S, Brown FD, De Tomaso AW. Regeneration and stem cells in ascidians. In: Bosh TC, editor. Stem cells from hydra to man. London: Springer; 2008. p. 95–112.
12. Berrill NJ. The Tunicata with an account of the British species. London: Quaritch; 1950.
13. Nakauchi M. Oozooid development and budding in the polyclinid ascidian, Parascidia flemingii (Urochordata). J Zool. 1986;208:255–67.
14. Millar RH. Budding in the ascidian Aplidium petrense Michaelsen. J Nat Hist Ser. 1962;13:337–40.
15. Brien P. Blastogenesis and morphogenesis. Adv Morphog. 1968;7:151–203.
16. Berrill NJ. Regeneration and budding in tunicates. Biol Rev. 1951;26:456–75. https://doi.org/10.1111/j.1469-185X.1951.tb01207.x.
17. Manni L, Zaniolo G, Cima F, Burighel P, Ballarin L. Botryllus schlosseri: a model ascidian for the study of asexual reproduction. Dev Dyn. 2007;236:335–52.
18. Nunzi MG, Burighel P, Schiaffino S. Muscle cell differentiation in the ascidian heart. Dev Biol. 1979;380:371–80. https://doi.org/10.1016/0012-1606(79)90211-2.
19. Tunicata B, Manni L, Zaniolo G, Burighel P. Ultrastructural study of oogenesis in the compound ascidian. Acta Zool. 1994;75:101–13.
20. Akhmadieva AV, Shukalyuk AI, Aleksandrova YN, Isaeva VV. Stem cells in asexual reproduction of the colonial ascidian *Botryllus tubaratus* (Tunicata: Ascidiacea) text. Rus J Mar Biol. 2007;33:181–6.
21. Watanabe H, Tokioka H. Two new species and one possibly new race of social styelids from Sagami Bay, with remarks on their life history, especially the mode of budding. Publ Seto Mar Biol Lab. 1972;XIX(5):327–45.
22. Kawamura K, Fujiwara S, Sugino YM. Budding-specific lectin induced in epithelial cells is an extracellular matrix component for stem cell aggregation in tunicates. Development. 1991;113:995–1005.
23. Kawamura K, Nakauchi M. Homeostatic integration of stem cell dynamics during palleal budding of ascidians. Zool Sci Zool Soc Jpn. 1991;8:p11–22.
24. Selys-Longchamps M. Sur le bourgeonnement des Polystiélinés Stolonica et Heterocarpa avec quelques notes sur l'anatomie de ces deux genres. Bull Sci Fr Belg. 1917;L(3):170–276.
25. Berrill NJJ. The development, morphology and budding of the ascidian Diazona. J Mar Biol Assoc UK. 1948;27:389–99
26. Gutierrez S, Brown FD. Vascular budding in Symplegma brakenhielmi and the evolution of coloniality in styelid ascidians. Dev Biol. 2017;423:152–69. https://doi.org/10.1016/j.ydbio.2017.01.012.
27. Oka H, Watanabe H. Vascular budding, a new type of budding in botryllus. Biol Bull. 1957;112:225–40.
28. Brunetti R, Mastrototaro F. The non-indigenous stolidobranch ascidian polyandrocarpa zorritensis in the Mediterranean: description, larval morphology and pattern of vascular budding. Zootaxa. 2004;528:1.
29. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9:671.
30. Freeman G. The role of blood cells in the process of asexual reproduction in the tunicate Perophora viridis. J Exp Zool. 1964;156:157–83.
31. Mukai H, Koyama H, Watanabe H. Studies on the reproduction of three species of *Perophora* (Ascidiacea). Biol Bull. 1983;164:251–66.
32. Ricci L, Cabrera F, Lotito S, Tiozzo S. Redeployment of germ layers related TFs shows regionalized expression during two non-embryonic developments. Dev Biol. 2016;416:235–48.
33. Ricci L, Chaurasia A, Lapébie P, Dru P, Helm RR, Copley RR, et al. Identification of differentially expressed genes from multipotent epithelia at the onset of an asexual development. Sci Rep. 2016;6:27357.
34. Prünster MM, Ricci L, Brown FD, Tiozzo S. De novo neurogenesis in a budding chordate: co-option of larval anteroposterior patterning genes in a transitory neurogenic organ. Dev Biol. 2018. https://doi.org/10.1016/j.ydbio.2018.10.009.
35. Prünster MM, Ricci L, Brown FD, Tiozzo S. Modular co-option of cardiopharyngeal genes during non-embryonic myogenesis. Evodevo. 2019;10:3. https://doi.org/10.1186/s13227-019-0116-7.
36. Laird DJ, De Tomaso AW, Weissman IL. Stem cells are units of natural selection in a colonial ascidian. Cell. 2005;123:1351–60.
37. Dale Beers C. Some points in the bud development of a simple ascidian, Ecteinascidia turbinata Herdman. J Elisha Mitchell Sci Soc. 1923;39:92–4.
38. Brien P, Brien-Gavage E. Contribution à l'etude de la blastogenèse des Tuniciers. III. Bourgeonnement de Clavelina lepadiformis. Miiller Rec Inst Zool Torley Rousseau. 1927;1:31–81.
39. Burighel P, Brunetti R, Zaniolo G. Hibernation of the colonial ascidian botrylloides leachi (Savigny): histological observations. Boll di Zool. 1976;43:293–301.
40. Tatzuke Y, Sunanaga T, Fujiwara S, Kawamura K. RACK1 regulates mesenchymal cell recruitment during sexual and asexual reproduction of budding tunicates. Dev Biol. 2012;368:393–403.
41. Fujiwara S, Isozaki T, Mori K, Kawamura K. Expression and function of myc during asexual reproduction of the budding ascidian polyandrocarpa misakiensis. Dev Growth Differ. 2011;53:1004–14.
42. Fujimoto H, Watanabe H. Studies on the asexual reproduction in the polystyelid ascidian, polyzoa vesiculiphora Tokioka. J Morphol. 1976;150:607–21. https://doi.org/10.1002/jmor.1051500302.
43. Berrill NJ, Cohen A. Regeneration in Clavelina lepadiformis. J Exp Biol. 1936;13:352–62.
44. Brien P. Embranchement des Tuniciers. Trait Zool Anat Syst Biol. 1928;11:553.
45. Turon X. A new mode of colony multiplication by modified budding in the ascidian Clavelina gemmae n. sp. (Clavelinidae). Invertebr Biol. 2005;124:273–83. https://doi.org/10.1111/j.1744-7410.2005.00025.x.
46. Rost-Roszkowska MM, Poprawa I, Chachulska-Zymełka A. Apoptosis and Autophagy in the Midgut Epithelium of *Acheta domesticus* (Insecta, Orthoptera, Gryllidae). Zool Sci. 2010;27:740–5.
47. Lipovšek S, Letofsky-Papst I, Hofer F, Leitinger G, Devetak D. The evidence on the degradation processes in the midgut epithelial cells of the larval antlion Euroleon nostras (Geoffroy in Fourcroy, 1785) (Myrmeleontidae, Neuroptera). Micron. 2012;43:651–65.
48. Lobo-Da-Cunha A, Calado G. Histology and ultrastructure of the salivary glands in Bulla striata (Mollusca, Opisthobranchia). Invertebr Biol. 2008;127:33–44.
49. Sato K, Nakamura M, Tomita T, Toda M, Miyamoto K, Nozu R. How great white sharks nourish their embryos to a large size: evidence of lipid histotraphy in lamnoid shark reproduction. Biol Open. 2016;5:1211–5.
50. Angermuller S, Fahimi HD. Imidazole-buffered osmium tetroxide: an excellent stain for visualization of lipids in transmission electron microscopy. Histochem J. 1982;14:823–35.
51. Cima F, Peronato A, Ballarin L. The haemocytes of the colonial aplousobranch ascidian Diplosoma listerianum: structural, cytochemical and functional analyses. Micron. 2017;102:51–64.
52. Hirose E, Shirae M, Saito Y. Ultrastructures and classification of circulating hemocytes in 9 botryllid ascidians (chordata: ascidiacea). Zool Sci. 2003;20:647–56.
53. Ballarin L, Franchi N, Schiavon F, Tosatto SCE, Mičetić I, Kawamura K. Looking for putative phenoloxidases of compound ascidians: haemocyanin-like proteins in polyandrocarpa misakiensis and Botryllus schlosseri. Dev Comp Immunol. 2012;38:232–42.
54. Nakauchi M. Asexual development of ascidians : its biological significance, diversity, and morphogenesis. Am Zool. 1982;22:753–63.
55. Turon X. Periods of non-feeding in Polysyncraton lacazei (Ascidiacea: Didemnidae): a rejuvenative process? Mar Biol. 1992;655:647–55.
56. Mastrototaro F, D'Onghia G, Tursi A. Spatial and seasonal distribution of ascidians in a semi-enclosed basin of the Mediterranean Sea. J Mar Biol. 2008;88:1053–61.
57. Cáceres CE. Dormancy in invertebrates. Invertebr Biol. 1997;116:371–83.
58. Tiozzo S, Copley RR. Reconsidering regeneration in metazoans: an evo-devo approach. Front Ecol Evol. 2015;3:1–12.

Chapter 3

Investigating the role of putative stem cells, the hemoblasts, during vasal budding in Polyandrocarpa zorritensis*.*

During the life cycle of solitary and colonial ascidians, the circulating mesenchymal cells - called hemocytes - are the actors of various essential processes. Different hemocytes have been characterized based on their morphology, ultrastructure and cytochemical proprieties. One of these morphotypes, the hemoblasts, have been suggested to be putative stem cells involved in hematopoiesis and other somatic cell lineages in some types of ascidians' NED

As presented in the previous Chapters, the colonial styelid *Polyandrocarpa zorritensis* shows a unique type of NED, the vasal budding, characterized by swelling and folding of the vascular epidermis. Clusters of undifferentiated hemocytes were reported in association with the invaginating epidermis at the onset of vasal budding, but their nature and their role was not elucidated.

In this Chapter, I characterized the hemocytes in *Polyandrocarpa zorritensis*. Based on external morphology and ultrastructure I identified eight morphotypes. The cells belonging to the "hemoblast" morphotype are recognized as the undifferentiated hemocytes forming clusters during vasal budding. By immunohistochemistry with anti-phosphohistone H3 and EdU assays, we observed that hemoblast aggregates actively proliferate at different stages of vasal budding. The proliferating hemoblasts may be integrated into the budding tissues and participate with the invaginating epidermis to the formation of inner vesicle. Preliminary data suggest that hemoblast express Integrin-alpha 6, a stem cells marker expressed in putative stem cells responsible for WBR in other species. Pilot experiments towards further characterization and isolation of hemoblasts are also presented. These results lay the bases for further investigations to elucidate the role of hemoblasts during vasal budding in *P. zorritensis*.

Introduction

In tunicates, the cells of mesodermal origin that circulate in the mesenchymal space are commonly called hemocytes (Hartenstein, 2006). These coelomic cells are suspended in a colorless hemolymph, isotonic with sea water (Cima et al., 2016). Except for the characterization in few thaliacean species (Cima et al., 2014) most of the information about tunicate hemocytes concerns ascidians. Ascidians hemocytes participate to different process such as immunity response, storage and transport of nutrients, storage and elimination of catabolites, production of tunic and - in some colonial species – triggering of NED and bud formation (Cima et al., 2018; Freeman, 1964; Fujimoto and Watanabe, 1976b; Kassmer et al., 2019a; Kawamura and Sunanaga, 2010; Smith, 1970).

Different authors attempted to identify the sites where hemocytes originate, both in solitary and colonial ascidians. In the solitary species *Ciona robusta,* the presumptive hematopoietic organs and hematopoietic stem cell niches have been proposed to be at the level of the pharyngeal region. These niches are called lymph-glands or lymphatic nodules (Ermak, 1976; Wright, 1981) and are located around the transversal bar of the branchial basket. In another solitary ascidian, *Styela plicata*, the intestinal submucosa has been identified as a putative stem cell niche, where mesenchymal undifferentiated proliferating cells expressing *Piwi* are grouped in cluster (Jiménez-Merino et al., 2019).

In the colonial species *Botryllus schlosseri*, the presumptive hematopoietic sites are located at the level of the endostyle (Voskoboynik et al., 2008). Indeed, homologues of genes commonly expressed in the human hematopoietic bone marrow are upregulated in the endostyle of *Botryllus schlosseri* (Rosental et al., 2018), suggesting common mechanisms of stem cell homing. In the putative niche located in the anterior-ventral region of the endostyle, the stem cells actively proliferate (Voskoboynik et al., 2008) and migrate ventrally to other putative stem cell niches distributed on both sides of the endostyle, the cell islands, where they continue to proliferate and express genes of the germline multipotency program (GMP) such as *Piwi* and *Vasa* (Rinkevich et al., 2013). Depending on the blastogenetic site, the stem cells can migrate from the cell islands to the budding area, where they are suggested to be integrated in the developing tissues. Once in the vasculature, these cells can also contribute to the germline and the hematopoiesis.

In order to understand the role that hemocytes play in ascidian, particularly during asexual reproduction and regeneration, it's fundamental to characterize and compare the repertoire of the different types of hemocytes encountered in various species. However using the published

data to perform a morphological comparison between hemocytes from different species can be difficult for two main reasons. First, the different methods of observation and fixative used can alter the morphology of the cells generating artifacts. Secondly, intermediate stages of differentiating haemocytes can lead to under- or over-estimations of morphotypes and can induce to misleading comparisons (Hirose et al., 2003). In addition, a unified terminology is lacking, and even in the same species the literature is not concordant, as for *Botryllus schlosseri* (Ballarin and Cima, 2005a) (Table 3.1, Figure 3.1).

Classification from Cima et al 2016	**Classification from previous reports**
Undifferentiated cells	Hemoblasts, Lymphocytes, Lymphocyte-like cells
Immunocytes	
Hyaline amoebocytes	Microgranular amoebocytes
Macrophage-like cells	Vacuolated phagocytes, Macrophages, Signet-ring cells
Granular amoebocytes	Macrogranular amoebocytes
Morula cells	
Storage cells	
Pigment cells	Granular pigment cells
Nephrocytes	Purinic cells

Table 3.1: Comparative classification of *Botryllus schlosseri* hemocytes. The table report all the nomenclature commonly used for the different hemocyte morphotypes. The three main functional groups (undifferentiated cells, immunocytes and storage cells) stated in Cima et al., 2016 are represented with different colors. The literature consulted to fill out this table is the following : (Ballarin et al., 1993; Burighel et al., 1983; Cima et al., 2016; Milanesi and Burighel, 1978; Rinkevich and Rabinowitz, 1993; Sabbadin, 1955; Schlumpberger et al., 1984).

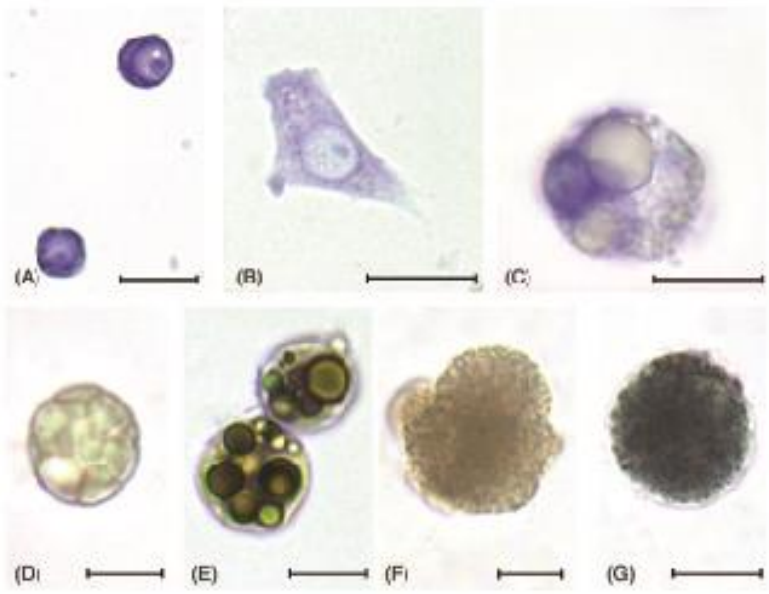


Figure 3.1: Circulating hemocytes of the colonial ascidian Botryllus schlosseri. (A–C, E) fixed hemocytes stained with Giemsa solution; (D, F, G) living hemocytes. (A) hemoblasts; (B) hyaline amoebocytes; (C) macrophage-like cell; (D) living morula cell; (E) fixed morula cells; (F) nephrocyte; (G) blue-pigment cell. Scale bar: 10 mm. (Mofidied from Cima eta al 2016). (H) Postulated differentiation pathways of Botryllus schlosseri hemocytes (From Ballarin and Cima 2005).

A conservative classification of hemocytes according to Cima and colleagues (2016) distinguish three main functional group of: undifferentiated cells, immunocytes and storage cells (Cima et al., 2016) (Table 3.1; Figure 3.1). In this chapter, I will base the characterization on this terminology, and I will mostly refer to data collected on colonial ascidian.

3.1 Immunocytes

As the name suggests, these cells are responsible for the innate immunity in ascidians (Ballarin, 2008). The immunocytes can be functionally subdivided in phagocytes and cytotoxic cells

3.1.1 Phagocytes

Phagocytes are responsible for the ingestion of exogenous particles and cells or endogenous apoptotic cells and debris. They comprise hyaline amoebocytes and macrophage-like cells, which represent two different stages of the same morphotype (Cima et al., 1996). They in fact show the same cytochemical proprieties and contain similar enzymes, as shown in *Botryllus schlosseri* and *Botrylloides diegensis* (Ballarin and Cima, 2005a; Cima et al., 2001). The hyaline amoebocytes (HA) (Figure 3.1B) are actively implicated in the engulfment process, migrating where the material to ingest is and emitting pseudopodes able to connect and trap it. At this point they become rounded big cells and acquire the characteristics of macrophage-like cells

(MLC) (Figure 3.1C). The ingested material can be visible in vacuoles called phagosomes, where lipofuscins and hydrolytic enzymes such as acid phosphatase are released for the digestion (Cima et al., 1996). Using flow cytometry and RNA-seq in *B. schlosseri*, macrophage-like cells were subdivided in two types: myeloid cells and large phagocytes, responsible respectively for phagocytosis and rejection reaction between non compatible colonies (Rosental et al., 2018).

3.1.2 Cytotoxic cells

Cytotoxic cells are responsible for non-self-recognition and inflammatory reaction, which lead to cells lysis. They are represented by morula cells and in some species by granular amoebocytes.

Granular amoebocytes (GA) are granulated cells considered precursors of the morula cells. Morula cells (MC) are generally the most abundant hemocyte morphotype in colonial species, for example, in *B. schlosseri* they can reach the 40-60% of the total hemocytes (Ballarin, 2008). MCs are large (between 10-15 μm in diameter, Figure 3.1D, E) and show a berry-like shape, filled with large vacuoles containing the cytotoxic enzyme phenoloxidase (PO) (Ballarin et al., 2012; Shirae and Saito, 2000). When contained in the MCs, the PO is in an inactive form. This pro-enzyme is activated once released in the hemolymph by degranulation of the vacuoles, which occurs when MCs recognize non-self-molecules or cells. In addition to PO, other humoral factors as cytokines, lectins and complement factors are released, inducing the cytotoxic response (Franchi and Ballarin, 2017). The active PO produces reactive oxygen species (ROS) and quinones, which induce oxidative stress and cytotoxicity in the foreign cells inducing their death (Ballarin et al., 2002; Franchi et al., 2015). At the same time, the humoral factors released such as the cytokines have a chemotactic activity, recruiting on site phagocytes for the digestion of the exogenous material (Ballarin, 2008).

In colonial ascidians, MCs are also implicated in the allorejection , e.g. in *Botryllus schlosseri* or *Botrylloides simodensis* (Cima et al., 2004; Shirae et al., 2002), an immunoreaction that occurs when incompatible colonies get in contact with part of their external ampullae and avoids the fusion and the formation of chimeras (Cima et al., 2004; Corey et al., 2016).

3.2 Storage cells

These hemocytes contain cytoplasmic granules of different nature. Depending on the substances accumulated they can be categorized in vanadocytes, pigment cells, nephrocytes or trophocytes.

The vanadocytes are present in some species of Phlebobranchia, such as *Phallusia mammillata* (Michibata et al., 2003). They are called also signet ring cells, since they contain single large

electron dense vacuole that occupies most of the cell volume (Yamaguchi et al., 2006). Vanadocytes contain high levels of vanadium, but their role still remains unclear.

The pigment cells are usually large cells that contain pigment granules of different color (carotenoids, purines and melanin-like substances), which can variate in different species or in different individuals of the same species (Burighel et al., 1983, Hirose et al., 1998)). (Figure 3.1G).

Nephrocytes are responsible for the storage and elimination of nitrogenous wastes and are characterized by large vacuoles containing urate crystals (Figure 3.1F). Nephrocytes and pigment cells are morphologically very similar, differing only by the nature of the inclusions. In some species the urate crystals acquire a geometric shape - i.e. hexagonal structure in *Diplosoma listerianum* (Cima et al., 2017) or hourglass shape in *Botrylloides diegensis* (Cima et al., 2001).

Trophocytes are large hemocytes, containing granules of different sizes filled with nutritive material, like lipid droplets or glycogen granules. Trophocytes have been reported in many colonial species such as *Polyandrocarpa misakiensis* (Sugino, 1993) or *Polyzoa vesiculiphora* (described as granular amoebocytes) (Fujimoto and Watanabe, 1976b).

3.3 Undifferentiated cells: hemoblasts

Undifferentiated hemocytes are usually small rounded cells (4-8 um). They are characterized by a high nuclear/cytoplasmic ratio, a prominent nucleolus and a cytoplasm that can contain few mitochondria, Golgi apparatus, RER or ribosomes. (Cima et al., 2017; Hirose et al., 2003) (Figure3.1A). These cells are commonly called hemoblasts or lymphocyte-like cells and many authors suggested a role of circulating putative stem cells (CPSCs) in both solitary and colonial species (Freeman, 1964; Jiménez-Merino et al., 2019; Kassmer et al., 2019a; Kawamura and Sunanaga, 2010).

Hemoblasts have been proposed to differentiate into all the other types of hemocytes (Figure3.2A). The expression of a CD34-like antigen - an hematopoietic molecular marker in mammals (Civin et al., 1996) - has been reported in hemoblasts of *Botryllus schlosseri* and *Styela plicata* (Ballarin and Cima, 2005a; Medina et al., 2015), suggesting that hemoblasts may multipotent hematopoietic progenitors. However, the detailed pattern of hemoblast differentiation into the other hemocytes is not described yet

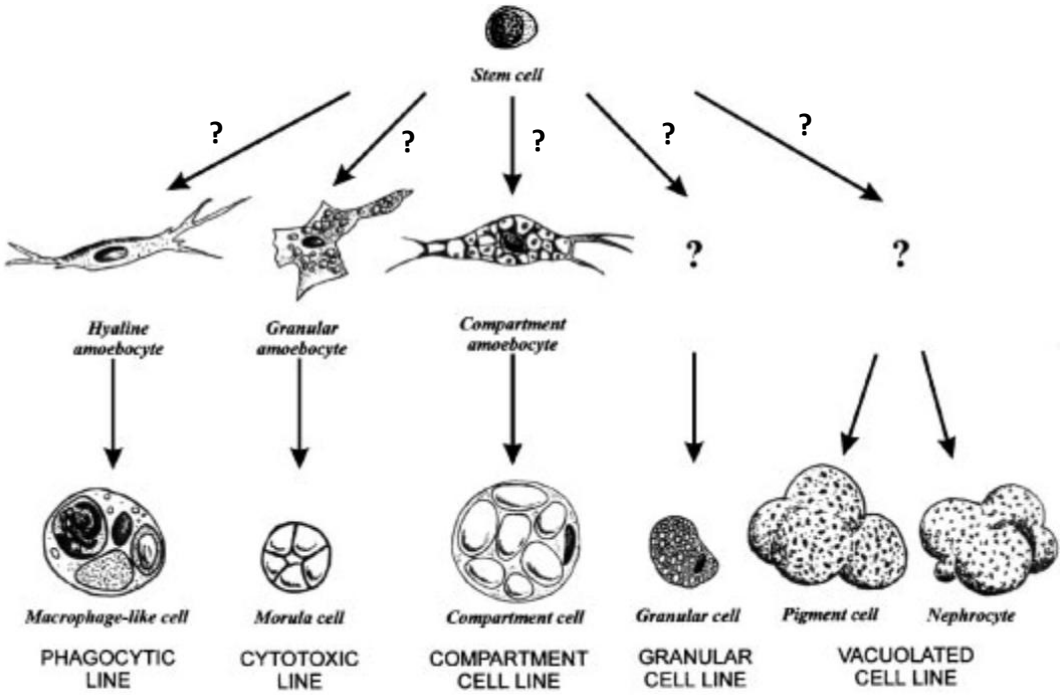


Figure 3.2: Postulated differentiation pathways of *Botryllus schlosseri* haemocytes (Modified from Ballarin and Cima, 2005).

In the solitary ascidian *Styela plicata,* hemoblasts migrating to the wound site after dissection of the cerebral ganglion and they seem to participate to the regeneration central nervous system , (Medina et al., 2015; da Silva et al., 2015). Some authors suggested that undifferentiated mesenchymal cells are responsible of CNS regeneration also *Ciona intestinalis* (reviewed in Jeffery, 2015) (Bollner, 1989; Bollner et al., 1992). In Botryllinae, during peribranchial and vascular budding, mass of mesenchymal undifferentiated cells form the testicular epithelium, the follicular cells and both male and female germ cells (Kawamura and Sunanaga, 2010; Manni et al., 1993; Mukai and Watanabe, 1976; Sunanaga et al., 2006). These germline precursors migrate through the bloodstream to the gonadal primordium of the new blastogenic generation (Mukai and Watanabe, 1976; Sabbadin and Zaniolo, 1979) and express germ line cells markers such as *vasa* and *piwi* (Brown and Swalla, 2007; Kawamura and Sunanaga, 2010 , 2011; Sunanaga et al., 2006).

3.4 The role of hemoblasts during NED

As highlighted in the paragraph 1.6.1, hemoblasts have been reported to be involved in different types of NED, resulting essential to budding initiation, whole-body regeneration or organogenesis across all the ascidian classes (Alié et al., 2020; Kassmer et al., 2019b).

For example, in the Phlebobranchia *Perophora viridis* and in the Aplousobranchia *Clavelina lepadiformis,* the main budding tissue is a stolonial septum which is composed of hemoblasts organized in a sort of monolayer epithelium (Alie' et al 2020, Brien and Brien-Gavage 1927, 1928). Freeman (1964) demonstrated that after irradiating colonies of *P. viridis* and blocking the budding, the reinjection of non-irradiated hemoblasts was sufficient to restore the budding ability. This very important and elegant experiments showed that, at least in this species, hemoblasts act as pluripotent stem cells (Freeman, 1964).

During peribranchial budding in some species of Stolidobranchia, hemoblasts are probably involved in organogenesis, as aggregates of hemoblast are observed in the vicinity of the forming organs, such as during cardiac differentiation in *Symplegma reptans* (Sugino et al., 2007) and *Botryllus schlosseri* (Nunzi et al., 1979), or in *Polyandrocapa misakiensis*, where Kawamura and Nakauchi (1991) suggested an integration of hemoblasts in the organogenetic area of the inner vesicle by mesenchymal to epithelial transition (Kawamura et al., 1991). If the interaction between inner vesicle and hemoblasts is inhibited, the organogenesis does not occur (Tatzuke et al., 2012).

During vascular budding, the role of hemoblasts in the budding process is even more evident. In fact, in Botryllinae, the very first sign of vascular budding observed is the formation of an hemoblast clusters (Gutierrez and Brown, 2017; Oka and Watanabe, 1957; Ricci et al., 2016) that eventually gives rise to the inner vesicle enclosed by the vascular epithelium. In *Botrylloides digenesis*, the hemoblasts actively proliferate and express markers such as *piwi*, *vasa* and *integrin-alpha 6* (Kassmer et al., 2019a). In a very recent study, Kassmer et al. (2019) provided the very first demonstration of hemoblatst pluripotency in *Botrylloides*. By injecting a single hemoblast, they rescued the regenerative capabilities of a colony in which vascular budding had been blocked by mitosis inhibitors (Kassmer et al., 2019).

In Chapter 2, I recently described vasal budding in *Polyandrocarpa zorritensis* and showed that this budding mode appeared independently from peribranchial and vascular budding in Styelidae. Vasal budding primarily arises by invagination of the epidermal vascular wall. However, the morphological observations suggest that hemocytes may also take part to the bud formation. In fact, at the earliest stages of the vasal bud formation, aggregates of hemocytes are visible in the vicinity of the invaginating tissues (Scelzo et al., 2019 – Chapter 2). Yet, the

different types of hemocytes, and their participation to bud formation in *P. zorritensis* have not been characterized.

In order to characterize the nature of the hemocytes aggregates in the budding tissues, I described the hemocytes in *P. zorritensis,* identifying eight morphotypes by histological stains and electron microscopy. Then, in order to elucidate their role during vassal budding, I investigated the distribution of hemocytes at different stages of the life cycle, as well as their proliferative pattern by assaying EdU incorporation and histone H3 phosphorylation. Finally, in order to identify a molecular marker specific for hemocytes participating to budding in *P. zorritensis*, I tested the expression of Iα6, known to be expressed in clonogenic hemocytes in other species (Kassmer et al 2015, 2019).

Results

3.5 Identification of eight hemocyte morphotypes in *Polyandrocarpa zorritensis*

Comparing the morphology and the ultrastructure of *Polyandrocarpa zorritensis* hemocytes with the descriptions made in other colonial species (as in Hirose et al 2003, Ballarin and Cima 2005, Gutierrez et al 2018) I identified eight hemocyte morphotypes in *P. zorritensis*.

3.5.1 Hyaline amoebocytes

Hyaline amoebocytes (8-9 µm) have an amoeboid shape with many cytoplasmic protrusions (pseudopodes) (Figure 3.3 A-D). Their cytoplasm contains small electrondense granules and empty-looking vacuoles (Figure 3.3 D), which acquire a pink-violet coloration after Giemsa staining, showing their basic nature (Figure3.3 A, B).

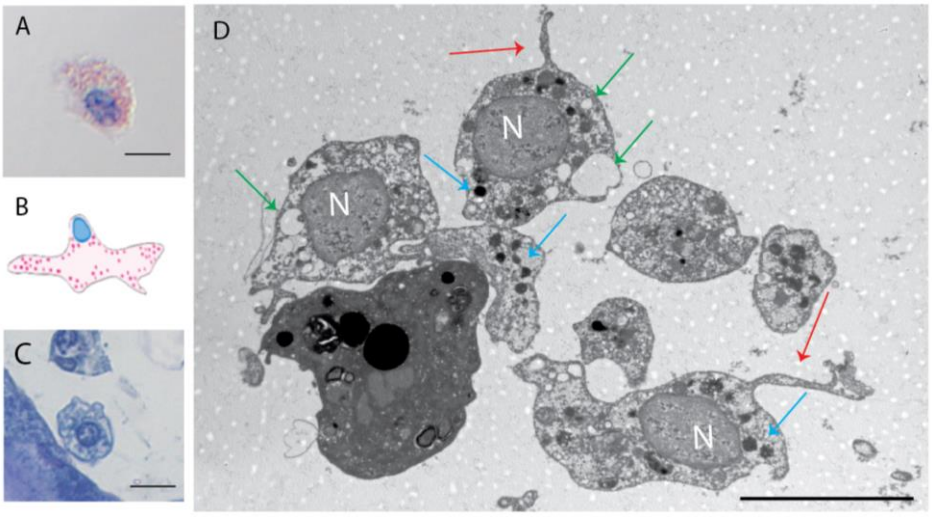


Figure 3.3: Morphological (A-C) and ultrastructural (D) description of hyaline amoebocytes (HA). A) Giemsa stain. B) Graphical schematization. C) Methylene blue stain on semi thin sections. D) A group of HA engulfing a cell. Pseudopods (red arrows), electrondense granules (blue arrows) and vacuoles (green arrows) are visible. (Scale bar: A, C, D: 5 µm).

3.5.2 Macrophage-like cells

Macrophage-like cells are big cells (more than 15 µm), with a shape that changes depending on the phagocytic activity (Figure 3.4). The cytoplasm contains one or more phagosomes, strongly stained with Giemsa (Figure 3.4 A, B), in which the engulfed material can be visible (Figure 3.4 C,

D, F). When the phagosome occupies most of the volume of the cell, the nucleus is often peripheral and flattened, while the cytoplasm is reduced to a thin layer around the phagosome (Figure 3.4 F).

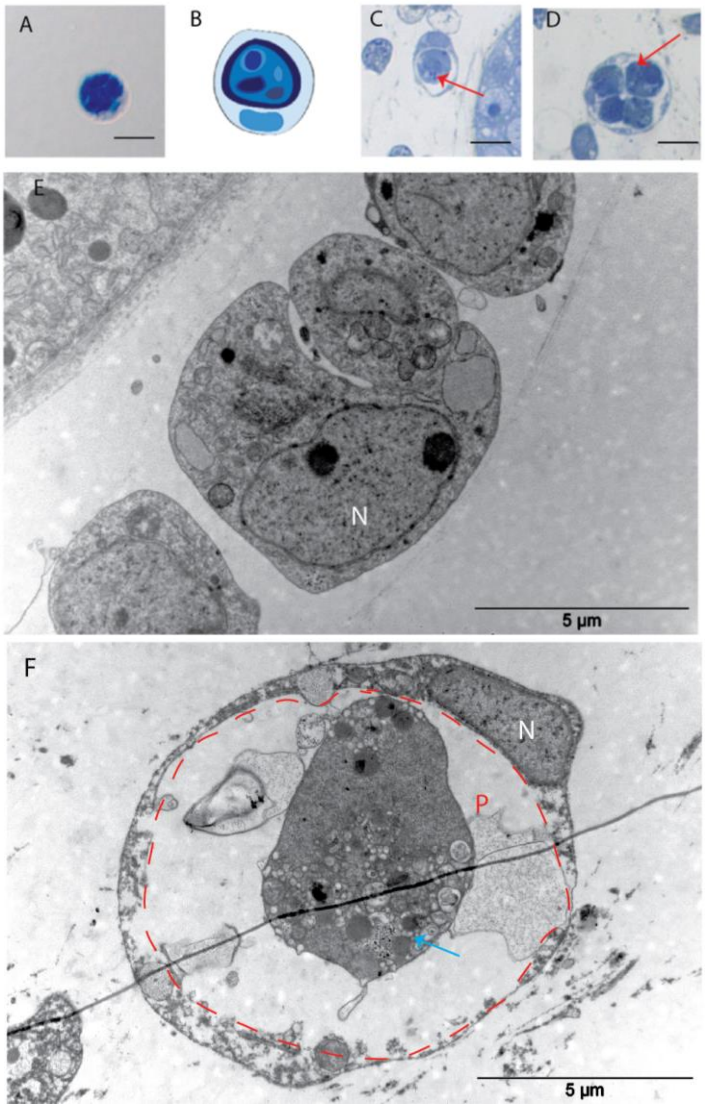


Figure 3.4: Morphological (A-D) and ultrastructural (E-F) characterization of macrophage-like cells (MLC). A) Giemsa stain. B) Graphical scheme of Giemsa stained MLC; C-D) Methylene blue on semi thin sections. The phagosome is pointed with a red arrow. In D, the phagosome contains multiple cells. E) MLC during the process of engulfment. F) Ultrastructure of a MLC with engulfed material (blue arrow) in the phagosome (P, dotted red line). The nucleus (N) is located in the periphery and the cytoplasm is segregated in a thin layer delimitating the phagosome. (Scale bar: A, C, D: 10 µm).

3.5.3 Granular amoebocytes

Granular amoebocytes are about 10 µm in length (Figure 3.5). Similarly to hyaline amoebocytes, granular amoebocytes show amoeboid shape, but unlike HA their cytoplasm is completely filled by small electrondense granules (about 0,5 µm) (Figure 3.5D) that turn to a yellow-brown color after Giemsa staining (Figure 3.5 A, B).

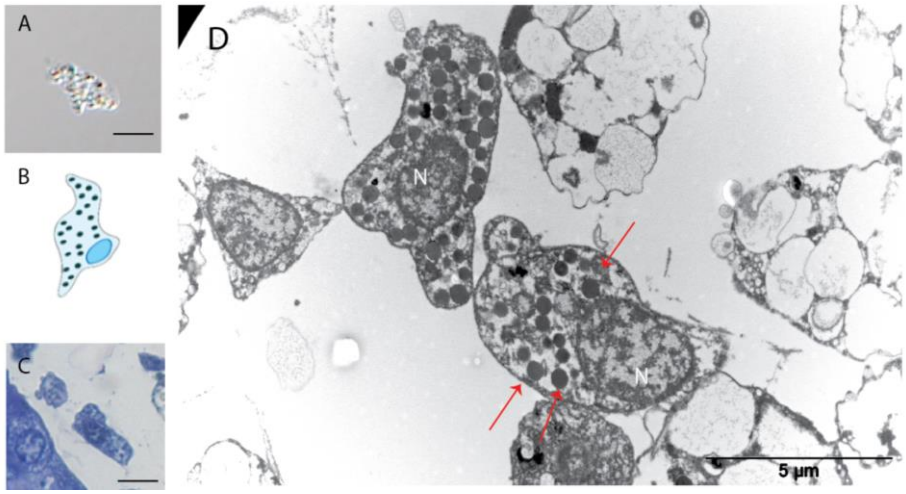


Figure 3.5: Morphological (A-C) and ultrastructural (E) characterization of granular amoebocytes (GA). A) Giemsa stain. B) Graphical scheme of Giemsa stained GA. C) Methylene blue on semi thin sections. D) Two GA showing an amoeboid shape. The cytoplasm is filled of electrondense granules (red arrows). N: nucleus. (Scale bar: A, C, D: 5 µm).

3.5.4 Morula cells

Morula cells are large hemocytes, ranging from 10 to 18 µm (Figure 3.6). The nucleus is peripheral and the cytoplasm is filled by a variable number of granules of about 2 µm of diameter. These granules contain PO and after Giemsa staining their color variates from pink to yellow (Figure 3.6 A-C), while after osmium counterstaining they can appear more or less electrondense (Figure 3.6 G-I). This difference depends if they released or not the PO in response to an immuno stimuli: when the MCs are not involved in the defensive reaction, the vacuoles show a homogeneous electrondense content in TEM images (Figure 3.6 G), while when they start to release the PO the electrondense material looks disorganized and flaking (Figure3.6 H, I).

3.5.5 Granulocytes cells

Granulocytes cells (around 8 um) are filled of electrondense granules, bigger than the ones in GA and HA (Figure3.6 E, J). Granulocytes do not show amoeboid movements and may possibly represent an intermediate stage between granular amoebocytes and morula cells.

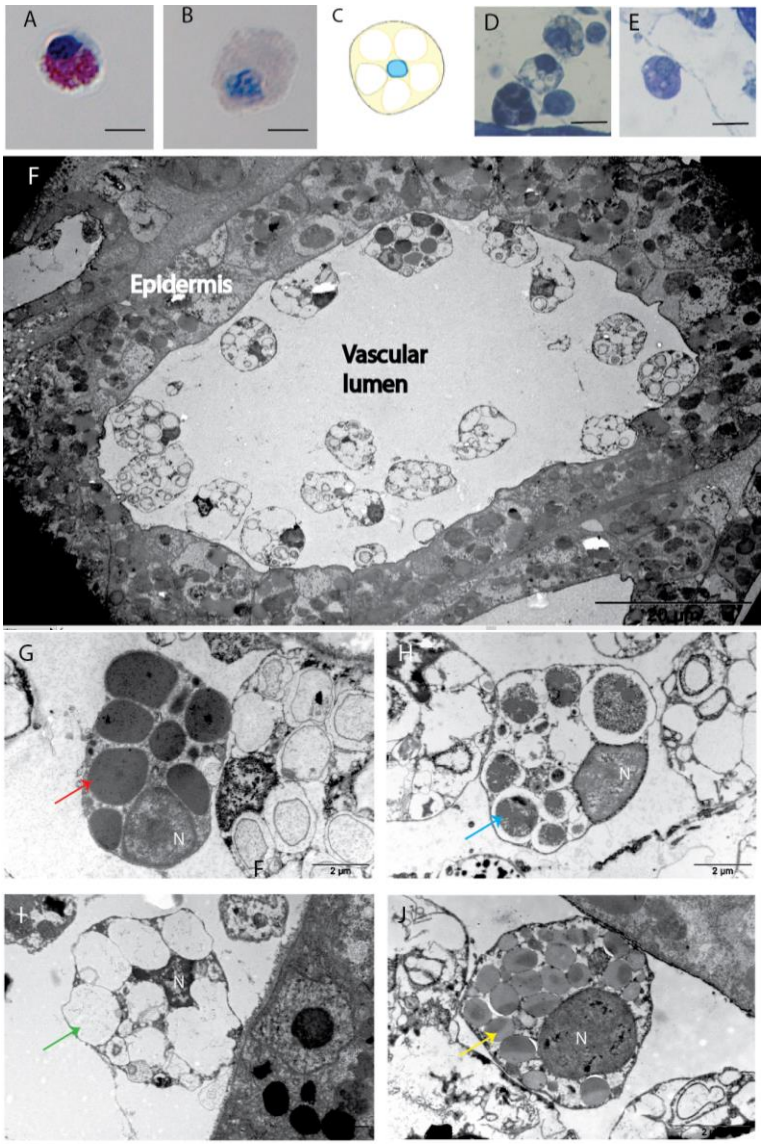


Figure 3.6: Morphological (A-C, D-E) and ultrastructural (F-J) characterization of morula cells (MC) (A-D, F-I) and granulocytes (E, J). A-B) Giemsa stain on two MC with different vacuole contend; C) General scheme of Giemsa stained MC; D) Three MC stained with methylene blue on semi thin sections. The granules appear more or less stained depending on the PO content; E) Granulocyte stained with methylene blue. The cytoplasm is filled by blue granules, smaller than the vacuoles present in MC; F) Ultrastructure of an ampulla containing various MC. In the vacuoles, the electrondense material – corresponding to PO- is not heterogeneous; G-I) Three MC at different stages of PO release. G: the electrondense granules (red arrow) are filled with PO. H: the content (blue arrow) starts to be released. I: the vacuoles (green arrow) lookempty. J) Granulocytes containing small electrondense granules (yellow arrow). (N: nucleus). (Scale bar: A, B: 5 µm; ED, E: 10 µm; D: 20 µm; G-J: 2 µm).

3.5.6 Storage cells (nephrocytes and pigment cells)

Storage cells are big cells up to 20 µm and show an irregular shape (Figure 3.7). The cytoplasm contains few (1-4) big vacuoles with granules that turn to purple with Giemsa staining (Figure 3.7 A-B). The ultrastructure of pigment cells (Figure 3.7 D) and nephrocytes (Figure 3.7 E) is similar. The urate crystals are bigger than the pigment granules, with irregular shape (Figure 3.7 F, G).

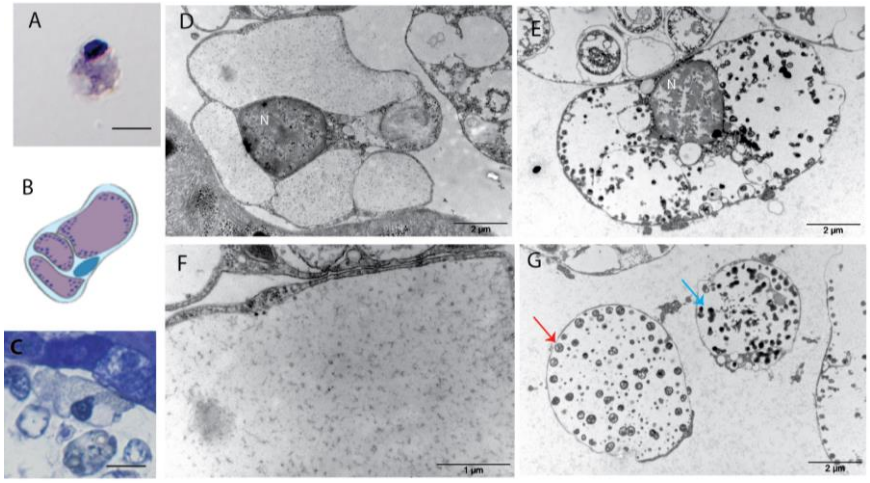


Figure 3.7: Morphological (A-C) and ultrastructural (D-G) characterization of storage cells. A) Giemsa stain; B) Graphical scheme of a Giemsa stained storage cell; C) methylene blue stain on semi thin sections; D-E) TEM images of a pigment cell (D) and a nephrocyte (D). F) Aspect of pigments contained in the vacuoles of pigment cells. G) Ural crystals contained in the vacuoles of nephrocytes. Those crystals show heterogeneity in shape (red arrow: circular crystals, blue arrow: elongated). (N: nucleus). (Scale bar: A, B 10 µm, D, E, 2 µm, F 1 µm).

3.5.7 Hemoblasts

Hemoblasts are small cells (5-6 µm) (Figure 3.8). The big nucleus shows dense granules of chromatin and it is strongly stained in blue by Giemsa (Figure 3.8 A, B). In the cytoplasm, organelles like mitochondria, endoplasmic reticulum, and small electrondense granules can be present (Figure 3.8 D, yellow, red and blue arrows). Interestingly, some elongated cells with pseudopods have been observed in the vascular lumen, often connected to the ampullar epidermis (Figure 3.9 A). The nucleus of these cells occupies half of the cell diameter and few small electrondense granules (Figure 3.9 A, blue arrow), extended RE (Figure3.9 A, red arrow) and mitochondria are present in the cytoplasm. One or two pseudopodes (Figure 3.9 B, blue

arrows) are visible. This morphotype may represent a stage of hemoblast differentiation, which probably will give rise to GA or HA.

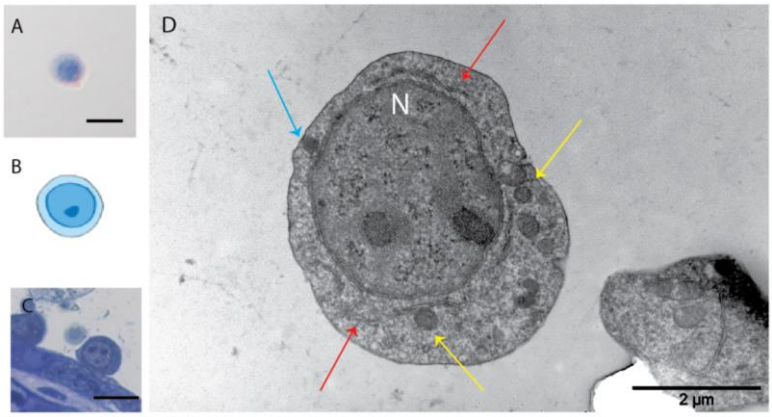


Figure 3.8: Morphological (A-C) and ultrastructural (D) characterization of hemoblasts. A) Giemsa stain. B) Graphical scheme of a Giemsa stained hemoblast. C) Methylene blue staining on semi thin section. D) Hemoblast ultrastructure. The big nucleus (N) occupies more than half of the cellular volume. Mitochondria (yellow arrows), endoplasmic reticulum (red arrows) and small electrondense granules (blue arrow) are visible. (Scale bar: A, C 5 µm, D 2 µm).

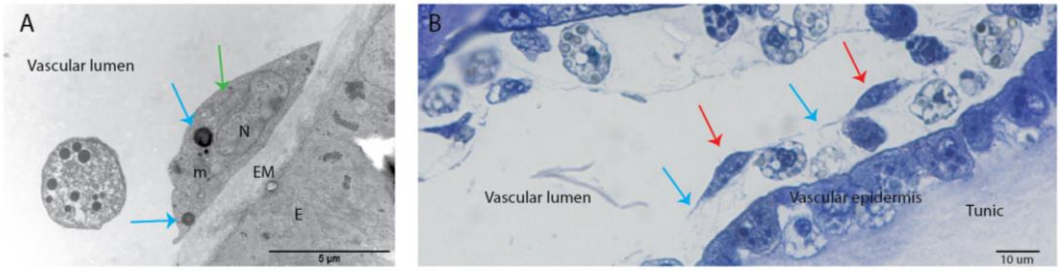


Figure 3.9: Characterization of amoeboid cells, probably an intermediate stage of hemoblast differentiation. A) ultrastructure of amoeboid cell present in the lumen of the stolon. The nucleus N is elongated and big, mithocondria (m) and extensive reticulum (green arrow) are present. The cell is in contact with the external matrix (EM) of an epithelial cell (E). B) Two amoeboid cells (red arrows) in the stolonial lumen. Pseudopodes (blue arrows) are emitted by the amoeboid cells) (Scale bars: A 5 µm, B 10 µm).

3.6 Relative distribution of hemoblast localization in budding tissues

The morphological characterization proposed in the previous paragraph has been used to describe the distribution of hemoblasts during the life cycle of *P. zorritensis.* The presence of hemoblasts have been screened in four stages and tissues: the stolon, the budding nest (before triggering the bud), in the spherule (dormant stage) and in the bud at the stage “double vesicle”

(Figure 3.10). The relative frequency of hemoblasts has been calculated in non-budding tissues (stolon, bud nest and spherules) and in budding tissues (double vesicle). Haemoblasts represent the 9,9±1,7% of all hemocytes in the double vesicle, while in the other stages they occurred at a slightly lower frequency in thither stages (5,2±3,4% in the stolons, 7,3±1,3% in the budding nests, 6,9±1,5% in the spherules).

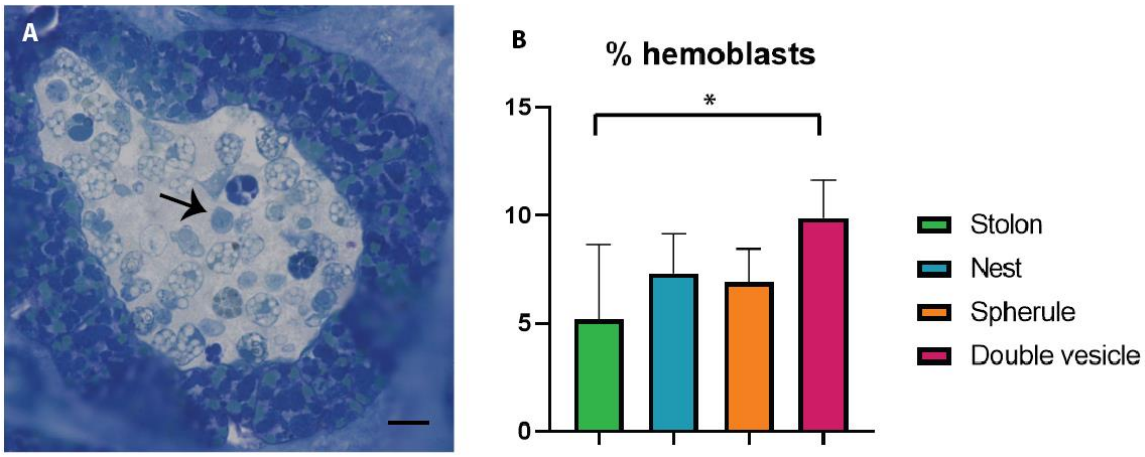


Figure 3.10: A) Transversal semithin section stained with methylene blue showing a vascular ampulla containing a hemoblast (arrow) (Scale bar: 10 um); B) Schematic representation of Proportions of hemoblasts at different stages of the life cycle in *Polyandrocarpa zorritensis*, calculated as reported in material a method chapter. The error bars represents the standard deviations for each average. Green: stolon stage; Blue: budding nest stage; Orange: spherule stage. Pink: double vesicle stage. The asterisk represents the significant differences using the one-way ANOVA with post-hoc Tukey's multiple comparisons test (p>0,05).

The presence of hemoblasts has been detected also in aggregates during vasal budding (See Chapter 2, Scelzo et al., 2019). Hemoblasts have been observed in association to the budding epidermis during invagination (Figure 3.11 A, red arrows), fusion and double vesicle (Figure 3.11 B, C, red arrows). In some of these cells, centrioles are visible, suggesting mitotic activity (Figure 3.11 D, E).

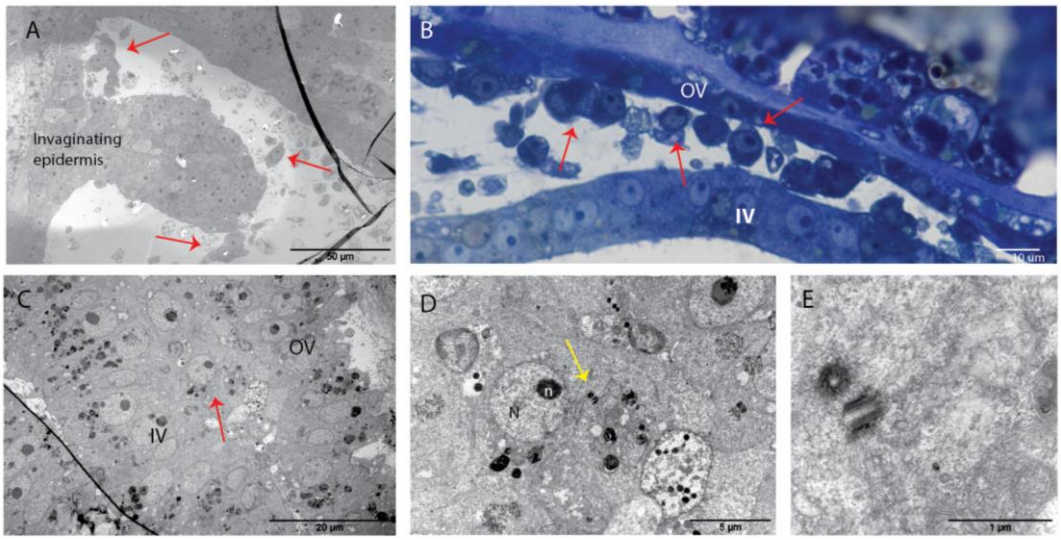


Figure 3.11: Localization of hemoblasts during vasal budding. A) Hemoblasts (red arrows) clustering close to the invaginating site. B-C) Hemoblasts aggregates (red arrows) located between outer vesicle (OV) and inner vesicle (IV). B is stained with methylene blue, C is a TEM image. D) Close up on the hemoblast aggregate. The yellow arrow point out the centrioles showed in figure E. (N: nucleus; n: nucleolus). (Scale bar: A 50B 10 µm,

3.7 Patterns of epithelial and hemocytes proliferation during vasal budding

In order to assess the role of epithelial and mesenchymal proliferation during the formation of the vasal bud, I performed proliferating assays by EdU incorporation and localization of the phosphorylated histone H3 (PHH3) in the early stages of vasal budding, from the triggering of the bud to the double vescicle stage (Scelzo et al., 2019). The EdU staining and EdU pulse-chase experiments are summarize in Figure 3.12. During long periods of incubation, i.e. from the abscission to the double vesicle stage (24 hours post abscission, hpa) EdU is integrated in both the outer and inner vesicle (Figure 3.13), suggesting the active role of proliferation in bud formation. EdU positive hemocytes are also localized in the vascular lumen and between the inner and the outer vesicle (Figure 3.13). In order to elucidate exactly at which stage the cell proliferation occurs and in which cells, I restricted the EdU incubation windows and I specifically targeted each budding stage. In the very early stages of vasal budding (4 hours after abscission), proliferating cells have not been detected in the epidermis, suggesting that proliferation is not involved in the firsts steps of the swelling process. However, some circulating hemocytes are EdU positive (Figure 3.14).

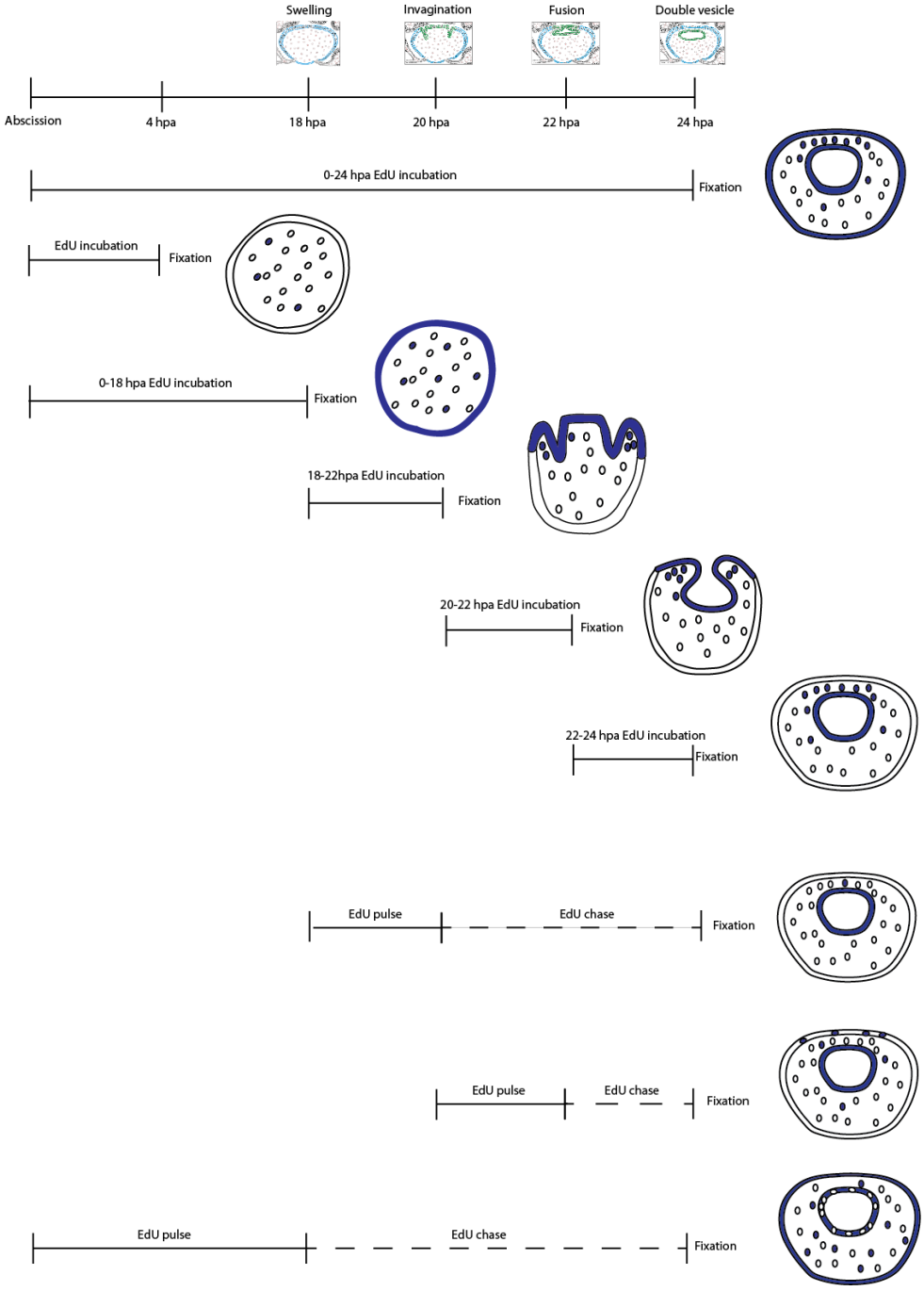


Figure 3.12: Scheme summarizing the experimental design and the results obtained in the experiments aimed to localize the cell proliferation dynamics with EdU. The stages at which the specimens were observed are schematically represented. The EdU negative epidermal and mesenchymal cells are left in white. The EdU positive epidermis and mesenchymal cells are represented in blue.

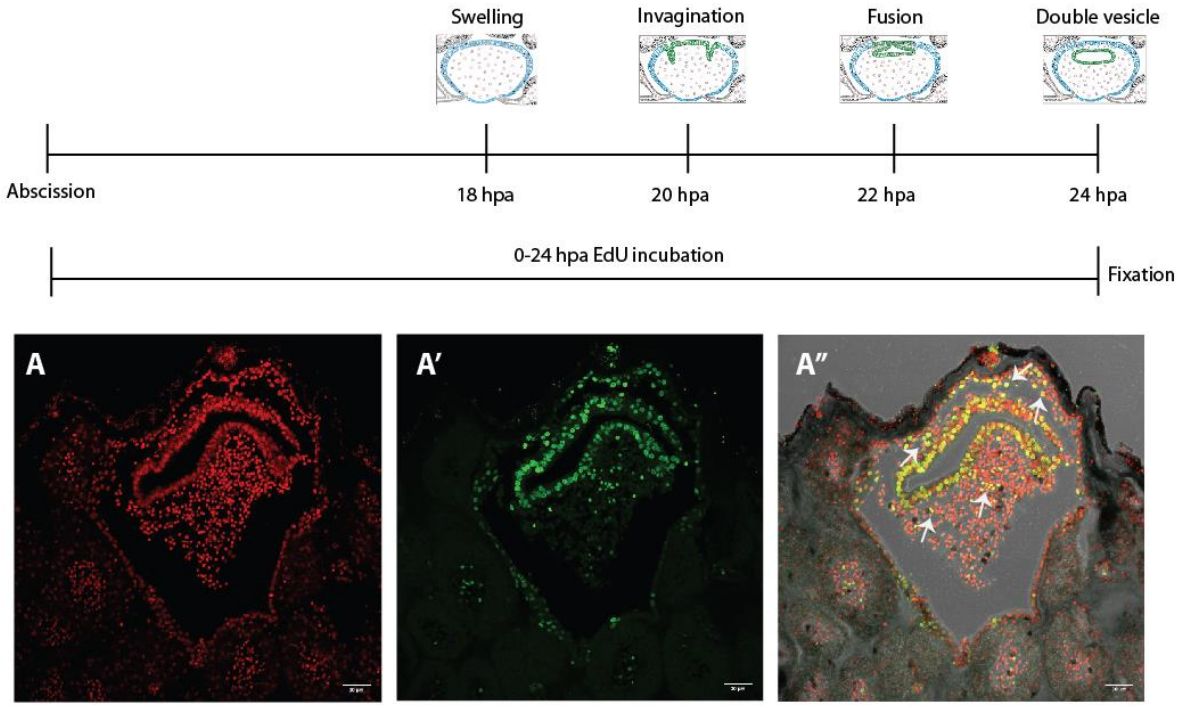


Figure 3.13: Buds incubated with EdU from abscission to double vesicle (0-24hpa). Inner vesicle, outer vesicle (figure A'', white arrows) are EdU positive. (A: Hoechst; A': EdU; A'': merged) (Scale bar: 30µm).

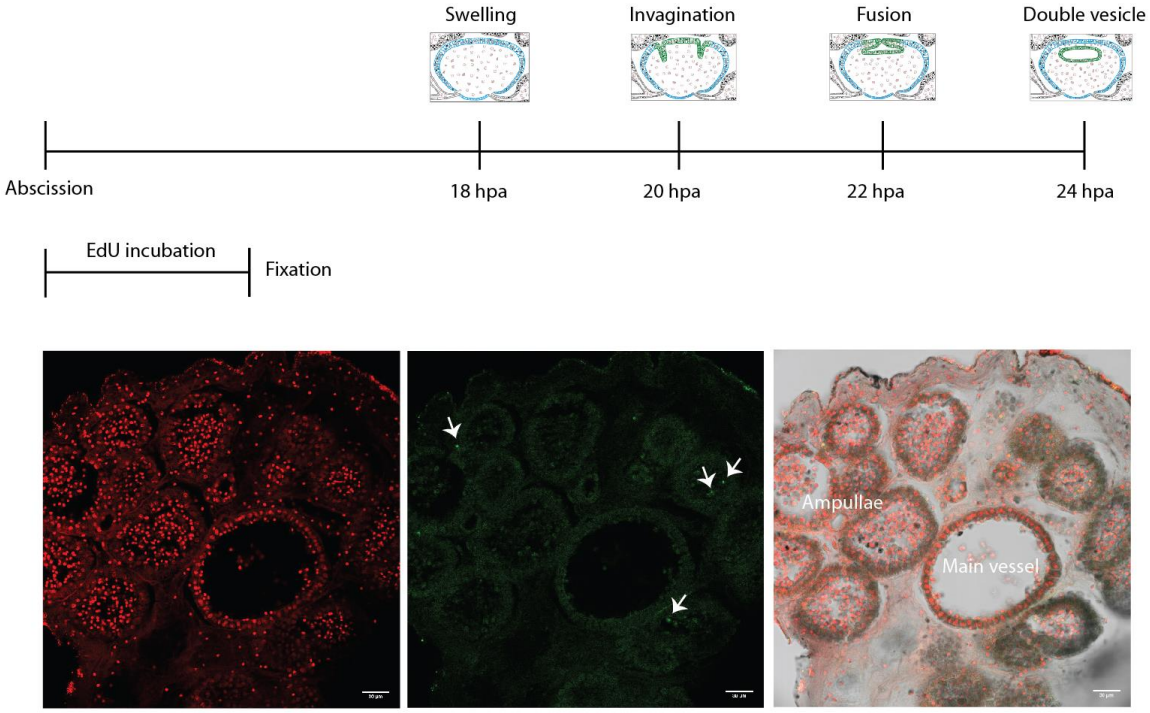


Figure 3.14: Short EdU incubation (4 hours) performed in the early stages of swelling (0-4hpa). The main vessel just started inflating. The epidermal cells of the vessel look thinner than the cells in the surrounding ampullae. Some EdU positive cells are localized in the ampullar lumen (arrows) but not in the epidermal cells of the vessel. (Red: Hoechst; Green: EdU; Yellow: merged) (Scale bar: 30 µm).

When increasing the incubation to the end of the swelling stage (0-18 hpa), EdU positive cells are detected in the vascular epidermis and in hemocytes circulating in the lumen (Figure 3.15), showing an early proliferating activity in the haemocytes but also that proliferation is involved in the swelling process.

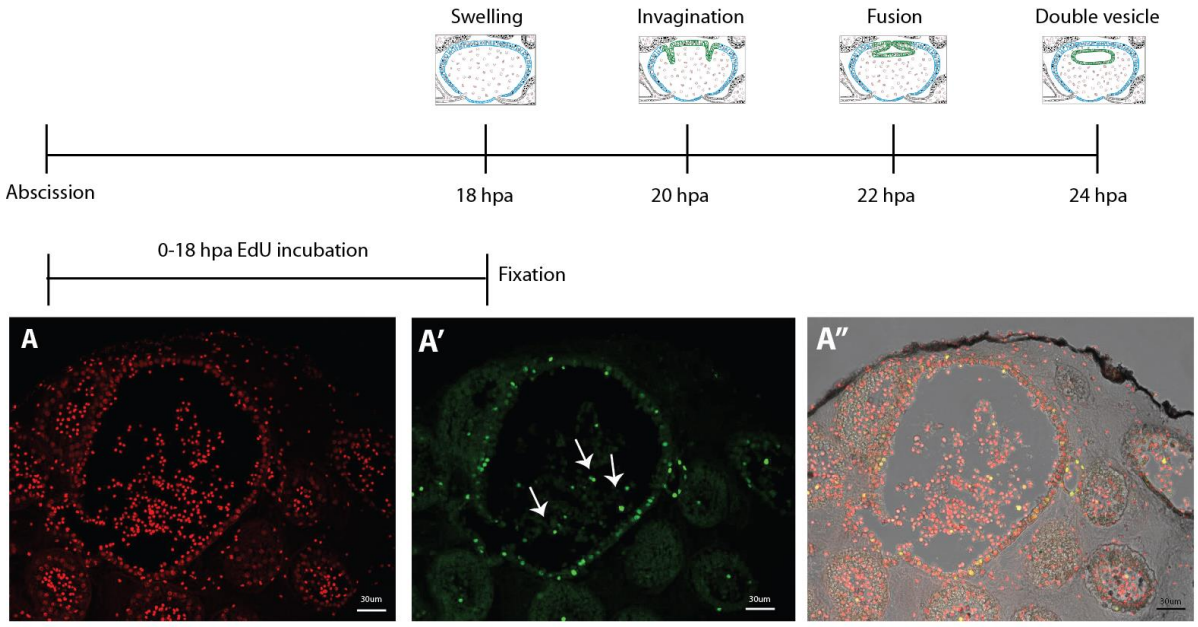


Figure 3.15: Bud incubated with EdU during swelling (0-18hpa). The swelled vessel is EdU positive, as some hemocytes in the lumen (figure A', arrows) (A: Hoechst; A': EdU; A'': merged) (Scale bar: 30µm).

When the samples are treated with EdU during the invagination stage (from 18 to 20 hpa), EdU positive cells in the epidermis are concentrated in the site that is undergoing to folding (Figure 3.16 arrows), as well as in the hemoblasts clustering close to the invaginating area (Figure 3.16 stars).

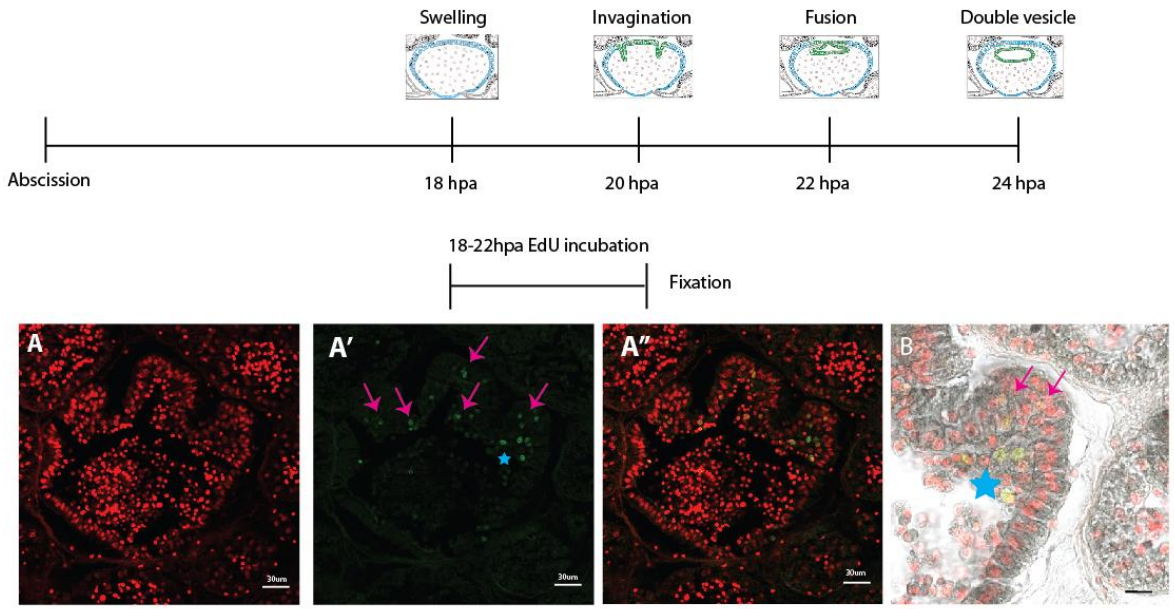


Figure 3.16: Bud incubated with EdU during invagination (18-20 hpa). The epidermal cells in the invaginating areas (figure A', B pink arrows) are EdU positive. Hemoblast aggregates (figure A, B star) are positive too. (Red: Hoechst; Green: EdU; A'', B: merged) (Scale bars: A-A'' 30um, B 10 µm).

From 20 to 22 hpa (Figure 3.17 A-B), the EdU has been incorporated in hemoblasts localized at the invaginating edges - which are fusing together at this stage – (Figure 3.17 pink arrows) and in the epithelial cells that will form the outer vesicle (Figure 3.17 B white arrows, the future lumen of the inner vesicle is pointed with a blue star). Localization by immunohistochemistry of phosphorylated histone H3 (PHH3) confirms an active proliferation of cells in the hemoblast clusters and in epithelial cells of the future inner vesicle (Figure 3.17 C-C'').

When the EdU is incubated at the end of the fusion process up to the formation of the inner vesicle (from 22 to 24 hpa) almost all the cells that compose the inner vesicle are EdU positive (Figure 3.18). The cells of the outer vesicle result positive only in the region closer to the inner vesicle (Figure 3.18 B white arrows), while hemocytes located between the inner and outer vesicle are also positive to EdU (Figure 3.18 B blue arrows). Localization by immunohistochemistry of phosphorylated histone H3 (PHH3) shows an active proliferation of cells in the inner vesicle (Figure 3.18 C white arrow) and in hemoblasts aggregating between the inner and outer vesicle (Figure 3.18 D' white arrows).

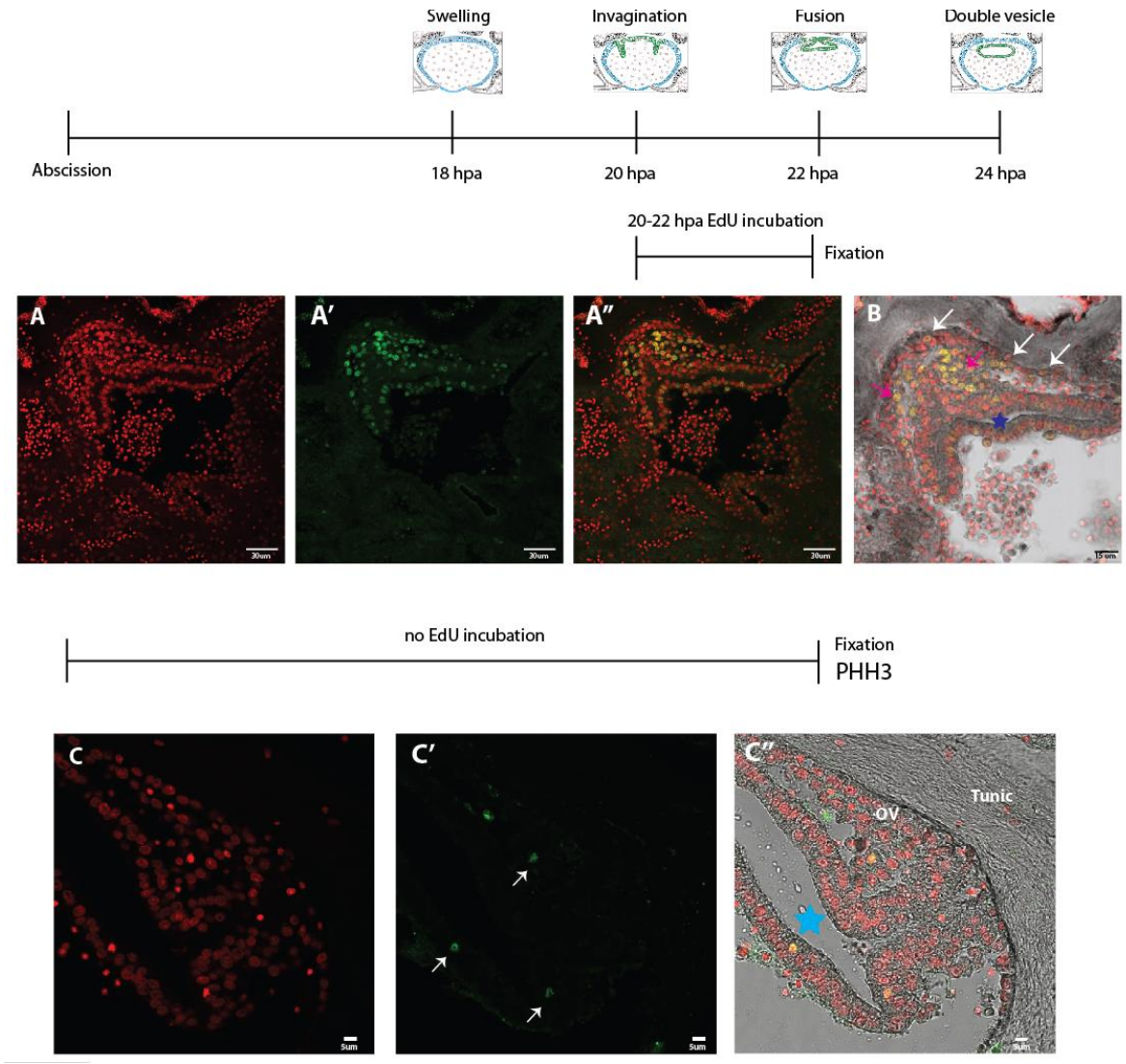


Figure 3.17: EdU incubation between 20 and 22hpa (A-B) and anti-PHH3 immunohistochemistry at the fusion stage (C-C"). A-B) EdU incubation. Buds incubated with EdU during fusion stage (incubation between 20 and 22hpa) (A-B). Epidermal cells (white arrows in B), hemoblast clusters (pink arrows in B) and cells in the future inner vesicle are EdU positive. The future lumen of the inner vesicle is pointed out with a star in B (A: Hoechst; A': EdU; A", B: merged); C-C'') Immunohistochemistry anti phosphorylated histone H3 (PHH3) at the fusion stage. PHH3 positive cells (arrows) are localized in the future inner vesicle and in hemoblasts aggregates. The lumen of the future inner vesicle is pointed out with a star in C" (C: Hoechst; C': PHH3; C, D, G: merged; ov: outer vesicle). (Scale bars: A-A", C-C": 30um, B: 10 µm).

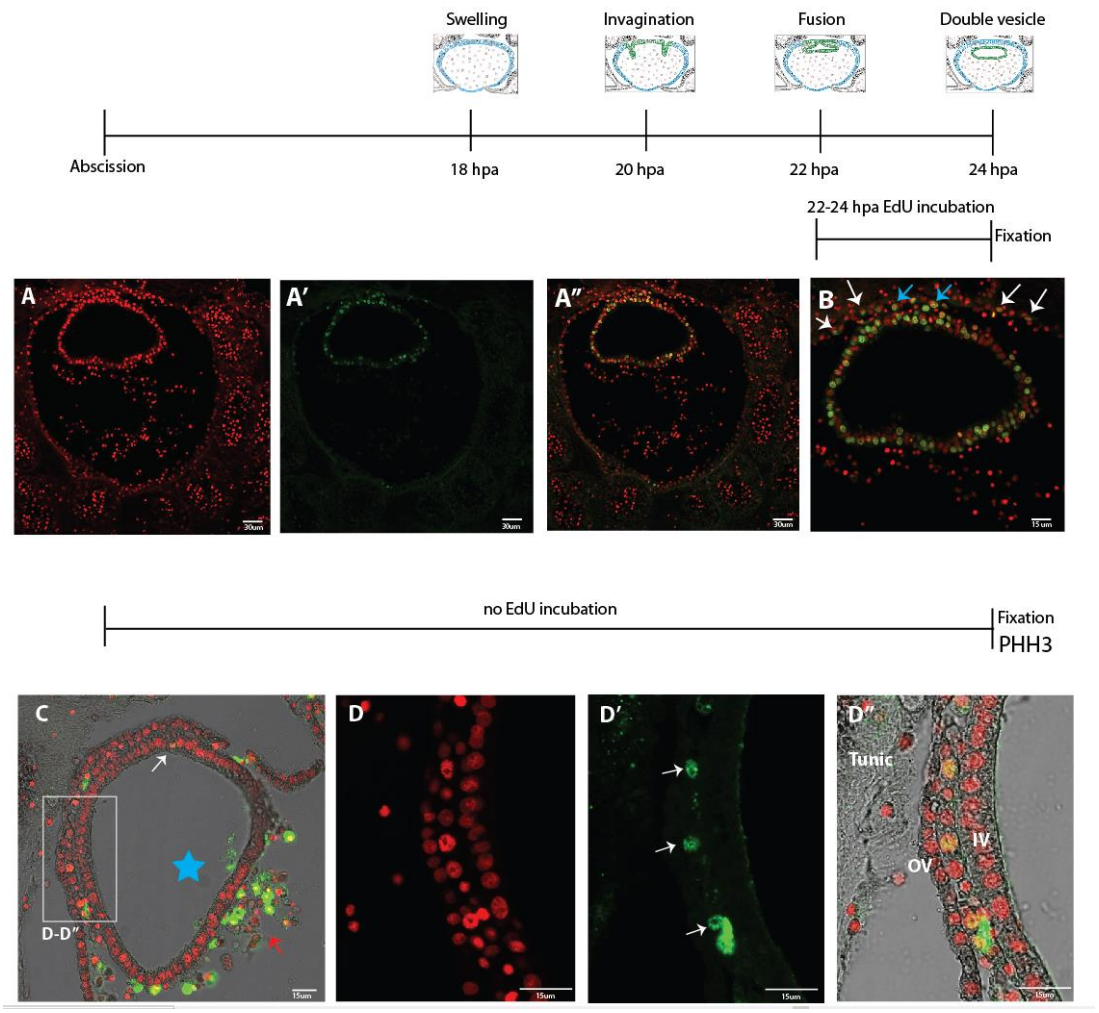


Figure 3.18: EdU incubation between 20 and 24hpa (A-B) and anti-PHH3 immunohistochemistry at the double vesicle stage (C-D''). A-B) EdU incubation. The cells in the inner vesicle, the cells of the outer vesicle in the area close to the inner vesicle (white arrows in B) and hemobast aggregates (blue arrows in B) are EdU positive. (A: Hoechst; A': EdU; A'-B merged. B: detail of the inner vesicle as shown in A'') (scale bars: A-A'': 30um; B: 15um); C-D'') Immunohistochemistry anti-PHH3 at stage double vesicle. PHH3 is localized in cells of inner vesicle (Figure C, white arrow) and in the hemoblats aggregating between inner vesicle (IV) and outer vesicle (OV) (Figure D' arrows). The other hemocytes stained in the vascular lumen (Figure C, red arrow) are morula cells showing a non-specific signal in the vacuoles. (D: Hoechst; F: PHH3; C, D, G: merged). (Scale bars: C-D'': 15 µm).

3.8 Hemocytes dynamic during vasal budding

In order to further dissect the proliferating events and investigate if the inner vesicle is originated from proliferating epithelia or from proliferating hemoblasts, pulse-chase EdU assays were performed (as summarized in figure 3.12). Bud undergoing invagination stage (~18hpa) (Figure 3.19 A-A'') and fusion (~ 20hpa) (Figure 3.19 B-B'') were incubated with EdU for 2 hours (pulse), transferred in sea water and let developed until double vesicle (24hpa – chase). The specimens incubated during invagination show EdU positive cells in the inner vesicle and few

mesenchymal cells (Figure 3.19 A'). In addition to EdU positive inner vesicle and mesenchymal cells (Figure 3.19 B' white arrows), the specimens incubated during fusion show also EdU positive cells in the outer vesicle, in the region closest to the inner vesicle (Figure 3.19 B' yellow arrows).

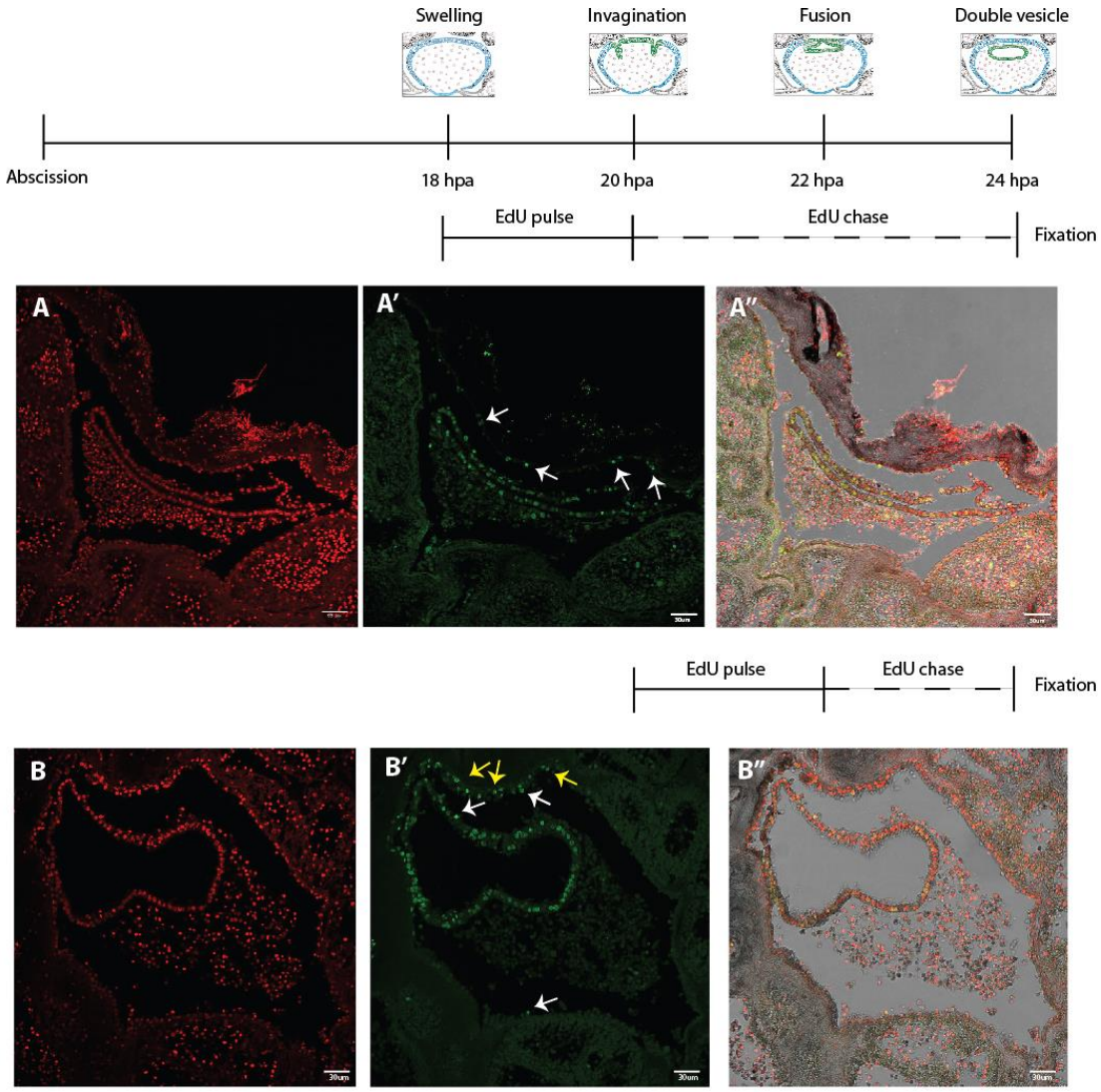


Figure 3.19: EdU pulse-chase assays. A-A") EdU incubated during invagination stage (18-20hpa). Cells in the inner vesicle and few hemoblasts (white arrows) are EdU positive after a 4 hour chase. (A: Hoechst; A': EdU; A": merged (scale bar 30um); B-B") EdU incubated during the fusion stage (20-22hpa). After a pulse of 2 hours, the EdU cells are localized in the inner vesicle, in the outer vesicle corresponding to the invaginating edges (yellow arrows) and few mesenchymal cells (white arrows) (B: Hoechst; B': EdU; B": merged (Scale bar 30 µm).

When the bud is incubated right after abscission until the end of swelling stage (~ 18hpa) and then let developed in sea water until double vesicle (24hpa), outer and inner vesicle are EdU

positive (Figure 3.20). However, the inner vesicle is not completely positive. At this stage, EdU positive hemoblasts are scattered distributed in the lumen of the vessel (Figure 3.20, arrows).

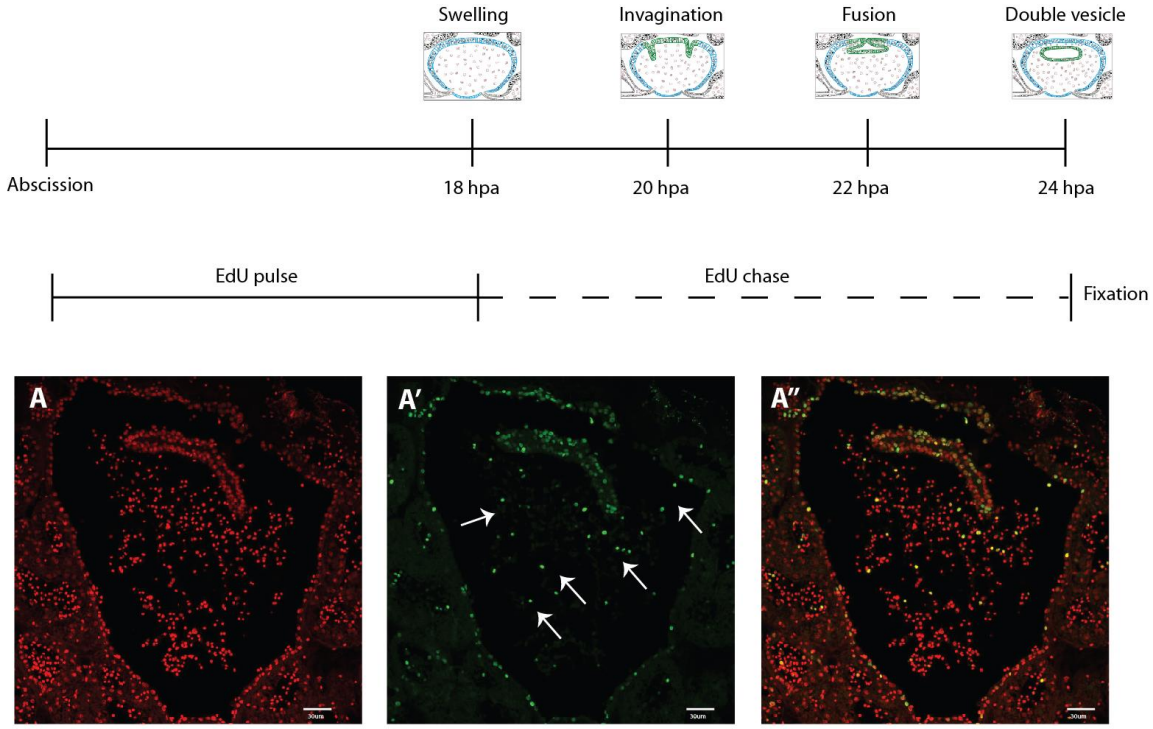


Figure 3.20: Long EdU pulse during the swelling stage (0-18hpa). The outer vesicle, some cells of the inner vesicle and hemocytes in the lumen (arrows) are EdU positive. (A: Hoechst; A': EdU; A": merged). (Scale bar: 30 µm).

3.9 Integrin-alpha 6 positive hemocytes are present in the developing buds

Integrins are transmembrane proteins that mediate cell adhesion (Albelda and Buck, 1990; Alberts et al., 2002b). Integrin-alpha 6 (Iα6) is a known marker of stem cells and tumor cells in vertebrates (Docheva et al., 2007; Krebsbach and Villa-Diaz, 2017). Recently, Iα6 have been shown to be a specific marker of hemoblasts responsible for WBR in *Botrylloides diegensis* (Kassmer et al., 2019). To assess if Iα6 is a hemoblast marker in *P. zorritensis* and localize its expression during budding, immunohistochemistry with an antibody anti integrin-alpha6 was performed at the double vesicle stage. Preliminary data (n=2) suggest the presence of Iα6 positive hemocytes in the budding region at the double vesicle stage (Figure 3.21). These cells are spherical and small (5 µm) and are located between the inner and outer vesicle, where usually hemoblast clusters are present (Figure 3.21 A', B' white arrows).

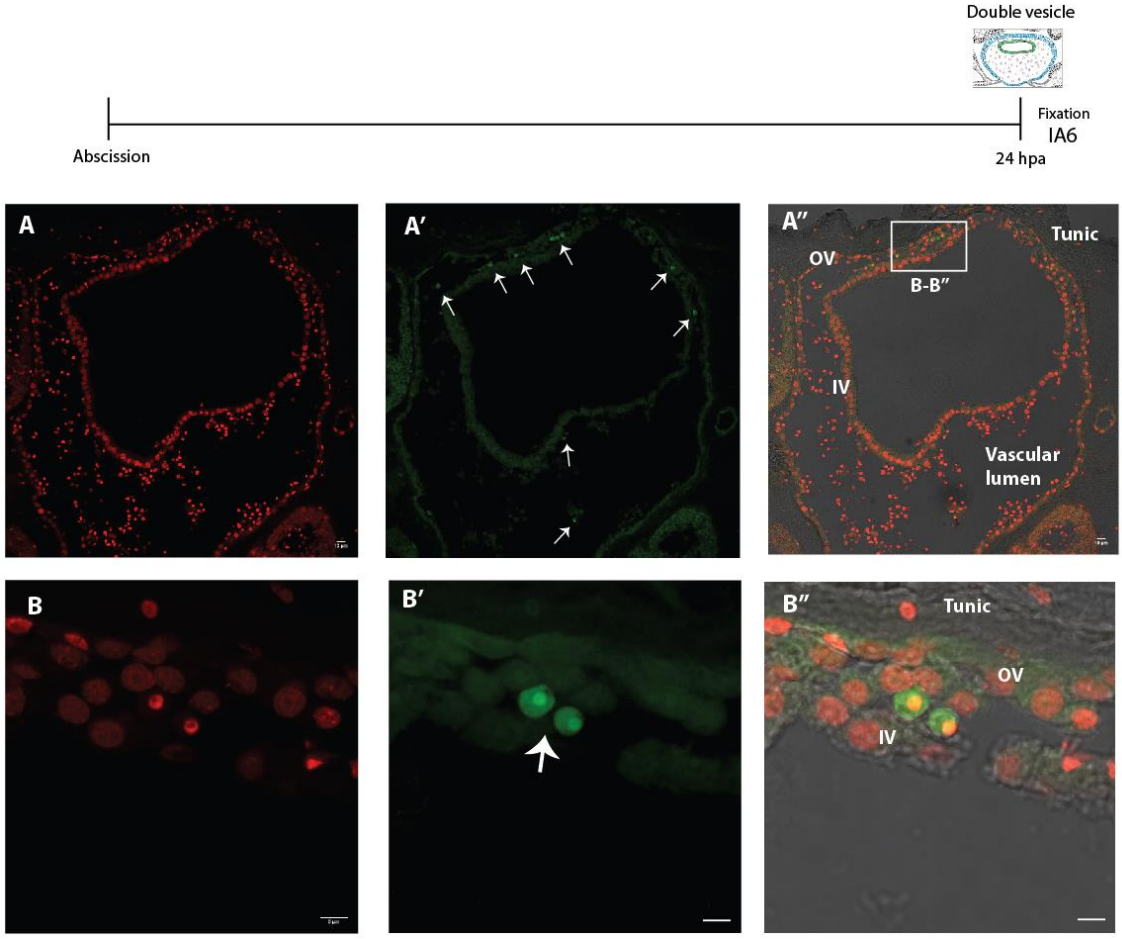


Figure 3.21: Anti-Integrin-alpha6 immunostaining performed at the double vesicle stage. A-C) Iα6 positive hemocytes are located in the lumen of the vessel and in hemoblast aggregates (figure B, arrows). D-F) Detail of Iα6 positive hemoblasts aggregating between inner (IV) and outer (OV) vesicle. (A,B: Hoechst; A',B': Iα6; A'', B'': merged) (Scale bars: A-A'' 10um; B-B'' 5 µm).

3.10 Flow cytometric characterization of hemoblasts

To further characterize the cytomorphology of the different hemocyte populations and identify specific markers as already performed in other species, such as *Halocynthia* (Donaghy et al., 2017) or *Botryllus* (Rosental et al., 2018), the hemocytes of *P. zorritensis* were sorted by flow cytometry. Four main Hoechst-positive populations were defined based on a Hoechst threshold and the SSC (Side Scatter, granularity) and FSC (Forward Scatter, size) profiles as described in material and methods section (Figure 3.22 A-A'). The population identified as P5 (Figure 3.22 B' blue) comprises cells with a low SSC and low FSC, meaning that they are relatively small and less differentiated. Cell-sorting set up beads with a diameter of 6µm (±10%) were run in parallel, showing that the cells in the P5 and the beads have a comparable FSC (see Material and methods section 3.20, Figure 3.24). Electron microscopy on the sorted P5 confirmed the size (around 5

µm in diameter) and revealed that the ultruastructure corresponds to the morphotype identified as hemoblasts (Figure 3.22 C): roundish shape and a simple cytoplasm containing only few mitochondria.

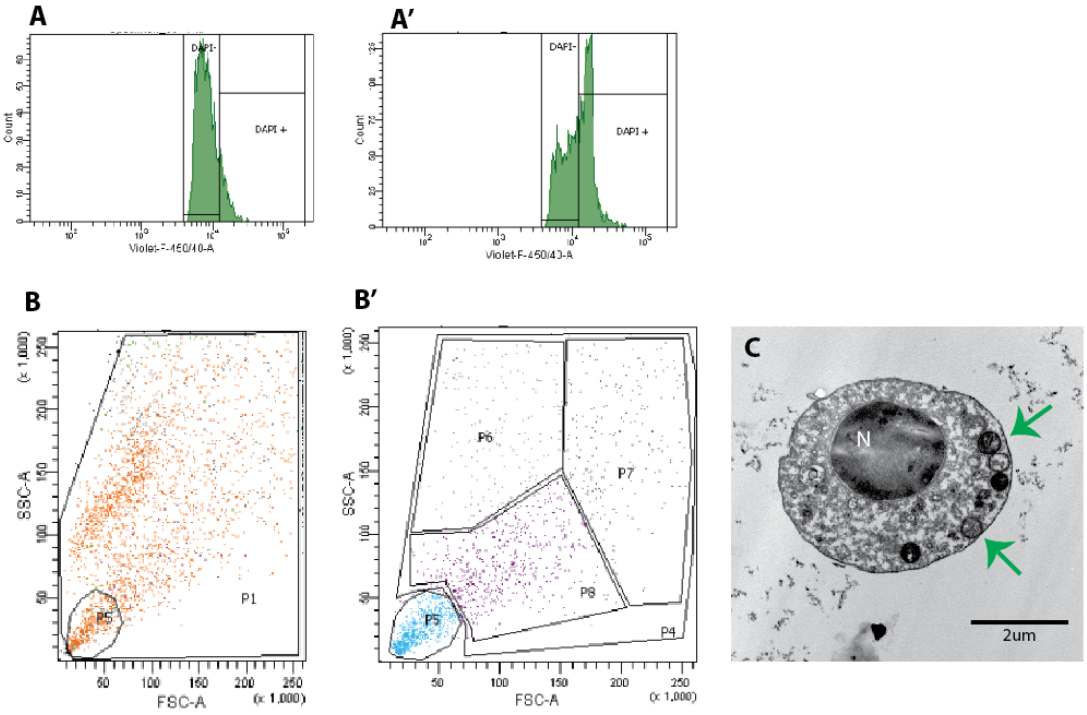


Figure 3.22: FACS plots and TEM on P5 population. A) Fluorescence intensity emission of not-stained hemocytes excited by the violet laser (excitation 405 nm), showing an autofluorescence pic of hemocytes in this channel; A') Fluorescence intensity emission of hemocytes stained with Hoechst excited by the violet laser (excitation 405 nm). The shift in the fluorescence pic corresponds to the Hoechst positive cells; B) Forward and side scatter plot of unstained hemolymph used as control; B') Forward and side scatter plot of hemocytes stained with Hoechst. Four populations (P5 in blue, P6 in grey, P7 in black, P8 in purple) were identified; C) Electron microscopy of a sorted P5 cell. Mitochondria are present in the cytoplasm (green arrows) (N: nucleus) (Scale bar: C 2 µm).

In order to obtain a population specifically isolated for the expression of Iα6, the hemocytes extracted were sorted based on the Iα6 enrichment. Spherules were used for the hemocyte extraction. Very preliminary experiments (n=1) show a low percentage (around 2-3%) of living cells expressing Iα6 in the spherules (Figure 3.23).

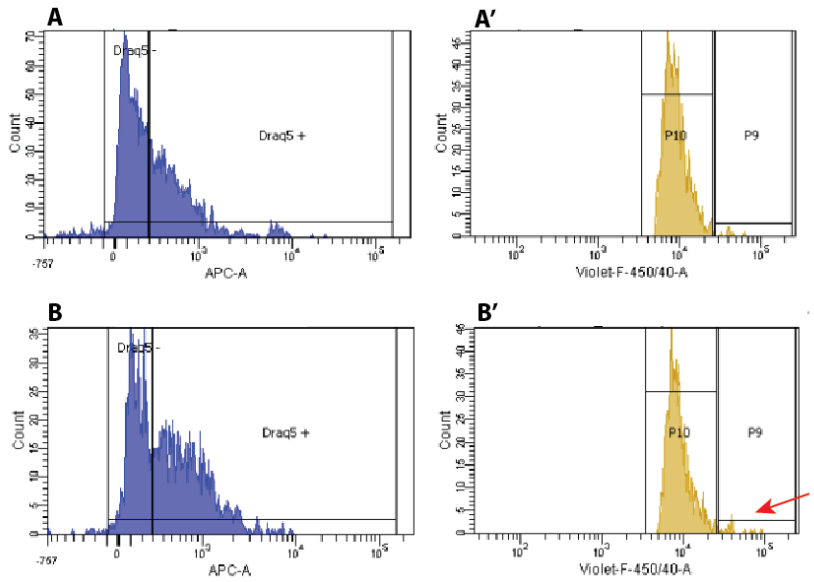


Figure 3.23: Identification of Iα6 positive cells with fluorescence intensity measurement by FACS. A, A') Un-treated specimens. *Polyandrocarpa zorritensis* hemocytes show autofluorescence in the APC-A channel (excitation 594-633 mm) (A) and in the violet channel (excitation 450 nm) (A'); B-B') Sorted cells treated with the live DNA stain DRAQ5 and the antibody anti-integrin alpha 6 conjugated with a fluorochrome. B reports the measurement of fluorescence intensity emitted by DRAQ5 positive cells, while B' reports the measurement of fluorescence intensity emitted by integrin-alpha6 positive cells. The pic emitted by DRAQ5/Iα6 positive cells is visible in the P9 plot (B' arrow).

Discussion

3.11 Conserved (and not conserved) role of hemocyte morphotypes in *Polyandrocarpa zorritensis*

The description of eight different morphotypes confirms the classification made in other colonial styelids, such as *Botryllus schlosseri* (Ballarin and Cima, 2005b) or *Polyandrocarpa misakiensis* (Ballarin and Kawamura, 2009). Despite species-specific differences on the number of hemocyte morphotypes, on the morphotypes proportions and the cell size or content, three main functional categories appear conserved among these different taxa: undiffentiated cells, immunocytes and storage cells (Cima et al., 2016). This functional conservation is probably strictly related to the general physiology of tunicates.

In *P.zorritensis,* two categories of immunocytes have been identified, namely phagocytes (comprising hyaline amoebocytes and macrophage-like cells) and cytotoxic cells (with granular amoebocytes and morula cells). Granulocytes were also suggested as an intermediate stage of differentiation into cytotoxic cells. Phagocytic activity and PO release were observed in ultrastructural analyses, suggesting a conserved role of these cells in the innate immunity of *P. zorritensis*. In *Polyandrocarpa zorritensis* it remains to elucidate if, when, and how immunocytes participate in vasal budding. Hemocytes here characterized as morula cells have been previously reported in contact with the ampullar wall at the double vesicle stage (Scelzo et al., 2019). From this stage, the organogenetic movements begin, and the ampullae regress in parallel to the formation of the new zoois. Morula cells may induce a cytotoxic reaction in the ampullar epithelial cells releasing PO, and at the same time they may recruit phagocytes to digest these cells. The histological and ultrastructural description of ampullar regression events during organogenesis could help to assess the role of immunocytes during vasal budding in *P. zorritensis*. Enzymatic assays aimed to detect the peroxidase, phenoloxidase and phagocytic activity (Cima, 2010) at different stages of vasal budding can also clarify this point.

Hemocytes responsible for storage and transport of nutrients have been reported in some colonial styelidae such as *Polyzoa vesiculiphora* (Fujimoto and Watanabe, 1976b) and *Polyandrocarpa misakiensis* (Kawamura and Watanabe, 1982). These cells called trophocytes are in charge of the nutrient supply of the developing bud before siphons opening. Interestingly, a hemocyte morphotype comparable to trophocytes has not been observed in *Polyandrocapa zorritensis* in this work. The lack of trophocytes during the early stages of vasal budding can be explained by the fact that bud nutrition is ensured by the epidermal wall of the ampullae surrounding the budding nest (see Chapter 2). Indeed, epidermal cells of the ampullae contain

lipid and glycogen granules, which start to accumulate at the onset of the nest formation and later disintegrate in the course of the budding process (Scelzo et al., 2019). It is intriguing to speculate that the morula cells interacting with the epidermis may be responsible for cytotoxic response in the cells and release of nutrients, which are eventually digested by the phagocytes recruited on site. Further investigations are necessary for validating this hypothesis.

3.12 Interplay between vascular epithelia and hemoblasts during vasal budding

In the Chapter 2, I first observed the presence of hemocyte clusters associated to vascular epidermis during early stages of vasal budding, in the areas that undergoes invagination and inner vesicle formation. At the ultrastructural level, these cells look undifferentiated, small, with a high nucleus:cytoplasm ratio. In this Chapter, I provided a description of these cells, which I identified as hemoblats based on the description reported in other ascidian species.

Hemoblasts have been considered by several authors as circulating putative stem cells in both solitary and colonial species (Freeman, 1964, Kassmer et al., 2019, Jimenez-Merino et al., 2019). In colonial species they seem to play a role in different stages of non-embryonic development (see section 3.4). In *Polyandrocarpa zorritensis*, the hemoblasts aggregates may play a role in early stages of vasal budding, leading to the formation of inner vesicle, or in later stages, participating to the organogenetic process as in *P. misakiensis* or *B. schlosseri*. In the latter case, we can speculate based on their position that these cells may participate to nervous system or siphon development, as already described for other species (Sugino et al., 2007).

Some authors suggested that, during *Polyandrocarpa misakiensis* peribranchial budding, the integration of hemoblasts in the inner vesicle occurs trough mesenchymal-to-epithelial transition (MET) (Nakauchi 1971, Tatzuke et al 2012). In *P.zorritensis* the attraction of hemoblasts and in the invaginating region and their accumulation may be due to production of chemotactic molecules by the vascular epidermis. To determine if MET occurs, the expression of epithelial markers such as E-Cadherin (Li et al., 2011, 2010; Pei et al., 2019) in hemoblasts aggregates can be tested by *in situ* hybridization.

During both vascular and peribranchial budding of *Botryllus spp.*, hemoblasts aggregates have been described as germline precursor cells, responsible for the formation of the gonadal primordia and freely circulating oocytes (Mukai and Watanabe 1976, Brown et al 2009, Kawamura and Sunanaga 2011 Sunanaga et al 2006, Brown and Swalla 2007, Brown et al 2009, Rosner et al 2009). The hemoblasts aggregating in the buds of *Polyandrocarpa zorritensis* may be

germ cells precursors. *In situ* hybridizations to test the expression of *piwi*, *vasa*, *nanos* and other GMP genes can be performed at different budding stages.

3.13 Proliferating activity leads to the double vesicle formation

The spatio-temporal distribution of mitosis during NED has been studied in different Styelidae species. In particular, it has been questioned if the mitotic activity of the budding tissue is the sourcing of the inner vesicle. During vasal budding in Botryllinae the inner vesicle develops from proliferating hemoblasts (Ricci et al. in prep; Rinkevich et al., 2007). On the other hand, during peribranchial budding of *Polyandrocarpa misakiensis*, transdifferentiation of the peribranchial epithelium rather than of cell proliferation has been proposed as the main actor of inner vesicle formation (Kawamura et al 2008): in this species, the peribranchial epithelium shows reduced mitotic activity in the early stages of peribranchial budding –i.e. when the bud is still attached to the parental zooid and the epidermis is still evaginating – while during organogenesis the cells constituting the inner vesicle actively proliferate (Kawamura and Nakauchi 1986, Kawamura and Nakauchi 1991) (see section 1.5.4).

In *Polyandrocarpa zorritensis*, the vasal epidermis undergoes drastic modifications during the early stages of budding (Scelzo et al., 2019). Upon the start of the budding process, in a budding nest, the epidermal cells of the main vessel are morphologically indistinguishable from the ampullar cells. After abscission, the morphology of the cells in the main vessel changes. During swelling, the number of cells increases and the vascular cavity expands considerably; then, the folding of the epidermal cells lead to the formation of the inner vesicle. Right after abscission, cell proliferation has not been detected in the cells composing the small vessel lumen. However, during the process of swelling the proliferation assays revealed active proliferation in the vascular epidermis. These results suggest that the first event occurring during vasal budding is the reorganization of epidermal cells induced by abscission. Once all the morphological changes have occurred, the proliferating activity starts and the vascular lumen enlarges as result of the increase of cell number. The distribution of EdU positive cells following 18-22hpa incubation shows that when the vesicle reaches its maximum size, most of the epidermis stops or reduces the proliferating activity, except in the area where the inner vesicle will form. These results suggest two events of epidermal proliferation: the first is leading to the swelling of the vascular lumen; the second, restricted to one side of the vesicle, may lead to the invaginating movements responsible for the double vesicle formation. I previously showed that invagination of the future inner vesicle is accompanied by apical constriction of the cells (Chapter 2). Results from the present work suggest that, in addition to apical constriction, cell proliferation is another force

driving the invagination. Such an interplay between apical constriction and mitosis increases the ability of an epithelium to rapidly change is shape, as described for instance in the tracheal pits of Drosophila (Pearl et al., 2017).

In this chapter, the haemocytes have been reported as actively proliferating in early stages of vasal budding. During the swelling, some hemocytes freely circulating in the lumen proliferate (Figure 3.15). From the invagination stage, EdU and PHH3 positive hemocytes are located in the clusters of hemoblasts associated to the forming inner vesicle (Figures 3.16, 3.17). This raises the question if the proliferating hemoblasts participate to the genesis of the inner vesicle – as during the vascular budding and WBR of other Styelidae. The pulse-chase EdU assays were performed to elucidate this point. When EdU is added for a chase of 18 hours during swelling and then removed for a pulse of 6 hours (up to the formation of double vesicle), part of the inner vesicle is EdU positive (Figure 3.20). From the chase-only experiments we know that the epidermal cells actively proliferate during swelling, and part of the inner vesicle may originate from this epidermal division. However, the inner vesicle is not completely EdU positive, and a regionalized EdU signaling pattern has not been observed. We can speculate on the participation of hemoblasts in the inner vesicle development arguing that these EdU negative cells in the inner vesicle derived by hemoblast integration: the hemoblasts actively proliferating in association with the epidermis during the invagination and fusion stage do not integrate the EdU during the chase step, and are not detected as EdU positive. On the other hand, during the short pulse-chase experiments (carried in buds at invagination and fusion stages), EdU positive cells are localized in the inner vesicle, hemocytes and (when the EdU is incubated at the fusion stage) in the region of the outer vesicle originated by fusion of the invaginating edges (Figure 3.19). Also in this case, the inner vesicle is not completely EdU positive; the EdU negative cells may represent the contribution of the epidermal cells proliferating before EdU incubation, during the process of vasal swelling. During fusion stage, the organization of the vascular epidermis is lost at the fusion site (as observed at the ultrastructural level in Chapter 2). It may be possible that some of the hemoblasts proliferating in close contact to the fusing edges are integrated within the epidermal cells that will form the outer vesicle; this will explain why some epidermal cells of the inner vesicle are EdU positive during pulse at the fusion stage. However, these results do not show clear evidences of hemoblast recruitment in the forming inner vesicle. Tracking experiments performed injecting EdU-incubated hemoblasts in developing buds could be useful to determine the role of hemoblasts in the early stages of vasal budding.

3.14 Iα6 as a potential marker for stem cells in vasal budding

The preliminary data presented in this Chapter show that *Polyandrocarpa zorritensis* hemoblasts aggregating in the budding areas actively proliferate and - at least at the double vesicle stage - express integrin-alpha6. A population of Iα6 enriched hemocytes was also identified by flow cytometry at the stage of spherule.

In *Botryllus schlosseri*, Iα6 positive cells sorted by flow cytometry express genes known to be markers for germline progenitors - such as *vasa*, *pumilio* and *piwi* - and sphingosine 1-phosphate receptor (S1pr1), a molecule involved in stem cell homing and germ cell mobility in mammals (Li et al., 2009). These Iα6 positive cells were localized in the developing gonads and have been identified as germline-committed mesenchymal cells (Kassmer et al., 2015). In *Botrylloides diegensis* the role of Iα6+ hemoblasts go beyond the germline, as they are responsible for WBR. In fact, Kassmer and colleagues showed that a single hemoblast being capable to regenerate all the tissues of the new organism (Kassmer et al., 2019).

Kassmer and colleagues (2019) proposed different hypotheses on the nature of Iα6 hemoblasts. In the first, hemoblasts are pluripotent stem cells, able to produce germline and somatic tissues. These cells can be considered Primordial Stem Cells (PSC, described in Solana 2013) and express both pluripotency genes and genes related to germline multipotency program. In the second hypothesis, the Iα6 cells are germline-committed stem cells that can be able to "switch" to the somatic potential under certain condition. Indeed, the hemoblast population, even the Iα6 positive, may be heterogeneous, comprising both germline and somatic stem cells (as proposed by Laird and colleagues, 2005).

The preliminary data obtained in *Polyandrocarpa zorritensis* do not permit to define the role or the commitment of Iα6 positive hemocytes during vasal budding. However, the presence of Iα6 hemoblasts in this new type of budding provide first clues and allow to speculate on the existence of conserved molecular mechanisms during different types of NED in the two clades of the Styelidae family, where NED is independently evolved.

3.15 Towards a functional characterization of hemoblasts in *P.zorritensis* NED

The data presented in this Chapter identify a population of mobile mesenchymal cells, the hemoblasts, which seems actively participating to vasal budding in *P. zorritensis*. However, a molecular characterization of hemoblasts during all the stages of vasal budding and a functional

analysis that will elucidate their role during NED are lacking. The experiments described in the next paragraphs are proposed to clarify these aspects.

3.15.1Control of the EdU chase efficiency

In the paragraph 3.8, EdU pulse-chase assays were performed at different budding stages. However, it is not clear for how long the EdU can be retained in the vasculature of *P. zorritensis* and if it continues to stain proliferating cells also during the chase period (see Material and Methods). The efficiency of the chase was tested compared the signal with samples at the same stage incubated with the EdU until the end of the experience (only pulse). The signal intensity was observed to be less intense in the pulse-chase sampled, suggesting a "dilution" through time of the EdU signal between daughter cells. While the confocal parameters have been kept constant in all experiments, a quantification of this difference in intensity has not been performed. In order to test the efficiency of the chase period, an additional control is needed. EdU will be incubated in budding nests in which the budding process has not been triggered yet for 18 hours (timing representing the longest pulse formed) (pulse); subsequently, the EdU will be removed and the bud maintained in sea water for 2 hours (timing representing the shorted chase performed) (chase). After this, the abscission will be triggered and the bud will be let developed until double vesicle stage. If the chase worked correctly, we expected that no EdU positive cells will be detected in the budding tissues.

3.15.2 Identification of hemoblast markers with immunostaining, flow cytometry and in situ hybridization

A first molecular marker, the Iα6, has been tested. Iα6 has been chosen since putative stem cells in *B. schlosseri* and *B. diegensis* express it during NED (Kassmer et al., 2015, 2019a). By immunostaining on sections shows that Iα6 is expressed in hemocytes between the inner and the outer vesicles at the double vesicle stage. Further experiments are necessary to assess if Iα6 cells are present in other stages of vasal budding and in which regions of the developing bud they are localized. Because the budding nests and the developing buds are not transparent, it will be suitable to pursuit the characterization of Iα6 cells during these stages on sections. We can expect that the hemoblasts aggregates present at the invaginating site and at the fusion site will be Iα6 positive too.

Preliminary data suggested that sorted hemocytes extracted from spherules are enriched with Iα6. However, the nature of these cells has not been clarified yet. The spherule stage was preferred to other stages of budding for technical reasons. For instance, the limiting factor for flow cytometry experiments was to obtain enough hemocytes to reach the optimal cell

concentration for the sorting machine (around 10 million for milliliter). The cell extraction protocol was tested at different stages (spherules, budding nests and double vesicle stage buds) and the highest harvesting rate was obtained with spherules (about 1.5 million for milliliter using 10 spherules). The quantity of cells obtained can be increased using more specimens. After collection, spherules are available in quantity in the laboratory and can be maintained stable in a resting form for long periods, while it is not possible yet to induce the production of budding nests or buds in the laboratory and their availability is dependent on the zooidal health. Since the hemocyte composition of spherules is comparable to the other stages and hemoblasts are present (see section 3.6), we can conclude that the deployment of spherules will be representative for the other stages. In order to identify if the Iα6 enriched cells sorted by FACS are hemoblasts, the further step will be to use an imaging flow cytometer. This will permit to capture photos of the cells positive to Iα6 and compare their morphology to the different morphotypes described in this chapter. Once the sorting and imaging technique will be completely set up, different antibodies can be tested to identify morphotype-specific cell surface markers as performed in other species (Rosental et al., 2018). RNA sequencing (RNA-seq) and targettedRT-qPCRon sorted enriched Iα6 cells can lead to identification of other marker of hemoblasts. The expression of candidate genes already reported in Iα6 positive cells responsible for NED in *Botrylloides diegensis*, as *pou3* and *piwi*, will be tested (Kassmer et al., 2019a)

In other colonial styelidae, genes of the germline multipotent program GMP result express in circulating putative stem cells (see section 1.6) during different stages of NED. Assays targeted to develop a protocol for *in situ* hybridizations on cryosections have been carried. Once this protocol will be completely set up, the expression of genes as *vasa*, *piwi*, *nanos, pou3* will be tested during different stages of vasal budding. We expect the expression of all or some of these genes in the hemoblast aggregates. Double ISH could be also performed to co-localize *Iα6* and these markers.

3.15.3 In vivo haemocytes tracking

In order to assay the contribution of the proliferating hemoblasts in the formation of the new bud, hemoblasts will be stained with EdU buy pulsing at different time (see below) then collected from the bloodstream as in Kassmer et al., 2019. The cells will be counted with hemocytometer and reinjected in the buds at different stages of development. Buds belonging from the same colony (i.e. showing the same genotype) will be used for the cell extraction and reinjection. The presence of EdU positive cells in the budding tissues will clarify at which stage and where the hemoblasts are integrated. We expect to find EdU cells in the inner vesicle and later, during organogenesis, in different organs, suggesting that proliferating hemoblasts participate to inner vesicle production and organogenesis. Injecting the EdU positive hemoblasts

at the invagination stage (~20 hours post abscission) and the fusion stage (~22 hours post abscission) - when the hemoblasts cluster with the vascular epidermis and proliferate - can elucidate the participation of hemoblasts in the inner vesicle formation, while the injection of these cells from the double vesicle stage (~24 hours post abscission) can define if hemoblasts are integrated in forming organs.

Hemoblasts EdU positive can be obtained in two different ways. A first possibility is to incubate the hemoblast with EdU before extraction. Since we know from the EdU assays that hemoblasts incorporate EdU during early stages of budding (for example, at the invagination or fusion stage), the cell extraction can be performed on developing buds incubated with EdU. If the incubation occurs between 18 and 22 hpa (i.e. from the end of the swelling stage to the end of the fusion stage) we expect that the hemoblasts forming clusters will be EdU positive and will be present in the extracted hemocytes fraction. At this point, the hemoblasts EdU positive can be sorted by FACS before transplantation. A second possibility is to extract the hemoblast before EdU incorporation. The hemoblasts extracted from developing buds (for example, at the invagination or fusion stage) can be sorted by FACS and isolated based on their enrichment in Iα6 (Kassmer et al 2019, Kassmer et al 2015). These cells will be subsequently incubated with EdU before reinjection. Different incubation times need to be experimentally tested, in order to obtain the high viability of EdU positive hemoblasts. The development of an efficient protocol for hemoblast reinjection is also necessary to accomplish this task. During the injection, a needle is mechanically inserted into the receiving bud. This delicate procedure can damage the budding tissue. In addition, the hemoblasts could obstruct the needle, adhere to the needle walls or get damaged. Different needle diameters, injection settings (such as flow rate) or "injection buffers" in which hemoblasts are suspended may be tested to avoid tissue damages and loss of hemoblasts.

3.15.4 NED inhibition and rescue experiments

To functionally test if Iα6 positive hemoblasts or other hemocytes are self-renewing, and to assay the grade of potency (uni-, oligo-, multi-, plury-potent) FACS-sorted hemoblasts can be reinjected in colonies in which the developmental process has been arrested. These tests will mimic the experiments performed by Freeman in *Perophora viridis* – which blocked the budding by irradiation (Freeman, 1964)- or by Kassmer and colleagues in *Botrylloides diegensis* – which blocked the WBR by Mitomycin C (MMC) treatment (Kassmer et al., 2019a). Radiations and MMC induce the budding arrest causing DNA damage and inhibiting the cell proliferation. We expect that the colonies transplanted with the proliferating hemoblasts will be able to restore the vasal budding. In order to block the budding activity, several technical adjustments are necessary. Using irradiations or chemical treatments, preliminary tests are needed in order to define the

sub-lethal irradiation dosage or the MMC concentration able to block the budding. The growth of treated colonies under a range of conditions will give information on the best settings. Based on the literature, two type of ionizing radiations, X-Ray and ϒ-Ray (Freeman, 1964; Laird and Weissman, 2004) can be tested, as well as different mitotic inhibitors, such as Hydroxyurea (HU), which blocks budding in other invertebrate species such as cnidarians (Balcer and Black, 1991).

Materials and methods

3.16 Hemocyte extraction and characterization

All the material (needles, glass pipettes, petri dishes) were rinsed in BSA before use to prevent cell adhesion. Spherules and budding nests were punctured with a needle at the level of the tunic and gently pressed with dissecting forceps to let the hemolymph go out. Using a glass pipette, the hemolymph was collected and transferred to a Superfrost ultra plus glass slide (Thermo Fisher Scientific). The hemocytes were let to attach to the slide for 30 minutes at room temperature before staining. The Giemsa staining was performed following the protocol for *Botryllus schlosseri* (Cima et al 2010). The slides were treated with a 10% solution of Giemsa stain for 5 minutes, and then fixed in a solution of 1% glutaraldehyde and 1% sucrose in FSW for 30 minutes on ice, washed in PBS sand mounted with Permount mounting medium. The hemocytes were observed with an optique microscope Zeiss Axio, connected to a camera Axiocam 506. Preparation of samples for electron microscopy was performed as described in Chapter 2.

3.17 Cell counting

The quantification of undifferentiated cells has been carried on semi thin sections (1um), stained with methylene blue following the protocol reported in Scelzo et al 2019 (Chapter 2). Five stages (stolon, budding nest, invagination, double vesicle and spherule) were analyzed. For each stage, three specimens were considered (biological replicates), and for each specimen three histological sections randomly chosen were observed (technical replicates). The number of undifferentiated hemocytes (hemoblasts) and "differentiated hemocytes" (comprising all the other hemocyte morphotypes) were counted using the software ImageJ (tool "multi-point").

Statistical analyses were performed with Prism8 (GraphPad Software, San Diego). One-way ANOVA with post-hoc Tukey's multiple comparisons test was used to determine significant

differences of hemoblast percentages between different stages, with a p-value of p>0,05. The output is reported in the following table (Table 3.2).

Number of families	1							
Number of comparisons per family	6							
Alpha	0,05							
Tukey's multiple comparisons test	Mean Diff.	95,00% CI of diff.	Significant	Summary	Adjusted P Value			
Stolon vs. Spherules	-1,343	-5,348 to 2,662	No	ns	0,7932	A-B		
Stolon vs. Budding nest	-2,593	-6,471 to 1,285	No	ns	0,2794	A-C		
Stolon vs. DV	-4,443	-8,448 to -0,4379	Yes	*	0,0257	A-D		
Spherules vs. Budding nest	-1,25	-5,128 to 2,628	No	ns	0,8117	B-C		
Spherules vs. DV	-3,1	-7,105 to 0,9050	No	ns	0,1715	B-D		
Budding nest vs. DV	-1,85	-5,728 to 2,028	No	ns	0,564	C-D		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Stolon vs. Spherules	5,457	6,8	-1,343	1,456	7	7	1,304	25
Stolon vs. Budding nest	5,457	8,05	-2,593	1,41	7	8	2,601	25
Stolon vs. DV	5,457	9,9	-4,443	1,456	7	7	4,315	25
Spherules vs. Budding nest	6,8	8,05	-1,25	1,41	7	8	1,254	25
Spherules vs. DV	6,8	9,9	-3,1	1,456	7	7	3,011	25
Budding nest vs. DV	8,05	9,9	-1,85	1,41	8	7	1,856	25

Table 3.2: GraphPad-Prism8 output of the one-way ANOVA with post-hoc Tukey's multiple comparisons test performed on the data obtained from the cell counting.

3.18 Cell proliferation assays

To localize dividing cells, immunohistochemistry on paraffin sections were performed using phospho histone H3 as mitosis marker. Samples were fixed in PFA4% in PBS1x overnight at 4C, then rehydrated in a graded series of alcohol and butanol and embedded in paraffin (as detailed in Scelzo et al. 2019, Chapter 2). Sections of 10 um were cut with a manual microtome and placed on glass slides. After deparaffinization and rehydration, the sections were washed in 0,1% Triton PBS to increase the permeability and blocked with 5% normal goat serum in PBS at room temperature for at least one hour. The samples were incubated overnight at 4C with the anti-phospho histone H3 antibody Ser10 (Millipore) at the concentration of 1:500 in PBS containing 5% normal goat serum. After three washes in PBS, the secondary antibody conjugated with a fluorochrome (Alexa Fluor 488 Anti Rabbit, Jackson ImmunoResearch) was incubated at 1:200 for two hours at room temperature. Then, after three washes in PBS, the sections were incubated 15 minutes at room temperature with 1 mg/ml Hoechst in PBS and washed again three times in PBS. The stained slides were dehydrate with bath in alcohol and xylene and mounted with Permount xylene-based mounting medium (Fisher) before confocal imaging.

In order to measure the DNA synthesis and identify spatio-temporal dynamic of cell proliferation, the buds were incubated with 5-ethynyl-2-deoxyuridine (EdU) - an analogous of thymidine - at 100uM in sea water. Different pulse and chase experiments were carried as described in the results (Figure 3.12). The treated buds were fixed, embedded in paraffin and cut as previously described. After deparaffinization and rehydration, the sections were washed three times in PBS 1x. Then the slides were placed in 0,5% Triton in PBS for one hour and

washed twice in 3% BSA in PBS for 10 minutes. The EdU was detected with the Click-iTEdU Imaging Kit (488, Invitrogen Molecular Probes) following the click reaction protocol. After washes in PBS, the samples were incubated with 1 mg/ml Hoechst in PBS 15 min at room temperature and mounted in glycerol before observation.

Confocal images were taken with a Leica SP8 confocal microscope. Adjustments of brightness and contrast and Z projections (maximum projection) were performed with ImageJ.

3.19 Localization of Integrin-alpha 6 positive cells by immunohistochemistry on cryosections

In order to preserve the integrin proteins, it was preferred to embed the samples in OCT instead of paraffin. Buds were fixed in 4% PFA in PBS1x overnight at 4C. After washes in PBS, the samples were soaked for one hour in 15% sucrose and for one hour in 30% sucrose before embedding in OCT. The samples were cut with a cryostat in 8um sections and stored at -80C. Before performing the immunohistochemistry the slides were kept at room temperature for at least 30 minutes. After three washes in PBS, the sections were selected and surrounded with a hydrophobic barrier using a marking pen. Blocking in 5% goat serum in PBS was performed for 1 hour at room temperature, and then the sections were incubated with the anti-Integrin-alpha6 primary antibody (P2C62C4, Developmental Studies Hybridoma Bank, Iowa City) overnight at 4C. As positive control, sections of the same specimens were incubated with anti-acetylated tubulin antibody (Sigma-Aldrich) and anti-tyrosinated α tubulin antibody (Sigma-Aldrich) overnight at 4C (Figure 3.24). As negative control, sections of the same specimens were incubated with blocking buffer without antibody. Then, all the slides were washed three times in PBS and incubated with the secondary antibody (Alexa Fluor 488 Anti Mouse) (Jackson ImmunoResearch, Ely) for two hours at room temperature. After washes in PBS, the samples were incubated with 1 mg/ml Hoechst in PBS 15 min at room temperature and mounted in Vectashield Antifade Mounting Medium H-1000 (Vector Laboratories, Burlingame) before confocal observation.

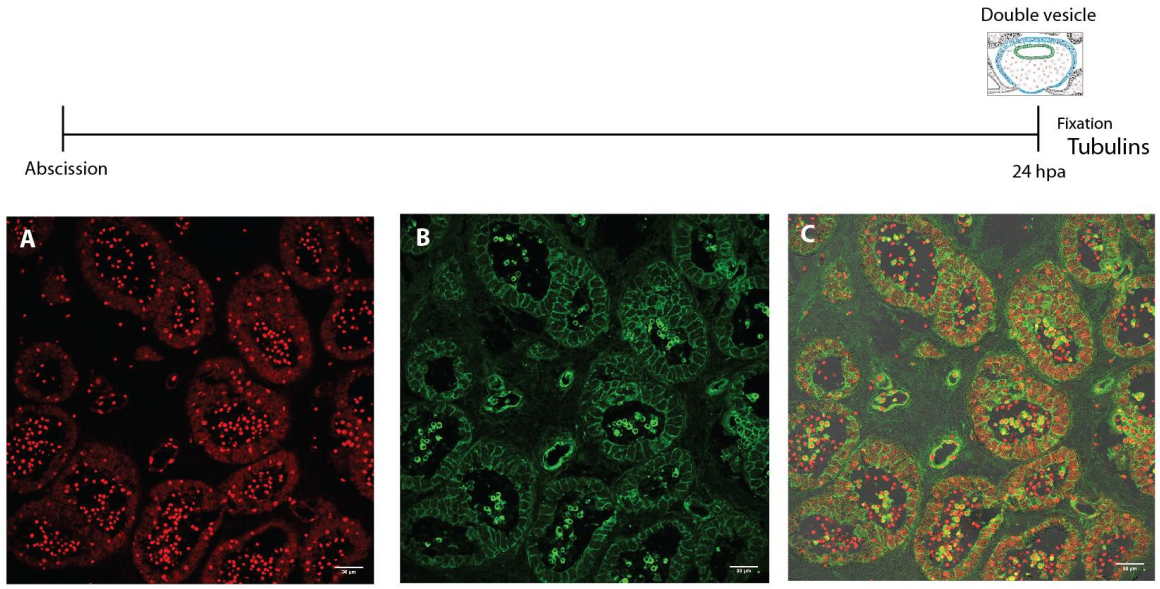


Figure 3.24: Anti- tyrosinated α tubulin and anti-acetylated tubulin immunostaining (positive control) performed at the double vesicle stage. In these sections, only vascular ampullae are visible. A) Hoechst; B) Tubulins; C) Merged (Scale bars: 30 µm).

3.20 Flow cytometry

Spherules (10-30) were transferred in sorting buffer (PBS 3.3x, 10mM HEPES, 2% FCS, from Rosental et al 2018). Hemolymph was collected pressing gently the spherules with dissecting forceps and passed through a 40µm cell strainer. The fluorescence activated cell sorting (FACS) was performed using FACS Aria III (BD Biosciences-US). Live cell gate was selected based on a Hoechst-threshold, comparing Hoechst stained and not stained cells: Hoechst (10mg/ml) was added to the hemocyte suspension and incubated 10 min on ice before sorting. Live cells were sorted with the fluorescent DNA dye DRAQ5 (Biostatus), incubated at concentration of 20µM for 10 minutes at room temperature. To these cells the anti-CD49f (Integrin alpha 6) monoclonal antibody (Super Bright 436, clone eBioGoH3, eBioscience, Invitrogen) was added in a dilution 1:50 an incubated for 30 minutes on ice. In order to approximatively define the size of the P5, cell-sorting set-up beads (BD Biosciences-US) with a known diameter of 6µm were sorted with the same sorting parameters than the Hoechst stained hemocytes. The Forward and Side Scatter dot plot in Figure 3.25 shows that the hemocytes belonging to the population P5 have a comparable SSC.

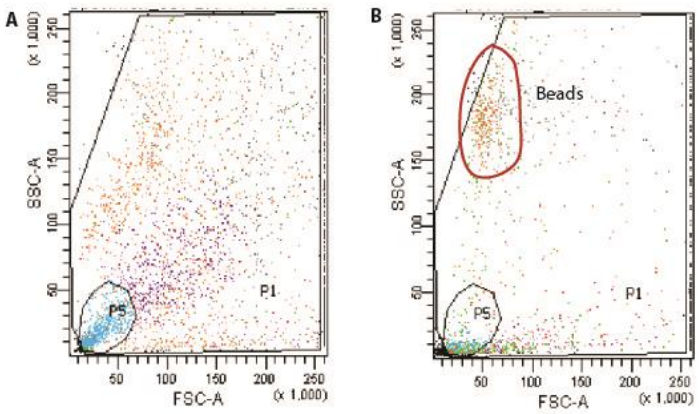


Figure 3.25: A) Forward and side scatter plot of hemocytes stained with Hoechst. The hemocyte population P5 is encircled with a black circle; B) Forward and side scatter plot of calibration beads of 6 µm in diameter. The dots representing the beads are encircled in red, while the gating corresponding to the P5 in (A) is encircled in black.

Chapter 4

Introduction

4.1 Dormancy: definitions and generalities

A strategy that an organism can adopt to cope with adverse environmental conditions is to enter into a resting state characterized by a reduced metabolic rate (Withers and Cooper, 2010). The term dormancy has been commonly used to define this state of metabolic depression (Cáceres, 1997; Hand, 1991; Mansingh, 1971). Dormancy can be used to assure the synchronization of biorhythms with seasonal rhythms (for example, to align the breeding season with maximal food availability) and/or as a defense to a wide spectrum of hostile conditions (Alekseev and Pinel-Alloul, 2019).

Dormancy encompasses a variety of hypometabolic processes, divided into two main categories based on the nature of the inducing stimuli: quiescence and diapause. Quiescence is controlled by variation in environmental conditions, with unfavorable conditions stimulating the entrance into quiescence. Various types of quiescence exist and are defined by the nature of the stimulus, such as hibernation or aestivation (cold and hot temperatures respectively), anaerobiosis (anoxia), or anhydrobiosis (desiccation). When favorable environmental conditions are restored, the exit from dormancy occurs. Diapause, on the other hand, is regulated by endogenous physiological factors rather than by the environment. Rescue from diapause occurs after exposure to a specific cue that does not necessarily correspond to optimal environmental conditions. Quiescence and diapause can occur simultaneously, and in some species, they can represent two consecutive stages of the life cycle: for instance, insect diapause is often followed by a period of quiescence, which allows for synchronization of developmental resumption to tolerable environmental conditions (Belozerov 2009). In some organisms, metabolic activity is extremely reduced that some authors call this extreme state cryptobiosis ("hidden life") and distinguish it from dormancy, arguing that dormancy is a less severe form of inactivity compared to cryptobiosis (Clegg, 2001; Keilin, 1959; Withers and Cooper, 2010). In this manuscript I will refer to all the hypometabolic states (diapause, quiescence, and cryptobiosis) with the general term "dormancy", without distinguishing between them, unless necessary.

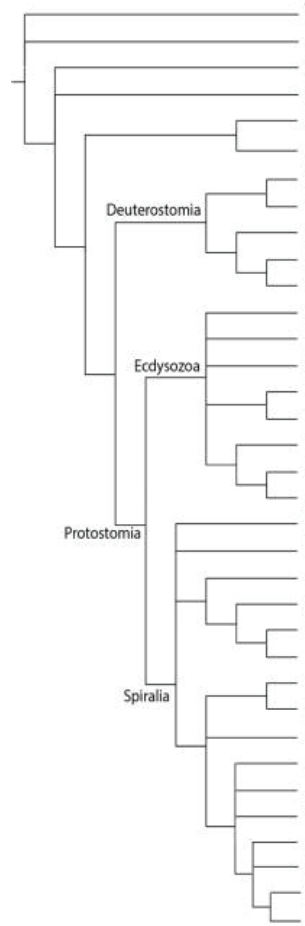
4.1.1 Dormancy: evolution and ecology

Dormancy is distributed among different unicellular and multicellular taxa, including Bacteria (Jones and Lennon, 2010), plants (Viemont and Crabbe, 2000), Protozoa (Corliss, 2001) and Metazoa (García-Roger et al., 2019) (Figure 4.1). Among the metazoans, vertebrates are known to show substantial diversity in dormant forms (Bickler and Buck, 2007; Geiser, 2010, 2013; Richards, 2010; Secor and Lignot, 2010); however, in this Chapter, I will mostly focus on

dormancy in invertebrates. The environmental conditions that regulate dormancy and the related physiological and metabolic modifications have been deeply investigated only in a handful of invertebrate taxa, such as Rotifera, Nematoda, Tardigrada and some arthropod species (Bertolani et al., 2019; Guidetti et al., 2011; Hahn and Denlinger, 2011; Ragland et al., 2010; Wang et al., 2009). In most other invertebrate taxa, the presence of dormancy as well as the stimuli and mechanisms involved remain unexplored.

Just like budding and whole-body regeneration, the study of the evolutionary history of dormancy can be approached by comparing phylogenetically closely related species able to undergo dormancy and species incapable of producing such resting forms. So far, studies aimed to examine dormancy in a comparative way have been carried in few groups, yielding only few insights into the evolutionary and ecological forces that drive the origins of dormancy. Comparative studies in tardigrades (Guidetti et al., 2011) and rotifers (Caprioli and Ricci, 2001; Ricci, 1998; Ricci and Fontaneto, 2009) illustrate how closely related species may show completely different abilities to enter into a dormant state or may enter dormancy upon exposure to completely different stimuli. In addition, distantly related species can show convergent dormancy regimes. In these latter cases, the distribution of dormancy and its stimulus are related to the habitat. These examples suggest that dormancy is a phylogenetically plastic trait and is not constrained by phylogenetic relationships. In bdelloid rotifers, Ricci (1998) demonstrated that species living in more stable habitats show lower resistance capabilities than closely related species living in variable environments. However, there is not enough evidence to conclude whether dormancy in these groups has been acquired multiple times as an adaptive response to inconstant and unfavorable conditions (as proposed by Cáceres, 1997) or if it has been lost in species living in relatively stable environments (Radzikowski, 2013). Nevertheless, such studies indicate that loss and gain of dormancy is likely due in large part by selection pressures to cope with habitat variability.

Species able to undergo dormancy are more common in terrestrial and freshwater habitats than in marine habitats (Cáceres, 1997; Crowe, 1971). This observation may suggest that the ability to undergo dormancy has evolved when the first organisms colonized the inland water habitats (Radzikowski, 2013). However, the reduced number of resting forms known in the marine environment may be simply due to a lack of information; as already mentioned above, the true extent of the presence of dormancy among invertebrates is widely under-studied.



Phylum	Dormancy	Type	Oceanic example	Continental example	Species
Ctenophora	NO	NO	NO	-	187
Porifera	YES	Both	*Chalinula loosanoffi*	*Spongilla lacustris*	8659
Placozoa	NO	NO	NO	-	1
Cnidaria	YES	Quiescence	*Cyanea lamarcki*, *Paracoryne huvei*	*Hydra vulgaris*, *Craspedacusta sowerbii*	10203
Xenoturbellida	NO	NO	NO	-	2
Acoelomorpha	NO	NO	NO	-	428
Echinodermata	YES	Quiescence	*Holothuria theeli*	-	7550
Hemichordata	NO	NO	NO	-	103
Cephalochordata	NO	NO	NO	-	33
Urochordata	YES	Both	*Botryllus schlosseri*, *Clavelina lepadiformis*	-	2804
Craniata	YES	Both	*Notothenia coriiceps*	*Nothobranchius guentheri*, *Glis glis*	65458
Loricifera	NO	NO	NO	-	30
Kinorhyncha	NO	NO	NO	-	196
Priapulida	NO	NO	NO	-	19
Nematoda	YES	Both	NO	*Panagrolaimus davidi*, *Anguina tritici*	25033
Nematomorpha	NO	NO	NO	NO	339
Tardigrada	YES	Both	*Echiniscoides sigismundi*	*Paramacrobiotus spatialis*, *Milnesium* sp.	1167
Onychophora	YES	Quiescence	-	Eastern Australian Peripatopsidae	183
Arthropoda	YES	Both	*Heteropsyllus nunni*, *Labidocera aestiva*	*Artemia salina*, *Onychodiaptomus sanguineus*	1257040
Orthonectida	NO	NO	NO	-	29
Rhombozoa	NO	NO	NO	-	107
Chaetognatha	NO	NO	NO	-	170
Gnathostomulida	NO	NO	NO	-	97
Micrognathozoa	YES	Diapause	-	*Limnognathia maerski*	1
Rotifera (with Acanthocephala)	YES	Both	*Brachionus plicatilis*	*Macrotrachela quadricornifera*, *Adineta vaga*	3246
Gastrotricha	YES	Diapause	NO	*Lepidodermella squamata*, *Chaetonotus maximus*	794
Platyhelminthes	YES	Diapause	*Monocelis longistyla*	*Bryioplana xerophila*	29487
Cycliophora	YES	Quiescence	*Symbion pandora*	-	2
Mollusca	YES	Quiescence	NO	*Eremina desertorum*	84977
Annelida	YES	Both	NO	*Aulodrilus acutus*, *Trigaster calwoodi*	17733
Nemertea	YES	Quiescence	NO	*Argonemertes dendyi*	1358
Bryozoa	YES	Both	*Bugula neritina*, *Steginoporella* sp.	*Cristatella mucedo*, *Plumatella* sp.	6008
Entoprocta	YES	Both	*Barentsia matsushimana*, *Urnatella gracilis*	*Urnatella gracilis*, *Loxosomatoides sirindhornae*	171
Brachiopoda	NO	NO	NO	-	392
Phoronida	NO	NO	NO	-	16

Figure 4.1 (following page): Overview of the occurrence of dormancy across the 35 currently recognized metazoan phyla. For each phylum, there is a column indicating the presence or absence of dormancy, type of dormancy (three groups: diapause, at a specific developmental stage, e.g. resting egg, dormant embryo etc; quiescence, at any life stage, e.g. desiccation, encystation, estivation, etc.; or both), examples of species with dormancy in oceanic and continental habitats, and total number of species. The phylogenetic tree is modified from Giribet and Edgecombe, 2020. The example columns with "NO" means that the phylum is present in the habitat but without dormancy, whereas the columns with a dash means that the phylum is not present in the habitat (from García-Roger et al., 2019).

4.2 Physiological and morphological changes of dormancy in invertebrates

Despite being referred to as a "resting state", dormancy is a dynamic process that implies radical morphological, physiological, and biochemical changes. The eco-physiological process of dormancy has been described as an alternative developmental pathway and as a distinct part of the animal life cycle (Hahn and Denlinger, 2007; Koštál, 2006; Koštál et al., 2011; MacRae, 2010). Mainly basing his observation on insects, Koštál (2006) chronologically subdivided the process of dormancy into five successive phases: induction, preparation, initialization, maintenance, termination. Each of these phases are characterized and regulated by specific molecular mechanisms (Denekamp et al., 2009; Qiu et al., 2007). The subdivision proposed by Koštál can be loosely generalized for other invertebrate species and always begins with the moment when the environmental conditions deteriorate and produce the stimuli that triggers the dormancy process (induction). After induction of dormancy the arrest of the ongoing developmental or growing processes (preparation) and the initiation of drastic metabolic and morphological changes (initialization) take place. Dormant eggs and embryos usually present thick egg envelopes or shells, which rend the dormant stage recognizable from subitaneous eggs or developing embryos (as in the case of the crustacean *Daphnia*) (Dharani and Altaff, 2004; Seidman and Larsen Jr., 1979). In juveniles and adult forms that already present a cuticle layer, such as tardigrades, nematodes and insects, the composition of the pre-existing cuticle can undergo modification and one or more new layers can be added to increase the stiffness (Cassada and Russell, 1975; Guidetti et al., 2006, 2011; Morita et al., 1999). These layers protect the dormant form from UV irradiation, exposure to chemicals, desiccation and predation (Green et al., 2005; Radzikowski, 2013; Rezende et al., 2008; Tanguay et al., 2004). Changes in the cuticle composition can also occur. For example, in the nematode *Pristionchus pacificus*, the dormant juvenile produces a lipidic layer external to the cuticle, which reduces the risk of water lost and increases the chances of propagation by attaching to a motile host (Penkov et al., 2014). The initialization stage is also characterized by the onset of the hypometabolic period, where

often there is a reduced rate of protein synthesis and general suppression of gene expression (Storey and Storey, 2010). However, the expression of genes and proteins involved in stress response increases, enhancing stress tolerance and stabilizing the cellular components. The molecular mechanisms of the cellular stress responses can be independent of the causing stress (Kültz, 2003).

During the fourth stage (maintenance) proteins regulating the cell-cycle, molecular chaperons, antioxidants, proteins responsible for DNA and chromatin stabilization or repair and degradation of damaged molecules are produced. Since the dormant stages are often non-feeding, the accumulation of reserves of energy before entering into dormancy is necessary to survive during the resting phase and recover normal functions in the post-dormancy period. The amount of nutrients stocked can influence the duration of dormancy (Hahn and Denlinger, 2007) and in some species of Cnidaria - as *Rhopilema esculentum* - the rate of dormant form production is higher when the non-dormant forms are constantly fed (Arai, 2009). Also, during the resting period, part of these reserves can be converted into cryoprotectant compounds such as glucose, trehalose, or glycerol (Hahn and Denlinger, 2007; Hand, 1991; Pauwels et al., 2007). These molecules limit the changes in cell volumes and maintain the osmotic equilibrium stabilizing the cellular components during the resting phase (Storey, 1997; Storey and Storey, 1988). Metabolic pathways such as glycolysis and gluconeogenesis are upregulated for this conversion into cryoprotectant compounds (Denlinger et al., 2012; Ragland et al., 2010). The composition of nutritive reserves can vary from dormant and non-dormant stages of the same species (Gilbert, 2004; Hahn and Denlinger, 2011) and different taxa - i.e lipids in nematodes (Wang and Kim, 2003), carbohydrate and proteins in snails (Horne, 1973).

Molecular chaperons, such as the disaccharide trehalose, glycerol or heat shock proteins, have been identified in various invertebrates like crustaceans *i.e Artemia* (MacRae, 2010; Qiu et al., 2007), *Daphnia* (Hengherr et al., 2008, 2011), rotifers (Denekamp et al., 2009), and insects (King and MacRae, 2015). During anhydrobiosis (resistance to desiccation) in Tardigrada the role of these molecules has been well studied (Crowe et al., 1992). When the tissue loses water, tardigrades contract their bodies and retract their limbs to generate a dormant form called a "tun"; in this way, the evaporation surface is reduced (Wright, 1989). The disaccharide trehalose replaces the water in the bindings with hydrogen and forms a glassy matrix in the cytoplasm (Hengherr et al., 2008; Wolkers et al., 2002). During the rehydration phase, the heat stress proteins Hsp70 and Hsp90 appear to stabilize the cell components and are probably involved in the repair of DNA damage (Schill et al., 2004; Wełnicz et al., 2011). In crustaceans (i.e *Artemia* McRae, 2003; Qui et al., 2007; *Daphnia* Pauwels et al., 2007; Hengher et al., 2011), rotifers

(Denekamps et al., 2009), nematodes (Morley and Morimoto, 2003) or insects (King and MacRae, 2015) these heat-shock proteins have also been identified.

Regardless of the nature of the signals that terminate the dormancy, during the final phase, termination, oxygen metabolism is often activated and accompanied by an increased production of reactive oxygen species (ROS), which can lead to oxidative cellular damages (Ferreira-Cravo et al., 2010). Therefore, antioxidant defense systems are activated with the production of enzymatic and non-enzymatic antioxidants: levels of superoxide dismutase, catalase, glutathione peroxidase, glutathione and other antioxidants are higher in resting stages than in non-resting forms - as in tardigrades (Rizzo et al., 2010) or rotifers (Denekamp et al., 2009, 2011). During this phase, development and morphogenesis restart. Developmental pathways like *Wnt* signaling seem to be enhanced at the end of dormancy in some insects to restore the developmental activity (Koštál et al., 2017).

4.3 Dormancy and development in invertebrates

Depending on the species, dormancy can occur during embryonic, larval, juvenile, and/or adult stages. For example, tardigrades can undergo dormancy at any stage of the life cycle, like egg, embryo and adult stages (Jönsson, 2005), while other species can produce resting forms only in a specific stage, such as some crustaceans in which only the eggs undergo dormancy (Strachan et al., 2015) or in nematodes, in which only the juvenile stage undergoes dormancy forming a so-called dauer larva (Ogawa and Brown, 2015).

4.3.1 Dormancy and sexual development

Embryonic dormancy is characterized by a developmental arrest at different embryonic stages. Dormant eggs or embryos are produced in many species of crustaceans, rotifers, or tardigrads and other metazoan groups (Domenici and Gremigni, 1977; Hand, 1991; Marcus, 1996). The resting eggs can be accumulated in the sediments or transported in the ballast waters, increasing the dispersal range (Briski et al., 2011). Resting eggs avoid predation by resisting digestion during passage through the digestive system of the predator (Alekseev and Vinogradova, 2019; Lopez et al., 2002). Interestingly, some rotifers and arthropods that in normal conditions reproduce parthenogenetically can produce resting eggs by sexual reproduction (Fryer, 1996; Gilbert, 1974, 2004). Photoperiod and food concentration (in *Daphnia spp)* (Alekseev and Lampert, 2004; LaMontagne and McCauley, 2001; Stross and Hill, 1968) or food quality (i.e. vitamin E intake in some rotifers) (Gilbert, 1983; Gilbert and Litton, 1978; Gilbert and Thompson, 1968) have been proposed as main inducers for this “sexual shift”.

4.3.2 Dormancy as a form of NED in colonial invertebrates

In colonial invertebrates able to reproduce by budding or fission, such as cnidarians or bryozoans, the dormant forms (when present) can be considered as non-embryonic stages of the life cycle. Indeed, when adverse environmental conditions undermine the survival of the individuals, dormant forms capable of survival are produced by asexual reproduction. They assure the continuity of the colony and its spatial and temporal dispersal (Arai, 2009; Bilton et al., 2001; Gerber and Kokko, 2018). Thus, dormancy can be considered, together with asexual reproduction and whole-body regeneration, a form of non-embryonic development (NED) (see Chapter 1). Gemmules in sponges (Hand, 1991), podocysts in scyphozoan cnidarians (Ikeda et al., 2011), statoblasts and hibernacula in bryozoans (Francis, 2001) and survival buds in ascidians (Nakauchi, 1982, see Chapter 1 section 1.5) are some of the most studied examples of dormant forms generated by asexual development. These structures show peculiar morphological characteristics, such as the accumulation of nutritive reserves and the presence of an external protective layer. Besides the morphological descriptions of dormant forms in some species, little is known about the biochemical and molecular pathways involved in their formation, maintenance and reactivation.

In sponges, gemmules are covered by a collagenous capsule and siliceous spicules and are filled by mono- or bi-nucleated cells called thesocytes. The morphology of these cells can vary in different species, but usually thesocytes contain vitelline platelets and lipid inclusions (Simpson, 2012; Simpson and Fell, 1974). Studies in different species have aimed to identify the variables regulating dormancy. The mechanisms vary among different species but also within different populations of the same species. An example is given by the species *Haliclona loosanofi*, registered in a wide geographic range (Fell, 1974). Populations located at the cold latitudes show a hibernating phase, while the populations registered in warm areas undergo aestivation (Manconi and Pronzato, 2008). In sponges, dormancy is commonly induced by temperature, and in laboratory conditions the gemmules can be kept in a dormant status for years (such as in the species *Ephydatia ramsayi*, Fell 1994). While the mechanisms underlying dormancy in gemmules is poorly known, an increase of osmotic pressure in the thesocytes or high levels or cAMP have been proposed to maintain the gemmules in dormancy (Harrison, 2012; Loomis, 2010; Simpson and Rodan, 1976).

In cnidarians, some scyphozoan polyps produce encysted dormant protrusions on the pedal disc called podocysts that can survive in the resting stage for years. The podocysts are composed of a layer of peripheral cells covered by a cuticle and amoebocytes containing nutrients such as proteins, carbohydrates, and lipids (Chapman, 1968; Ikeda et al., 2011). The nutrients are used by the peripheral cells at the end of the dormant period, when they reform a new polyp (Black,

1981; Chapman, 1968). Cysts can be also produced from planula larvae (planulocysts) or the whole polyp but less frequently than from the pedal disc. Podocyst formation has been described as a way of asexual propagation responsible for scyphozoan blooms (Arai, 2009; Ikeda et al., 2011). Despite the morphological studies describing the gemmule structure in different species, less is known about the factors influencing formation, maintenance, and excystment of podocysts. Different environmental cues, such as temperature, salinity, light exposure, and availability of oxygen and nutrients, can regulate dormancy in cnidarians (reviewed in Arai, 2009).

In bryozoans two types of dormant buds, statoblasts and hibernacula, are produced (Bushnell and Rao, 1974; Hand, 1991). Hibernacula originate as outgrowths of the colony, particularly along the stolon (Brushnell and Rao, 1974). Hibernacula are also called winter buds because in some species they represent the overwintering resting forms (Massard and Geimer, 2008). The statoblasts are produced inside the colony and can be subdivided into floatoblasts, sessoblasts, and piptoblasts depending on the presence of an air sac that permits to float (Francis, 2001; Mukai and Kobayashi, 1988; Wood, 2001). Statoblasts and hibernaculae contain so-called yolk cells, filled with nutritive substances such as glycoproteins, lipids, and glycogen (Terakado and Mukai, 1978). The effects of temperature, desiccation, light intensity, and wavelength on statoblast viability and germination have been studied by Mukai (1974) in the species *Pectinatella gelatinosa*. Mukai identified a substance called blastostasin, which is proposed to be responsible for inhibiting the statoblast reactivation, but its mechanism of action and presence in other species remain unclear.

4.4 Dormancy in ascidians

Dormancy has been reported in all the three classes of ascidians, exclusively in colonial species (Figure 4.2). Some authors (Mukai et al., 1983b; Nakauchi, 1982) functionally classified dormancy as a developmental process responsible for the survival of the colony (survival budding). The dormant forms called survival buds temporarily arrest blastogenesis and sexual reproduction until the environmental conditions become favorable. Interestingly, after the return to tolerable conditions the development of the new zooids is identical to that occurring during propagative budding. It remains unclear whether the molecular and cellular mechanisms are shared with asexual reproduction and whole-body regeneration, and if these mechanisms are conserved between colonial species.

Thermal stress seems to be the main factor inducing dormancy in colonial ascidians. Dormant forms are well described morphologically in few species, but very little is known about the

molecular and metabolic mechanisms involved. For example, in Perophoridae (Huxley, 1921) and Clavelinidae (Huxley 1926) dormant stolons are produced under various unfavorable conditions. Mesenchymal cells called trophocytes accumulate nutritive reserves that are used during the development of the new zooid (Brien and Brien-Gavage 1927, Mukai et al 1983).

In the family Styelidae, dormancy has been described in *Stolonica socialis*, *Distomus variolosus,* and *Botrylloides diegensis* (see paragraph 1.5.4). The morphological characterization of resting stages is well described in *Botrylloides diegensis*, in which changes in hemocyte populations and expression of *Piwi* and *PCNA* (proliferation marker) at different stages of dormancy were reported (Hyams et al., 2017). In *Polyandrocarpa zorritensis* we recently reported the presence of dormant forms, the spherules, connected to the zooids of the colonies through a vascular stolon (Scelzo et al., 2019, Chapter 2). Once separated from the colonies, the spherules stored at 11°C or lower temperatures remained inactive. When the spherules were transferred to 24°C, they sprouted and formed new zooids. These observations suggest that temperature is one environmental cue regulating dormancy in *P. zorritensis*. In addition, other yet undetermined cues maybe be involved, such as salinity, food availability, or dissolved oxygen.

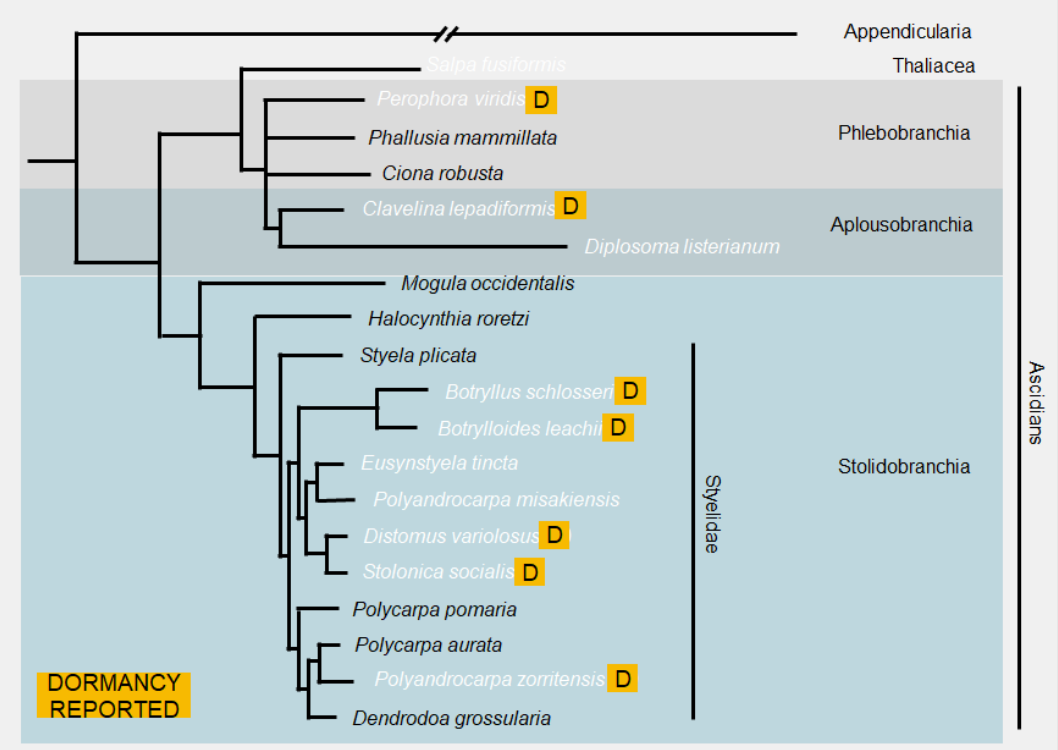


Figure 4.2: Phylogenetic tree showing the phylogenetic relationships between tunicates, with a focus on Styelidae. Colonial species are reported in white, while solitary forms are reported in black. Orange D represents the species where dormancy has been reported.

4.5 Aims of the study

The first part of this study aimed to better describe the environmental variables involved in the induction, maintenance, and release of dormancy in *P. zorritensis* and *C. lepadiformis.* We decided to carry our an ecological, morphological and molecular comparison between dormancy in these two species since *C. lepadiformis* is able to produce dormant forms but it distantly related to *P. zorritensis* and acquired NED convergently. Seasonal observations on the natural population of *P. zorritensis* located in La Spezia (Italy) and experimental manipulations of the environmental parameters under laboratory conditions for both species were conducted to generate a staging scheme for dormancy and led to the production of dormant forms in the lab. Once the dormancy of the two species was induced in laboratory conditions, the second part of this study aimed to define the morphology and ultrastructure of dormant tissues at different stages of the resting period. All the experiments were carried in collaboration with Laurel S. Hiebert during her post-doc in the laboratory. Finally, the third part aims to describe the molecular mechanisms governing dormancy to explore the similarity and differences between the two species. Gene expression profiles of dormant (spherules and winter buds) and non-dormant (stolon and zooid) stages of *P. zorritensis* and *C. lepadiformis* have been generated by RNAseq analysis. In addition, gene expression profiles of spherules and winter buds that were reactivated at different temperatures were also analyzed to identify mechanisms involved in exit of dormancy in both species. Differences in gene expression between dormant and non-dormant stages of the same species and between the similar stages of the two species are under analysis.

Results

4.6 Identification of field conditions regulating dormancy in P. zorritensis

The natural population of *P. zorritensis* located in La Spezia (Italy) was monitored over two years (from August 2017 to May 2019) with approximately one observation per season. In summer, fall, and winter the colonies formed dense clusters of zooids, and spherules were connected to the colony by stolons on the bottom side of these clusters (Figure 4.3 A-B). During spring (March 2018 and May 2019) no zooids were observed and the colony was constituted by isolated spherules, attached to the ropes, and covered by epibionts. Colonies were collected at three different sites (i.e. on three different docks of the marina "Porto Mirabello") during fall (October 2018), winter (January 2019), and spring (May 2019) and spherule size was measured. Interestingly, the average spherule size was significantly larger during fall than in winter, and significantly larger in winter than spring (RM-ANOVA, $p<0.03$; Figure 4.3 C-D). These data suggest that nutrients within the spherules are consumed over the year (from fall to winter to spring). Alternatively, it may mean that the larger spherules are produced during the cold month in order to survive the degeneration of zooid that occurs in spring.

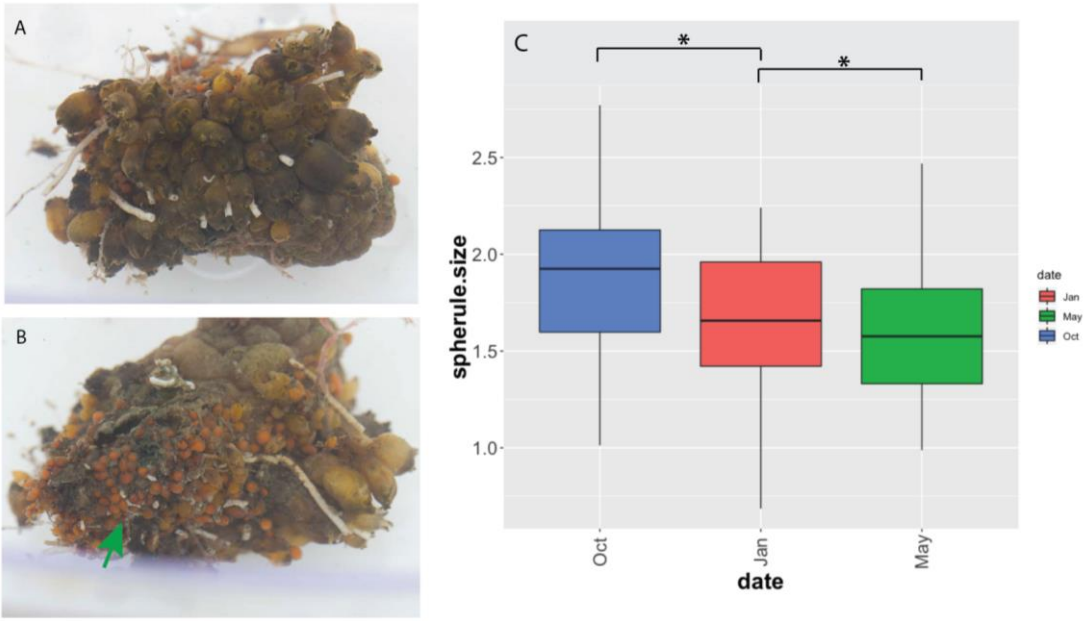


Figure 4.3 (previous page): Observations on the natural population of *P. zorritensis*. A) *Polyandrocarpa zorritensis* colony; B) Bottom side of the same colony as in (A). A dense network of spherules is visible (green arrow); C) graph reporting the spherule sizes measured in fall (Oct., blu), winter (Jan., red) and spring (May, green). The asterisk depicts the significant difference using the Tukey multiple comparison test;

4.7 Effect of salinity and temperature on dormancy in P. zorritensis in laboratory condition

In order to study the environmental cues responsible for spherule production, maintenance in the resting state, and withdrawal from dormancy, specimens of *P. zorritensis* were exposed to different conditions of temperature and salinity.

4.7.1 Production of Polyandrocarpa zorritensis *spherules induced by temperature*

Small colonies of field-collected *Polyandrocarpa zorritensis* (comprising zooids, budding nests, developing buds, and stolons) were maintained in a flow-through aquarium system at 24°C and fed with a diet of living algae (*Tisochrysis lutea* and *Chaetoceros*) and concentrated algae (Isochrysis 100 and Shellfish Diet 1800, Red Mariculture Inc). In order to determine if temperature would induce dormancy, we removed a few small colonies (number of zooids=17; number of stolons=24; number of developing nests=5; number of non-abscissed nests=5) from the aquarium system and placed them at lower temperatures (10°C). Over the course of two months, we observed the production of dormant spherules (Figure 4.4 A-B). The zooids, when still alive, showed closed siphons and a low response to mechanical stimulation, such as tactic stimulation (n=15) (Figure 4.4 C', white arrows). The buds that we experimentally detached from their "parent" zooid (abscised), which had already started the budding process, arrested development and degenerated (n=5) (Figure 4.4 C', red arrow). Stolonial growth continued (Figure 4.4 C-C'), allowing the growth of the already present budding nests (n=5) and the development of new ones (n=5) (Figure 4.4 C', blue arrows; Figure 4.4 A). These structures were externally similar to spherules: they were dome-shaped and the tunic was so thick that it was impossible to observe the internal vascular ampullae through it (Figure 4.4 A). To determine if these structures are effectively spherules, ultrastructural observations were performed (see later paragraph 4.8).

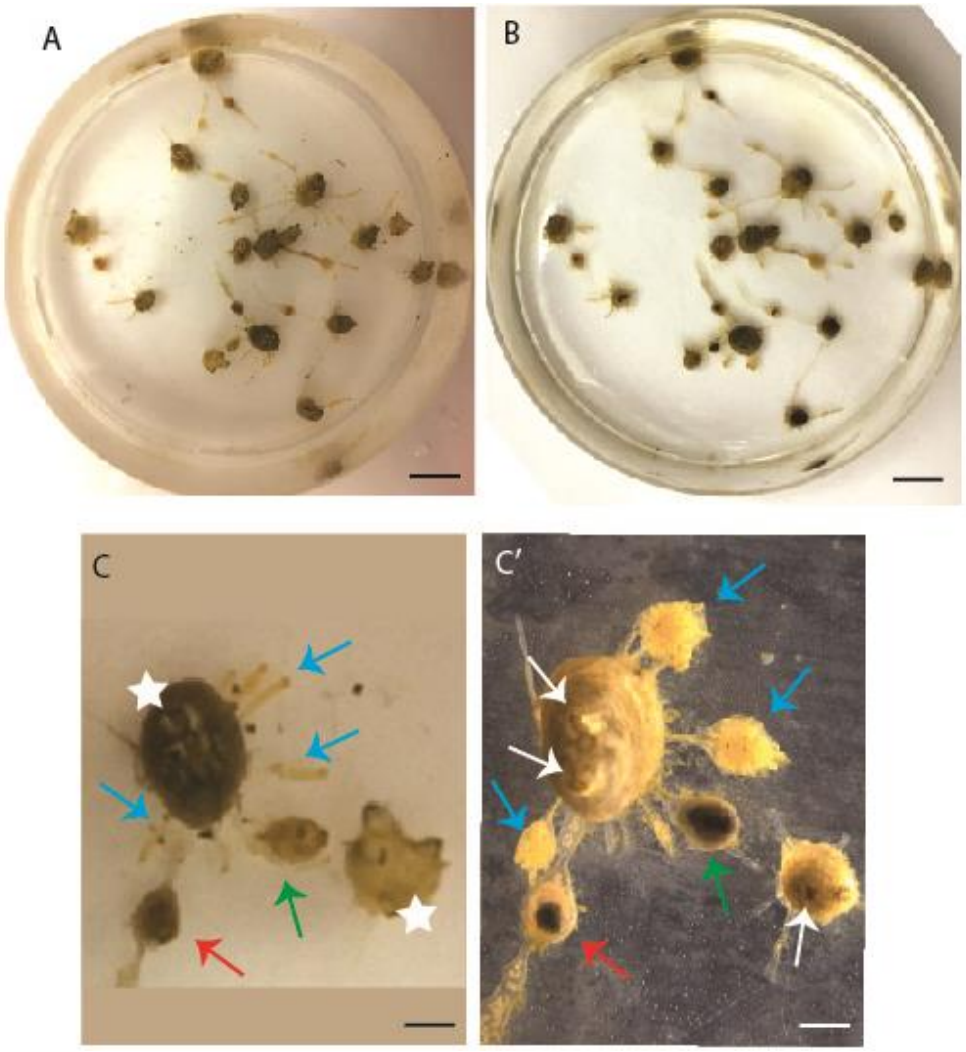


Figure 4.4: Production of *Polyandrocarpa zorritensis* spherules under laboratory conditions. A) Colonies maintained at 24°C; B) the same colonies as (A) after two months at 10°C; C) Small colony before transferring at 10°C. Two zooids (white star) and one small zooids freshly produced by vasal budding (green arrow) are present. The red arrow points a developing bud. Many stolons (blue arrows) are sprouted on the slide; C') the same colony than C after 1 month at 10°C. The zooids show closed siphons (white arrows) while the young zooid (green arrow) and the developing budding nests (red arrow) are degenerating. Spherule-like structures originated along the stolons (blue arrows) (Scale bars: A-B: 1cm, C-C': 1mm).

4.7.2 Temperature dynamics of spherule production and reactivation

To identify the range of temperatures involved in dormancy induction and activation, we exposed the following groups to four different temperatures (8°C, 12°C, 17°C, 22°C) and documented their development for 6 weeks: (i) spherules collected in the field (n=10) stored at 10°C, (ii) zooids with spherules (n=3) produced in laboratory after transfer at 10°C, and (iii) budding nests (n=3) produced in the laboratory after sprouting of spherules and stored at 24°C. At the lowest temperature (8°C) all the zooids died while the spherules and the budding nests did not germinate. At 12°C, half of the zooids died or detached from the glass slide. The stolonial growth was arrested; new budding nests or developing buds did not form after 6 weeks. At this temperature all the spherules (collected in the field and produced in the laboratory) were activated and produced new zooids in 30-35 days. At 17°C the zooids stayed alive and well-attached to the glass slide, all spherules were activated (after about 20 days) and new stolons, budding nests, and spherules were produced. At 22°C the spherules and budding nests were rapidly activated (after two weeks new zooids were already visible). However, half of the zooids died or were detached and few new stolons and nests were produced. The results suggest that the budding nests transferred at 8°C, 12°C, 17°C can form spherules, and that the increasing temperature leads to reactivation of the resting stage. In summary, when the spherules (collected in the field or produced in the laboratory, but both stored at 10°C) were transferred at lower temperatures (from 10°C to 8°C) they do not activate; however, when they are transferred at slightly higher temperatures (from 10°C to 12°C) the germination starts and new zooids are generated. Spherule activation is faster at higher temperatures. Thanks to these observations, the life cycle of P. zorritensis can be schematized as reported in Figure 4.5.

In order to test if the developmental events that lead to the production of a new zooid after spherule reactivation are comparable to those occurring during vasal budding in budding nests, spherules maintained at 10°C were transferred to a higher temperature (24°C) and fixed after 48 hours for observations. In the developing spherule, the vascular epidermis starts invaginating in the same way as occurs in developing budding nests (as described in Chapter 2) (Figure 4.6). These results suggest that the epidermal movements occurring in reactivated spherules are comparable to the vasal budding described in budding nests. Further investigations aimed to compare the later stages may be necessary.

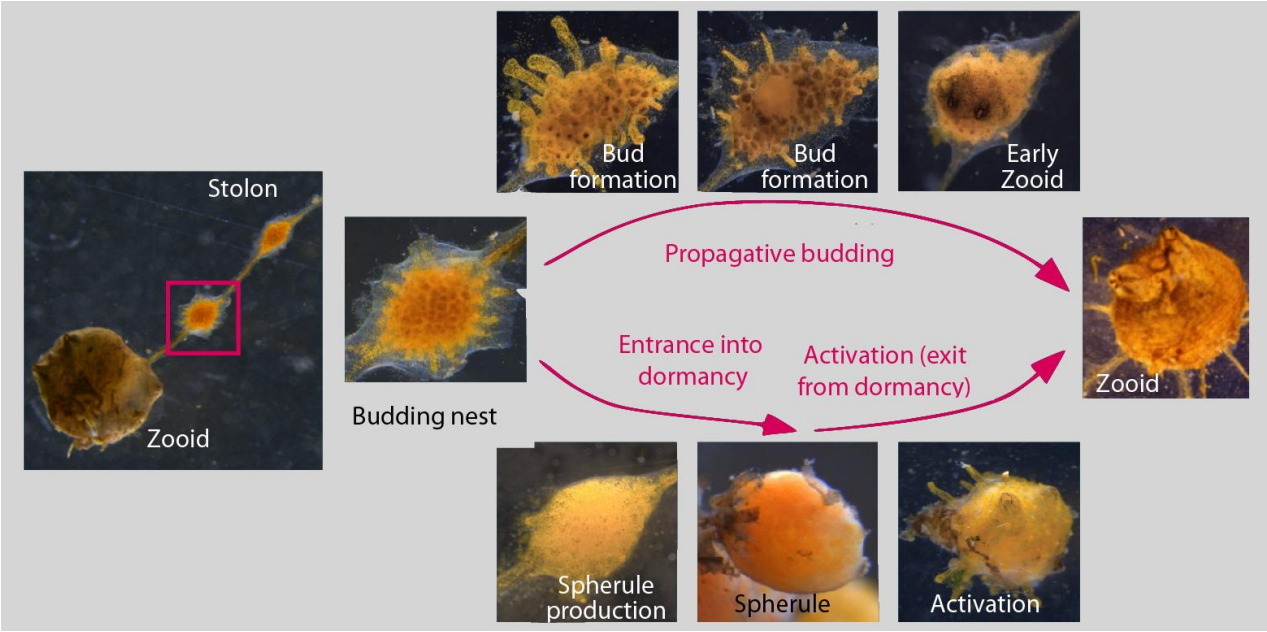


Figure 4.5: Scheme summarizing the NED-life cycle of *P. zorritensis*. The blue arrow shows the vasal budding, the red arrow shows the entrance into dormancy and the orange arrow shows the exit of dormancy.

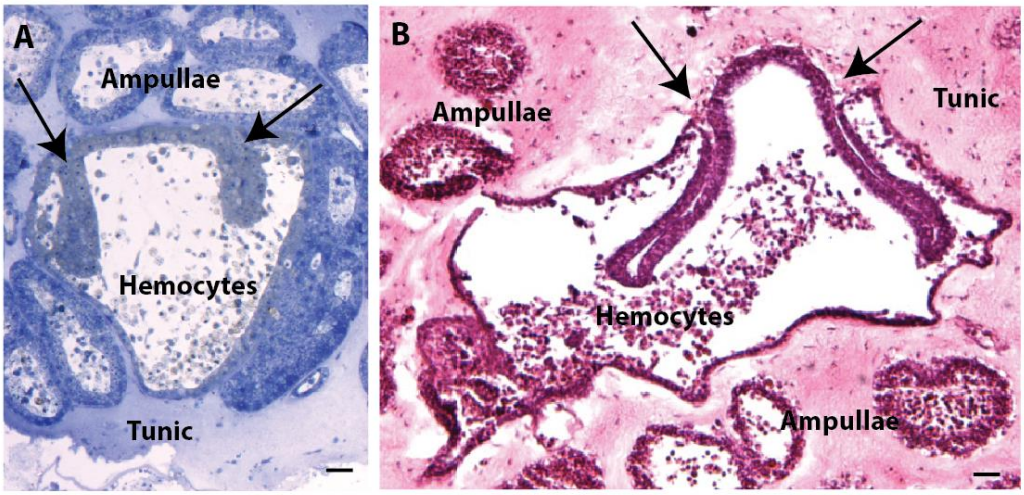


Figure 4.6: Comparison between the development of a vasal bud (A) and a reactivated spherule (B). A) transversal section stained with methylene blue of a developing bud at 20 hours post abscission (see Chapter 2) (modified from Scelzo et al 2019); B) transversal section stained with hematoxylin-eosin of an activated spherule after 48 hours at 24°C. In both cases, the vascular epidermis is invaginating (arrows). (Scale bars: 10µm). In both sections, circulating mesenchymal cells (hemocytes) are visible in the vessel lumen.

4.7.3 Viability of spherules during exposure to long cold periods

After collection, the spherules obtained in the field can be stored at 10°C for up to 12 months without affecting their viability (Scelzo et al., 2019). The viability of artificially-produced spherules (i.e. obtained transferring budding nests at low temperatures under laboratory conditions) and their capability to hatch and reform zooids when transferred at higher temperatures were tested. Artificially-produced spherules were kept 6 months at 10°C and 3 months at 8°C and, at the end of this period, they were transferred at 17°C and 22°C. All the spherules were still viable and reactivated in 17-19 days after long-continued periods at low temperature. These experiments demonstrated that the spherules produced by manipulating the temperature in the laboratory can be maintained at least for 6 months in a dormant stage without affecting the viability, exactly like the spherules produced by colonies in the field.

4.7.4 Range of resistance to environmental cues of adult zooids and spherules in P. zorritensis

In order to determine which conditions the dormancy stages are resistant to, and compare how the feeding zooid stage resists to the same conditions, we exposed these stages of *P. zorritensis* to stressful conditions. Zooids and spherules were exposed for 24h to a wide range of temperatures and water salinity, as well as desiccation (number of spherules and zooids for each condition n=2). Then, the specimens were transferred at 24°C and the viability of zooids and the reactivation of dormant forms were observed after two weeks. The results are resumed in table 4.1. *Polyandrocarpa zorritensis* zooids survived to a short-range of conditions (between 18 and 28°C and between 30-38 ppt), while spherules resisted a wider range of conditions (temperature fluctuation between 0 and 32°C and salinity between 15 and 45 ppt) (Figure 4.7). Both zooids and spherules did not survive to desiccation.

	Polyandrocarpa	
	Zooid	Spherule
18C (control)	2	2
-80	0	0
-20	0	0
0C	0	2
4C	0	2
7C	0	2
28C	2	2
32C	0	2
37C	0	0
10ppt	0	2
15ppt	0	2
30ppt	0	2
45ppt	0	2
desiccation	0	0

Table 4.1: Summary table reporting the viability test results in adults and dormant forms of *Polyandrocarpa zorritensis* (blue column: zooid; orange column: spherule). Numbers represent the specimens viable (i.e. zooids alive, dormant forms activated) after 2 weeks from the treatment. The "0" means that the specimens were not viable (i.e. zooids dead, spherules not reactivated) after 2 weeks from the treatment.

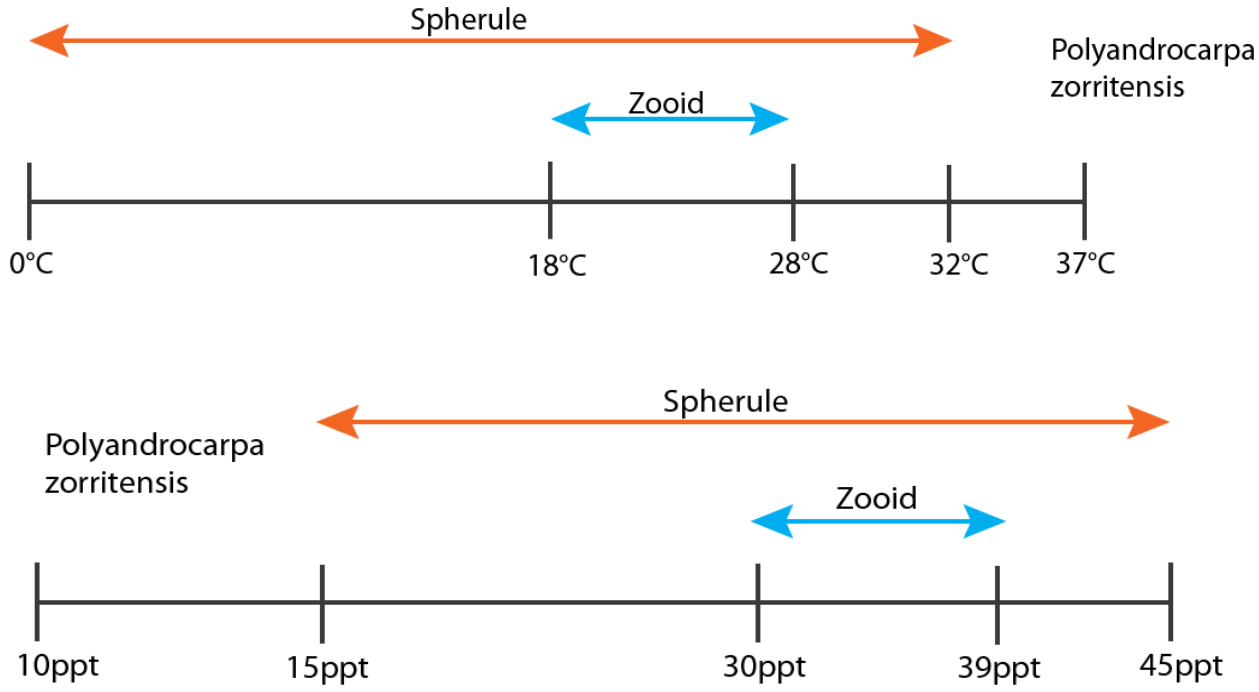


Figure 4.7: Schematic representation the range of resistance for adults and spherules. A) Temperature ranges of survival of adults (blue line) and dormant forms (orange line) in *Polyandrocarpa zorritensis;* B) salinity ranges of survival of adults (blue line) and dormant forms (orange line) in *Polyandrocarpa zorritensis.*

4.8 Morphological description of laboratory-produced *P. zorritensis* spherules

The spherule-like structures (Figure 4.8 A) were fixed and analyzed histologically and ultrastructurally to compare them with the spherules collected in the field (described in Scelzo et al., 2019, Chapter 2). In the lab-produced spherules, tunic fibers were thicker than in the budding nests (about 20 µm thickness) (Figure 4.8 B-C). The cytoplasm of the ampullar epithelial cells was filled with three types of electron-dense inclusions: small electron-dense granules of glycogen inclusion (0.5 -1 µm), uniform electron-dense granules of lipids (2-5 µm) and heterogeneous electron-dense granules containing remnants of organelles (probably autophagosomes, 1-2 µm) (Figure 4.8 D-E). Endoplasmic reticulum and mitochondria were visible (Figure 4.8 D-E green and yellow arrows). The morphology of these structures resembled the spherules collected in the field. These results allowed us to obtain laboratory-produced spherules by transferring the colonies to low temperatures that were functionally and morphologically equivalent to the naturally-produced spherules.

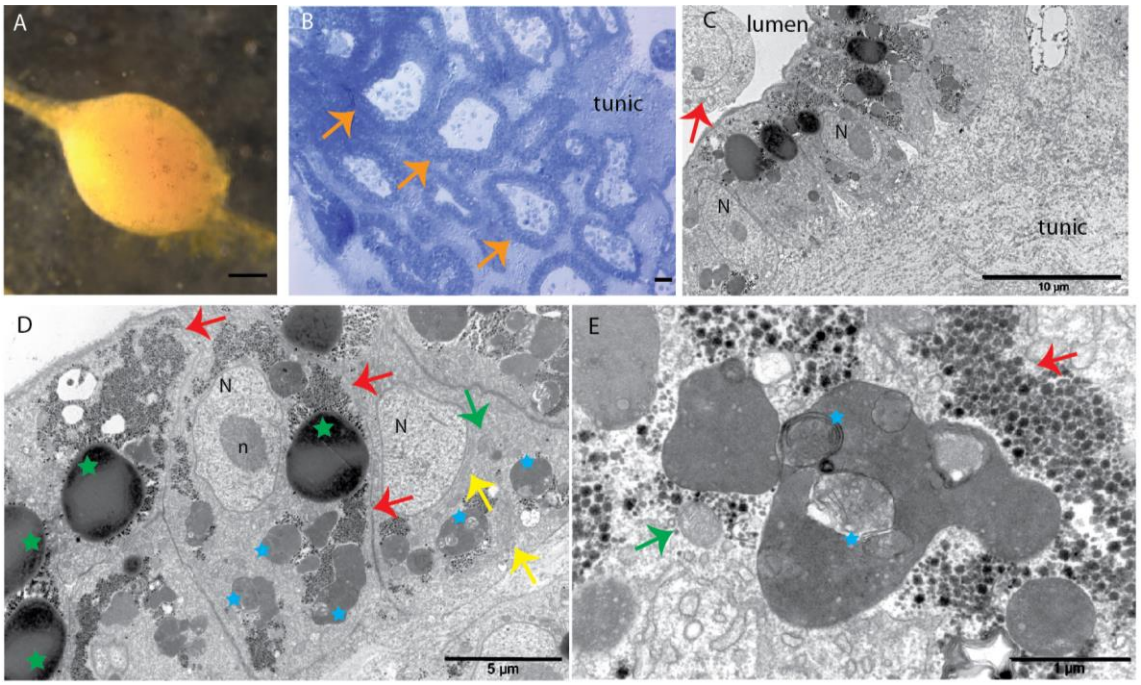


Figure 4.8: Histology and ultrastructure of spherules produced under laboratory conditions. A) Spherule-like structures produced at 10°C; B) Transversal semi-thin section of a spherule-like structure (transversal ampullae shown with orange arrows); C) Detail of the vascular epidermis. Circulating hemocytes are present in the vascular lumen (red arrow); D) Detail of epithelial cells, showing the electron-dense uniform granules (green stars), the heterogeneous granules (blue stars), the small glycogen granules (red arrows). Mitochondria (green arrow) and endoplasmic reticulum (yellow arrows) are also shown; E) Detail of the heterogeneous granules, with mitochondria-like content (blue stars). The glycogen granules (red arrow) and mitochondria (green arrow) are also visible. (Scale bars: A) 200µm; B) 10µm).

4.9 Production of dormant forms in *Clavelina lepadiformis*

4.9.1 Observations on winter buds production in specimens of C. lepadiformis *bred in the laboratory*

The ability to produce dormant forms under laboratory conditions was tested in another colonial ascidian, *C. lepadiformis*. Specimens of *Clavelina* were collected on the same site as *Polyandrocarpa* and were let develop on glass slides at 18°C. To study the process of dormancy, external observations were first carried. As previously described by other authors (Giard and Caullery 1896, Della Valle 1915, Huxley 1926) the zooids emit vascular stolons as prolongations of the postabdomen (white and blue arrows, Figure 4.9 A). The stolonial extremity can bulge forming one or more lobes, which look more pale and whitish than the rest of the stolon. These structures represent the budding chambers (green arrow Figure 4.9 B, Figure 4.9 C), in which the budding process will take place once the hemocytes circulation is interrupted between the

zooid and the budding chamber. At this temperature, other structures similar to budding chambers but more compact and whitish are produced along the stolon: they represent the winter buds, able to resist to cold temperature according to other authors (Berrill, 1951; Berrill and Cohen, 1936) (white arrow Figure 4.9 B, Figure 4.10 D). Similarly to budding chambers, the winter buds can be activated by isolation from the stolon (Figure 4.9 E). According to the above-mentioned authors, the milky coloration of winter buds is coloration is given by accumulation of trophocytes, i.e. hemocytes specialized for storage and transport of nutrients. These results evidence the production by *C. lepadiformis* of dormant forms, the winter buds, also at a constant temperature (18°C). Thanks to these observations, the life cycle of *C. lepadiformis* can be schematized as reported in Figure 4.10.

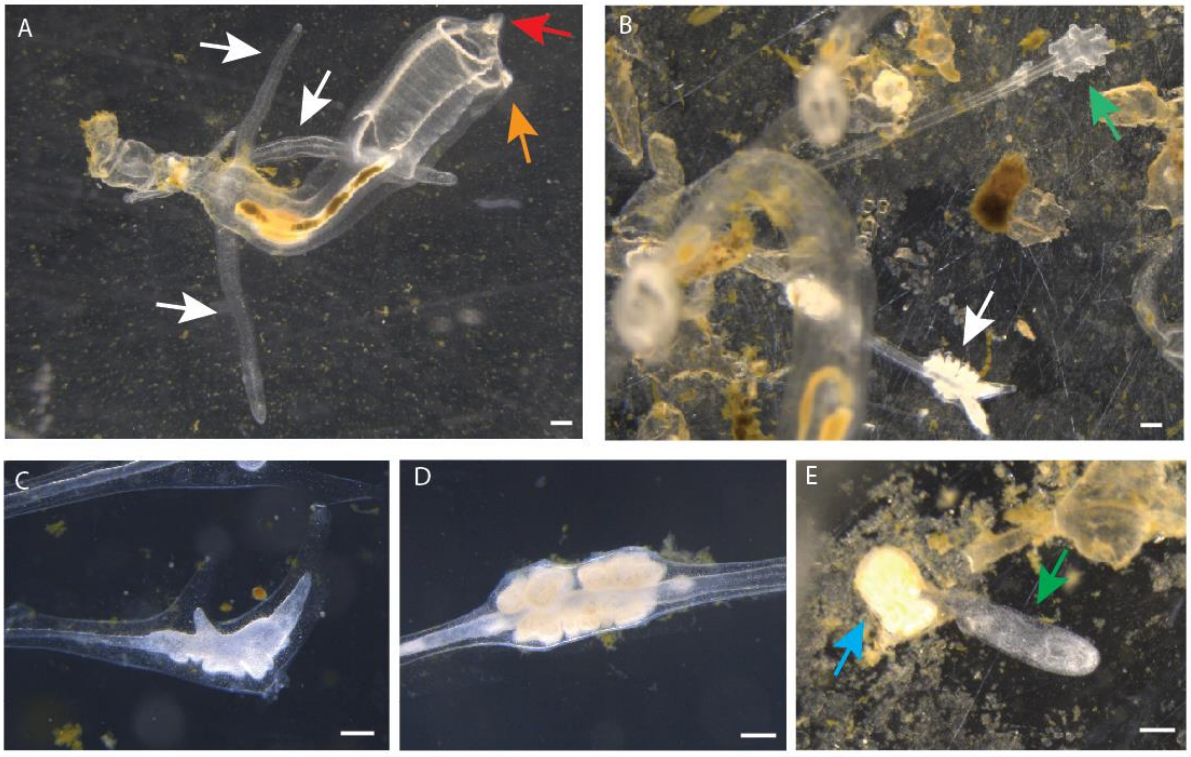


Figure 4.9: A) Zooid of *Clavelina lepadiformis.* Stolons (white arrows) sprout on the glass slide; B) two stolons showing a budding chamber (green arrow) and a winter bud (white arrow); C) detail of a budding chamber; D) detail of a winter bud; E) winter bud (blue arrow) during the dormancy breakout. The new zooid is pointed out with a green arrow. (Scale bars: A-E 10 μm).

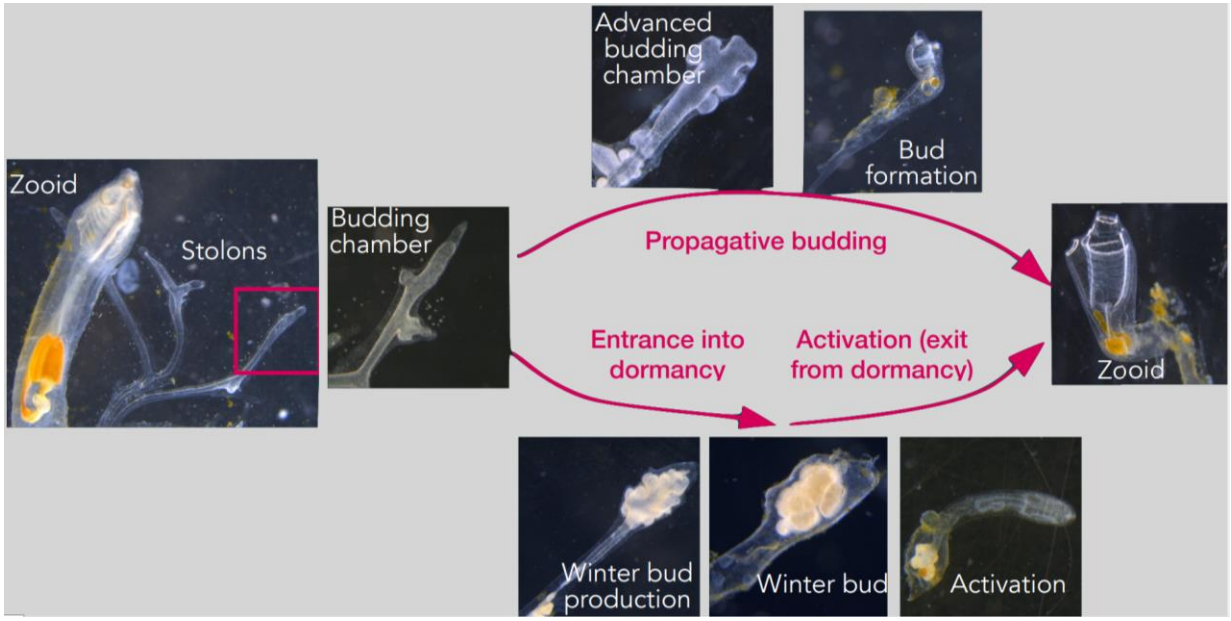


Figure 4.10: Scheme summarizing the NED-life cycle of *C. lepadiformis*.

4.9.2 Range of resistance to environmental cues of adult zooids and spherules in C. lepadiformis

In order to determine which conditions the dormancy stages are resistant to, and how the dormancy stage compares in resistance abilities to the feeding zooid stage, buds and zooids of *C. lepadiformis* were exposed for 24h to a wide range of temperatures and water salinity, as well as desiccation. Then, the specimens were transferred to 24°C and the viability of zooids and the reactivation of dormant forms were observed after one week. The results are resumed in the table 4.2. *Clavelina* zooids are more resistant to lower temperatures (between 0 and 28°C) and higher salinity (30-45 ppt) than *P. zorritensis*. Winter buds can resist a wider range of conditions than zooids (temperature fluctuation between 0 and 32°C and salinity between 15 and 45ppt), exactly like *P. zorritensis* spherules (Figure 4.11).

	Clavelina	
	Zooid	Winter Bud
18C (control)	2	2
-80	0	0
-20	0	0
0C	0	2
4C	2	2
7C	2	2
28C	2	2
32C	0	2
37C	0	0
10ppt	0	2
15ppt	0	2
30ppt	0	2
45ppt	2	2
desiccation	0	0

Table 4.2: Summary table reporting the viability test results in adults and dormant forms of *Clavelina lepadiformis* (blue column: zooid; orange column: winter bud). Numbers represent the specimens viable (i.e. zooids alive, winter buds activated). The "0" means that the specimens were not viable (i.e. zooids dead, winter buds not reactivated) after 2 weeks from the treatment.

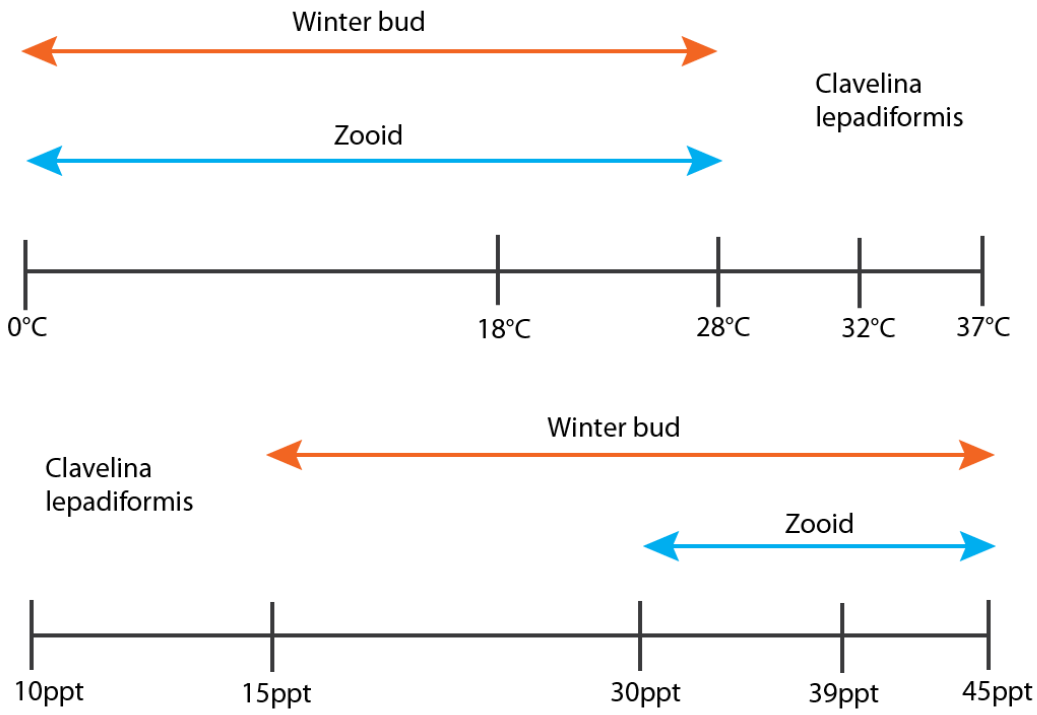


Figure 4.11: Schematic representation the range of resistance for adults and winter buds. A) Temperature ranges of survival of adults (blue line) and dormant forms (orange line) in *Clavelina lepadiformis*; B) Salinity ranges of survival of adults (blue line) and dormant forms (orange line) *Clavelina lepadiformis*.

4.10 Ultrastructural description of dormant and non-dormant tissues of *Clavelina lepadiformis*

The ultrastructure of a non-dormant stolon (Figure 4.12), a resting winter bud (Figure 4.13), and a reactivated winter bud (Figure 4.14) were described, showing morphological differences. A regular stolon is delimited by a thin monolayered epidermis covered by the tunic (Figure 4.12 A). In the lumen, the mesenchymal septum is visible. Hemocytes circulating in the lumen look similar to hemocytes described in other species, like morula cells and hemoblasts (see Chapter 3) (Figure 4.12 B).

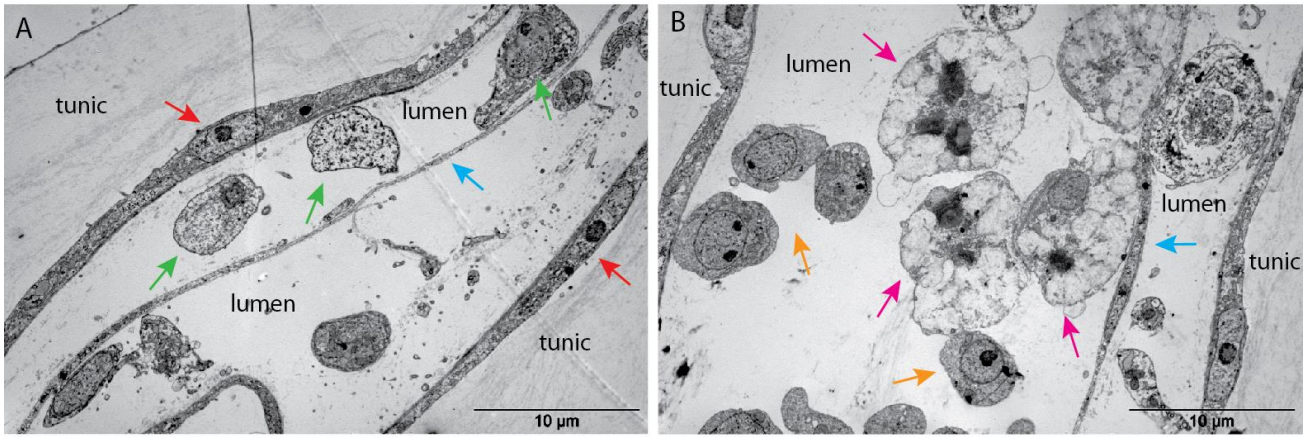


Figure 4.12: Ultrastructure of a *Clavelina lepadiformis* stolon. A) Transversal section of a *C. lepadiformis* stolon. The thin vascular epidermal cells are indicated with a red arrow. The mesenchymal septum (blue arrow) divides the blood vessel into two vascular lumens, where hemocytes (green arrows) circulate in opposing directions; B) Detail of the mesenchymal cells present in the stolonial lumen (mesenchymal septum: blue arrow). Undifferentiated cells (orange arrows) and cells similar to the morula cells of other species (pink arrows) are recognizable. (Scale bars: 10µm).

In a winter bud, the epidermal cells underlying the vascular lumen are filled with electron-dense lipid inclusions. Similarly to the spherules of *P. zorritensis*, different inclusions (small glycogen granules, homogeneous electrondense granules, and heterogeneous electrondense inclusions) are present in the cytoplasm (Figure 4.13 A-B). The hemocytes' content is completely different from the normal stolon: the vascular lumen contains sparse cellular material and large cells of more than 20 um that appear to be macrophages (Figure 4.13 C). The phagosomes of these macrophage-like cells are filled with material containing electron-dense inclusions (Figure 4.13 D).

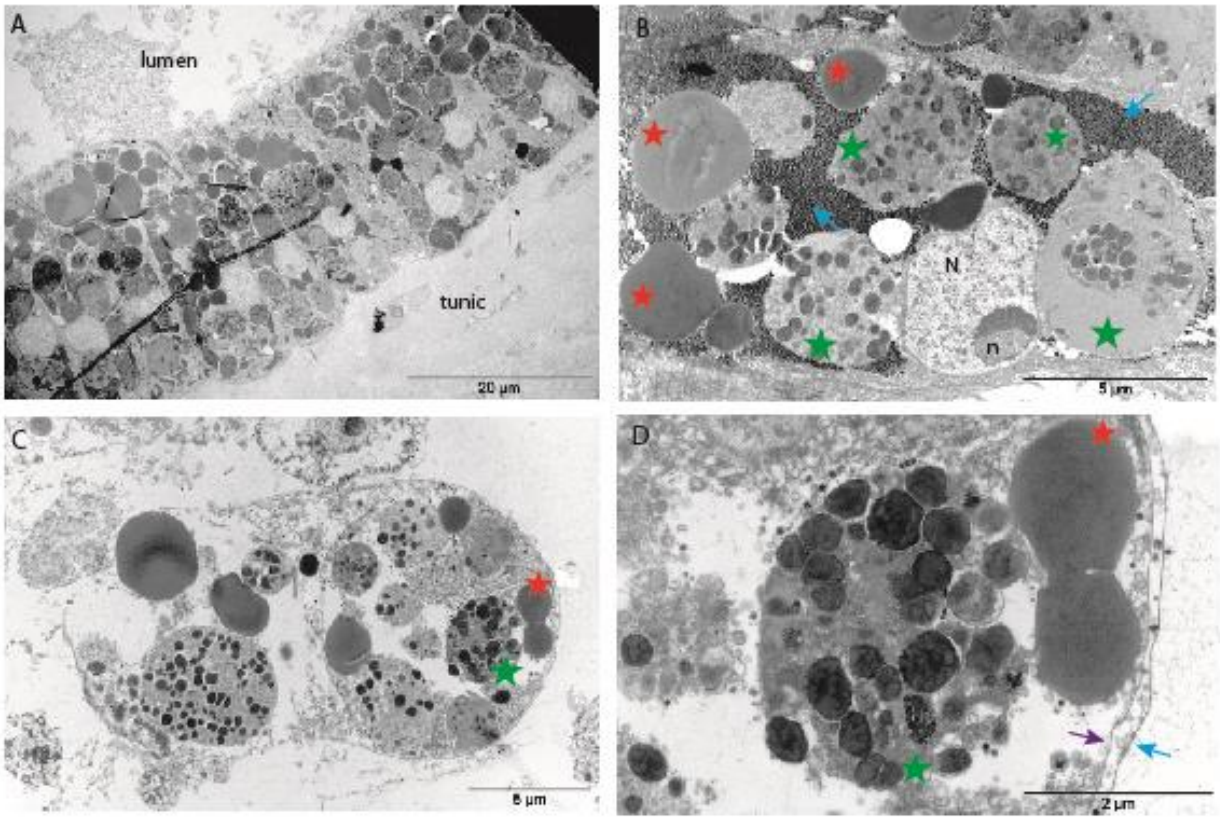


Figure 4.13: Ultrastructure of a *Clavelina lepadiformis* winter bud. A) Vascular epithelium in a winter bud of *C. lepadiformis*. B) Detail of a vascular epithelial cell. The cytoplasm is completely packed by granules of diverse nature: small glycogen granules (blue arrows), electrondense granules (red stars), and heterogeneous inclusiosn (green arrows), potentially auto-phagosomes; C) Hemocyte circulating in the winter bud lumen. The cytoplasm contains different electrondense inclusions; D) Detail of the cytoplasm and the inclusions in the same hemocyte as (C). A heterogeneous inclusion (green star) and a homogeneous big granule (red star) similar to the ones present in the vascular epithelial cells are shown. The inclusions are enveloped by a double membrane (blue and violet arrows). (Scale bars: A 20µm, B-C 5µm, D 2µm).

When the winter bud is activated, the developing zooid is visible on one side (Figure 4.9 E). At this stage, the mesenchymal content of the winter bud is even more disorganized. The lipids, glycogen, and autophagosomes previously contained in the epithelial cells are reversed in the lumen and are engulfed by huge macrophages (Figure 4.14 A-B). The macrophages are also observed in the mesenchymal space of the developing zooid (Figure 4.14 C-D). The results suggest that the nutrients stored during the dormant stage are transferred to the developing zooids by macrophage-like cells.

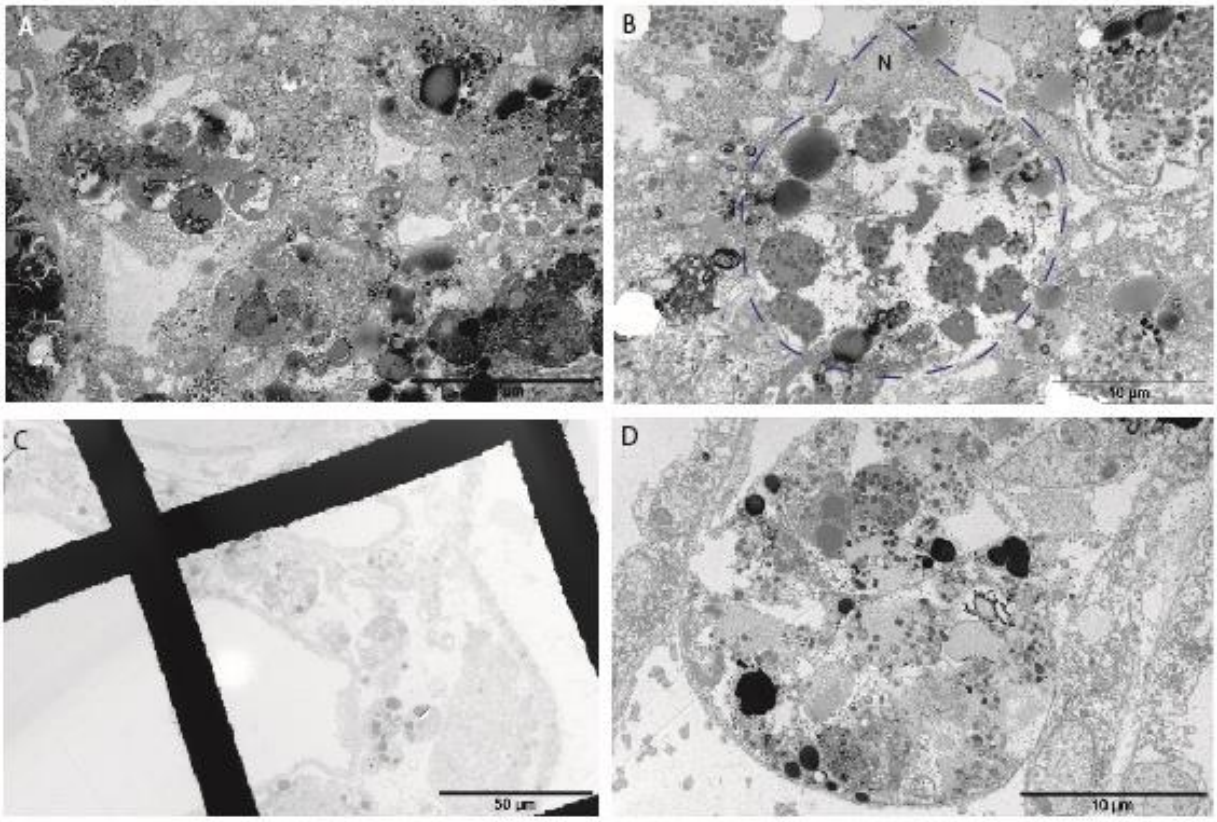


Figure 4.14: Ultrastructure of a *Clavelina lepadiformis* activated winter bud. A) Cellular material present in the mesenchymal space. It is hard to clearly define hemocyte borders; B) Macrophage-like cell presents in the mesenchymal space. The cell membrane is outlined with a purple dotted line; C) View of the mesenchymal space of a zooid developing from a winter bud; D) Detail of a mesenchymal cell - probably a macrophage-like cell- circulating in the developing bud. (N: nucleus). (Scale bars A 20µm, B 10µm, C 50µm, D 10µm).

4.11 Dynamics of gene expression between dormant and non-dormant stages

In order to elucidate the molecular mechanisms underlying the dormancy process, we examine gene expression across life stages in both *Polyandrocarpa zorritensis* and *Clavelina lepadiformis.* The aforementioned experiments allowed us to define stages of the dormancy process for both species (Figure 4.5 and Figure 4.10). Based on this staging scheme we selected different stages/tissues for each species that encompassed a range of morphologies with particular emphasis on the entrance into and exit from dormancy (Figure 4.15). More specifically we selected six stages for *P. zorritensis*, three non-dormant (zooid, stolon and budding nests) and four dormant (freshly collected spherules - referred from now as "dormant" -, spherules stored for 48 hours after collection at 8°C -referred from now as "stored" - , and spherules reactivated - referred from now as "reactivated"), while for *C. lepadiformis* five stages were selected, two non-dormant (zooid and stolon) and three dormand (winter buds still attached to the parental zooid

at 18°C - refferred from now as “dormant”-, winter buds stored for 48 hours at 8°C - referred from now as “stored”-, reactivated winter buds- referred from now as “reactivated”) (figure 4.15). The reactivated stages were obtained maintaining spherules and winter buds at 18°C and 24°C for 48 hours. In the analyses, the reactivated stages obtained at the two different temperatures were pooled together.

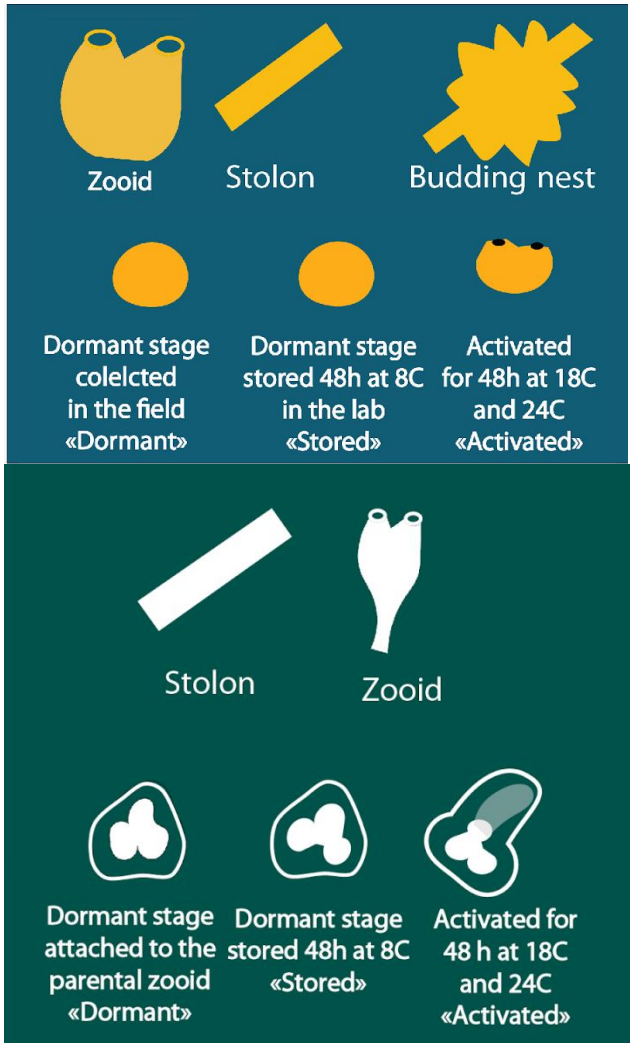


Figure 4.15: Schematic representation of the stages selected for the RNA-seq analysis in *P. zorritensis* (A) and *C. lepadiformis* (B).

The RNASeq data processing, transcriptome assembling, and annotation and the differential expression analysis were performed following the pipeline shown in figure 4.16 (see also Material and Methods, section 4.20). At this moment, the data analysis is still in progress and the results are not complete. In the following sections I present some steps of the analysis and some

Analysis Pipeline

Preprocessing

Preprocessing follows recommendations on this site: https://informatics.fas.harvard.edu/best-practices-for-de-novo-transcriptome-assembly-with-trinity.html

- FastQC
- Use rCorrector to flag unfixable reads
- Remove unfixable reads with FilterUncorrectablePEfastq.py
- Use TrimGalore to trim low-quality reads, Illumina adapters
- FastQC
- Remove rRNA by mapping reads to SILVA database with Bowtie2 and keeping only those unmapped
- FastQC
- Remove overrepresented reads with RemoveFastqcOverrepSequenceReads.py

Transcriptome Assembly and Assessment of Completeness

- Use Trinity to assemble, with In Silico read normalization (—normalize_by_read_set)
- Assess assembly by looking at basic summary metrics and assess completeness with BUSCO

Annotation

Trinotate:

- Build Boilerplate SQLite database
- Using the Trinity.fasta contigs, detect similarities to known proteins with BLASTX search of UniProt databases
- Predict coding regions using TransDecoder, then
  - BLASTP against Uniprot for homologies (Hmmer and PFam)
  - HMMER for protein domains using Pfam database
  - SignalP for conserved signaling peptides
  - Use TmHMM to detect transmembrane regions
  - Use RNAMMER to detect rRNA
- Load data into the SQLite database and write annotation report

Mapping/Quantifying

https://github.com/trinityrnaseq/trinityrnaseq/wiki/Trinity-Transcript-Quantification

- Use Kallisto to "pseudo"map reads from each replicate to the Trinity.fasta file, using 100 bootstraps (two steps: prep reference and then map reads)
- Construct expression matrix

QC of Samples and Biological Replicates

Note: run both "gene" files and"transcripts (isoforms)"

- Use PtR script packaged with Trinity to create sample correlation matrix and PCA (two steps: compare replicates within condition and compare replicates between): https://github.com/trinityrnaseq/trinityrnaseq/wiki/QC-Samples-and-Biological-Replicates
- Combine treatments and remove outliers

Combine Annotations and Expresion Matrix

- Add functional annotations to expression matrix with rename_matrix_feature_identifiers.pl from https://github.com/trinityrnaseq/trinityrnaseq/wiki/Functional-Annotation-of-Transcripts

Tissue Enrichment Analysis/ DEG Composition Dynamics

- Sample Specific Expression to identify tissue-enriched transcripts: https://github.com/trinityrnaseq/trinityrnaseq/wiki/Sample-Specific-Expression
- Venn diagram, upset plot

Differential Expression Analysis

https://github.com/trinityrnaseq/trinityrnaseq/wiki/Trinity-Differential-Expression

- Identify DE features with voom using Trinity scripts (run_DE_analysis.pl and analyze_diff_expr.pl). P = 0.001, C = 1 (Fold change >2) Use the —examine_GO_enrichment option
- Extract and cluster DEGs using define_clusters_by_cutting_tree.pl and 20% tree height

preliminary results that highlight potential molecular mechanisms linked to the dynamics of the dormancy process.

Figure 4.16 (previous page): Schematic overview of the bioinformatics pipeline used for the analysis.

4.11.1 Sample quality controls

Once the transcriptomes were assembled, we performed a quality control in order to compare the samples, assuring that the biological replicates are well correlated in terms of similarity in the levels of expression. To accomplish this, we conducted principal component analysis (PCA) and a hierarchical clustering analysis. PCA plots show the correlation in terms of gene expression levels among all the samples in 2-D graph space (Figure 4.17), while with the hierarchical cluster analysis, a tree-like structure (dendrogram) is produced to show the relationships between samples. Hierarchical clusters are often associated with heatmaps in order to better visualize the results (Figure 4.18). Both quality control analyses showed a good correlation between the replicates.

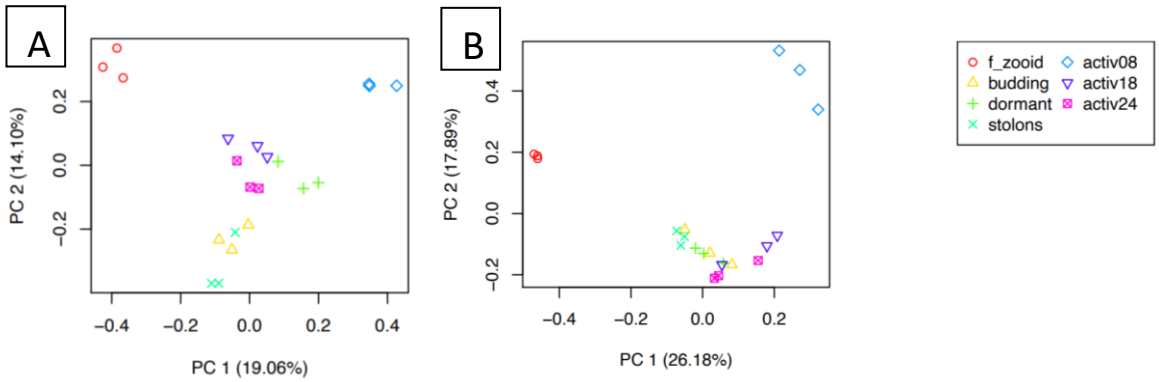


Figure 4.17: PCA plot showing correlations between samples of *P. zorritensis* (A) and *C. lepadiformis* (B). Each sample is represented by a different symbol and the biological replicates of the same stages are indicated with the same colors. Zooids: red circle; Budding nests: yellow triangle; Dormant: green cross; Stolons: green x; Stored (here called "active08"): blue rectangle; Reactivated (here separated in specimens activated at 18°C and 24°C): purple triangle and pink rectangle.

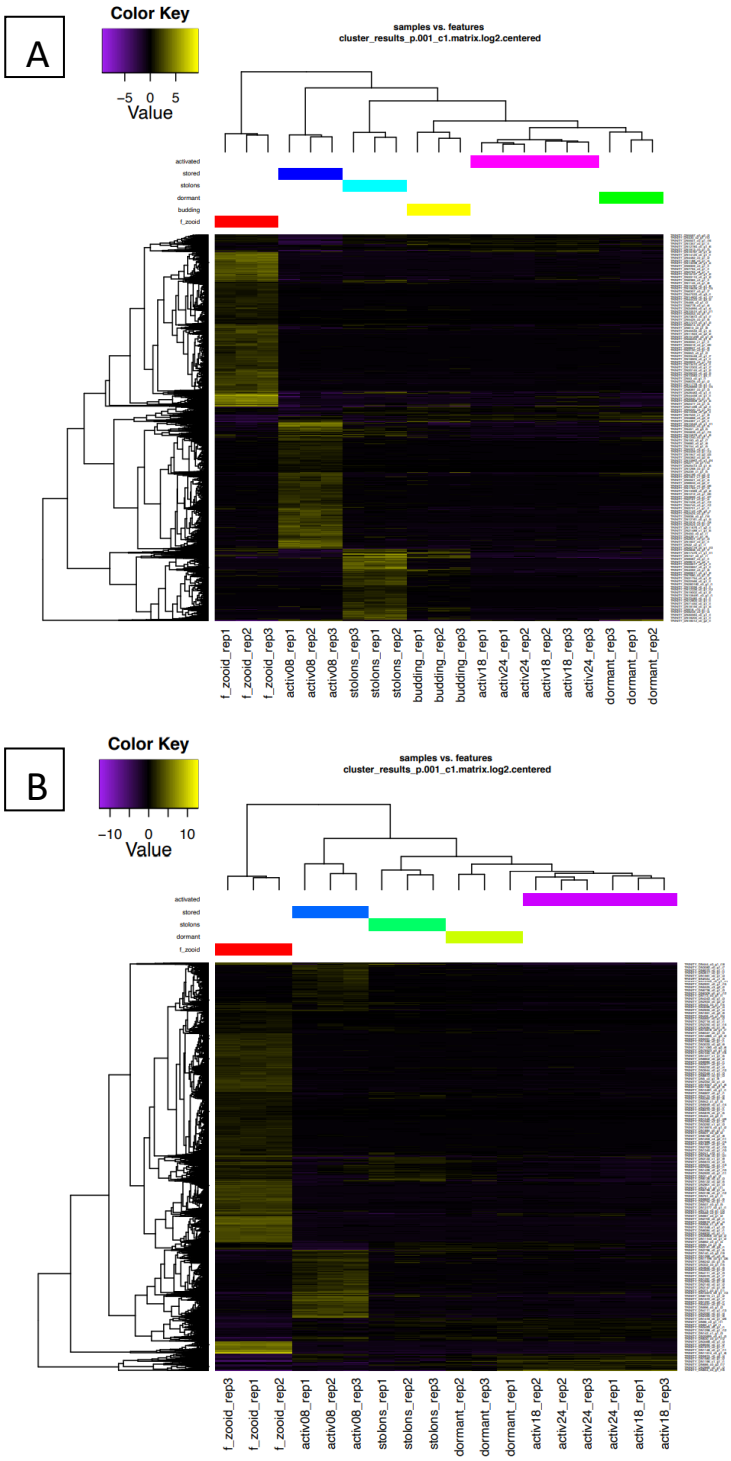
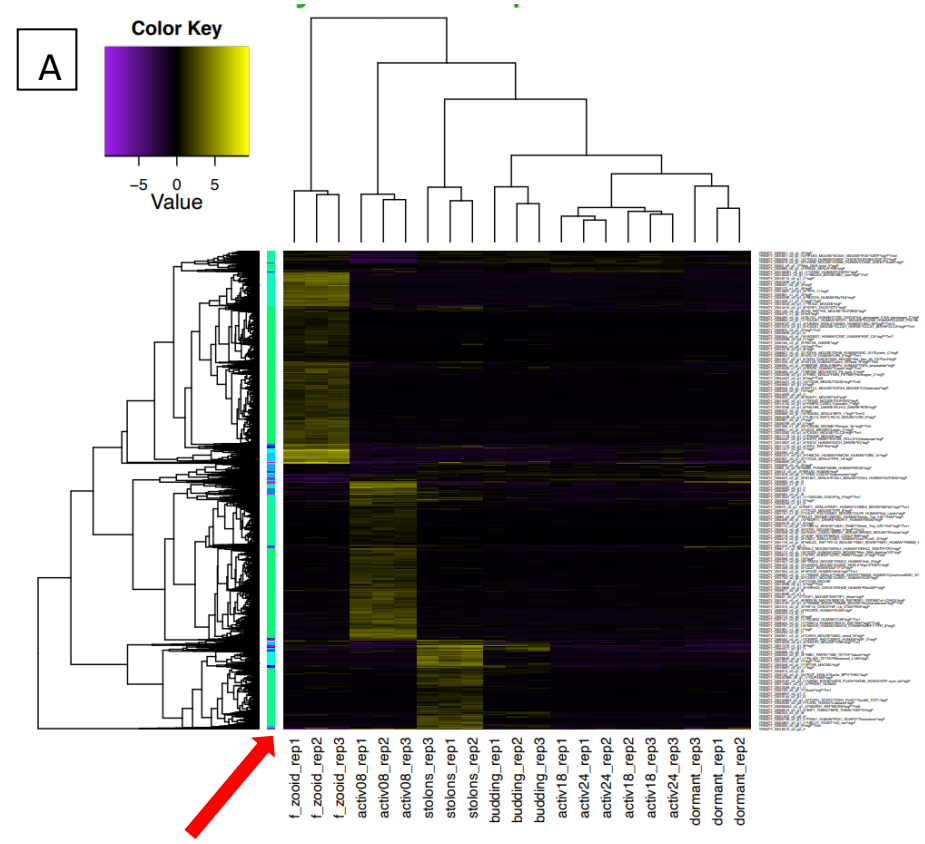


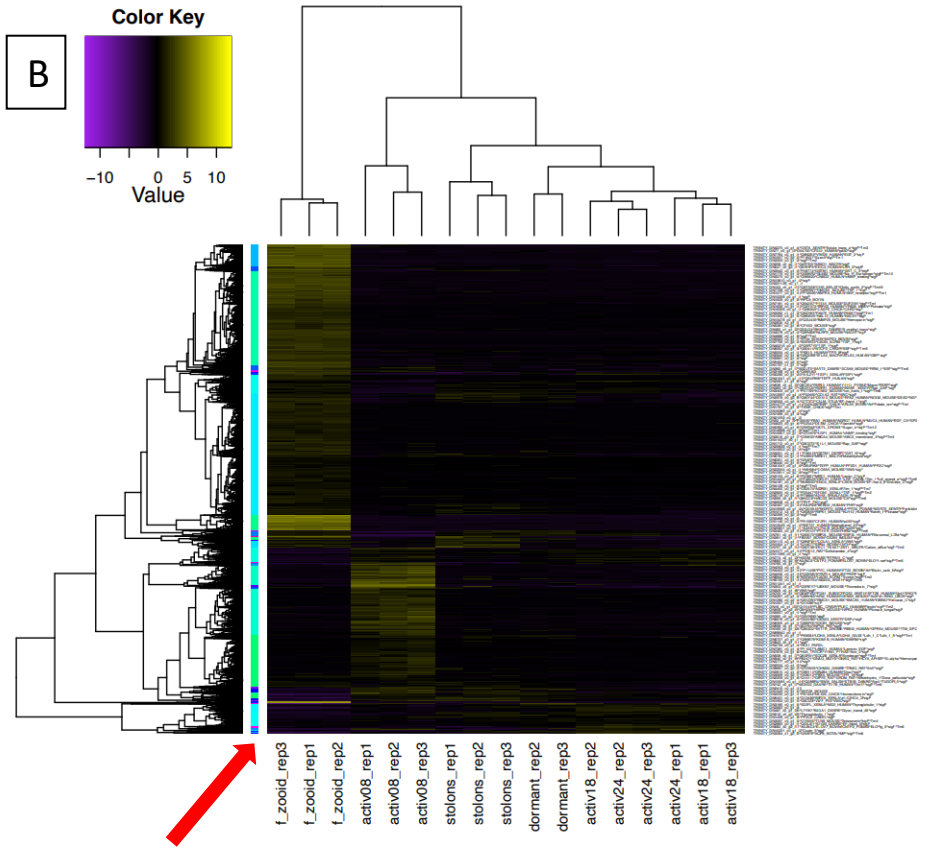
Figure 4.18: Hierarchical clustering plots showing the correlations between samples using the isoform data from *P. zorritensis* (A) and *C. lepadiformis* (B). Each column represents a specimen, while each row represents a gene. The different stages are represented on the dendrogram with different colors: zooids in red, budding in yellow (only in P. zorritensis plot), dormants in green, stolons in light blue, stored in dark blue, activated in pink. The highly expressed genes are visualized in yellow, while the lowly expressed are visualized in violet.

4.11.2 Differential expression analysis and production of co-expression clusters

Once the samples passed the quality control, we performed the differential expression analysis to identify the statistically differential expressed genes (DEGs). Then, we repeated the hierarchical analysis including only the DEGs (Figure 4.19). Using the isoform data, we identified 5347 DEGs in *P. zorritensis* and 11691 DEGs in *C. lepadiformis*. We subdivided these DEGs in clusters based on their similar levels of expression across stages. Using the isoform data we formed 102 gene clusters in *P. zorritensis* and 65 in *C. lepadiformis* (Figure 4.19 arrows, Figure 4.20).

Figure 4.19 (following page): Expression matrix showing the significant DEGs in each stage of *P. zorritensis* (A) and *C. lepadiformis* (B). Each column represents a specimen, while each row represents a differential expressed gene. The highly expressed genes are visualized in yellow, while the lowly expressed are visualized in violet. The gene clusters are represented individually with a different color (arrows).


A
Color Key
−5 0 5
Value
f_zooid_rep1
f_zooid_rep2
f_zooid_rep3
activ08_rep1
activ08_rep2
activ08_rep3
stolons_rep3
stolons_rep1
stolons_rep2
budding_rep1
budding_rep2
budding_rep3
activ18_rep1
activ24_rep1
activ24_rep2
activ18_rep2
activ18_rep3
activ24_rep3
dormant_rep3
dormant_rep1
dormant_rep2


B
Color Key
−10 0 5 10
Value
f_zooid_rep3
f_zooid_rep1
f_zooid_rep2
activ08_rep1
activ08_rep2
activ08_rep3
stolons_rep1
stolons_rep2
stolons_rep3
dormant_rep2
dormant_rep3
activ18_rep2
activ24_rep2
activ24_rep3
activ24_rep1
activ18_rep1
activ18_rep3

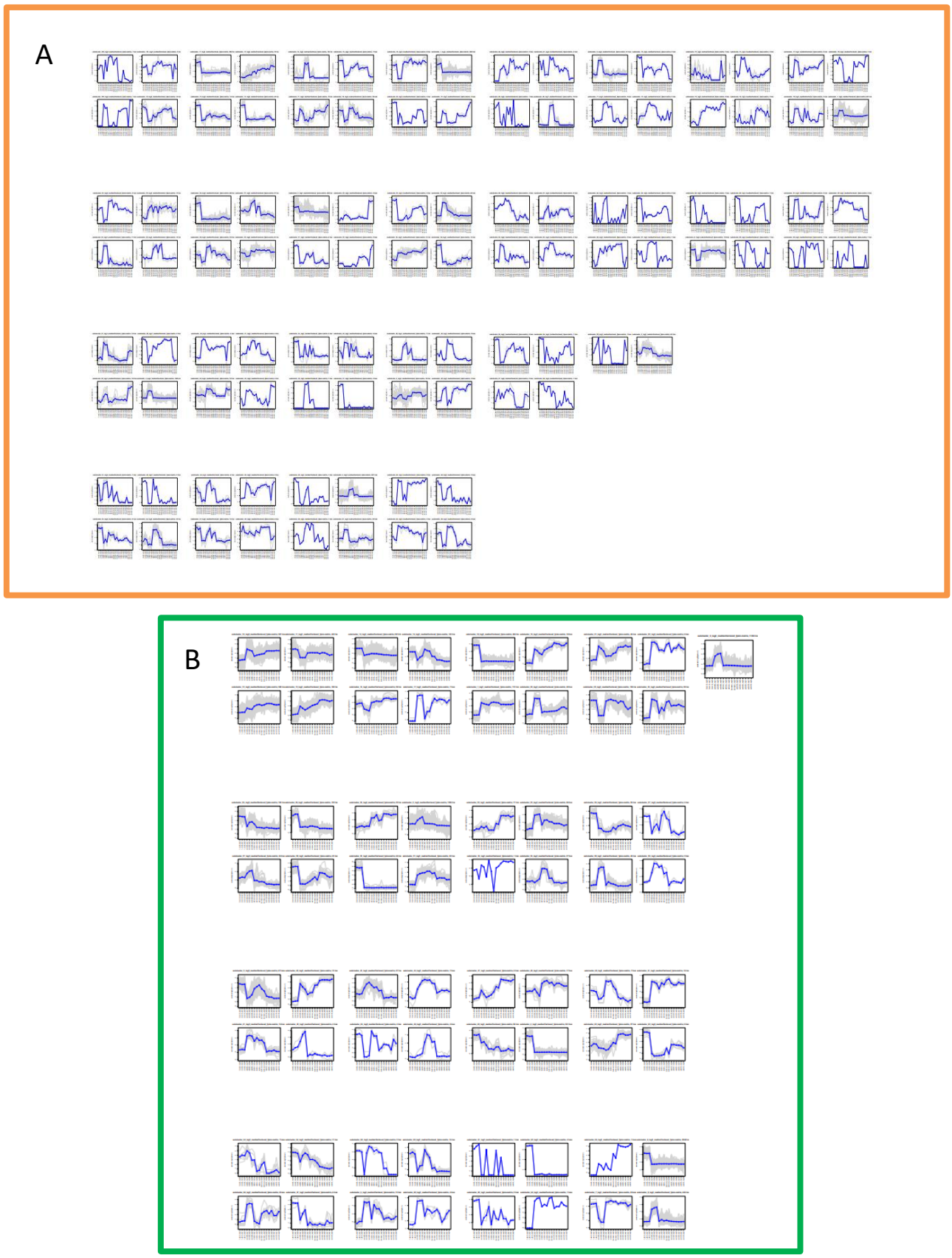
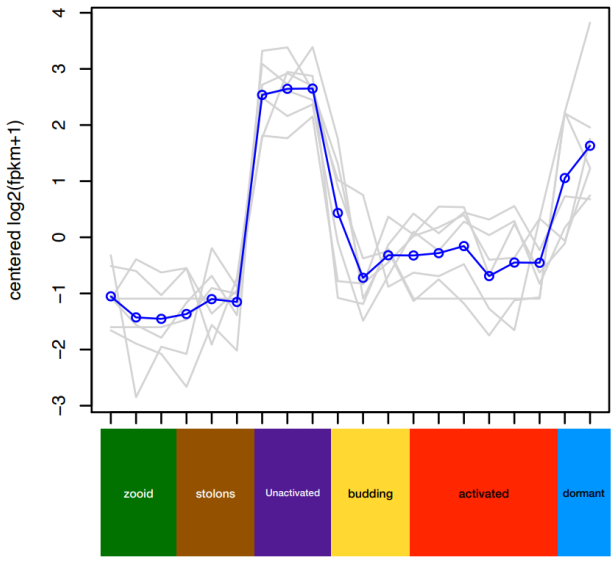


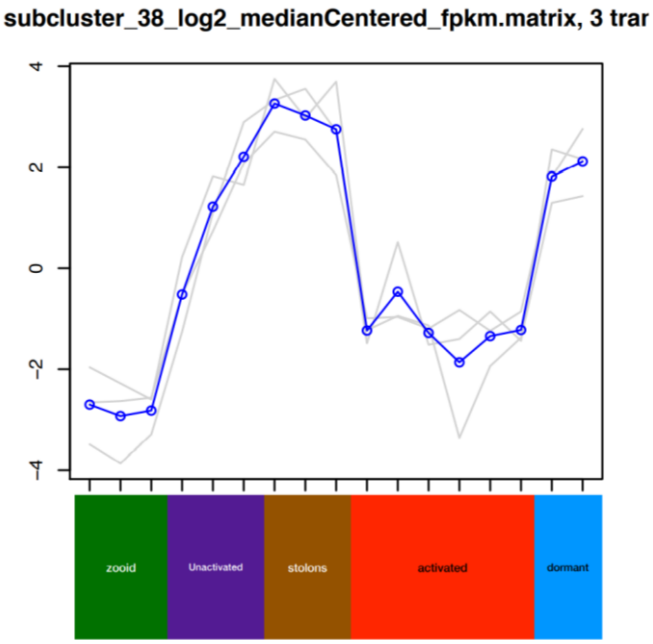
Figure 4.20: Upregulated clusters in *P. zorritensis* (A, orange rectangle) and in *C. lepadiformis* (B, green rectangle) obtained analyzing the genes isoforms. A) The 102 expression matrices of the upregulated gene clusters in *P. zorritensis*. B) The 65 expression matrices.

Combining the results of the differential expression with the annotation, it is possible to add functional information on the genes composing the clusters. Each cluster can be divided in sub-subclusters representing the different isoforms of one gene (Figure 4.21, Figure 4.22).



Gene	Blastx result
TRINITY_DN4642_c0_g1	perilipin-2 isoform X2 [Ciona intestinalis]

Figure 4.21: Subcluster corresponding to the gene encoding for the protein Periplin-2 in *P. zorritensis.* Each isoform is represented with a gray line and the mean expression profile is represented with a blue line. The expression levels are reported on the y axis, while the samples are reported on the x axis in different colors (zooids: green; stolons: brown; stored: purple; budding nests: yellow; activated: red; dormant spherules: blue). The gene is upregulated in the spherules stored for 48h at 8°C (renamed in this graph as “unactivated”) and in the dormant spherules.



TRINITY_DN6489_c0_g1_i3	LYAM3_HUMAN	P-selectin. Ca^{2+}-dependent receptor for myeloid cells that binds to carbohydrates on neutrophils and monocytes. Mediates the interaction of activated endothelial cells or platelets with leukocytes.
TRINITY_DN6489_c0_g1_i2	SVEP1_RAT	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1. May play a role in the cell attachment process

Figure 4.22: expression matrix of the subcluster corresponding to the gene *Lyam3* encoding for the protein P-selectin in *C. lepadiformis.* Each isoform is represented with a gray line and the mean expression profile is represented with a blue line. The expression levels are reported on the y axis, while the samples are reported on the x axis in different colors (zooids: green; stolons: brown; stored: purple; budding nests: yellow; activated: red; dormant spherules: blue). The gene is upregulated in the spherules stored for 48h at 8°C (renamed in this graph as "unactivated") , in the stolons and in the dormant spherules.

4.11.3 Tissue/sample enrichment analysis

In order to identify genes that are consistently upregulated in each tissue/sample-type, we performed a tissue-specific expression analysis. This involves comparing the expression in each sample type with the average across all samples. Results show that *P. zorritensis* spherules have 17 genes that are consistently upregulated specifically in the dormancy stage. No dormancy-specific genes were found for *C. lepadiformis*. For both species, the zooids show the highest number of sample-specific genes, followed by the stored samples. (Figure 4.23).

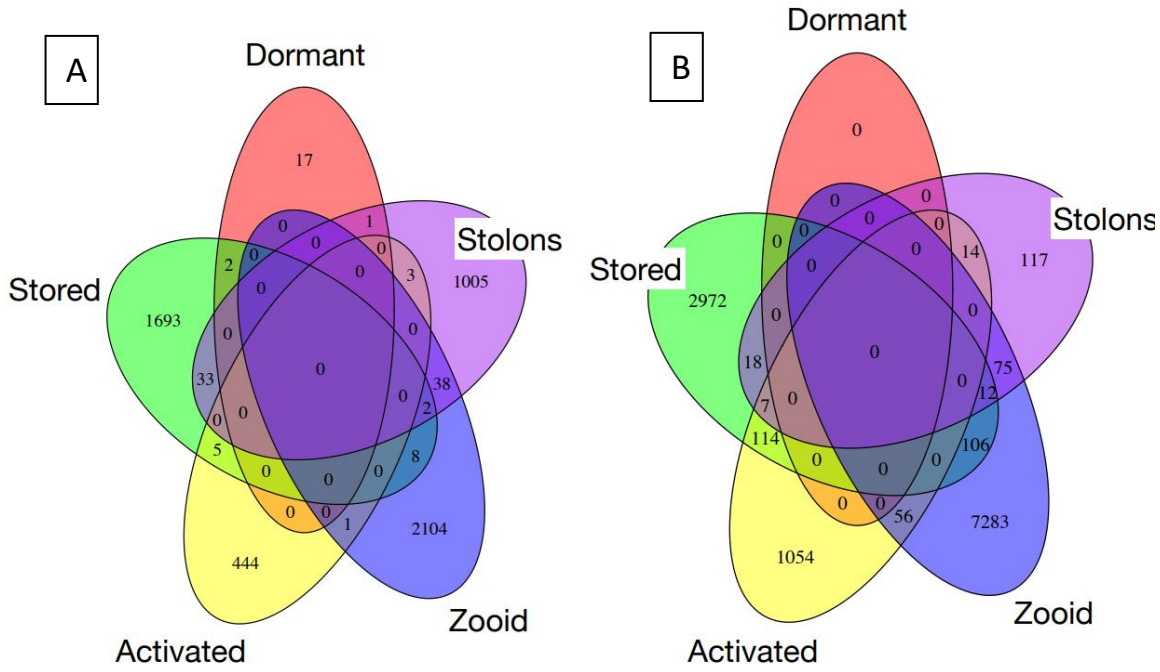


Figure 4.23: Venn diagram showing the number of the tissue-specific upregulated genes in P. zorritensis (A) and C. lepadiformis (B). Different colors identify the different stages/tissues: blue zooid; purple stolon; orange dormant spherules, greed stored spherules; yellow activated spherules.

Discussion

4.12 Production of spherules as a strategy for colony survival and dispersion in *P. zorritensis*.

The *in situ* observations of the natural population located in La Spezia and the *ex situ* experiments carried out in the laboratory show that *Polyandrocarpa zorritensis* produces resting forms, the spherules. These spherules resist a wide range of temperatures and salinities, including conditions in which the zooids normally degenerate. After months in the resting stage, spherules can reactivate and produce new adults, and consequently new colonies.

Polyandrocarpa zorritensis, described for the first time in Peru (Van Name, 1931), has been reported in the last decades in other sites all over the world, especially harbors due to the international shipping traffic (Carman et al., 2011; Lambert and Lambert, 2003; Nishikawa, 1993). In the Mediterranean Sea, *P. zorritensis* has been recorder in Italy (Brunetti, 1978; Brunetti and Mastrototaro, 2004; Mastrototaro et al., 2008; Stabili et al., 2015), Spain (Turon and Becerro, 1992) and very recently in France, in the Thau pond (reported on the DORIS website https://doris.ffessm.fr/Especes/Polyandrocarpa-zorritensis-Polyandrocarpe-de-Zorritos-5004). The tolerance to different conditions of this species has been proposed as one of the reasons of its invasive success. The physiological responses to abiotic parameters as light, pressure, and salinity have been investigated in the larva of *P. zorritensis* (Sumida et al., 2015; Vázquez and Young, 1996, 1998) but these results were unsufficient to explain the rapid expansion of this species. The successful colonization of this species in non-native environments may be explained by the capacity to undergo dormancy. *P.zorritensis* spherules are capable of resisting a wide range of environmental extremes, and a single spherule accidentally transported in a new site may be capable to restore a new colony.

During the *in situ* observations in La Spezia, we described seasonal variations in the zooidal biomass, since the zooids "disappear" during spring. Despite this seasonality of the zooids, the resistant spherules are present in the field all over the year. Mastrototaro and colleagues (2008) reported an increase in *P. zorritensis* zooidal biomass in fall in the Mar Piccolo of Taranto (Mastrototaro et al., 2008). Combining our observations with the monthly temperature ranges of seawater in La Spezia (Figure 4.24) it is clear that the biggest spherules are obtained in the coldest months, right before the zooidal degeneration that occurs in spring, when the water temperatures start to increase. This suggests that, during the cold season, the spherules accumulate nutrients and increase in size, getting prepared to survive after zooidal regression. These spherules will be able to reconstitute the entire colony and assure its continuity.

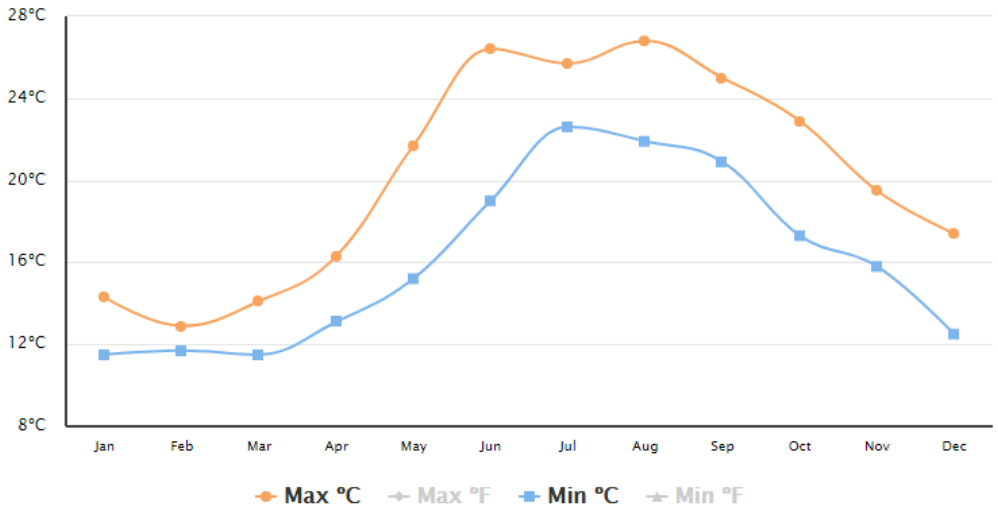


Figure 4.24: Graph showing the monthly average sea water temperature in La Spezia harbor. The range derived from historical sea surface temperature data collected by satellite (from the web site https://www.seatemperature.org/europe/italy/la-spezia.htm).

4.13 Production of winter buds in *Clavelina lepadiformis* and comparison with *P. zorritensis*

Under laboratory conditions, we observed that the winter buds of *C. lepadiformis* can be produced when the temperature is constantly maintained at 18°C and the zooids are not degenerating. *Clavelina* winter buds have been reported in winter (Orton 1919, 1921, Giard and Callery 1896, Kerb 1908, Della Valle 1914) in the Mediterranean and North Sea. However, Huxley (1926) reported the presence of winter buds during summer, when the zooids were still present in the colony, and he hypothesized that cues different from temperatures could lead to winter bud formation. Further investigations are necessary to identify other environmental cues responsible for winter bud formation in *C. lepadiformis*.

The experiments aimed to test a wide range of conditions on zooidal survival highlighted differences between *C. lepadiformis* and *P. zorritensis*. *Clavelina* zooids can resist lower temperatures and higher salinity compared to *Polyandrocarpa*. The natural geographic distribution may be related to this difference between the species. *Polyandrocarpa zorritensis* has been reported for the first time on the Pacific coasts of South America, and only subsequently has been stated in other sites (see section 4.12.1). On the other hand, *Clavelina lepadiformis* is a native of the Mediterranean Sea, Atlantic subarctic regions (i.e. Norwegian Sea and Greenland), and Bay of Biscay, and it has been introduced on the Atlantic coasts of North and Central America, in Brazil (Pyo and Shin, 2011; Reinhardt et al., 2010; Turon et al., 2003).

The fact that *Clavelina* is present in subarctic regions may be linked to a greater resistance to temperature and salinity of the zooids compared with *P. zorritensis*.

4.14 Dealing with temperature: does dormancy represent the seasonality of NED?

Environmental conditions regulate the seasonality of reproductive cycles in marine invertebrates (Lawrence and Soame, 2004; Munro et al., 1990). In particular, the temperature has been proposed as one of the main environmental parameters influencing the seasonality of sexual reproduction in different species, including ascidians (Bates, 2005; Orton, 1920). The breeding cycle can vary in different species or different populations of the same species according to the geographical range: ascidians living at latitudes where the seasonal temperature fluctuations are more constant, for example at the tropics, can be sexually reproducing throughout the year (van Duyl et al., 1981; Goodbody, 1961), while where the fluctuations are more important (for example, at high latitudes), the seasonality of the breeding cycle appears temperature-dependent (Millar, 1958). World-wide distributed species as *Ciona spp.* can show variations in the reproductive periodicity depending on the latitude: in more temperate zones, such as Japan and the Mediterranean Sea, *Ciona spp* can continuously breed all year round (Nomaguchi, 1974; Yamaguchi, 1975) while at higher latitudes the development usually decline in the cold months (Harris et al., 2017). Goodbody (1961) defined the spawning as a parameter to assess the seasonality of the breeding cycle, instead of presence of gonads and gametes. The periodicity of the spawning may be a consequence of the thermosensibility of pelagic ascidian embryos and larvae, which show like other invertebrates a linear relationship between growth rate and temperature (Nomaguchi et al., 1997; Trudgill et al., 2005). The seasonality in the spawning activity can be seen as a temperature adaptation that leads planktonic survival of the germline, the embryos and larvae in favorable environmental conditions.

In colonial ascidians, the production of new buds can be continuously through all the year, also if the budding rate can variate in the different seasons, such as in *Metandrocarpa taylori* (Haven, 1971). However, when the environmental conditions (and in particular the temperature) are unfavourable, budding is arrested and the colony can undergo dormancy. Orton (1920) compared the capability to hibernate in invertebrates with the seasonality of breeding, saying that "*phenomena similar to those of breeding are observable in forms which hibernate*" (Orton, 1920, pag. 351). Interestingly, he took as example the colonial ascidian *Clavelina lepadiformis* and reported the observation of hibernating periods of the population located in the Gulf of

Naples. In this light, the ability to produce dormant stages can be seen as an adaptation of somatic cell lines to temperature changes, and it may represent the seasonality of NED.

In *P. zorritensis* and *C. lepadiformis*, the dormant forms originate from budding tissues and can be considered as specialized buds able to cope with environmental changes. These "seasonal buds" are morphologically different from the buds produced in favourable conditions, and the preliminary data presented in Chapter 4 show also a specific molecular signature. These evidences may be introduced in a broader consideration on the environmental control of reproduction.

4.15 Dormant forms of *P. zorritensis* and *C. lepadiformis* are specialized buds

In *P. zorritensis* and *C. lepadiformis*, the dormant forms can be considered as specialized buds produced to resist adverse conditions. Spherules and winter buds acquire characteristics of stiffness (as increased accumulation of nutrients and a thicker tunic) that permit to functionally distinguish them from the budding nests and chambers, but externally the morphological difference between active asexual and dormant forms is very subtle. The result of the development of dormant and non-dormant forms (that is the formation of a functional zooid) is identical, and the zooids produced by reactivation of dormant form and by budding are indistinguishable. Also, the stages of development and the organogenetic tissue seem to coincide (i.e. invagination of the vascular epidermis in *P. zorritensis,* see section 4.7.2). Dormant forms participate in the dispersal of benthic asexually reproducing species, such as in sponges (Manconi and Pronzato, 2016). In this perspective, spherules and winter buds may be seen as propagative buds, equivalent to budding nests and budding chambers, responsible for colony growth but on a larger environmental scale. In addition, our observations revealed that in these species dormant forms can be produced even when environmental conditions are stable (as in *Clavelina* colonies maintained constantly at 18°C) or can be maintained in a resting state also if the environmental conditions are tolerable for colony formation (as spherules produced all over the year in field conditions). We can speculate that the dormant asexual forms are just specialized buds constantly produced, ready at any moment to resist changes in the environment or able to assure colony propagation in case of detachment and dispersal. When the environmental conditions suddenly change into an unfavorable state, the tissues with budding potential can quickly respond acquiring the dormant phenotype.

4.16 Potential insights from the differential gene expression

Within invertebrates, changes in gene expression in response to dormancy have been studied particularly in insects. Genes expressed in resting eggs or embryos can be analyzed and compared within different species (Ragland et al., 2010), under different triggering conditions (Poupardin et al., 2015; Rinehart et al., 2007; Yocum, 2001) and/or at different stages of dormancy (Ragland et al., 2011). Recently, transcriptomic emerged as a useful tool to investigate the molecular regulation of dormancy and compare the stress responses (Hayward, 2014). Comparative transcriptomic have been used to trace the evolution of dormancy (and in particular diapause) in insects. Different species of insects show various dormant strategies (i.e. dormancy at different stages of the life cycle, responses to different stimuli, etc). However, physiological responses are common, and some authors hypothesized the presence of a genetic "toolkit" of dormancy for each dormant stage shared between different species (Poelchau et al., 2013; Poupardin et al., 2015; Yocum et al., 2015). Analyzing together the transcriptomic data of differentially expressed genes during diapause, a few percentages of these genes is commonly differentially regulated across the different species (Ragland and Keep, 2017). The differentially expressed genes can be clustered in functional categories, associated with the physiological changes responsible for dormancy: for example, cell cycle-related transcripts appear commonly down-regulated during dormancy in different species, consistently with the cell division control that occur during dormancy (Ragland and Keep, 2017). Koštál and colleagues (2017) proposed that "*the evolutionary conservation/convergence of different diapause responses is unlikely to be observable at the level of transcriptional patterns of isolated genes. Rather, a genetic toolkit of diapause may be reflected in the activation/inhibition of whole signaling pathways regulating the hallmark diapause-linked physiological phenotypes*"(Koštál et al., 2017).

The differential expression analysis on *C. lepadiformis* and *P. zorritensis* are still in progress, but we expect to find, between the two species, conserved genes expression profiles up-regulated during the dormancy process. For example, the thickening of the tunic observed in both spherules and winter buds suggest the up-regulation of genes involved in tunicin synthesis. The accumulation of lipids and glycogen granules in the vascular epidermis and in trophocytes indicate the possible up-regulation of genes involved in the metabolisms of these compounds in the stages preceding dormancy. For instance, the preliminary results obtained in *P. zorritensis* reported in the dormant spherules the up-regulation of the gene encoding for Perilipin 2, a lipid droplet protein regulating the storage and utilization of lipids (Sztalryd and Brasaemle, 2017; Yang et al., 2010). The comparison between genes up-regulated in dormant stage in both *P. zorritensis* and *C. lepadiformis* may identify dormancy-specific genes involved in a convergent origin of dormancy in these two species not closely-related and showing different modes of NED.

Material and methods

4.17 Sample collection and specimen husbandry

Colonies of *Polyandrocarpa zorritensis* and *Clavelina lepadiformis* were collected in the harbor of La Spezia (Assonautica Benedetti, 44°06′10.7″N 9°49′34.5″E) (Italy). Colonies and spherules of P. zorritensis were maintained in the laboratory as reported in Scelzo et al., 2019 (see Chapter 2). Colonies of *C. lepadiformis* were maintained at 18°C in a closed sea water system and fed with living algae (Tisochrysis lutea and Chaetoceros) and concentrated algae (Isochrysis 100 and Shellfish Diet 1800, Red Mariculture Inc). In order to clearly observe the stolons and the winter bud production, Clavelina zooids were separated from the colonies and let attach on a glass microscope slide with the help of a thread at 18°C for one week.

4.18 Light and transmission electron microscopy (TEM)

Inclusion of Polyandrocarpa zorritensis specimens for paraffin sectioning and hematoxylin/eosin staining were performed as described in Alié et al., 2018 (see Annex 2). Samples of *P. zorritensis* and *C. lepadiformis* for semi-thin and ultra-thin sectioning were fixed, embedded and treated as reported in Scelzo et al., 2019 (Chapter 2).

4.19 Effects of different environmental cues on colonies of *P. zorritensis* and *C. lepadiformis*

In order to define the temperature dynamics of spherules production and reactivation in *P. zorritensis* (section 4.7.2), four incubators at different constant temperatures (8°C, 12°C, 17°C, 24°C) were set up. For the whole experiment, the animals were maintained in aquarium tanks filled with sea water for a volume of ~700mL and fed 3 times a week with a mix of living algae (Tisochrysis lutea and Chaetoceros) and concentrated algae (Isochrysis 100 and Shellfish Diet 1800, Red Mariculture Inc). Water changes and animal cleaning were carried two times a week.

To test the temperature resistance range of zooids and resistant forms in both species (section 4.7.2 for *P. zorritensis*, 4.9.2 for *C. lepadiformis*), the specimens were transferred to 50mL Falcon tubes (Corning, Glendale, Arizona) in filtered sea water and exposed to different temperatures in incubators (for the 18°C, 28°C, 32°C, 37°C conditions), fridge (for the 4°C condition) and freezers (for the -20°C and -80°C conditions) or on ice (for the 0°C condition) for 24 hours. Then, the *P.*

zorritensis and *C. lepadiformis* specimens were gradually transferred respectively to 24°C and 18°C. For the following two weeks, their survival or activation was observed and they were cleaned and fed as the non-treated specimens.

To test the salinity resistance range of zooids and resistant forms in both species (section 4.7.2 for *P. zorritensis*, 4.9.2 for *C. lepadiformis*), the suited salinity was obtained adding deionized water to filtrated sea water at 39 ppt (for the 10 ppt, 15 ppt and 30 ppt conditions) or preparing artificial sea water (for the 45 ppt condition) (Red Sea Salt, Red Sea). For each condition, the specimens were transferred to a 50mL Falcon tube (Corning, Glendale, Arizona) and incubated at 24°C (*P. zorritensis*) or 18°C (*C. lepadiformis*) for 24 hours. Then, they were gradually transferred to filtrated sea water at 39 ppt and observed for the following two weeks.

4.20 Differential expression analysis

For each stage (reported in section 4.11, Figure 4.15), we extracted RNA from three replicate samples. Tissue from three or more individuals had to be pooled to obtain enough RNA for sequencing. For the dormant stage *P. zorritensis*, spherules were collected from the field in the spring (the time of the year in which only dormant stages were present) and the tissue was immediately flash-frozen. Additional material from the same rope was transported back to the lab, and activated to generate new zooids, stolons, and buds, from which we extracted RNA for those stages. For *C. lepadiformis*, we extracted all the material from the same colony in the lab. RNA was extracted with the NucleoSpin RNA extraction kit (Machery-Nagel) and sample quality and quantity was verified with the B2100 BioAnalyzer Instrument and Bioanalyzer RNA 6000 Nano assay (Agilent). Frozen RNA was sent for cDNA library preparation (using cDNA libraries were prepared using NEB Next® Ultra RNA Library Prep Kit for Illumina) and Illumina paired-end 150bp sequencing at Novogene Bioinformatics Technology Co. Ltd (Tianjing, China). Sequencing was done on a Novaseq 6000 PE150 Illumina sequencing platform.

Raw sequencing reads were cleaned and trimmed. First, the program rCorrector was used to correct random sequencing errors and to eliminate read pairs where reads were flagged as 'uncorrectable' (Song and Florea, 2015). Next, Trim Galore! was used to remove low-quality reads from the dataset (with a quality Phred score cutoff of 5, a minimum read length threshold of 36 bp, a stringency parameter of 1 for adapter sequence overlap, and a maximum allowed error rate of 0.1) and to trim Illumina adapters sequences from raw sequencing reads (Martin, 2011). Ribosomal RNA was removed by eliminating reads that mapped to the SILVA large and small subunit databases (SILVA_132_LSUParc_tax_silva.fasta and SILVA_138_SSURef_NR99_tax_silva.fasta) using Bowtie2 (Langmead and Salzberg, 2012). Finally,

we used the software FastQC to identify overrepresented sequences from the read set (Andrews, 2010).

Transcriptomes were assembled using the software Trinity with in silico read normalization (Grabherr et al., 2011). For each species, raw read data from all conditions and replicates were pooled to create a single assembly per species. Assemblies were assessed by looking at basic summary matrices and by quantifying completeness using the program BUSCO (v3) by searching for a curated set of single copy orthologs present in all metazoans (metazoa odb9 database) (Waterhouse et al., 2018). Transcriptomes were assembled using the software Trinity with in silico read normalization (Grabherr et al., 2011). For each species, raw read data from all conditions and replicates were pooled to create a single assembly per species. Assemblies were assessed by looking at basic summary matrices and by quantifying completeness using the program BUSCO (v3) by searching for a curated set of single copy orthologs present in all metazoans (metazoa odb9 database) (Waterhouse et al., 2017).

Transcripts were functionally annotated by using the program Trinotate (Bryant et al., 2017; Griffith et al., 2015). Trinotate prepares an annotation database using homology searches to known sequence data (BLAST+/SwissProt databases), protein domain identification (HMMER/PFAM), protein signal peptide identification (SignalP), and transmembrane domain prediction (tmHMM). Gene Ontology (GO) assignments for all genes were also added to this database (by blasting to the SwissProt database and selecting the top hit).

The program Kallisto was used to quantify abundances of transcripts and align raw reads onto the assembled contigs (using 100 bootstraps) (Bray et al., 2016). Transcript and gene expression matrices were built using these quantifications. The Trinity analysis program 'PtR' was used to compare biological replicates across samples, by constructing a correlation matrix for each sample in an assembly, and principal components analysis plots, labeled by stage and replicate. Outliers were removed and treatments were combined as necessary at this step.

Differential transcript expression was assessed using the program *voom* (Law et al., 2014). All pairwise comparisons of sample type within species were tested. Transcripts were considered differentially expressed if they showed a 2-fold average difference in expression between sample types with a false discovery rate (FDR) p-value of <0.001. Using these criteria, the analysis found 5,347 differentially expressed genes (DEGs) in the *P. zorritensis* dataset and 11,1691 genes in the *C. lepadiformis* dataset. Using only the DEGs, we repeated the hierarchical clustering analysis. By dividing the tree by 20% tree height, we formed 102 The gene expression analysis revealed the presence of 61 gene clusters in *P. zorritensis* and 6540 in *C. lepadiformis* differentially expressed (Figure 4.19).

Sample-specific expression patterns were identified first finding the average expression across stages and then identifying tissue-enriched genes using the average as a reference. These values were computed using perl scripts packaged with Trinity and venn diagrams were produced in R.

Chapter 5
General remarks and conclusions

5.1 Experimental models for evo-devo: tunicate clades and tunicate species

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 represent great models to investigate the evolution of developmental processes because these comparisons can be explored at different levels.

First, colonial tunicates can perform both embryonic and non-embryonic development in the same species to generate similar bodies using different cellular sources and strikingly different morphogenesis. In fact, despite the differences in developmental processes, all lead to the formation of similar post metamorphic bodies (i.e. adult bodies). All these divergent developmental processes can be analyzed side-by-side in the same species to explore how morphologically similar features can arise from completely different developmental pathways. For instance, previous studies in *Botryllus schlosseri* revealed that some developmental genes are co-opted from embryogenesis during peribranchial budding (Ricci et al., 2016, Pruenster et al., 2019 a, b). Comparisons of developmental processes with similar phenotypic outcomes in the same organism could reveal how gene regulatory networks are rewired during distinct phases of the life cycle of an organism and, more in general, during the evolution of each independent developmental process.

Second, different species of tunicates also present an array of different NED modes that can be defined according to the nature of the budding tissues and the different budding ontogenesis, which result in the formation of zooids with similar body plans (Alie' et al 2020, Chapter 1 section 1.5). The combination of transcriptomic and functional approaches has the potential to identify conserved modules (genes, portions of gene regulatory networks, or particular cell or tissue "types") that control budding. The comparison of similar and different NED modes in distinct species has the potential to uncover homologies in NED mechanisms.

Finally, phylogenetic reconstructions in the tunicates revealed multiple gains and losses of NED, providing the possibility to explore the underlying mechanisms guiding the acquisitions of NED and perhaps their evolutionary trajectories. One intriguing direction to follow is to explore the function of undifferentiated mesenchymal cells (hemoblasts), which have been reported in both solitary and colonial ascidians, yet with different potency and ontogenic roles (see section 5.2).

5.2 Can the hemoblasts plasticity lead to NED in colonial ascidians?

An undifferentiated hemocyte morphotype has been characterized in both solitary and colonial tunicates. These cells, commonly named hemoblasts, show a stereotyped morphology (i.e. small, spherical shape and high nuclear-cytoplasmic ratio) and have been proposed as circulating putative stem cells (CPSCs). In solitary species - i.e. species with restricted regenerative potential - hemoblasts have been proposed to be involved in tissue-specific regeneration. In *Ciona* the siphons repair and regeneration is triggered by the mobilization of multipotent progenitors that migrate from "niches" located in the branchial sac (Jeffery, 2018). These putative stem cells from the branchial sac are presumably multipotent and responsible for the regeneration of the central nervous system (Jeffery, 2018). Recently, circulatory ALDH hemoblast-like cells and the presence of proliferating *Piwi* positive hemoblasts in the intestinal submucosa of *Styela plicata* (another solitary species) suggest the existence of another potential stem cell "niche". While the role and dynamics of these hemoblasts have not been thoroughly studied, they seem to play a role in nervous system regeneration (Jiménez-Merino et al 2019, da Silva et al., 2015, F. Brown personal communications).

On the other hand, several studies in colonial species have suggested hemoblasts as key players of NED (Alie et al. 2020, see Chapter 1 paragraph 1.6), but it is in *Perophora viridis* (Freeman 1964) and *Botrylloides diegensis* (Kassmer et al 2019), that the pluripotency of hemoblasts have been functionally tested. In these two species one single clonogenic hemoblast is sufficient to trigger NED (stolonial budding in *P.viridis* and vascular budding in *B.diegensis*) and build the whole zooid.

Weissman (2000) defined stem cells as "*units of biological organization, responsible for the development and the regeneration of tissue and organ system*" (Weissman 2000). Because solitary and colonial tunicates show a different regenerative potential, it is intriguing to hypothesize that hemoblasts are stem cells both in adult solitary and colonial species but with different degrees of potency (Brown and Swalla, 2012).

Exploring the level of hemoblast potency and comparing their roles in the different developmental scenarios, represents a new approach to the study of stem cells biology and their evolution, and it can be an entry point to provide a molecular portrait of a major life history transition like the emergence of NED.

5.3 Dormancy, budding and WBR: the "*NED triad*"

Clavelina lepadiformis and *Polyandrocarpa zorritensis* are two colonial ascidians that have convergently acquired NED. These species show different modes of budding, stolonial and vasal budding respectively. Both species also produce dormant forms from the same tissues responsible for budding. Other ascidian species like *Perophora spp*., which undergo stolonial budding (Brien and Brien-Gavage, 1927, Barth and Barth, 1966, Mukai et al., 1983), and *Botrylloides diegensis*, which can undergo vascular budding (Hyams et al., 2017), can survive to thermal stress through remnants of external vasculature. The vascular tissues are also in charge to restore the developmental process at the end of dormancy, and the growth of the new zooid in the activated dormant form occurs following the same morphogenetic events that take place during NED. This suggests that when the conditions are unfavourable for survival, the colony sacrifices all the unnecessary structures and maintains only the budding tissues, together with the necessary structures for protection and nourishment. In the examples provided here, the dormant forms take place in vascular tissues. For both *P. viridis* and *B. diegensis* NED originates from pluripotent hemoblasts (Freeman 1964, Kassmer et al 2019). It is also likely that *C. lepadiformis* and *P. zorritensis* retain putative stem cells in their dormant forms. *Clavelina* retain the cells that form the "pluripotent" septum during its stolonial budding, and *P. zorritensis* retain the same population of hemoblasts that play a role in vasal budding (see Chapter 3)

Since the budding tissues seem to have a role also during dormancy, the correlation between these two developmental processes seems clear. While dormancy has not been reported in solitary ascidians (except in *Rhopalaea*, F. Brown personal communication), several colonial species that have convergently acquired NED show dormancy. It is interesting to look at the capacity of budding/dormancy in the same way that Weismann and Morgan have suggested for regeneration (Weismann 1893, Morgan 1898): dormancy can be considered as a adaptive characters that evolved in response to environmental cues, or as an epiphenomenon of asexual development. In this sense, dormancy may have co-opted molecular modules (e.g. pluripotent stem cells) of budding and evolved mechanisms of protection and avoidance in harsh environmental conditions. However, from the sparsely documented dormancy in tunicates, we cannot draw any conclusions about the ancestral state of this character.

5.4 A star is born*: Polyandrocarpa zorritensis* as key species for investigating NED mechanisms and evolution

The reconstruction of phylogenetic relationships between solitary and colonial species and the description of the morphological events occurring during the early stages of different types of NED already provided a potential evolutionary scenario in the family of Styelidae (Alie' et. al 2018, Scelzo et al. 2019), which as proposed at the beginning of this manuscript (Chapter 1 paragraph 1.6) is a good representative model of tunicate NED distribution.

During my doctoral dissertation I have generated basic biological understanding of a new organism suitable for laboratory research and for comparative studies in tunicates, *Polyandrocarpa zorritensis*. The description of a new form of NED in *Polyandrocarpa zorritensis* (Chapter 2) permitted the identification of an independent acquisition of NED within the Styelidae. The elucidation of the occurrence of hemoblasts during NED in *P. zorritensis* may bring out new insights on the function of these cells during NED (Chapter 3). Thanks to the presence of overwintering forms in its life cycle, *P. zorritensis* is also a suitable species to study dormancy, which can be considered a survival mode of budding (Nakauchi 1982, Mukai et al 1983) (Chapter 4).

In the last four years, I have helped develop new tools in order to work with this species, such as the establishment of a breeding system that allows to constantly obtain new buds or the optimization of techniques (such as cytological stains, EdU assay, immunostaining, *in situ* hybridization, hemocytes cells sorting by FACS). Despite the fact that further toolsets and datasets for molecular analyses (e.g. a stage-specific transcriptome for a molecular comparison with other species) are necessary, *Polyandrocarpa zorritensis* has all the potential to become a key species for better understanding the mechanisms and the evolution of NED. In particular, *Polyandrocarpa zorritensis* is a relevant species to study the role of the PCSCs during NED evolution. During vasal budding of *Polyandrocarpa zorritensis*, hemoblasts interact with the vascular epidermis of buds. I have shown that these cells proliferate and express Iα6, a marker of pluripotent hemoblasts in other Styelidae. Further molecular characterizations are necessary in order to determine the role of these putative stem cells during vasal budding in *P. zorritensis*. The comparisons of molecular mechanisms (e.g. identifying differences in gene expression of isolated hemoblasts of different species) in colonial and solitary species may allow us to understand whether these cells are responsible for the high regenerative capabilities of colonial ascidians.

Annex 1

Received: 25 November 2019 | Revised: 21 February 2020 | Accepted: 25 February 2020

DOI: 10.1002/jez.b.22940

Check for updates

REVIEW

JEZ-B MOLECULAR AND DEVELOPMENTAL EVOLUTION WILEY

The eventful history of nonembryonic development in tunicates

Alexandre Alié[1] | Laurel S. Hiebert[1,2] | Marta Scelzo[1] | Stefano Tiozzo[1]

[1]Laboratoire de Biologie du Développement de Villefranche-sur-Mer, CNRS, Institut de la Mer de Villefranche, Sorbonne Université, Villefranche-sur-Mer, France

[2]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

Correspondence
Alexandre Alié and Stefano Tiozzo, CNRS, Institut de la Mer de Villefranche (IMEV), Laboratoire de Biologie du Développement de Villefranche-sur-Mer (LBDV), Sorbonne Université, 06230 Villefranche-sur-Mer, France.
Email: alie@obs-vlfr.fr (A. A.) and tiozzo@obs-vlfr.fr (S. T.)

Funding information
Center National de la Recherche Scientifique, Grant/Award Number: PICS 07679; Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Number: BEPE 2018/05923-3; Agence Nationale de la Recherche, Grant/Award Number: ANR-14-CE02-0019-01

The peer review history for this article is available at https://publons.com/publon/10.1002/jez.b.22940

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 nonembryonic 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 nonhomologous tissues and cells. In this review, we explore more than 150 years of literature 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 gray areas that need to be further explored 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 nonembryonic development, as well as to better understand how such a profound innovation in life-history has evolved in numerous metazoans.

KEYWORDS

ascidian, asexual propagation, budding, coloniality, evo-devo, regeneration, stem cells

1 | INTRODUCTION

Tunicates (Phylum Chordata) encompass a large group of marine filter-feeding animals that inhabit a wide variety of marine ecosystems around the world (Shenkar & Swalla, 2011; Stolfi & Brown, 2015). They typically have a biphasic life cycle, with a planktonic larva and a postmetamorphic phase that is benthic in ascidians and planktonic in thaliaceans (Brusca, Moore, & Shuster, 2016). While solitary tunicates reproduce strictly sexually, many can propagate both sexually and asexually. During asexual reproduction, somatic tissues undergo a budding process that, bypassing fertilization, embryonic development, larval stage, and metamorphosis, gives rise to one or several adult individuals, called blastozooids. Individuals generated asexually generally have a bauplan that is very similar to the individuals formed by embryonic development (named oozooids). In this paper, we use the term "budding" as a synonym of asexual development and therefore we will call "budding species" those able to reproduce asexually. Budding has also been referred to as blastogenesis (Manni et al., 2014), clonal replication (Hughes, 1989), asexual propagation (Kürn, Rendulic, Tiozzo, & Lauzon, 2011), and more recently we coined nonembryonic development or NED (Alié et al., 2018), to include also whole-body regeneration, that is budding induced by exogenous inputs

 © 2020 Wiley Periodicals, Inc.

(Tiozzo & Copley, 2015). In some budding tunicates blastozooids remain connected to each other and form colonies, and then we talk about colonial tunicates, as opposed to solitary species.

Budding and nonbudding species are scattered among the four main tunicate orders: Thaliacea, Phlebobranchia, Aplousobranchia, and Stolidobranchia, and their phylogenetic distribution unambiguously demonstrates that asexual reproduction arose convergently several times (Alié et al., 2018; Delsuc et al., 2018; Govindarajan, Bucklin, & Madin, 2011; Kocot, Tassia, Halanych, & Swalla, 2018; Pérez-Portela, Bishop, Davis, & Turon, 2009; Shenkar, Koplovitz, Dray, Gissi, & Huchon, 2016; Tsagkogeorga et al., 2009). Convergent acquisition of NED becomes even more evident by the variety of tissues and mechanisms involved in bud formation across the different species (Berrill, 1951; Godeaux, 1957; Oka & Watanabe, 1957; Scelzo, Alié, Pagnotta, & Tiozzo, 2019). In fact, in contrast to embryonic development, which shows an extraordinary level of conservation amongst almost all the main tunicate orders, nonembryonic developmental processes involve a variety of cells, tissues, and ontogenesis, all displaying different degrees of interaction between epithelial and mesenchymal cells (even within a single order).

While in the last few decades the study of tunicate budding has focused on a few stolidobranch model species (from Sabbadin, 1958 to Kassmer, Langenbacher, & De Tomaso, 2019, reviewed in Manni et al., 2018), much knowledge about the diversity of NED is scattered across almost two centuries of literature. This knowledge has been brilliantly reviewed by others (Berrill, 1951; Brown & Swalla, 2012; Kawamura, Sugino, Sunanaga, & Fujiwara, 2008; Nakauchi, 1982), but an explicit reconstruction of NED history based on a modern understanding of the tunicate phylogeny is still needed. In this review, we first introduce an up-to-date consensus phylogeny of Tunicata, which includes budding and nonbudding 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 NONBUDDING 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 20th century proposed various evolutionary scenarios regarding budding evolution, but in this precladistic 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 (Moreno & Rocha, 2008; Zeng & Swalla, 2005, Pérez-Portela et al., 2009, Alié et al., 2018; Shenkar et al., 2016). 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 15 years (Alié et al., 2018; Da Silva Oliveira, Michonneau, & da Cruz Lotufo, 2017; Delsuc et al., 2018; Govindarajan et al., 2011; Kocot et al., 2018; Pérez-Portela, Goodwin, Picton, & Turon, 2010; Shenkar et al., 2016; Tatián, Lagger, Demarchi, & Mattoni, 2011; Tsagkogeorga et al., 2009; Turon & Lopez-Legentil, 2004). From each study, we only retained the nodes that received maximal statistical support (PP ≥ 0.99 or BS ≥ 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 have 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. (a) Thaliacea, that are all able to propagate by budding; (b) all the Aplousobranchia with the exception of the solitary *Rhopalaea idoneta*; (c) the Stolidobranchia species *Polyandrocarpa zorritensis* and (d) all the other colonial Stolidobranchia. For the species traditionally classified in the paraphyletic "Phlebobranchia", budding species are (e) the species *Polyoctacnemus patagoniensis*; (f) 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 forms a monophyletic order of ascidians that comprises budding and nonbudding species (Figure 2a-d), and among which *Styelidae* is the only family where NED has been reported

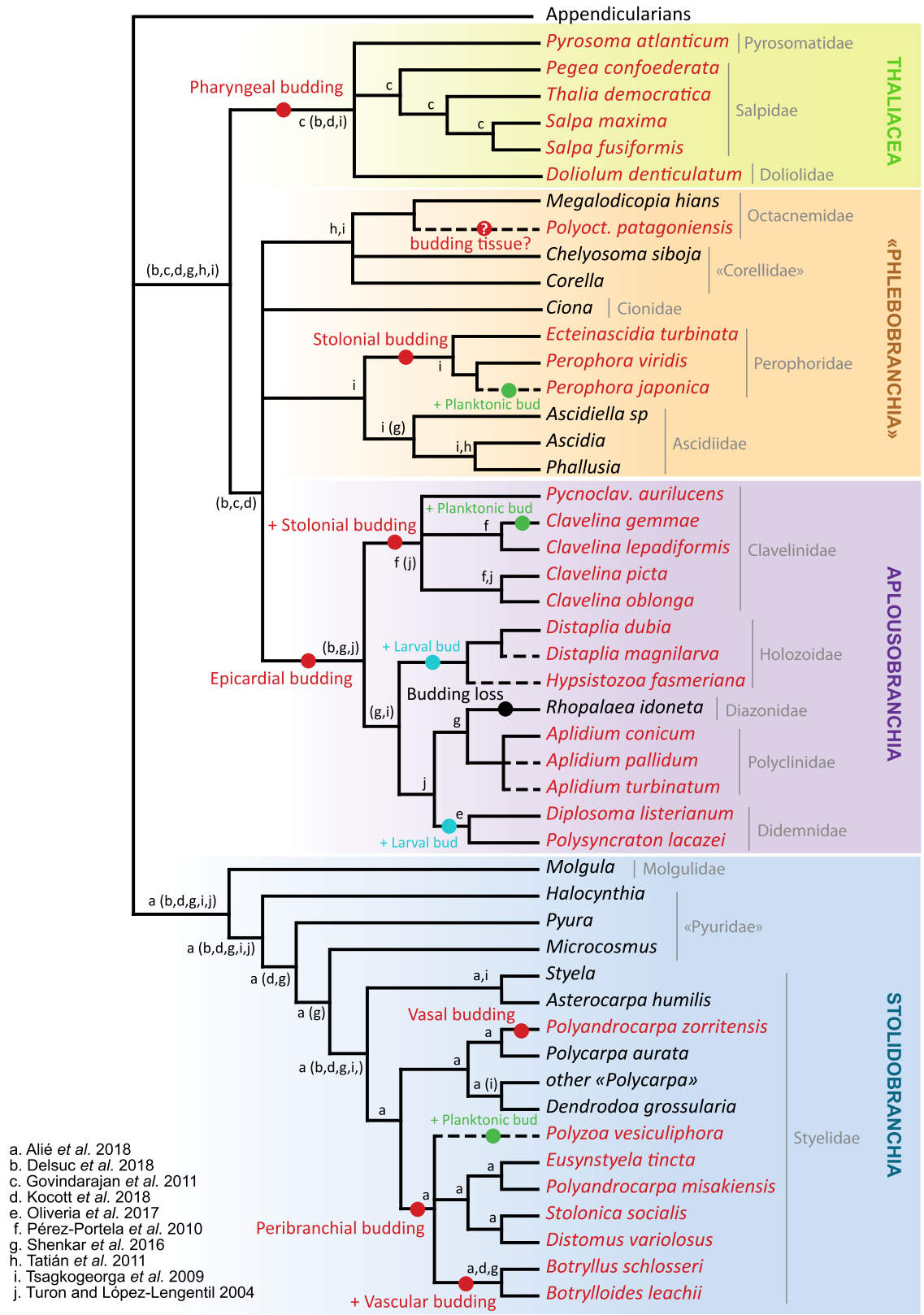


FIGURE 1 Phylogenetic relationships between budding and nonbudding tunicates. Budding species are represented in red, nonbudding species in black. For each branch, letters refer to the studies in which this clade was retrieved, including all the represented species. Letters between parentheses refer to studies that support the corresponding clade, that is 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 arised in budding taxa, it's preceded by "+" [Color figure can be viewed at wileyonlinelibrary.com]

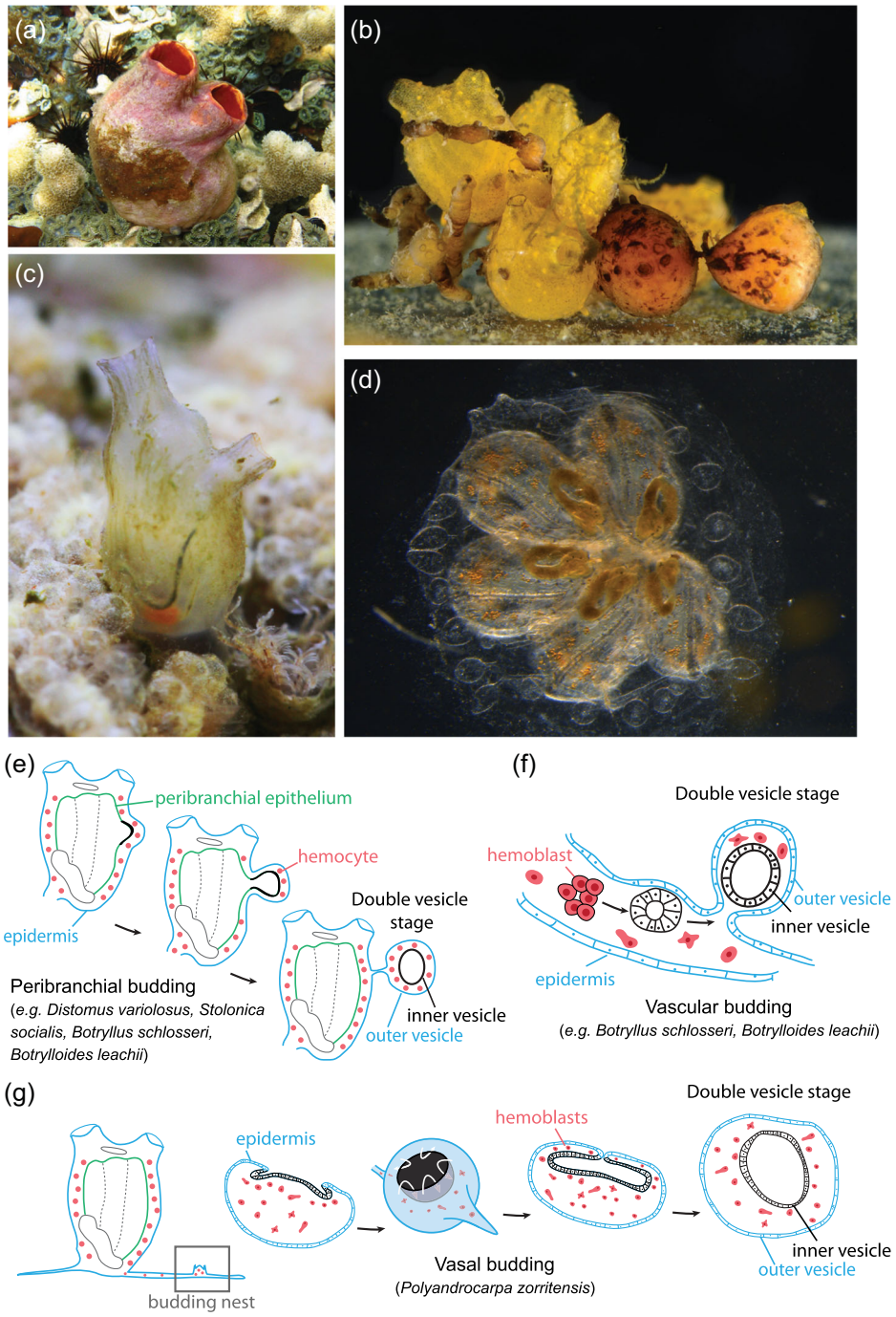


FIGURE 2 Stolidobranchia. a, *Polycarpa spongiabilis* (courtesy of Rosana Rocha). b, Colony of *Polyandrocarpa zorritensis* hatching from dormant spherules (courtesy of Alexandre Jan). c, *Styela plicata*. d, Small colony of *Botryllus schlosseri*. e, Schematic representation of peribranchial budding 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 vassal budding from nest formation to the double vesicle stage [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 1). Peribranchial budding, sometimes referred to as atrial, palleal, or pallial budding, is the most common form of nonembryonic development adopted by *Styelidae*. Since the first description of peribranchial budding by Metschnikoff (1869) in *Botryllus sp.*, it has been described in many styelids (Abbott, 1953; Akhmadieva, Shukalyuk, Aleksandrova, & Isaeva, 2007; Berrill, 1940, 1948a; Kawamura & Watanabe, 1981; Mukai & Watanabe, 1976; Pizon, 1893; Ritter, 1896; Selys-Longchamps, 1917; Watanabe & Tokioka, 1972). Peribranchial buds always arise from the 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 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 (Berrill, 1941, 1948a; Kawamura & Watanabe, 1981; Mukai & Watanabe, 1976; Selys-Longchamps, 1917; Watanabe & Tokioka, 1972). Germ cells are derived from circulating hemocytes (Brown & Swalla, 2007; Brown, Tiozzo, et al., 2009; Izzard, 1968; Kassmer, Rodriguez, Langenbacher, Bui, & De Tomaso, 2015; Kawamura & Sunanaga, 2010; Langenbacher & De Tomaso, 2016; Mukai & Watanabe, 1976; Sabbadin & Zaniolo, 1979; Sugimoto & Nakauchi, 1974; reviewed in Rodriguez, Kassmer, & De Tomaso, 2017).

The mechanisms regulating cell sources and lineages that lead to a fully functional blastozooid 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, Cabrera, Lotito, & Tiozzo, 2016). Some authors suggested that in *B. schlosseri* the nervous system and musculature may differentiate from a transient anatomical structure during inner vesicle morphogenesis (Burighel, Lane, Zaniolo, & Manni, 1998; Prünster, Ricci, Brown, & Tiozzo, 2018; Prünster, Ricci, Brown, & Tiozzo, 2019). In *Polyandrocarpa misakiensis* mesenchymal cells are integrated into the budding epithelium and may play a role in organogenesis (Kawamura, Fujiwara, & Sugino, 1991; Tatzuke, Sunanaga, Fujiwara, & Kawamura, 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 blastozooid organogenesis (Laird, De Tomaso, & Weissman, 2005; Rinkevich et al., 2013; Stoner & Weissman, 1996; Voskoboynik et al., 2008). 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 (Figures 1 and 2f). The existence of vascular budding was a controversial issue until Oka and Watanabe (1957) described this process in *Botryllus primigenus* and *Botrylloides violaceus* (Oka & 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 & Sunanaga, 2010; Figure 2f). Similar processes have been later reported in *Botryllus schlosseri* (Milkman & Therrien, 1965; Ricci, Cabrera, et al., 2016; Sabbadin, Zaniolo, & Majone, 1975; Voskoboynik et al., 2007; Watkins, 1958), *Botrylloides leachii* (Burighel, Brunetti, & Zaniolo, 1976; Milne-Edwards, 1841), *Botryllus delicatus* (Okuyama & Saito, 2001), and most recently in *Symplegma brakenhielmi* (Gutierrez & 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, Cabrera, et al., 2016).

Despite efforts in the last decade to characterize the cells building vascular buds (Brown, Keeling, Le, & Swalla, 2009; Rinkevich et al., 2010; Sunanaga, Inubushi, & Kawamura, 2010; Sunanaga, Saito, & Kawamura, 2006; Voskoboynik et al., 2007), 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 leachii* previously treated with a mitosis inhibitor can rescue budding capacities. The number of IA6+hemoblasts rapidly increases at the onset of vascular budding, participating in the formation of the bud tissues (Kassmer et al., 2019). Taken together, these results functionally show for the first time the presence of blood-borne multipotent stem cells in *Botrylloides leachii*. Indeed, the nature of these cells may differ from one species to the other. While in *Botrylloides leachii* they express the stem cell markers Piwi2 and Vasa (Kassmer et al., 2019), Vasa and Piwi1 are not expressed in vascular buds of *Botryllus primigenus* (Sunanaga et al., 2006, 2010), while Piwi proteins are only detected in some peripheral cells of vascular bud in *Botrylloides violaceus* (Brown, Keeling et al., 2009).

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

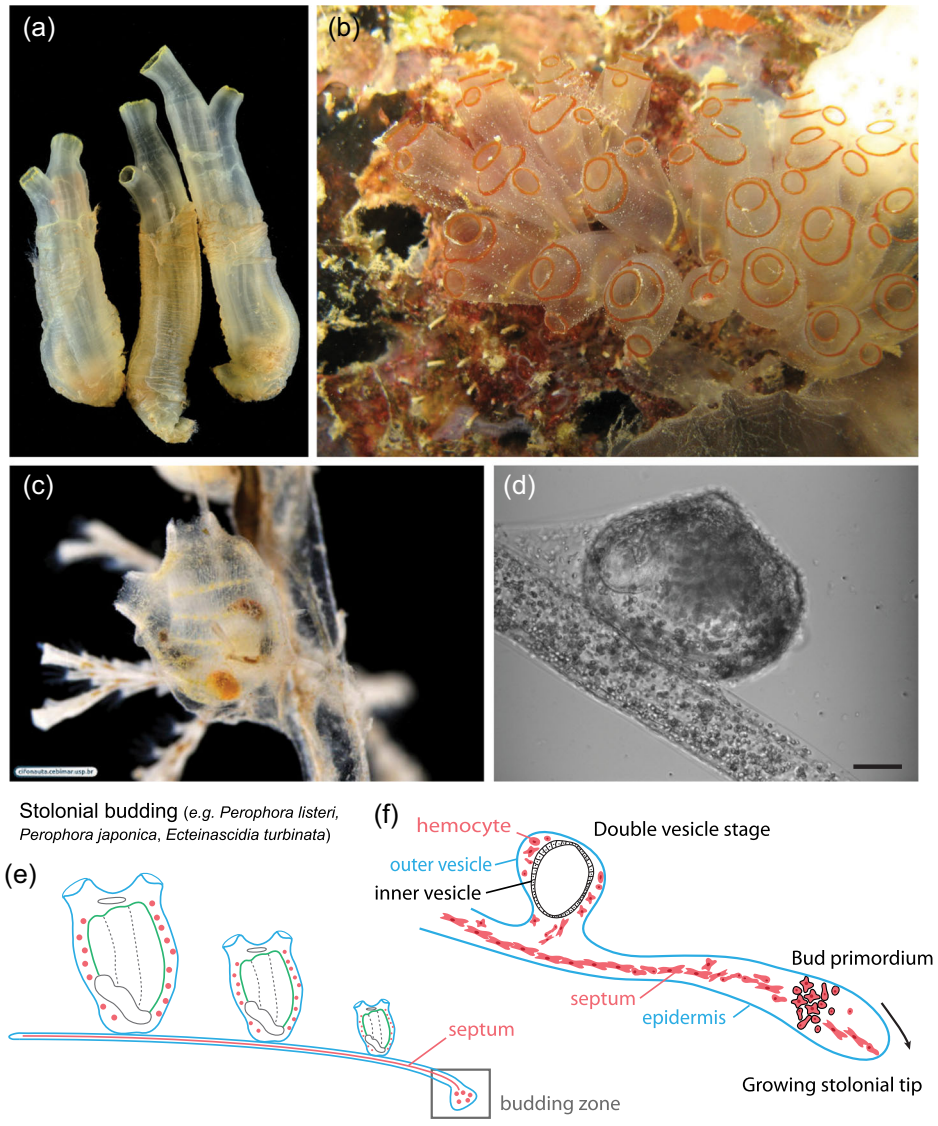


FIGURE 3 Phlebobranchia. a, *Ciona robusta* (courtesy of Alexandre Jan). b, *Ecteinascidia turbinata* (courtesy of Rosana Rocha). c, *Perophora sp.* (Cifonauta image database. Retrieved from http://cifonauta.cebimar.usp.br/media/10591/). d, Bud of *Perophora viridis*, scale bar: 75 μm (courtesy Rafaela Perez and Federico Brown). e,f, Schematic representation of stolonial budding from bud primordium to the double vesicle stage [Color figure can be viewed at wileyonlinelibrary.com]

4 | BUDDING IN "PHLEBOBRANCHIA": THE STOLONIAL AND THE UNKNOWN

Ascidians traditionally classified as Phlebobranchia (Figure 3) form a paraphyletic group with respect to Aplousobranchia, and due to conflictual results between recent phylogenomic studies, their deep relationships remain ambiguous (Figure 1). Budding "Phlebobranchia" are distributed in three distinct families: Perophoridae that are all budding *Perophora* and *Ecteinascidia*; Plurellidae, with solitary *Microgastra* and colonial *Plurella*; and Octacnemidae, 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 is 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 (Brien & Brien-Gavage, 1928; Dale Beers, 1923; Deviney, 1934; Freeman, 1964; Fukumoto, 1971; Kowalewsky, 1874; Lefèvre, 1897, 1898; Mukai, Koyama, & Watanabe, 1983; Ritter, 1896; Van Beneden & Julin, 1886). Buds arise along stolons that grow from the base of

each zooid and creep along the substrate (Figure 3c-f). 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). The Phlebobranchia stolon is an epidermally protruded vessel surrounded by a thin layer of the tunic. The stolonial lumen is separated into two longitudinal compartments by a central septum that is an epithelial-like arrangement of mesenchymal cells, on each side of which blood circulates in opposite directions (Brien & Brien-Gavage, 1928, Deviney, 1934; Lefèvre, 1897, 1898, 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 & 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 & Brien-Gavage, 1928; Koguchi, Sugino, & Kawamura, 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 (Dale Beers, 1923; Lefèvre, 1897). 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 the 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 1,500 described species, almost all colonial (Figure 4) with the exception of some *Rhopalaea* 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 postabdomen. It is the abdominal and the postabdominal region that are merely concerned with budding via different forms of strobilation (or strobilization), while one family, the Clavelinidae, also undergoes a divergent mode of stolonial budding (Figure 1).

5.1 | Epicardial budding across Aplousobranchia

In most Aplousobranchia, budding takes place in the abdominal and/or postabdominal regions, but the nature and behaviors of the budding tissues change 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 contacts the pericardium in its posterior-most extremity. Budding arises by epidermal constriction that encloses a piece of epicardium and other entrapped tissues, and therefore this budding mode has often been referred to as strobilization (or strobilation; Figure 4g). Here we will use the term epicardial budding (Figure 1), owing to the conserved role of the epicardium in this wide and complex array of nonembryonic developmental modes in Aplousobranchia.

Abdominal and postabdominal budding have been described in many *Aplidium* species (Figure 4a,b) (Nakauchi, 1982). *Aplidium turbinatum* is characterized by a very long postabdomen containing the epicardium, the gonads and the heart at its posterior-most extremity. During budding, the postabdomen detaches from the abdomen and then divides into a dozen pieces by strobilation, which begins in the most anterior part and proceeds posteriorly (Brien, 1948), with each of the strobilae giving rise to a bud. While the epidermis of the parent becomes the bud epidermis, each trapped fragment of the epicardium reshapes itself into a monolayered spherical vesicle that eventually gives rise to the internal organs (Figure 4g). Trapped between these two vesicles, the gonads become the future genital glands, while other mesenchymal cells function as storage of nutrients for the developing bud (Brien, 1948). A closely related species, *Aplidium pallidum*, has a very short postabdomen. Thus strobilation arises in the abdomen and buds contain not only an epicardial fragment but

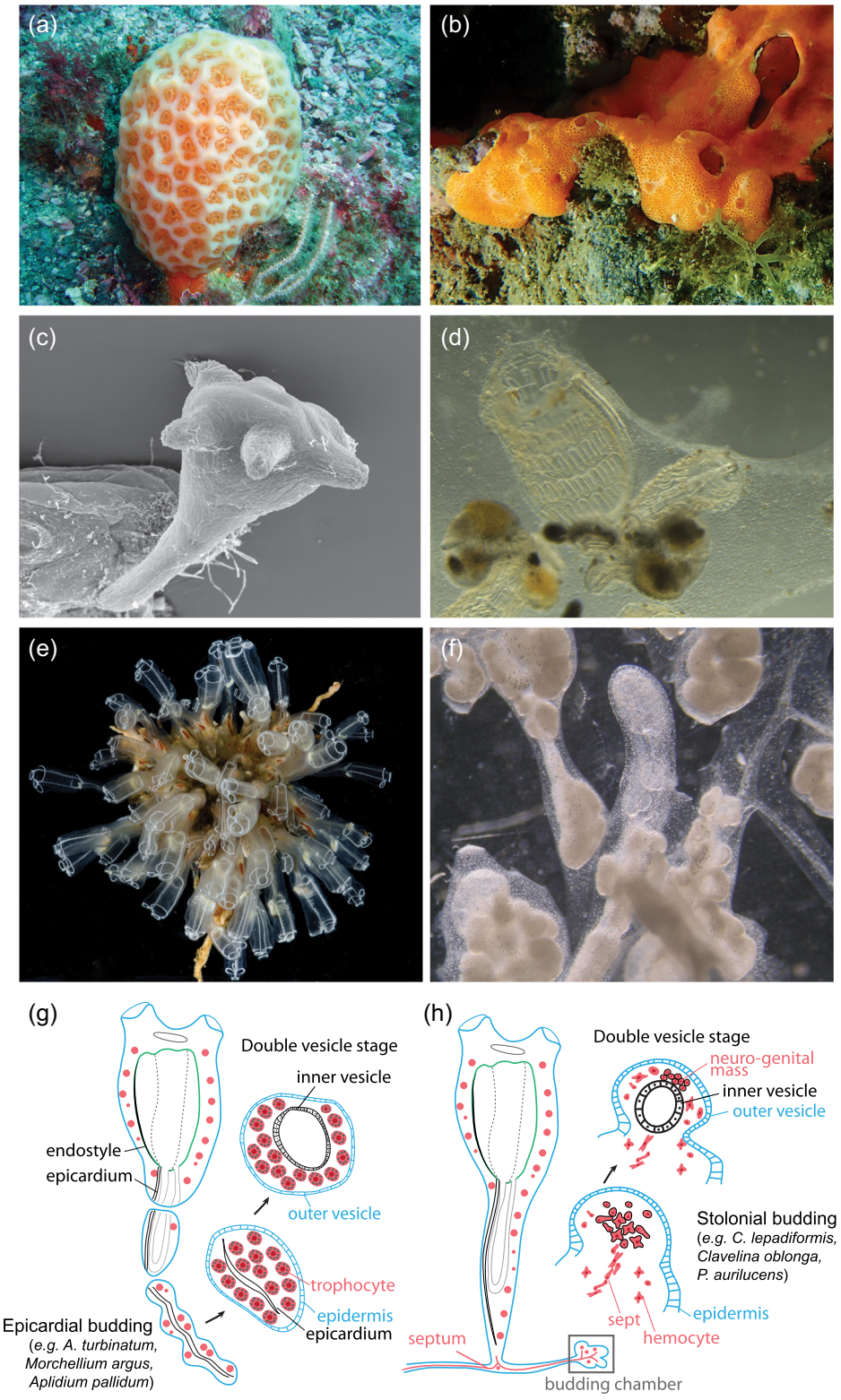


FIGURE 4 Aplousobranchia. a, Colony of *Aplidium conicum* and b, *Didemnum fulgens* (courtesy of Xavier Turon). c, Planktonic bud of *Clavelina gemmae* (courtesy of Xavier Turon). d, *Diplosoma listerianum* in the process of budding. e, Colony of *Clavelina lepadiformis* and (f) detailed view of budding chambers showing a forming zooid (courtesy of Kazuo Kawamura). g, Schematic representation of epicardial budding and detailed view of the postabdominal budding, from strobilization to the double vesicle stage. h, Schematic representation of stolonial budding in *Clavelinidae* [Color figure can be viewed at wileyonlinelibrary.com]

also different sections of the digestive tract, of gonads, and of a neuron-like strand of cells called the dorsal cord (Brien, 1925, 1948). The epicardium develops into the pharynx, the peribranchial chambers, and the heart. The fragments of the old digestive tube regenerate a new digestive system and the dorsal cord gives rise to the neural and germinal cells (Brien, 1925; Brien, 1948).

Brien (1948) has speculated that the presence of additional tissues may limit the organogenetic potentialities of the epicardium. Indeed several *Aplidium* are able to strobilate both in the abdominal and the postabdominal regions (Nakauchi, 1981, 1982, 1986; Nakauchi & Kawamura, 1978) but the relative role of the epicardium in those buds remains to be studied. Brien's hypothesis is nevertheless supported by experiments on *Aplidium turbinatum* and on *Clavelina lepadiformis*. When abdominal sectioning is performed in these species, the epicardium regenerates the thorax, while the digestive loop, is formed by the rectal and esophageal tissues. In contrast, if the animals are sectioned in the postabdominal regions, the epicardium ensures the regeneration of all the organs, including the digestive loop (Brien, 1930, 1948).

Budding in two other families, Didemnidae and Holozoidae represent two illustrations of the very diverse role that the epicardium plays in NED. In Didemnidae the epicardium is reduced in size and remains as a pair of small abdominal sacs. The most detailed description of budding in this family has been given by Pizon (1905) in *Diplosoma listerianum*, and despite some specific variations, it arises similarly in other *Didemnidae*, for example, *Polysyncraton lacazei* (Brien 1948), *Tridemnum cereum*, and *Leptoclinum gelatinosum* (Salfi, 1932). In these species, there is no sign of abdominal or postabdominal strobilation, and the organs of the bud first remain 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 stage in Didemnidae, by a process that, whereas likely similar to the above-described epicardial budding, remains largely unknown (Brien, 1948; Caullery, 1895; Pizon, 1905).

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* (Brien, 1948; Julin, 1896) and *Hypsistozoa fasmeriana* (Brewin, 1959) and in several other Holozoidea (Berrill, 1948b; Caullery, 1908; Ivanova-Kazas, 1967; Nishikawa, 1990). In the larva that is still brooded, the left epicardium elongates until it reaches the neighboring 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 (Brewin, 1959; Brien, 1948). 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 blastozooids 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 blastozooids 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 blastozooids after the 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).

5.2 | Stolonial budding in *Clavelinidae*

In addition to epicardial budding, *Clavelinidae* undergo another form of NED called stolonial budding (Figure 4e,f-h) Stolonial budding in *Clavelinidae* has been particularly well-studied in *Clavelina lepadiformis* (Brien, 1930; Brien & Brien-Gavage, 1927) and is essentially the same in *C. picta* (Berrill, 1935), *C. oblonga* (Brien, 1930), and *Pycnoclavella aurilucens* (Berrill, 1947). *Clavelinidae* possesses hypertrophied ventral stolons consisting of an epidermally derived circulatory vessel surrounded by a thin layer of tunic (Figure 4e,f). As in Perophoridea described above, each stolon is divided into two longitudinal sinuses by a mesenchymal septum, on each side of which blood flows in opposite directions. Brien and Brien-Gavage (1927) showed the mesenchymal nature of the septum, which consists of a thin wall of mesenchymal cells that is an extension of the adult hemocoel and is in physical continuity with the mesenchymal network of cells underlying the stolonial epidermis (Brien & Brien-Gavage, 1927). Along this stolon, opaque, and lobulated regions become budding chambers (Figure 4h), which are congested with mesenchymal cells called trophocytes that likely play a role in bud nutrition (Berrill, 1947; Brien, 1930; Brien & Brien-Gavage, 1927; Kerb, 1908; Seeliger, 1882). When the budding chamber is isolated from its parent, for example, by necrosis or after zooid regression, the mesenchymal septum forms ramifications that extend into the lobes of the budding chamber. In one of these lobes, the tip of a septal ramification enlarges by the accumulation of free hemocytes. Then, this cluster of mesenchymal cells gets organized into a monolayered inner vesicle surrounded by an epidermal outer vesicle (Figure 4h; Brien, 1930; Brien & Brien-Gavage, 1927; Giard & Caullery, 1896). The outer vesicle gives rise to the zooid epidermis and the inner vesicle to all the other organs, with the exception of the nervous system and the germline that, according to Brien and Brien-Gavage (1927) derive from a separate aggregate of mesenchymal cells that they call the "neurogenital mass" (Brien, 1948). One dated report suggests that the inner vesicle derives from an invagination of the stolonial epidermis (Kerb, 1908) but there are no other publications that support this observation.

Budding chambers produce zooids year-round, but they can also act as dormancy structures, allowing the colony to bypass seasonal adverse conditions (Berrill, 1935; Berrill, 1947; Brien, 1930; Brien & Brien-Gavage, 1927; De Caralt, López-Legentil, Tarjuelo, Uriz, & Turon, 2002; Mukai, 1977). During dormancy, the zooids regress while the budding chambers, typically called "winter buds", survive and go on to repopulate the colonies when conditions improve. It is not clear if there are morphological differences between normal budding chambers and winter buds, but it is possible that the trophocytes may be allowed to further accumulate in the chambers before hibernation, providing more reserve storage for the dormancy period (L. Hiebert, personal observations). Finally, zooids of *C. lepadiformis* can also arise from isolated fragments of stolon, or from budding chambers upon an artificial section of the adult postabdomen, probably resulting from the arrest of blood circulation (Berrill & Cohen, 1936; Brien, 1930; Della Valle, 1915). The regeneration process is histologically identical to the stolonial budding described above, but the ability of a stolon fragment to form a fully functional zooid is linked to its absolute size. For instance, in fragments smaller than a half-millimeter the epidermal cells change shape and begin to form the outer vesicle, but the inner cell mass never appears (Berrill & Cohen, 1936), likely because the number of septal cells is too low to initiate organogenesis.

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, 2015; WorMs, 2019). They are divided into three monophyletic groups: salps, pyrosomes, and doliolids (Figure 1). With the exception of doliolids, that retained 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; Conley, Lombard, & Sutherland, 2018; Lebrato et al., 2013; Phillips, Kremer, & Madin, 2009). So far, NED mechanisms have only been studied in few thaliaceans, sometimes leading to conflicting results regarding the nature of tissues involved in the bud constitution.

6.1 | Salps

Colonies of salps are long chains of blastozooids, sometimes several meters long, attached to each other by the tunic (Figure 5a), until the chain breaks off and blastozooids live their own lives. Each blastozooid houses a single egg, which once fertilized develops within a brooding chamber (Berrill, 1950), leading to an oozooid that in turn produces a budding stolon that will eventually give rise to several hundred blastozooids (Figure 5b,c). The stolon primarily arises as an invagination of the pharyngeal floor at the posterior extremity of the endostyle (Berrill, 1950; Brien, 1928; Brooks, 1893; Figure 5d). At the onset of stolon initiation, this innermost 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 blastozooids (Figure 5b). According to Brien (1928), who studied stolonial budding in *Thalia democratica*, *Salpa maxima*, and *Salpa fusiformis*, while the inner tube arises from the pharynx and the outer from the 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 stolonial 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.

6.2 | 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 cyathozooid, 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 cyathozooid organs are not even fully developed. The cyathozooid elongates posteriorly and rapidly strobilates by epidermal constrictions into four buds, in which each organ originates from its corresponding rudiment in the cyathozooid larvae (except the nervous system i.e., formed de novo; Brien, 1948; Godeaux, 1957; Julin, 1912; Salenski, 1890, 1891). The four blastozooids (called tetrazooids), formed by this primary budding, in turn, produce new buds by secondary budding. The secondary stolon contains 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 ectoderm while other organs derive from the inner pharyngeal tube.

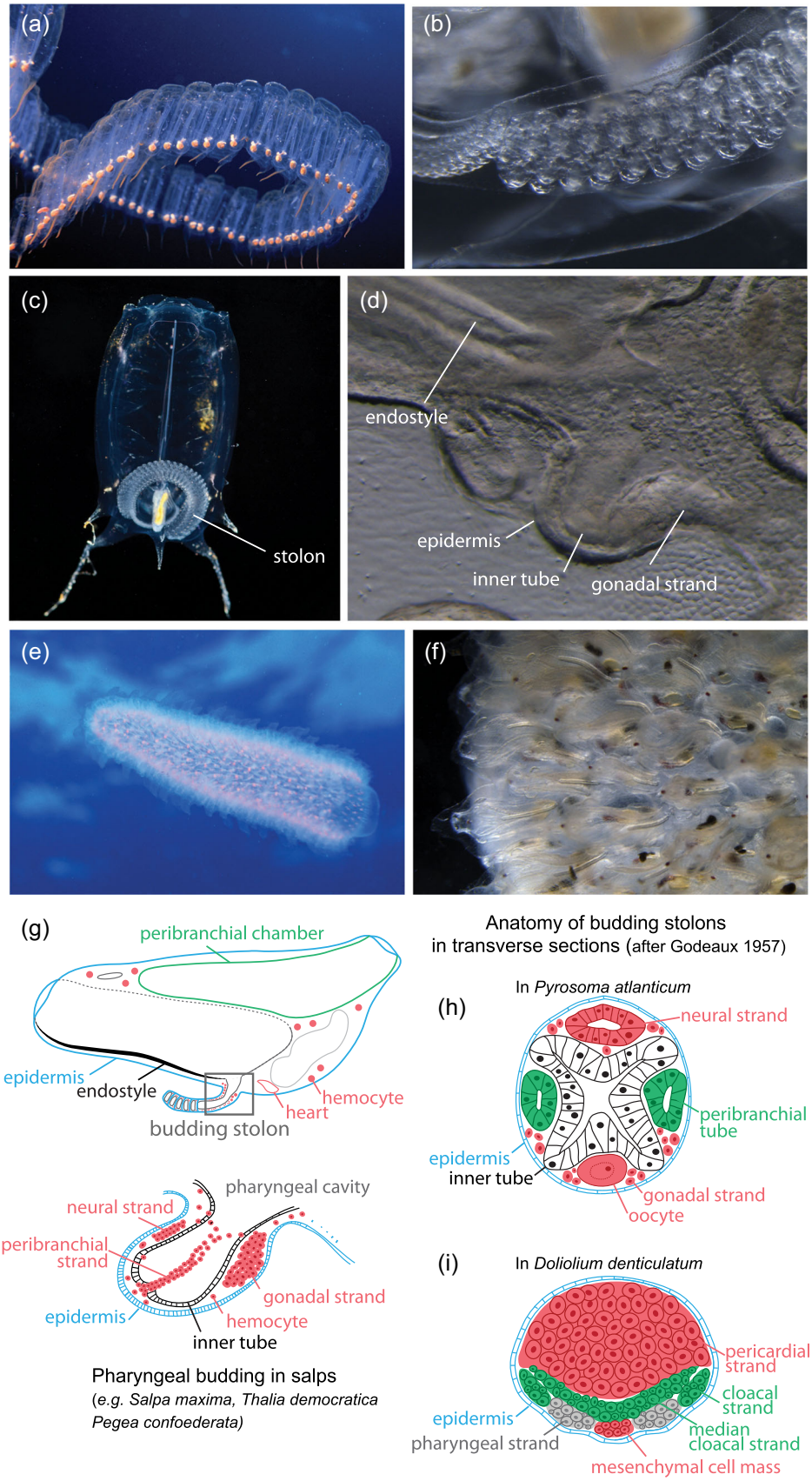


FIGURE 5 Thaliacea. a, Chain of *Pegea confoederata* (courtesy of David Luquet). b, Close-up view of a stolon in the process of strobilization in *Salpa fusiformis*. c, Oozoid of *Thalia democratica*, bearing a budding stolon (courtesy of Alexandre Jan). d, Microscope view of the early bud initiation 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* [Color figure can be viewed at wileyonlinelibrary.com]

6.3 | Doliolids

Colonies of doliolids are composed of a barrel-shaped oozooid, called a nurse, which 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 Bone, 1998; Piette & Lemaire, 2015). 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 (Godeaux, 1957; Grobben, 1882; Neumann, 1906, 1935; Uljanin, 1884) 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, will form the digestive system and the peribranchial chambers, and one mass of mesenchymal cells with unknown origin and fate (Figure 5i). According to this description, the detailed structure of the doliolid stolon may differ from those of pyrosomes and salps, but they all share an inner tube derived from the pharynx—in the same position—and therefore we named bud formation from this structure as pharyngeal budding and we propose it to be homologous across Thaliaceans (Figure 1).

7 | RECONSTRUCTING GAINS AND LOSSES OF NED: A PHYLOGENY AND HOMOLOGY ISSUE

The combination of a reliable phylogeny and detailed anatomical descriptions allows for the inference of gains and losses of NED across tunicates. A parsimonious reconstruction of solitary versus colonial clearly favors a solitary tunicate ancestor, and therefore NED most likely results from several convergent acquisitions during tunicate evolution. This view is strongly supported by the diversity of budding processes that involve disparate and in many occasions nonhomologous tissues. In fact, tunicate buds always contain an outer layer derived from the parental epidermis but their inner layer originates from a variety of tissues. The different budding modes have been traditionally classified upon the nature of the budding tissues (Berrill, 1951) as well as ecological aspects (Nakauchi, 1982; Turon, 1992). Following the descriptions of NED provided in the previous paragraphs, we distinguish six different budding modes based on the origin of the inner layer (see Table 1).

In Stolidobranchia, the position of the solitary *Pyuridae*, *Molgulidae*, and *Styelidae* strongly supports the hypothesis of a nonbudding ancestor. Thus, we can speculate that three acquisitions of budding arose in the evolutionary history of Styelidae (Alié et al., 2018): (a) vasal budding has been acquired in the species *Polyandrocarpa zorritensis*, (b) peribranchial budding in the last common ancestor of all peribranchial budders and (c) vascular budding was acquired secondarily in an ancestor that already possessed peribranchial budding (Figure 1). The evolutionary scenario of NED evolution in Stolidobranchia could prove even more complex, as NED in numerous colonial species is undescribed (e.g., *Styela complexa*, Kott, 1995; *Syncarpa composita*, Hasegawa & Kajihara, 2019).

In Aplousobranchia, the epicardial budding is shared by all colonial species and therefore it is very likely ancestral to this order (Moreno & Rocha, 2008; Shenkar et al., 2016). While different families of Aplousobranchia have particular variations of the epicardial budding (Moreno & Rocha, 2008), the presence of a multipotent epicardium involved in NED is not reported in other tunicates and likely represents a synapomorphy of this order (Figure 1). *Clavelinidae* combine epicardial and stolonial budding, and even if some authors have initially considered the stolonial septum as

TABLE 1 Six budding modes in Tunicates

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

derived from the epicardium or the pericardium (Giard & Caullery, 1896; Van Beneden & Julin, 1886) its mesenchymal nature has been firmly demonstrated (Brien & 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 & 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 & 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 (Berrill, 1935; Bonnevie, 1896; Brien, 1928; 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 nonembryonic 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 (Prünster et al., 2018, 2019; Ricci, Cabrera, et al., 2016; Ricci, Chourasia, et al. 2016; Tiozzo et al., 2005). 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 tissue "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 nonhomologous 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 the 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 postmetamorphic NED.

The multiple gains and losses of budding in tunicates provide 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 (Jimenez-Merino et al., 2019). In *Botrylloides leachii*, also a stylelid, cells with similar characteristics have been shown to initiate vascular budding (Kassmer et al., 2019). 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 thaliaceans, the bauplan of a zooid is relatively conserved across the different tunicate groups. On the other hand, the variations in shapes, colors, and sizes of colonies are remarkable, a diversity deeply rooted in the variability of NED ontogenic steps. For example, in Styelidae the variation in the length of the bud peduncle defines the degree of connection between the zooids, resulting in more or less integrated colonies. In Aplousobranchia, it is the buds

movements within the parental tunic that determine the shape of the colony. Therefore, the regulation of one structure or behavior may not change the bauplan of the zooid but influences the colony phenotype. Such changes can also affect the ecology of the colony, such as in species that convergently evolved planktonic buds (Figure 1), allowing for somatic dispersion (Fujimoto & Watanabe, 1976; Mukai et al., 1983; Turon, 2005).

In light of the techniques and genomic resources now available, the diversity of developmental modes characterizing Tunicata and the relative richness of anatomical knowledge clearly supply a well-stocked toolbox to explore developmental mechanisms and better understand their evolution.

ACKNOWLEDGMENTS

The authors want to thank Martine Fioroni, head of the library of the Institut de la Mer de Villefranche, for her invaluable help and her commitment to making the old and priceless literature accessible to everyone. This work was supported by ANR (ANR-14-CE02-0019-01) and CNRS (PICS 07679), FAPESP-BEPE fellowship (2018/05923-3) to Laurel S. Hiebert and ARC (DOC20190509164) to Marta Scelzo.

ORCID

Laurel S. Hiebert http://orcid.org/0000-0001-7756-1066
Stefano Tiozzo http://orcid.org/0000-0003-2822-2783

REFERENCES

Abbott, D. P. (1953). Asexual reproduction in the colonial ascidian *Metandrocarpa taylori* Huntsman, (61, pp. 1–78). Berkeley, CA: University of California Press.

Andersen, V. (1998). Salp and pyrosomid blooms and their importance in biogeochemical cycles. In Q. Bone (Ed.), *The biology of pelagic Tunicates*. Oxford, UK: Oxford University Press.

Akhmadieva, A. V., Shukalyuk, A. I., Aleksandrova, Y. N., & Isaeva, V. V. (2007). Stem cells in asexual reproduction of the colonial ascidian *Botryllus tubaratus* (Tunicata: Ascidiacea). *Russian Journal of Marine Biology*, *33*, 181–186.

Alié, A., Hiebert, L. S., Simion, P., Scelzo, M., Prünster, M. M., Lotito, S., & Tiozzo, S. (2018). Convergent acquisition of nonembryonic development in styelid ascidians. *Molecular Biology and Evolution*, *35*, 1728–4173.

Berrill, N. J. (1935). Studies in Tunicate development Part IV – Asexual reproduction. *Philosophical Transactions of the Royal Society B*, *225*, 327–379.

Berrill, N. J. (1940). The development of a colonial organism: *Symplegma viride*. *Biological Bulletin*, *79*, 272–281.

Berrill, N. J. (1941). The development of the bud in *Botryllus*. *Biological Bulletin*, *80*, 169–184.

Berrill, N. J. (1947). The structure, tadpole and budding of the ascidian *Pycnoclavella Aurilucens* Garstang. *Journal of the Marine Biological Association of the UK*, *27*, 245–251.

Berrill, N. J. (1948a). The gonads, larvae, and budding of the polystyelid ascidians *Stolonica* and *Distomus*. *Journal of the Marine Biological Association of the UK*, *27*, 633–650.

Berrill, N. J. (1948b). Budding and the reproductive cycle of *Distaplia*. *Journal of Cell Science*, *s3-89*, 253–289.

Berrill, N. J. (1950). Budding and development in Salpa. *Journal of Morphology*, *87*, 553–606.

Berrill, N. J. (1951). Regeneration and budding in tunicates. *Biological Reviews*, *26*, 456–475.

Berrill, N. J., & Cohen, A. (1936). Regeneration in *Clavelina lepadiformis*. *Journal of Experimental Biology*, *13*, 352–362.

Bone, Q. (1998). *The biology of pelagic tunicates*. Oxford, UK: Oxford University Press.

Bonnevie, K. (1896). On gemmation in *Distaplia magnilarva* and *Pyrosoma elegans*. The Norwegian North-Atlantic Expedition, 1876-78." 'Zoology, Tunicata,' 4

Brewin, B. I. (1959). An account of larval budding in the compound Ascidian, *Hypsistozoa fasmeriana*. *Journal of Cell Science*, *100*(3), 575–589.

Brien, P. (1925). Contribution à l'étude de la blastogénese des Tuniciers. Bourgeonnement chez *Aplidium zostericola* (Giard.). *Archives de Biologie*, *35*, 155–205.

Brien, P. (1928). Contribution à l'étude de l'embryogénese et de la blastogénese des Salpes. *Recueil de l'Institut zoologique Torley-Rousseau*, *2*, 5–116.

Brien, P. (1930). Contribution à l'étude de la régénération naturelle et expérimentale chez les Clavelinidae. *Annales de la Société royale zoologique de Belgique*, *61*, 19–112.

Brien, P. (1948). Embranchement des Tuniciers. In P. P. Grassé (Ed.), *Traité de Zoologie*, *Anatomie*, *Systématique*, *Biologie* (11, pp. 553–930). Paris, France: Masson et Cie.

Brien, P., & Brien-Gavage, E. (1927). Contribution à l'étude de la blastogenèse des Tuniciers. III. Bourgeonnement de *Clavelina lepadiformis* Müller. *Recueil de l'Institut zoologique Torley-Rousseau*, *1*, 31–81.

Brien, P., & Brien-Gavage, E. (1928). Contribution à l'étude de la blastogenèse des Tuniciers. IV. Recherches sur le Bourgeonnement de *Perophora Listeri* Weigm. Origine mésoblastique du septum stolonial. *Recueil de l'Institut zoologique Torley-Rousseau*, *1*, 123–151.

Brooks, W. K. (1893). Memoirs of the biological laboratory, *The genus Salpa*. Baltimore, MD: Johns Hopkins University.

Brown, F. D., & Swalla, B. J. (2007). Vasa expression in a colonial ascidian, Botrylloides violaceus. *Evolution and Development*, *9*(2), 165–177.

Brown, F. D., Tiozzo, S., Roux, M. M., Ishizuka, K., Swalla, B. J., & De Tomaso, A. W. (2009). Early lineage specification of long-lived germline precursors in the colonial ascidian *Botryllus schlosseri*. *Development*, *136*, 3485–3494.

Brown, F. D., Keeling, E. L., Le, A. D., & Swalla, B. J. (2009b). Whole body regeneration in a colonial ascidian, *Botrylloides violaceus*. *Journal of Experimental Zoology: Part B*, *312*, 885–900.

Brown, F. D., & Swalla, B. J. (2012). Evolution and development of budding by stem cells: Ascidian coloniality as a case study. *Developmental Biology*, *369*(2), 151–162.

Brunetti, R., & Mastrototaro, F. (2004). The non-indigenous stolidobranch ascidian *Polyandrocarpa zorritensis* in the Mediterranean: Description, larval morphology and pattern of vascular budding. *Zootaxa*, *528*(1), 1–8.

Brusca, R. C., Moore, W., & Shuster, S. M. (2016). *Invertebrates*. Sunderland, MA: Sinauer Associates, Inc., Publishers.

Burighel, P., Brunetti, R., & Zaniolo, G. (1976). Hibernation of the colonial ascidian *Botrylloides leachi* (Savigny): Histological observations. *Italian Journal of Zoology*, *43*, 293–301.

Burighel, P., Lane, N. J., Zaniolo, G., & Manni, L. (1998). Neurogenic role of the neural gland in the development of the ascidian, *Botryllus schlosseri* (Tunicata, Urochordata). *Journal of Comparative Neurology*, *394*, 230–241.

Caullery, M. (1895). Contributions à l'étude des Ascidies composées. *Bulletin Biologique de la France et de la Belgique*, *27*, 1–158.

Caullery, M. (1908). Recherches sur les Synascidies du genre Colella et considérations sur la famille des Distomidae. *Bulletin Scientifique de la France et de la Belgique*. *t*, *42*, 1–59.

Conley, K. R., Lombard, F., & Sutherland, K. R. (2018). Mammoth grazers on the ocean's minuteness: A review of selective feeding using mucous meshes. *Proceedings of the Royal Society B: Biological Sciences*, *285*: 20180056.

Dale Beers, C. (1923). Some points in the bud development of a simple ascidian, *Ecteinascidia turbinata* Herdman. *Journal of the Elisha Mitchell Scientific Society*, *39*, 92–94.

Da Silva Oliveira, F. A., Michonneau, F., & da Cruz Lotufo, T. M. (2017). Molecular phylogeny of Didemnidae (Ascidiacea: Tunicata). *Zoological Journal of the Linnean Society*, *180*, 603–612.

De Caralt, S., López-Legentil, S., Tarjuelo, I., Uriz, M. J., & Turon, X. (2002). Contrasting biological traits of *Clavelina lepadiformis* (Ascidiacea) populations from inside and outside harbours in the western Mediterranean. *Marine Ecology Progress Series*, *244*, 125–137.

Della Valle, A. (1881). Nuove contribuzioni alla storia naturale delle ascidie composte del Golfo di Napoli. *Atti della Accademia Nazionale dei Lincei*, *10*, 431–498.

Della Valle, P. (1915). Studii sui rapporti fra differenziazione e rigenerazione - 3. Lo sviluppo di segmenti isolati di stolone di *Clavelina* di calibro eguale e di lunghezza diversa. *Bollettino della Società' di naturalisti in Napoli*, *27*, 195–237.

Delsuc, F., Philippe, H., Tsagkogeorga, G., Simion, P., Tilak, M. K., Turon, X., & Douzery, E. J. P. (2018). A phylogenomic framework and timescale for comparative studies of tunicates. *BMC Biology*, *16*(39), 39.

Deviney, E. M. (1934). The behavior of isolated pieces of ascidian (*Perophora viridis*) stolon as compared with ordinary budding. *Journal of the Elisha Mitchell Scientific Society*, *49*(2), 185–224.

Freeman, G. (1964). The role of blood cells in the process of asexual reproduction in the tunicate *Perophora viridis*. *Journal of Experimental Zoology*, *156*, 157–184.

Fujimoto, H., & Watanabe, H. (1976). Studies on the asexual reproduction in the polystyelid ascidian, *Polyzoa vesiculiphora* Tokioka. *Journal of Morphology*, *150*, 607–621.

Fukumoto, M. (1971). Experimental control of budding and stolon elongation in *Perophora orientalis*, a compound ascidian. *Development, Growth & Differentiation*, *13*(2), 73–82.

Garstang, W. (1928). The morphology of the Tunicata and its bearing on the phylogeny of the Chordata. *Journal of Cell Science*, *72*, 51–77.

Giard, M. M. A., & Caullery, M. (1896). On the hibernation of *Clavelina lepadiformis*, Müller. *Journal of Natural History*, *18*, 485–486.

Godeaux, J. (1957). Contribution à la connaissance des Thaliacés (Pyrosome et Doliolum). *Annales de la Société Royale Zoologique de Belgique*, *85*, 103–118.

Govindarajan, A., Bucklin, A., & Madin, L. P. (2011). A molecular phylogeny of the Thaliacea. *Journal of Plankton Research*, *33*, 843–853.

Grobben, C. (1882). Doliolum und sein Generationswechsel. *Arbeiten aus dem Zoologischen Instituten der Universität Wien*, *4*. 201-198.

Gutierrez, S., & Brown, F. D. (2017). Vascular budding in Symplegma brakenhielmi and the evolution of coloniality in styelid ascidians. *Developmental Biology*, *423*, 152–169.

Hasegawa, N., & Kajihara, H. (2019). A redescription of Syncarpa composita (Ascidiacea, Stolidobranchia) with an inference of its phylogenetic position within Styelidae. *ZooKeys*, *857*, 1–15.

Hughes, R. N. (1989). *A functional biology of clonal animals* (p. 331). New York, NY: Chapman & Hall.

Izzard, C. S. (1968). Migration of germ cells through successive generations of pallial buds in *Botryllus schlosseri*. *The Biological Bulletin*, *135*, 424.

Ivanova-Kazas, O. M. (1967). Reproduction asexuée et cycle évolutif de l'ascidie *Distaplia unigermis*. *Cahiers de Biologie Marine*, *7*, 21–62.

Jimenez-Merino, J., Santos de Abreu, I., Hiebert, L. S., Allodi, S., Tiozzo, S., de Barros, C. M., & Brown, F. D. (2019). Putative stem cells in the hemolymph and in the intestinal submucosa of the solitary ascidian *Styela plicata*. *Evo-Devo*, *10*, 31.

Julin, C. (1896). Recherches sur la blastogenese chez *Distaplia magnilarva* et *D. rosea*. *Compte-Rendu des Séances du troisième Congrès International de Zoologie tenu à Leyde du 16 au 21 septembre 1985*, 507–524.

Julin, C. (1912). Recherches sur le développement embryonnaire de Pyrosoma giganteum Les: Aperçu général de l'embryogenèse, les cellules du testa et le développement des organs lumineux. *Zoologische Jahrbiic*, *15*(2), 775–863.

Kassmer, S. H., Rodriguez, D., Langenbacher, A. D., Bui, C., & De Tomaso, A. W. (2015). Migration of germline progenitor cells is directed by sphingosine-1-phosphate signalling in a basal chordate. *Nature Communications*, *61*, 8565.

Kassmer, S. H., Langenbacher, A. D., & De Tomaso, A. W. (2019). Primordial blasts, a population of blood borne stem cells responsible for whole body regeneration in a basal chordate. *bioRxiv*, 647578. https://doi.org/10.1101/647578

Kawamura, K., Fujiwara, S., & Sugino, Y. M. (1991). Budding-specific lectin induced in epithelial cells is an extracellular matrix component for stem cell aggregation in tunicates. *Development*, *113*, 995–1005.

Kawamura, K., Sugino, Y., Sunanaga, T., & Fujiwara, S. (2008). Multipotent epithelial cells in the process of regeneration and asexual reproduction in colonial tunicates. *Development Growth and Differentiation*, *50*(1), 1–11.

Kawamura, K., & Sunanaga, T. (2010). Hemoblasts in colonial tunicates: Are they stem cells or tissue-restricted progenitor cells? *Development Growth and Differentiation*, *52*(1), 69–76.

Kawamura, K., & Watanabe, H. (1981). Studies of Japanese compound styelid ascidians. III. A new, possibly asexual *Polyandrocarpa* from Shimoda Bay. *Publications of the Seto Marine Biological Laboratory*, *4/6*, 425–436.

Kerb, H. (1908). Biologische Beiträge zur Frage der Überwinterung der Ascidien. *Archiv für Mikroskopische Anatomie*, *72*(1), 386–414.

Kocot, K. M., Tassia, M. G., Halanych, K. M., & Swalla, B. J. (2018). Phylogenomics offers resolution of major tunicate relationships. *Molecular Phylogenetics and Evolution*, *121*, 166–173.

Koguchi, S., Sugino, Y. M., & Kawamura, K. (1993). Dynamics of epithelial stem cell in the process of stolonial budding of the colonial ascidian *Perophora japonica*. *Memoirs of the Faculty of Science Kochi University Series D Biology*, *14*, 7–14.

Kott, P. (1972). The ascidians of South Australia II. Eastern Sector of the Great Australian Bight and Investigator Strait. *Transactions of the Royal Society of South Australia*, *3*(3), 206–333.

Kott, P. (1985). The Australian Ascidiacea. Part 1, Phlebobranchia and Stolidobranchia. *Memoirs of the Queensland Museum*, *21*, 1–440.

Kott, P. (1995). Ascidians from the Solomon Islands. *The Beagle: Records of the Museums and Art Galleries of the Northern Territory*, *21*, 53–66.

Kowalewsky, A. (1874). Sur le bourgeonnement du *Perophora Listeri*. *Journal of Morphology*, *9*(1), 473–483. (Translated from russian by Giard, A.).

Kürn, U., Rendulic, S., Tiozzo, S., & Lauzon, R. J. (2011). Asexual propagation and regeneration in colonial ascidians. *Biological Bulletin*, *221*(1), 43–61.

Laird, D. J., De Tomaso, A. W., & Weissman, I. L. (2005). Stem cells are units of natural selection in a colonial ascidian. *Cell*, *123*(7), 1351–1360.

Langenbacher, A. D., & De Tomaso, A. W. (2016). Temporally and spatially dynamic germ cell niches in *Botryllus schlosseri* revealed by expression of a TGF-beta family ligand and vasa. *EvoDevo*, *7*, 9.

Lefèvre, G. (1897). Budding in Ecteinascidia. *Anatomischer Anzeiger*, *13*, 474–483.

Lefèvre, G. (1898). Budding in *Perophora*. *Journal of Morphology*, *14*, 367–424.

Lebrato, M., de Jesus Mendes, P., Steinberg, D. K., Cartes, J. E., Jones, B. M., Birsa, L. M., … Oschlies, A. (2013). Jelly biomass sinking speed reveals a fast carbon export mechanism. *Limnology and Oceanography*, *58*(3), 1113–1122.

Manni, L., Gasparini, F., Hotta, K., Ishizuka, K. J., Ricci, L., Tiozzo, S., … Dauga, D. (2014). Ontology for the asexual development and anatomy of the colonial chordate *Botryllus schlosseri*. *PLOS One*, *95*, e96434.

Manni, L., Anselmi, C., Cima, F., Gasparini, F., Voskoboynik, A., Martini, M., … Ballarin, L. (2018). Sixty years of experimental studies on the blastogenesis of the colonial tunicate *Botryllus schlosseri*. *Developmental Biology*, *448*, 293–308.

McNamara, K. J. (2012). Heterochrony: The evolution of development. *Evo Edu Outreach*, *5*, 203–218. https://doi.org/10.1007/s12052-012-0420-3

Metcalf, M. M. (1893). Notes upon apparently new species of Octacnemus, a deepsea Salpa-like Tunicata. *Johns Hopkins University Circular*, *12*, 98–100.

Metschnikoff, E. (1869). Uber die Larven und Knospen von Botryllus. *Bulletin de l'Académie Impériake des Sciences de Saint-Pétersbourg*, *13*, 293–298.

Milkman, R., & Therrien, E. (1965). Development and genetic studies on compound ascidian *Botryllus schlosseri*. *The Biological Bulletin*, *129*, 417.

Milne-Edwards, H. (1841). *Observations sur les ascidies composées des côtes de la Manche*. Paris, France: Fortine-Masson.

Moreno, T. T., & Rocha, R. M. (2008). Phylogeny of the Aplousobranchia (Tunicata: Ascidiacea). *Revista Brasileira de Zoologia*, *25*(2), 269–298.

Mukai, H. (1977). Comparative studies on the structure of reproductive organs of four Botryllid Ascidians. *Journal of Morphology*, *152*, 363–380.

Mukai, H., & Watanabe, H. (1976). Studies on the formation of germ cells in a compound ascidian *Botryllus primigenus* Oka. *Journal of Morphology*, *148*. 377-62.

Mukai, H., Koyama, H., & Watanabe, H. (1983). Studies on the reproduction of three species of *Perophora* (Ascidiacea). *Biological Bulletin*, *164*, 251–266.

Nakauchi, M. (1981). Development and budding in the oozoids of *Polyclinum aurantium* (a colonial ascidian) (Protochordata). *Journal of Zoology, London*, *194*, 1–9.

Nakauchi, M. (1982). Asexual development of ascidians: Its biological significance, diversity, and morphogenesis. *Integrative and comparative biology*, *22*(4), 753–763.

Nakauchi, M. (1986). Oozooid development and budding in the polyclinid ascidian, *Parascidia flemingii* (Urochordata). *Journal of Zoology*, *208*(2), 255–267.

Nakauchi, M., & Kawamura, K. (1978). Additional experiments on the behavior of buds in the ascidian, *Aplidium multiplicatum*. *The Biological Bulletin*, *154*(3), 453–462.

Neumann, G. (1906). Doliolum. *Wissenschaftliche Ergebnisse der Deutschen Tiefsee- Expedition*, *12*(2), 93–243.

Neumann, G. (1935). Cyclomyaria. Tunicata. *Handbuch der zoologie*, *5*, 24–400.

Nishikawa, T. (1990). The ascidians of the Japan Sea. I. *Publications of the Seto marine biological laboratory*, *34*(4-6), 73–148.

Oka, H., & Watanabe, H. (1957). Vascular budding, a new type of budding in botryllus. *Biological Bulletin*, *112*, 225–240.

Oka, H., & Watanabe, H. (1959). Vascular budding in Botrylloides. *The Biological Bulletin*, *177*(2), 340–346.

Okuyama, M., & Saito, Y. (2001). Studies on Japanese botryllid ascidians. 1. A new species of the genus Botryllus from the Izu Islands. *Zoological Science*, *18*, 261–267.

Pérez-Portela, R., Bishop, J., Davis, A., & Turon, X. (2009). Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, *50*(3), 560–570.

Pérez-Portela, R., Goodwin, C., Picton, B., & Turon, X. (2010). *Pycnoclavella stolonialis n. sp.* (Tunicata: Ascidiacea), with phylogenetic and distributional remarks on the genus in Europe. *Zootaxa*, *2407*, 51–66.

Phillips, B., Kremer, P., & Madin, L. P. (2009). Defecation by *Salpa thompsoni* and its contribution to vertical flux in the Southern Ocean. *Marine Biology*, *156*(2), 455–467.

Piette, J., & Lemaire, P. (2015). Thaliaceans, the neglected pelagic relatives of ascidians: A developmental and evolutionary enigma. *The Quarterly Review of Biology*, *90*(2), 117–145.

Pizon, A. (1893). Histoire de la blastogénèse chez les Botryllides. *Annales de Sciences Naturelles. ser 7*, 1–386. Zool.14.

Pizon, A. (1905). L'évolution des diplosomes: (Ascidies composées). *Archives de zoologie expérimentale et générale, ser*, *4*, 1–68.

Prünster, M. M., Ricci, L., Brown, F. D., & Tiozzo, S. (2018). *De novo* neurogenesis in a budding chordate: Co-option of larval anteroposterior patterning genes in a transitory neurogenic organ. *Developmental Biology*, *448*, 342–352.

Prünster, M. M., Ricci, L., Brown, F. D., & Tiozzo, S. (2019). Modular co-option of cardiopharyngeal genes during non-embryonic myogenesis. *EvoDevo*, *10*, 10–13.

Ricci, L., Cabrera, F., Lotito, S., & Tiozzo, S. (2016). Redeployment of germ layers related TFs shows regionalized expression during two non-embryonic developments. *Developmental Biology*, *4161*, 235–248.

Ricci, L., Chaurasia, A., Lapébie, P., Dru, P., Helm, R. R., Copley, R. R., & Tiozzo, S. (2016). Identification of differentially expressed genes from multipotent epithelia at the onset of an asexual development. *Scientific Reports*, *6*, 27357.

Rinkevich, Y., Rosner, A., Rabinowitz, C., Lapidot, Z., Moiseeva, E., & Rinkevich, B. (2010). Piwi positive cells that line the vasculature epithelium, underlie whole body regeneration in a basal chordate. *Developmental Biology*, *3451*, 94–104.

Rinkevich, Y., Voskoboynik, A., Rosner, A., Rabinowitz, C., Paz, G., Oren, M., … Rinkevich, B. (2013). Repeated, long-term cycling of putative stem cells between niches in a basal chordate. *Developmental Cell*, *24*(1), 76–88.

Ritter, W. E. (1896). Budding in compound ascidians based on studies on Goodsiria and Perophora. *Journal of Morphology*, *12*, 149–238.

Rodriguez, D., Kassmer, S. H., & De Tomaso, A. W. (2017). Gonad development and hermaphroditism in the ascidian *Botryllus schlosseri*. *Molecular Reproduction and Development*, *84*(2), 158–170.

Sabbadin, A. (1958). *Analisi sperimentale* della sviluppo delle colonie di *Botryllus* schlosseri (Pallas) [Ascidiacea]. *Archivio Italiano di Anatomia e di Embriologia*, *63*, 178–221.

Sabbadin, A., Zaniolo, G., & Majone, F. (1975). Determination of polarity and bilateral asymmetry in palleal and vascular buds of the ascidian *Botryllus schlosseri*. *Developmental Biology*, *46*(1), 79–87.

Sabbadin, A., & Zaniolo, G. (1979). Sexual differentiation and germ cell transfer in the colonial ascidian *Botryllus schlosseri*. *Journal of Experimental Biology*, *207*(2), 289–304.

Salenski, W. (1890). On the development of Pyrosoma. *Annals and Magazine of Natural History*, *33*(6), 236–244.

Salenski, W. (1891). Beitrage zur Embryonalentiwichlung der Pyrosomen. *Zool. Jahob. Anat*, *5*, 1–98.

Salfi, M. (1932). Il ciclo blastogenetico dei Didemnidi. *Bolletino di zoologia*, *3*, 185–193.

Scelzo, M., Alié, A., Pagnotta, S., & Tiozzo, S. (2019). Novel budding mode in *Polyandrocarpa zorritensis*: A model for comparative studies on asexual development and whole body regeneration. *EvoDevo*, *10*, 7.

Seeliger, O. (1882). Zur Entwicklungsgeschichte der Ascidien. Eibildung und Knospung von *Clavelina lepadiformis*. *Bayerische Akademie der Wissenschaften Wien*, *85*, 361–413.

Selys-Longchamps, M. D. (1917). Sur le bourgeonnement des Polystiélinés Stolonica et Heterocarpa avec quelques notes sur l'anatomie de ces deux genres. *Bulletin scientifique de la France et de la Belgique, L*, *3*, 170–276.

Shenkar, N., & Swalla, B. J. (2011). Global diversity of Ascidiacea. *PLOS One*, *6*(6):e20657.

Shenkar, N. (2013). A new species of the genus Rhopalaea (Class: Ascidiacea from the Red Sea. *Zootaxa*, *3599*(1), 51–58.

Shenkar, N., Koplovitz, G., Dray, L., Gissi, C., & Huchon, D. (2016). Back to solitude: Solving the phylogenetic position of the Diazonidae using molecular and developmental characters. *Molecular Phylogenetics and Evolution*, *100*, 51–56.

Stolfi, A., & Brown, F. D. (2015). Tunicata. In A. Wanninger (Ed.), *Evolutionary Developmental Biology of Invertebrates* (p. 6). Vienna, Austria: Springer.

Stoner, D.S., & Weissman, I.L. (1996). Somatic and germ cell parasitism in a colonial ascidian: Possible role for a highly polymorphic allorecognition system. *Proceedings of the National Academy of Sciences*, *93*(26), 15254–15259.

Sugimoto, K., & Nakauchi, M. (1974). Budding, sexual reproduction and degeneration in the colonial ascidian, *Symplegma reptans*. *The Biological Bulletin*, *147*(1), 213–226.

Sunanaga, T., Inubushi, H., & Kawamura, K. (2010). Piwi-expressing hemoblasts serve as germline stem cells during postembryonic germ cell specification in colonial ascidian, *Botryllus primigenus*. *Development, Growth and Differentiation*, *52*(7), 603–614.

Sunanaga, T., Saito, Y., & Kawamura, K. (2006). Postembryonic epigenesis of Vasa-positive germ cells from aggregated hemoblasts in the colonial ascidian, *Botryllus primigenus*. *Development, Growth and Differentiation*, *48*(2), 87–100.

Tatián, M., Lagger, C., Demarchi, M., & Mattoni, C. (2011). Molecular phylogeny endorses the relationship between carnivorous and filter-feeding tunicates (Tunicata, Ascidiacea). *Zoologica Scripta*, *40*(6), 603–612.

Tatzuke, Y., Sunanaga, T., Fujiwara, S., & Kawamura, K. (2012). RACK1 regulates mesenchymal cell recruitment during sexual and asexual reproduction of budding tunicates. *Developmental Biology*, *368*, 393–403.

Tiozzo, S., Christiaen, L., Deyts, C., Manni, L., Joly, J. S., & Burighel, P. (2005). Embryonic versus blastogenetic development in the compound ascidian *Botryllus schlosseri*: Insights from Pitx expression patterns. *Developmental Dynamics*, *232*(2), 468–478.

Tiozzo, S., & Copley, R. (2015). Reconsidering regeneration in metazoans: An evo-devo approach. *Frontiers in Ecology and Evolution*, *3*, 3.

Tsagkogeorga, G., Turon, X., Hopcroft, R. R., Tilak, M. K., Feldstein, T., Shenkar, N., … Delsuc, F. (2009). An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. *BMC Evolutionary Biology*, *9*, 187.

Turon, X. (1992). Periods of non-feeding in *Polysyncraton lacazei* (Ascidiacea: Didemnidae): A rejuvenative process? *Marine Biology*, *655*, 647–655.

Turon, X. (2005). A new mode of colony multiplication by modified budding in the ascidian *Clavelina gemmae n. sp.* (Clavelinidae). *Invertebrate Biology*, *124*, 273–283.

Turon, X., & Lopez-Legentil, S. (2004). Ascidian molecular phylogeny inferred from mtDNA data with emphasis on the Aplousobranchiata. *Molecular Phylogenetics and Evolution*, *33*(2), 309–320.

Uljanin, V. N. (1884). Die arten der gattung Doliolum im golfe von Neapel und den angrenzenden meeresabschnitten. *Fauna Und Flora Des Golfes Von Neapel*, *10*, 1–140.

Van Name, W. G. (1921). Budding in compound ascidians and other invertebrates, and its bearing on the question of the early ancestry of the vertebrates. *Bulletin of the American Museum of Natural History*, *4*, 275–282.

Van Beneden, E., & Julin, C. (1886). Recherches sur la morphologie des tuniciers. *Archives de Biologie*, *6*.

Voskoboynik, A., Simon-Blecher, N., Soen, Y., Rinkevich, B., De Tomaso, A. W., Ishizuka, K. J., & Weissman, I. J. (2007). Striving for normality: Whole body regeneration through a series of abnormal generations. *The FASEB*, *21*(7), 1335–1344.

Voskoboynik, A., Soen, Y., Rinkevich, Y., Rosner, A., Ueno, H., Reshef, R., … Weissman, I. L. (2008). Identification of the endostyle as a stem cell niche in a colonial chordate. *Cell Stem Cell*, *3*(4), 456–464.

Watanabe, H., & Tokioka, T. (1972). Two new species and one possibly new race of social styelids from Sagami Bay with remarks on their life history especially the mode of budding. *Publications of the Seto Marine Biological Laboratory*, *195*, 327–345.

Watkins, M. J. (1958). Regeneration of buds in Botryllus. *The Biological Bulletin*, *115*, 147–152.

WoRMS Editorial Board (2019). World register of marine species. Retrieved from http://www.marinespecies.org

Zeng, L., & Swalla, B. J. (2005). Molecular phylogeny of the protochordates: Chordate evolution. *Canadian Journal of Zoology*, *83*(1), 24–33.

How to cite this article: Alié A, Hiebert LS, Scelzo M, Tiozzo S. The eventful history of nonembryonic development in tunicates. *J Exp Zool (Mol Dev Evol)*. 2020;1–17. https://doi.org/10.1002/jez.b.22940

Annex 2

Convergent Acquisition of Nonembryonic Development in Styelid Ascidians

Alexandre Alié,[†,1] Laurel Sky Hiebert,[†,2,3] Paul Simion,[†,4] Marta Scelzo,[1] Maria Mandela Prünster,[1] Sonia Lotito,[1] Frédéric Delsuc,[4] Emmanuel J.P. Douzery,[4] Christelle Dantec,[5] Patrick Lemaire,[5] Sébastien Darras,[6] Kazuo Kawamura,[7] Federico D. Brown,[2,3,8] and Stefano Tiozzo*[,1]

[1]Sorbonne Université, CNRS, Laboratoire de Biologie du Développement de Villefranche-sur-mer (LBDV), 06230 Paris, France

[2]Departamento de Zoologia – Instituto Biociências, Universidade de São Paulo, São Paulo, Brazil

[3]Centro de Biologia Marinha (CEBIMar), Universidade de São Paulo, São Paulo, Brazil

[4]ISEM, Université de Montpellier, CNRS, IRD, EPHE, Montpellier, France

[5]CRBM, Université de Montpellier, CNRS, Montpellier, France

[6]Sorbonne Université, CNRS, Biologie Int[1]egrative des Organismes Marins (BIOM),Observatoire Oc[1]eanologique, Banyuls/Mer, 06230 Paris, France

[7]Laboratory of Cellular and Molecular Biotechnology, Faculty of Science, Kochi University, Kochi, Japan

[8]Instituto Nacional de Ciência e Tecnologia em Estudos Interdisciplinares e Transdisciplinares em Ecologia e Evolução (IN-TREE), Salvador, Brazil

[†]These authors contributed equally to this work.

***Corresponding author:** E-mail: tiozzo@obs-vlfr.fr.

Associate editor: Nicole King

Abstract

Asexual propagation and whole body regeneration are forms of nonembryonic development (NED) widespread across animal phyla and central in life history and evolutionary diversification of metazoans. Whereas it is challenging to reconstruct the gains or losses of NED at large phylogenetic scale, comparative studies could benefit from being conducted at more restricted taxonomic scale, in groups for which phylogenetic relationships are well established. The ascidian family of Styelidae encompasses strictly sexually reproducing solitary forms as well as colonial species that combine sexual reproduction with different forms of NED. To date, the phylogenetic relationships between colonial and solitary styelids remain controversial and so is the pattern of NED evolution. In this study, we built an original pipeline to combine eight genomes with 18 de novo assembled transcriptomes and constructed data sets of unambiguously orthologous genes. Using a phylogenomic super-matrix of 4,908 genes from these 26 tunicates we provided a robust phylogeny of this family of chordates, which supports two convergent acquisitions of NED. This result prompted us to further describe the budding process in the species *Polyandrocarpa zorritensis*, leading to the discovery of a novel mechanism of asexual development. Whereas the pipeline and the data sets produced can be used for further phylogenetic reconstructions in tunicates, the phylogeny provided here sets an evolutionary framework for future experimental studies on the emergence and disappearance of complex characters such as asexual propagation and whole body regeneration.

Key words: **asexual reproduction, Botryllinae, cross-contamination, *Polyandrocarpa*, regeneration, tunicate.**

Article

Introduction

Most animals reproduce sexually (White 1978). Male and female gametes combine their genetic material in a zygote, develop by embryogenesis, and produce individuals that are genetically different from their parents. This reproductive strategy is regarded as evolutionarily advantageous by allowing rapid adaptation to constantly changing environments; for example, it can efficiently eliminate deleterious mutations or rapidly incorporate beneficial mutations (reviewed by West et al. 1999). However, other reproductive modes coexist within the metazoans. Nearly half of all animal phyla contain species that are able to propagate asexually via agametic reproduction (i.e., cloning), and the number is higher if one includes parthenogenetic species (Bell 1982; Hughes and Cancino 1985; Nilsson Sköld et al. 2009; Brusca et al. 2016). Organisms that reproduce clonally mobilize somatic cells, either as undifferentiated progenitors or terminally differentiated cells, to produce new individuals genetically identical to their parent. Agametic asexual reproduction is often linked with extensive regenerative capacity, as many clonally propagating organisms can regenerate their body entirely, including the germline (Hughes 1989; Sanchez-Alvarado and Yamanaka 2014). This relationship strongly suggests that clonal propagation and regeneration are two

© The Author(s) 2018. Published by Oxford University Press on behalf of the Society for Molecular Biology and Evolution.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Open Access

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

processes of "nonembryonic development" (NED) that involve common cellular and molecular mechanisms (Martinez et al. 2005; Agata et al. 2007; Bosch 2007; Galliot and Chera 2010; Gurley et al. 2010).

Some animals undergoing NED can form colonies, where individuals remain either physically connected to each other or develop clusters of genetically identical modules termed zooids (e.g., in tunicates), ramets (e.g., in bryozoans), or polyps (e.g., in cnidarians; Boardman et al. 1973). Depending on the species, the modules can be either morphologically identical or different, so that physiological functions, like reproduction or feeding can be subdivided among all units of the colony. This type of growth and modular organization has important physiological and ecological consequences, such as allowing competitive dominance over solitary forms on space-limited hard substrata (Jackson 1977) or reducing gene flow compared with solitary species (Jackson and Coates 1986). Solitary organisms show an inverse relationship between body size and reproductive rate due to reduced metabolic rate with high body mass. However, colonial species escape this limitation and show mass-specific metabolic rates related to module size rather than colony size. Thus, colonial species can grow faster than solitary species and propagate indefinitely without loss in reproductive potential (Hughes 1989; Jackson and Coates 1986; Burgess et al. 2017). Therefore, widespread sexual reproduction by gamete production only represents one facet of species perpetuation and evolutionary diversification of metazoans.

Species employing asexual clonal reproduction are distributed across animal phyla, including nonbilaterian animals such as placozoans, cnidarians or sponges, many spiralian groups such as bryozoans, annelids or planarians, and deuterostome phyla such as hemichordates, echinoderms or tunicates, with the notable exception of vertebrates (Hughes 1989; Bely and Nyberg 2010; Brusca et al. 2016; Alié et al. 2015; Funayama et al. 2010). Regenerative abilities are also widespread and unevenly distributed across metazoans (reviewed in Tiozzo and Copley 2015). While many authors consider regeneration an ancestral trait for animals (Sánchez Alvarado 2000; Bely and Nyberg 2010), the more scattered phylogenetic distribution and diversity of agametic asexual reproductive mechanisms is suggestive of multiple independent acquisitions (Nilsson Sköld et al. 2009). For example, in annelids asexual reproduction by fission was gained at least 19 times (Zattara and Bely 2016). In anthozoan cnidarians fission has likely arisen from an aclonal ancestor at least four times (Geller et al. 2005). In scleractinian corals, on the other hand, coloniality is reported to have been lost at least six times (Barbeitos et al. 2010). It is difficult to reconstruct the cellular and molecular mechanisms underlying the evolution of NED by comparing distant phyla, first because in many cases phylogenetic relationships are unresolved, and because, the cell types involved and their behaviors are hardly comparable at such phylogenetic scales (Carlson 2007). Therefore, in order to understand how novel reproductive and regenerative strategies evolved and the directionality of these transitions (i.e., gains or losses) it is essential to compare closely related species undergoing NED or lacking such traits with well-defined phylogenetic relationships (Tiozzo and Copley 2015; Nogueira et al. 2016).

Ascidians belong to the subphylum Tunicata, the closest living relatives to vertebrates (Delsuc et al. 2006) in which it is possible to find strictly sexually reproducing species and others that combine sexual reproduction with different forms of budding and whole body regeneration (Kürn et al. 2011; Brown and Swalla 2012; Piette and Lemaire 2015). Whereas solitary ascidians reproduce only sexually, colonial species can develop both sexually and via NED. Interestingly, colonial and solitary species are scattered across the phylogenetic tree of ascidians, suggesting that NED, and therefore coloniality, has been acquired and/or lost several times independently (Brown and Swalla 2012). Ascidians are polyphyletic and classically divided in three orders—Aplousobranchia, Phlebobranchia, and Stolidobranchia—in each of which budding mechanisms are very different, further supporting convergent acquisitions of coloniality (Brown and Swalla 2012). The order Stolidobranchia comprises three traditionally recognized families (Molgulidae, Pyuridae, and Styelidae). Molgulidae and Pyuridae are solitary, whereas approximately one third of Styelidae are colonial species (WoRMS Editorial Board 2017; fig. 1*A*–*F*). Thus, Stolidobranchia has long been considered a suitable group to study the plastic evolution of NED and coloniality (Zeng et al. 2006; Pérez-Portela et al. 2009; Brown and Swalla 2012).

In colonial styelids, two budding modes have been described and sometimes coexist in the same species: peribranchial budding and vascular budding (fig. 1*G*–*H*). In peribranchial budding, a portion of the zooid epithelium lining the peribranchial chamber starts to bud in a stereotyped process (fig. 1*G*) and gives rise to new adult zooids (Selys-Longchamps 1917; Berrill 1935; Kawamura and Watanabe 1981; Fujiwara et al. 2011; Manni et al. 2014). In addition to peribranchial budding, several species also use vascular budding either for asexual propagation or in response to injury (fig. 1*H*). During this process circulatory cells cluster, proliferate, and develop into a new individual within the vasculature of the colony (Kawamura and Sunanaga 2010; Rinkevich et al. 2010; Ricci et al. 2016). In both processes, the surrounding epidermis envelops the bud, forming a double vesicle of monolayered tissue (fig. 1*G* and *H*) that will later undergo organogenesis. Therefore, in Styelidae the two described budding processes pass through a similar stage, the double vesicle, which has different cellular origins and is triggered by different mechanisms. Whereas peribranchial budding mainly involves epithelial morphogenesis, vascular budding is ensured by circulating putative stem cells (Laird et al. 2005).

Previous attempts to reconstruct the phylogeny of the Styelidae have not reached a consensus on the relationships between colonial versus solitary species. Kott (1985) splits the styelids into three subfamilies, solitary Styelinae, colonial Polyzoinae, and colonial Botryllinae based on morphological characters. However, molecular phylogenies based on ribosomal and mitochondrial markers have led to conflicting results, either retrieving all colonial species together in a single clade and suggesting a unique acquisition of asexual

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

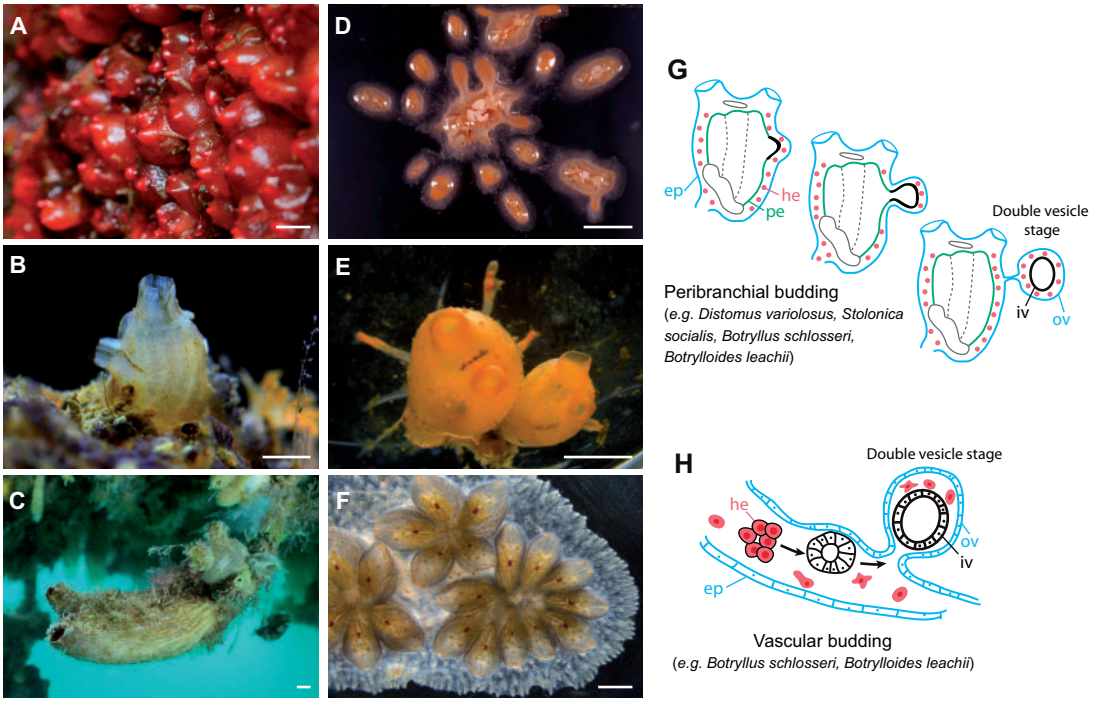


Fig. 1. (*A*–*C*) Solitary Styelidae. (*A*) *Dendrodoa grossularia*. (*B*) juvenile of *Styela plicata*. (*C*) *Styela clava* © Thomas Wilfried Station Biologique de Roscoff. (*D*–*F*) Colonial Styelidae. (*D*) *Polyandrocarpa misakiensis*. (*E*) *Stolonica socialis*. (*F*) *Botryllus schlosseri* (courtesy of L. Ricci). (*G*) Schematic representation of peribranchial budding at three successive stages, from peribranchial invagination to double vesicle. (*H*) Schematic representation of vascular budding at three successive stages, from hemocyte clustering to double vesicle. ep: epidermis, he: hemocytes, iv: inner vesicle, ov: outer vesicle, pe: peribranchial epithelium. Scale bars: (*A*–*C*) 1 cm, (*D*–*E*) 5 mm, (*F*) 1 mm.

reproduction (Zeng et al. 2006; Tsagkogeorga et al. 2009; Shenkar et al 2016), or retrieving several lineages of colonial species and thus proposing multiple convergent acquisitions of asexual reproduction (Pérez-Portela et al. 2009). Whereas the former studies included only a limited number of colonial styelids, Pérez-Portela et al. (2009) included more of them but the key branches were not statistically supported.

In the present work, we used Illumina-based RNA-seq comparisons to address phylogenetic relationships between colonial and solitary styelids. We used an original pipeline to combine de novo assembled transcriptomes of 18 stolidobranchs with 8 ascidian genomes to build a supermatrix of orthologous genes, which lead to a maximally supported tree topology. Our results showed that NED was acquired at least twice independently in the family Styelidae and led us to uncover a novel budding mechanism in *Polyandrocarpa zorritensis*. In light of these results, we discuss the evolution of asexual reproduction and coloniality emphasizing the evolutionary plasticity of asexual development in tunicates.

Results

Species Selection from 18S rRNA-COI Phylogeny

In order to a priori select species that span the diversity of colonial and solitary styelids, we first conducted a preliminary phylogenetic analysis based on a concatenated alignment of 18S rRNA and mitochondrial COI markers, combining data from Pérez-Portela et al. (2009), publicly available sequences, and sequences obtained from 12 specimens that we collected (see Materials and Methods and supplementary fig. 1, Supplementary Material online). Whereas most of the deep nodes were not statistically supported, the topology of the resulting tree was helpful to guide a posteriori selection of the colonial and solitary species to be used in the study. We used colonial ascidians from all different clades recovered in the phylogeny, as well as solitary species of *Styela*, *Polycarpa*, and *Asterocarpa* that were located in different phylogenetic lineages (supplementary fig. 1, Supplementary Material online).

In order to test the monophyly of styelids, we chose an outgroup that is a priori paraphyletic, including four Pyuridae, two Molgulidae, and four other distantly related species belonging to the genera *Ciona* and *Phallusia* (order Phlebobranchia). The final list of selected species is given in supplementary table 1, Supplementary Material online. Outgroup species of choice were mainly selected by the availability of the full genome sequence, which later simplified the steps of data set construction (supplementary table 1, Supplementary Material online).

A New Pipeline for the De Novo Assembly of Ascidian Transcriptomes

We generated cDNA libraries from the 16 species we collected, from most of which 30–50 million reads were sequenced using the Illumina HiSeq 4000 platform (table 1). The number of sequence reads, accession numbers, and other basic statistics to assess the quality of the transcriptomes are shown in table 1. The initial assembly of these reads using Trinity software (Grabherr et al. 2011) led to transcriptomes composed of between 76,825 and 410,479 contigs depending

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

Table 1. Species from Which De Novo Transcriptomes Were Assembled and Included in Our Study.

Species	Accession Number	Sampling Location	Tissues Used for De Novo Transcriptome	*n* Raw Reads	*n* Contigs	N50	Average Length	Busco Index
Asterocarpa humilis	*PRJNA422120*	Brest (France)	1 adult (no tunic, no stomach)	39,963,596	24,341	1,644	1,180.46	88%
Botrylloides leachii	SAMN04161391-98	Otago (New Zealand)	Embryos and regenerative tissues	315,587,750	42,036	1,365	903	96%
Botryllus schlosseri	SRX726446-59, 67-80	Santa Barbara (USA)	Whole colonies	621,300,622	65,586	1,224	905	95%
Dendroda grossularia	*PRJNA422120*	Roscoff (France)	1 adult + larvae (no tunic, no stomach)	42,386,509	57,564	1,194	852.74	95%
Distomus variolosus	*PRJNA422120*	Roscoff (France)	1 adult (no tunic)	39,810,612	44,516	1,458	1,004.97	92%
Eusynstyela tincta	*PRJNA422120*	São Sebastião (Brazil)	1 adult (no tunic)	33,225,966	39,494	837	694	74%
Microcosmus sabatieri	*PRJNA422120*	Banyuls (France)	Siphon	29,425,964	22,951	1,471	1,022.8	93%
Polyandrocarpa misakiensis	*PRJNA422120*	Misaki (Japan)	Asexual zooids and buds	39,178,452	52,545	1,194	851.48	96%
Polyandrocarpa zorritensis	*PRJNA422120*	La Spezia (Italy)	2 adults and buds	158,133,887	57,781	1,197	816.91	96%
Polycarpa aurata	*PRJNA422120*	Cebu (Philippines)	1 adult (no tunic, no stomach)	34,876,779	29,348	1,443	982.17	95%
Polycarpa mamillaris	*PRJNA422120*	Banyuls (France)	Oocytes	12,985,467	25,221	1,599	1,056.42	96%
Polycarpa pomaria	*PRJNA422120*	Brest (France)	1 adult (no tunic)	33,554,939	41,730	1,386	949.48	95%
Pyura dura	*PRJNA422120*	Banyuls (France)	Gonads	33,619,672	17,739	1,854	1,216.9	96%
Stolonica socialis	*PRJNA422120*	Roscoff (France)	1 adult	35,870,064	43,474	1,431	934.34	94%
Styela canopus	*PRJNA422120*	São Sebastião (Brazil)	1 adult (mantle)	44,826,592	42,420	1,047	815.45	87%
Styela clava	*PRJNA422120*	Brest (France)	1 adult (no tunic, no stomach)	47,686,633	26,553	1,647	1,164.48	89%
Styela plicata	*PRJNA422120*	Banyuls (France)	Embryos	38,186,700	19,288	1,870	1,253.51	97%
Polycarpa sp.	*PRJNA422120*	Roscoff (France)	1 adult (no tunic)	43,522,115	44,672	1,311	918.30	90%

on the species (supplementary table 1, Supplementary Material online). These values are far higher than expected from current knowledge of ascidian genome complexity (Dehal et al. 2002; Voskoboynik et al. 2013; Velandia-Huerto et al. 2016). We therefore sought to eliminate redundant and low-quality contigs by developing a custom pipeline (detailed in fig. 2*A* and Materials and Methods). Briefly, we used stringent parameters to filter reads, contigs and ORFs based on sequence quality, redundancy, length, and expression levels. We additionally detected and removed cross-contaminations between samples that were sequenced in the same batch using CroCo (Simion et al. 2018). An average of 5% of all filtered contigs were detected as cross-contaminants (supplementary table 1, Supplementary Material online), and most cross-contaminations arose between samples sequenced in the same Illumina run (supplementary fig. 2 and supplementary table 2, Supplementary Material online).

On average, our pipeline retained ~20% of initial contigs (supplementary table 1, Supplementary Material online). BUSCO indices ranged from 87% and 96% (except for *Eusynstyela tincta* for which it was 74%), showing that our pipeline did not massively reduce gene numbers but rather eliminated undesired redundancy and/or contaminations. As an example, whereas Trinity assembly of reads from *Polycarpa aurata* gave 118,198 contigs with BUSCO index of 95% we ended up with 29,348 contigs. Final transcriptomes are available at https://github.com/AlexAlie/styelidae, last accessed April 2018, and raw Illumina reads can be retrieved from NCBI BioProject PRJNA422120.

A New Data Set of Orthologous Genes for Ascidian Phylogeny

Combining our refined de novo transcriptome assemblies with sets of genes derived from genomes allowed us to build a high quality data set of orthologous tunicate genes (fig. 2). The contigs of our 18 transcriptomes (fig. 2*A*) were first attributed to 14,552 clusters that were built using 18 chordate genomes (fig. 2*B*, supplementary table 3, Supplementary Material online). Among them we selected 8,187 clusters with no apparent paralogy in tunicate genomes (fig. 2*C*). After removing all nontunicate species, the contigs within each cluster were aligned (fig. 2*D*) and we performed alignment curation by joining end-to-end fragments of single transcripts that were split due to imperfect transcriptome assembly (fig. 2*D*, step "fusion of nonoverlapping sequences"). The combination of high quality transcriptomes, fusion of fragmented sequences, and subsequent filtering (see Materials and Methods) led to the retention of 4,908 alignments devoid of apparent paralogy in tunicates (fig. 2*D*, TOTAL data set of 1,740,663 amino acid sites and 36.5% missing data). A reduced version of this data set comprising 1,306 genes was selected, in which virtually all species were present (fig. 2*D*, REDUCED data set of 605,265 amino acid sites and 14.4% missing data). Functional annotations showed that the genes belong to different families, functional groups, and have different kinds of protein domains (supplementary table 4, Supplementary Material online). Both TOTAL and REDUCED matrices are provided at https://github.com/AlexAlie/styelidae, last accessed April 2018.

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

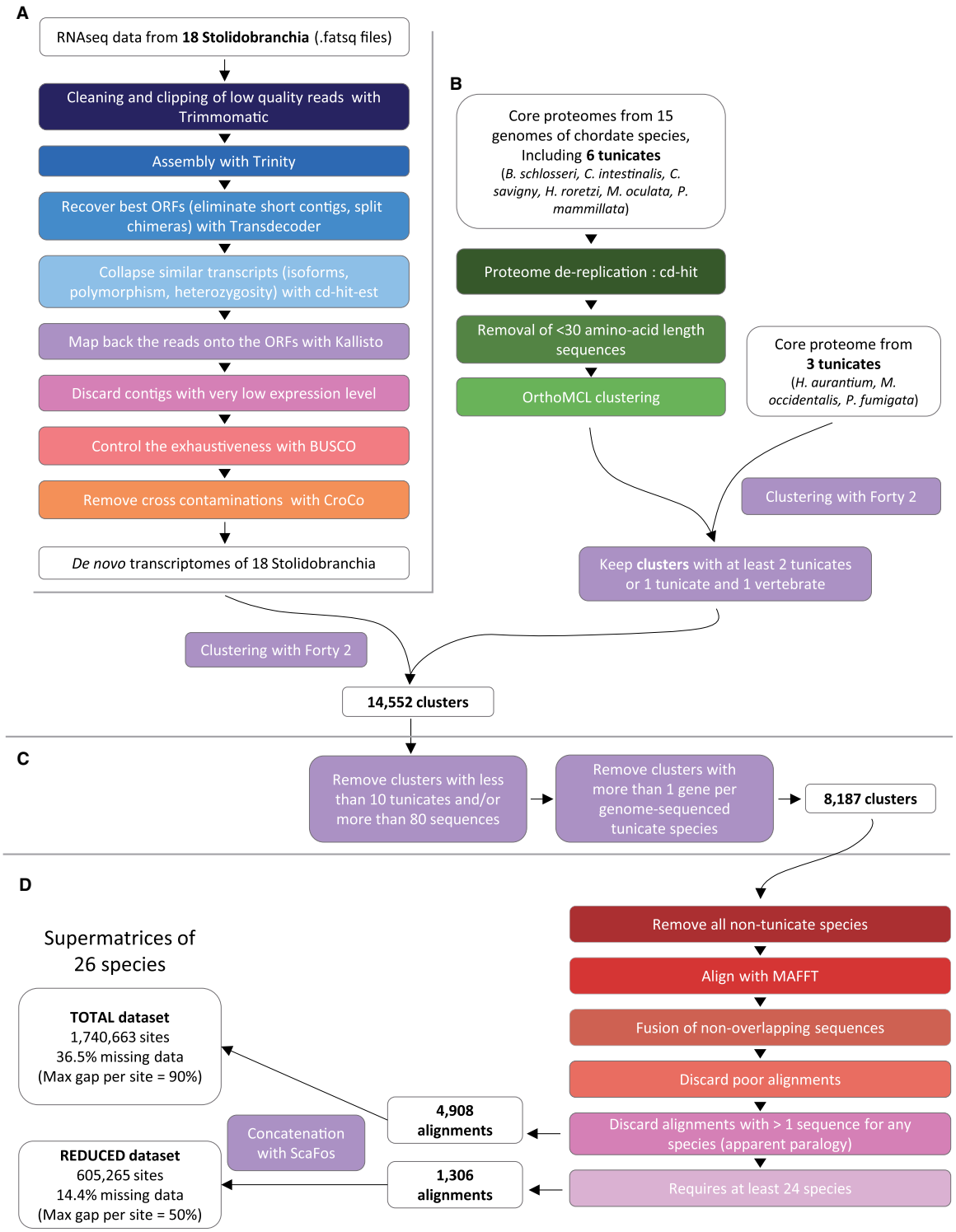


Fig. 2. Graphical summary of the data set construction protocol used in this study (see Materials and Methods for the details of each step). (*A*) Part of the pipeline corresponding to the de novo transcriptome assembly. (*B*) Part of the clustering pipeline resulting in 14,552 clusters deduced from 9 tunicate genomes and 18 de novo transcriptomes. (*C*) Part of the clustering pipeline leading to 8,187 clusters with no apparent paralogy in genome-sequenced species. (*D*) Part of the pipeline corresponding to alignment and building of the FULL and REDUCED data sets devoid of paralogy for any species.

A Robust Phylogeny of Styelid Ascidians

We conducted maximum likelihood analyses using models with (LG4X) and without (LG) site heterogeneity, as well as Bayesian inference under the site-heterogeneous CAT mixture model. All three strategies generated the same topology, with maximum bootstrap and maximum posterior probabilities obtained for all branches (fig. 3 and supplementary figs. 3–5, Supplementary Material online). We retrieved a

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

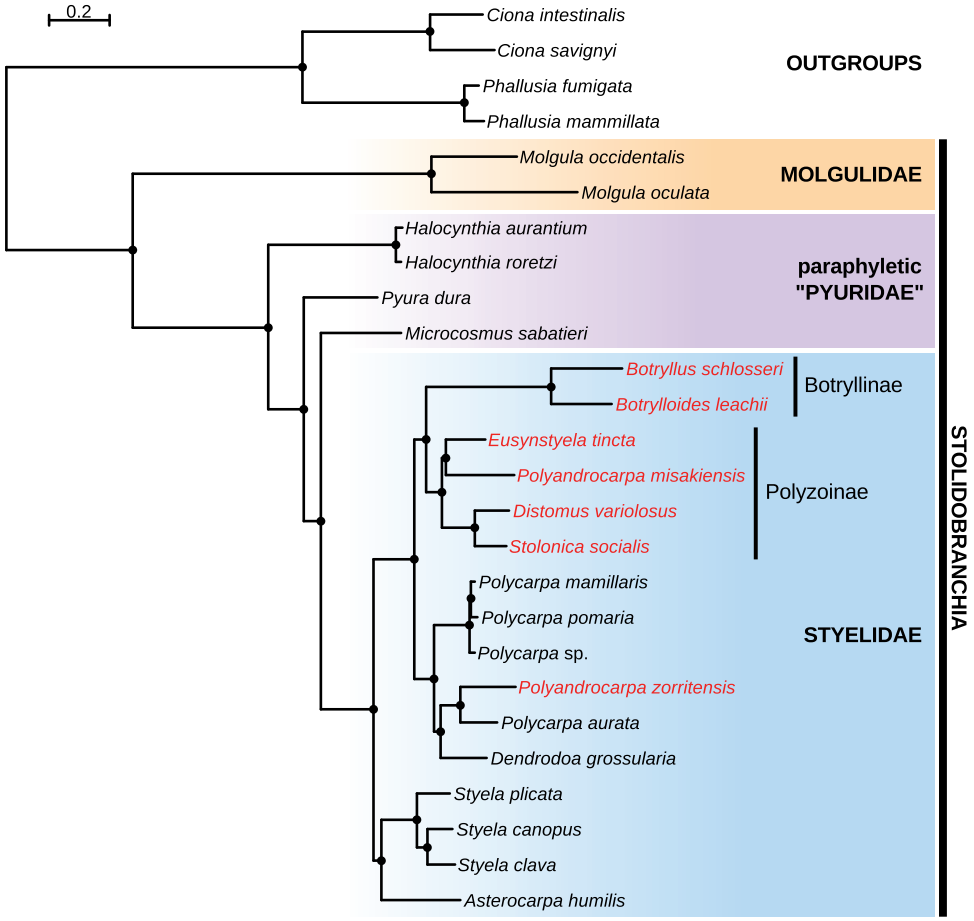


Fig. 3. Phylogenetic relationships between Stolidobranchia, with a focus on Styelidae, inferred from the REDUCED supermatrix (1,306 genes, 605,265 aa, 14.4% missing data) using the site-heterogeneous CAT + F81 + Γ4 model. All phylogenetic analyses in this study on both FULL and REDUCED data sets inferred the same topology with maximum bootstrap support and maximum posterior probabilities for all branches. Colonial species (capable of asexual reproduction via NED) are depicted in red, solitary species in black. The three other species trees obtained in this study are provided as supplementary figures 3–5, Supplementary Material online.

monophyletic Stolidobranchia, in which a monophyletic Styelidae was embedded within a paraphyletic Pyuridae, and in which a monophyletic Molgulidae was the sister-group to all other Stolidobranchia. Within Styelidae, a clade of solitary *Styela* and *Asterocarpa* was the sister-group to all the rest (fig. 3). Colonial species, that is, species adopting NED, fell in two distinct locations in our inferred phylogeny. First, we recovered a clade containing *Botryllus schlosseri*, *Botrylloides leachii*, *Polyandrocarpa misakiensis*, *Eusynstyela tincta*, *Distomus variolosus*, and *Stolonica socialis* (fig. 3). In all six species belonging to this clade, colonial growth occurs by peribranchial budding, and therefore we named this clade the peribranchial budding clade (PB clade). In this clade, *B. schlosseri* and *B. leachii* (Botryllinae) are also able to regenerate colonies through vascular budding (fig. 3). The Botryllinae was the sister group to a clade containing four Polyzoinae: *E. tincta* together with *P. misakiensis* and *S. socialis* together with *D. variolosus*. The fifth Polyzoinae and last colonial species of our sampling, *P. zorritensis* grouped together with solitary *Polycarpa* and *Dendrodoa grossularia*, as sister to *P. aurata* (fig. 3). Therefore, the subfamily Polyzoinae appeared as polyphyletic, with *P. zorritensis* being more closely related to solitary species than to other colonial styelids. The *Polycarpa* genus was also polyphyletic, with *P. aurata* being separated from other *Polycarpa* species. The distant phylogenetic position of *P. zorritensis* apart from the PB clade led us to further describe budding in this species.

Novel Budding in *P. zorritensis*

In a previous report, budding of *P. zorritensis* was described as vascular, involving "blood vessel wall and lymphocytes" (Brunetti and Mastrototaro 2004), but this was based on in vivo observations only. To gain more insights into the cellular processes leading to budding, we conducted histological observations of *P. zorritensis* budding. We observed that the budding process in this species differed from those known in other ascidians. After a few days in culture, the adult zooid emits stolons (fig. 4*A*, *B*) that consist of a single blood vessel bearing short blind ramifications, named ampullae, and surrounded by a thin layer of tunic (fig. 4*B*). Budding starts at several spots along each stolon, where ampullae become very numerous, short, and oriented in every direction, taking a flower-shape (fig. 4*C*). The growing bud appears in the center

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

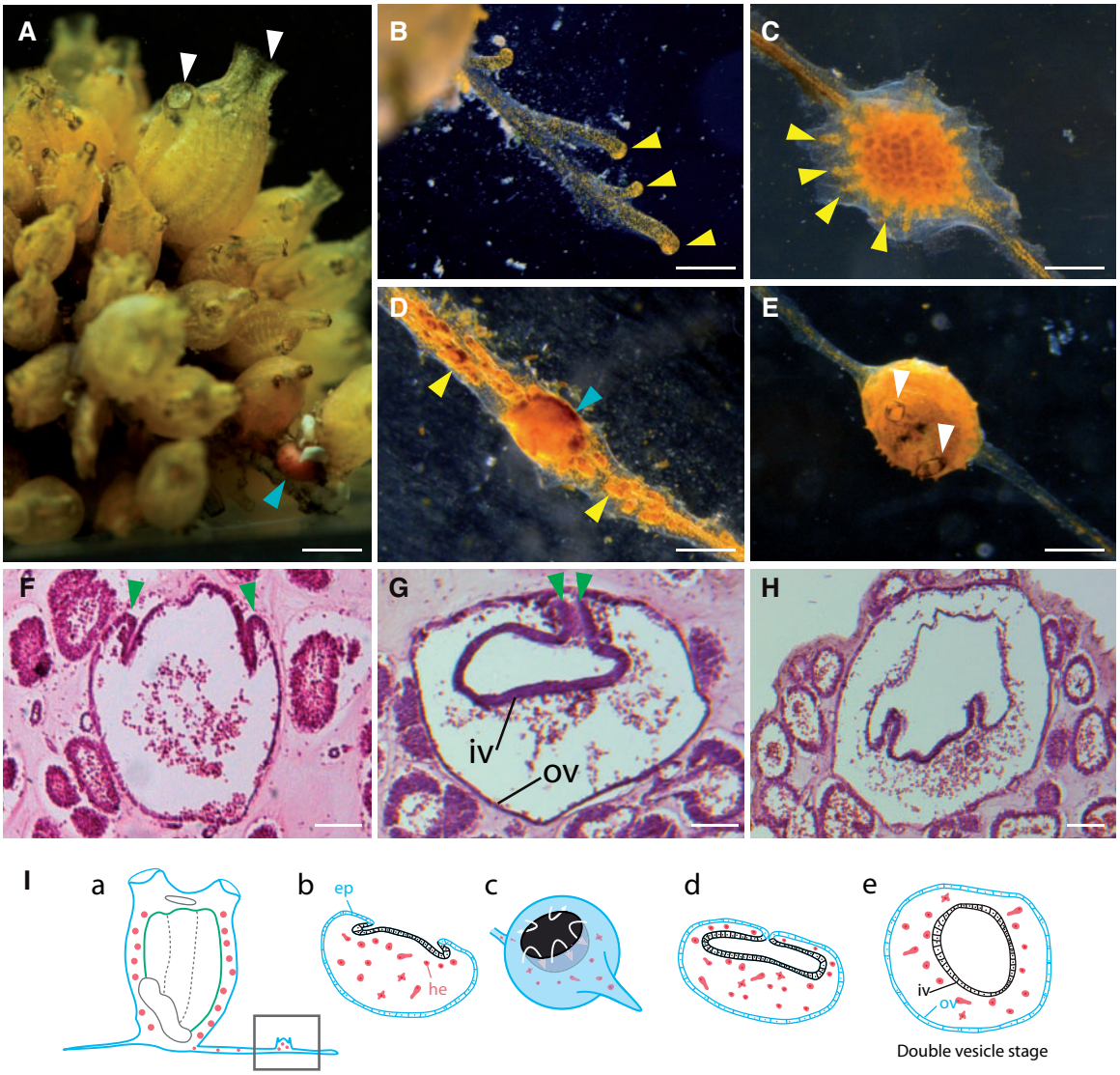


Fig. 4. Budding process in *Polyandrocarpa zorritensis*. (*A*) A colony of young adults of *P. zorritensis*, with a visible bud at the base of the colony (light blue arrowhead), white arrowheads: siphons. (*B*) A ramifying stolon, running onto the substrate (glass dish). (*C*) Budding site along a stolon, characterized by numerous ampullae oriented in every direction. (*D*) A new zooid in the process of forming (blue arrowhead), which externally appears as a round vesicle with no apparent organs. In (*B*)–(*D*) yellow arrowheads show ampullae. (*E*) A very young oozoid in which siphons recently opened (white arrowheads). (*F*–*H*) Histological sections of three successive budding stages. (*F*) Beginning of invagination. (*G*) Fusion of invaginating borders. Green arrowheads: invagination borders. (*H*) Beginning of organogenesis. (*I*) Diagram summarizing the process of budding: (*a*) A bud (squared) arises along a vascular stolon. (*b*) Initiation of invagination, the prospective inner vesicle is in black, whereas the stolon wall is in blue. (*c*) The same stage in a "3D" view, white arrows show the invagination movement. (*d*) Fusion of the inner invaginating borders. (*e*) Double-vesicle stage. ep: epidermis, he: hemocytes, iv: inner vesicle, ov: outer vesicle. Scale bars: (*A*) 2 mm, (*B*–*E*) 1 mm, (*F*–*H*) 25 µm.

of these ramifications in form of a round vesicle (fig. 4*D*) and later develops into a new functional zooid (fig. 4*E*). Histological sections at successive stages showed that the vascular epidermis of the central stolon invaginates (fig. 4*F*), until invagination borders come into contact and fuse with each other (fig. 4*G*). This process results in the formation of an inner vesicle, separated from the outer vesicle by mesenchymal space. A few hours later, the inner vesicle shows distinct foldings (fig. 4*H*) and later organ primordia become visible. Figure 4*I* summarizes the budding process until the double vesicle stage.

Expression of *NK4* Delineates the Prospective Inner Vesicle in *B. schlosseri* and *P. zorritensis*

In order to find molecular markers of budding tissues across styelids, we studied the expression of seven candidate genes based on known expression in *Botryllus* (supplementary table 6, Supplementary Material online) and identified the homeobox containing NK4, having a similar expression pattern in the prospective territory of the future inner vesicle in both *P. zorritensis* and *B. schlosseri* (fig. 5). NK4 is the name given to the ascidian orthologue of NkX2.5/2.6/Tinman family of homeobox cardiac determinants (fig. 5*A*, Wang et al. 2013).

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

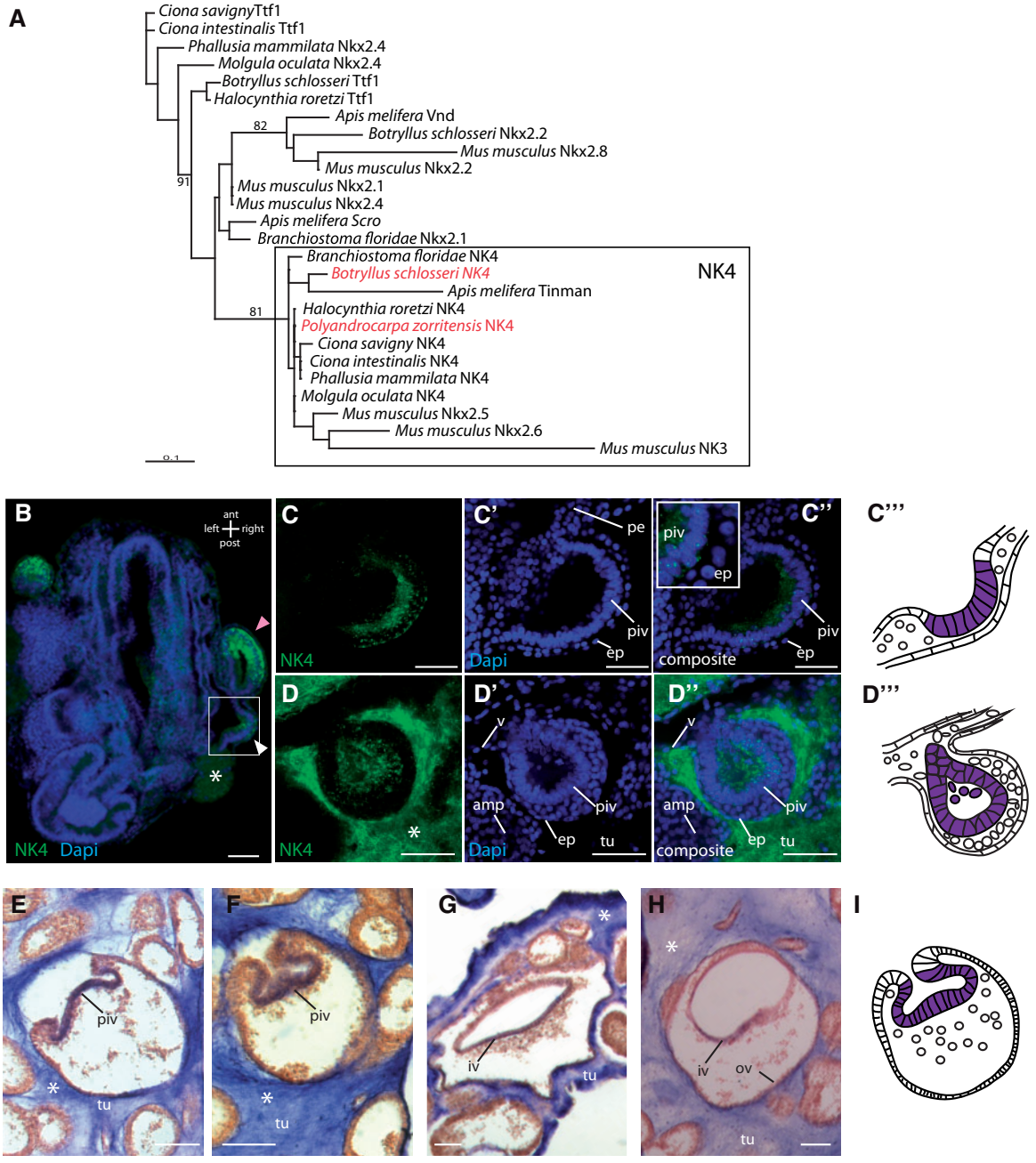


Fig. 5. Expression of the *NK4* gene in *Botryllus schlosseri* and *Polyandrocarpa zorritensis*. (*A*) Phylogenetic analysis of Nkx2.5/2.6 family members showing the orthology between *B. schlosseri* and *P. zorritensis NK4* genes (*NK4* orthology group was defined as the smallest group including *Botryllus NK4*, *Ciona NK4*, and mouse NKx2.5 and 2.6). (*B*–*C‴*) *NK4* expression in peribranchial bud of *B. schlosseri*. In (*B*) the zooid bears three buds at three different stages (white arrowhead shows the youngest one and pink arrowhead shows the oldest one). (*C*–*C″*) Close-up view of *NK4* expression in the early peribranchial bud, insert in (*C″*) is highly magnified to show the unlabeled epidermis. (*C‴*) Diagram of *NK4* expression in the peribranchial bud. (*D*–*D″*) Close-up view of *NK4* expression in a vascular bud at the double vesicle stage. (*D‴*) Diagram of *NK4* expression in the inner vesicle of a vascular bud. (*E*–*I*) Expression of *NK4* in bud of *P. zorritensis*. (*E*) Early invagination. (*F*) Closure of invagination borders. (*G*) Right after closure. (*H*) Late double vesicle stage. (*I*) Diagram showing *NK4* expression in the forming inner vesicle of *P. zorritensis*. Asterisks show nonspecific staining in the tunic, amp: ampullae. ep: epidermis, iv: inner vesicle, ov: outer vesicle, pe: peribranchial epithelium, piv: prospective inner vesicle, tu: tunic, v: vessel. Scale bars: (*B*) and (*D*–*D″*) 50 μm, (*C*–*C″*) 25 μm, (*E*–*H*) 25 μm.

During peribranchial budding in *B. schlosseri* this gene was first expressed in the thickened, evaginating peribranchial epithelium, neither beyond this region of budding nor in the surrounding epidermis (fig. 5*B*, *C‴* and supplementary fig. 6, Supplementary Material online). When the inner vesicle was fully formed, *NK4* became restricted to the anterior-right side of the budlet (fig. 5*B*). Similarly, during the earliest stages of vascular budding in *B. schlosseri*, *NK4* was specifically

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

expressed in the growing inner vesicle (fig. 5*D*–*D*‴). *NK4* was not detected either in circulating cells or in the vascular epidermis of the ampullae (fig. 5*D*–*D*″), but because the tunic stains nonspecifically, we cannot rule out expression in the epidermal outer vesicle of the budlet. In *P. zorritensis* the earliest detected expression of *NK4* began during epidermal invagination, and expanded throughout the whole invaginating region but not in the noninvaginating epithelium (fig. 5*E*). The expression persisted until the invagination borders fused to each other (fig. 5*F*–*H*). Thus, NK4 expression defines the onset of the budding epithelia in both *B. schlosseri* and *P. zorritensis* in three different budding modes.

Discussion

In this study, we generated 18 new transcriptomes of solitary and colonial ascidians belonging to the Styelidae family. We designed a bioinformatic pipeline that allowed us to conduct a phylogenomic analysis from heterogeneous sources of samples, including genomic and transcriptomic data. We provided a robust phylogeny of a family of chordates, which permitted us to trace the evolution of a complex character, that is, NED, and to highlight a previously undescribed mechanism of asexual development in colonial ascidians.

A Reference Set of Orthologous Genes for Tunicate Phylogeny

De novo transcriptome assembly often yields a high number of redundant contigs due to assembly artifacts, but also because of heterozygosity, isoforms, and polymorphism generated when several individuals are pooled together for RNA extraction (Cahais et al. 2012). Contaminations from other organisms, such as epibionts or micro and macroorganisms present in the ascidian pharynx, as well as poorly assembled rare transcripts, may also participate to increase the number of undesired contigs. In addition, some colonial ascidians form chimeric colonies (Kassmer et al. 2015), which adds another source of polymorphism to the transcriptome assembly. Reducing such redundancy and eliminating contaminations have proven essential for downstream analyses such as gene expression, functional enrichment, and orthology assessment (Davidson and Oshlack 2014; Philippe et al. 2017). In this paper, we designed an original procedure that was aimed at increasing transcriptome quality. The importance of good quality transcriptome assemblies only now is starting to be recognized (Cahais et al. 2012; Cabau et al. 2017) and therefore our new approach represents a straightforward solution to this problem with light computational demands. The merging step using CD-HIT-EST (fig. 2*A*) led to a drastic reduction of contig numbers (supplementary table 1, Supplementary Material online), showing that part of the redundancy comes from heterozygosity, assembly artifacts, or polymorphism of colonial species. Further drastic reduction of contig numbers was obtained from ORF selection, showing that many contigs assembled by Trinity had small ORF size (smaller than our threshold of 90 aa), possibly due to the inclusion of rare transcripts that split into multiple small ORFs.

Cross-contaminations in RNA-seq experiments are known to arise when RNA molecules from one sample accidentally pass to another sample, leading to a misattribution of sequencing reads to the contaminated sample (Ballenghien et al. 2017). Because of the Illumina sequencing depth, even extremely minor contamination events can be sequenced and result in unwanted assembled transcripts (Ballenghien et al. 2017; Simion et al. 2018). The level of cross-contamination was important in our samples, ranging between 0.07% and 24% of the contigs, depending on the species. Therefore removing cross-contaminants using recently developed CroCo software (Simion et al. 2018) turned out to be a crucial step. Interestingly, the level of cross-contamination was of the same order of magnitude regardless of whether RNA was extracted in the same or different laboratories, but it was much higher between samples sequenced on the same Illumina lane than between samples sequenced on different lanes performed at different dates (supplementary fig. 2, Supplementary Material online). These observations show that, even if all care is taken during tissue collection and RNA extractions, cross-contaminations mainly arise at the sequencing platform, when samples are handled for library preparation or Illumina sequencing. It is important to note that the amount of cross contaminants produced by sequencing facilities is drastically variable, as we observed an average of 0.2% of cross contaminants for samples handled by one company and an average of 7.3% of cross contaminants for samples handled by a different company (supplementary table 1, Supplementary Material online). Our study represents a typical case that highlights a recurrent problem that cannot be underestimated (Ballenghien et al. 2017; Simion et al. 2018), as it could produce artifacts not only for phylogenetic reconstruction, but also in any RNA-seq study.

Phylogenomics is believed to outperform single-gene phylogeny by adding a high number of genes that will supposedly convey sufficient phylogenetic signal (Philippe et al. 2011). However, if the genes selected for the data matrix are not conscientiously curated, a large amount of signal noise from sequences that deviate from the species tree can result in a fallacious phylogeny (Philippe et al. 2011). Careful selection of orthologous genes is therefore indispensable. The choice of the probabilistic model that best fits the data is also crucial in order to reduce systematic errors such as long-branch attraction between fast evolving taxa (Lartillot et al. 2007; Philippe et al. 2011). We built 4,908 alignments devoid of apparent paralogy by selecting single-copy genes thanks to a customized pipeline that was facilitated by genomic data available for eight ascidian species as well as by the elimination of spurious redundancy in the de novo assembled transcriptomes. All phylogenetic analyses conducted under site-homogeneous (LG) and site-heterogeneous (LG4X and CAT) models led to the same topology with maximum statistical support for all nodes. Therefore, the data set produced here turned out to be particularly appropriate to robustly recover phylogenetic relationships of Styelidae and relatives at the family level. It can thus be used as a reference set of orthologous genes in future tunicate phylogenetic studies. The REDUCED data set

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

of 1,306 alignments shared by at least 24 species provided the same resolution and can be used as an alternative when the computational power is limited, for instance when using elaborated evolutionary models.

Evolutionary and Developmental Implications for Asexual Reproduction and Coloniality

A diverse range of budding modes has been reported across the whole subphylum of tunicates. In planktonic thaliaceans, buds arise from strobilation of stolons that contain tissues from all the three germ layers (Godeaux et al. 1998; Piette and Lemaire 2015). In phlebobranch ascidians such as *Perophora viridis*, buds arise from a stolonial vessel composed of an epidermis and containing a transverse mesenchymal septum (Freeman 1964; Kawamura et al. 2007). In aplousobranch ascidians, budding involves different kinds of tissues and mechanisms, from epithelial epicardium in *Aplidium* during abdominal strobilation, to mesenchymal septum during stolonial budding in *Clavelina* (Berrill 1950; Nakauchi 1982). NED mechanisms in different orders are so diverse that unifying principles are difficult to identify and study. Moreover, a full understanding of the evolution of asexual reproduction and regeneration in tunicates is limited by the current state of the tunicate phylogeny, in which the relationships among orders have only recently started to be resolved (Turon and López-Legentil 2004; Tsagkogeorga et al. 2009; Tatián et al. 2011; Shenkar et al. 2016; Kocot et al. 2018, Delsuc et al. 2018). However, focusing on one particular order or family allows one to decipher phylogenetic relationships between closely related solitary and colonial species and thus to reconstruct the evolution of NED at the intrafamily level (Shenkar et al. 2016).

In the phylogeny presented here, the solitary *Styela* and *Asterocarpa* form an early-diverging clade together with the families Pyuridae and Molgulidae, the latter being exclusively composed of solitary species. Therefore, the most parsimonious interpretation is that the last common ancestor of Styelidae was a solitary animal, and as a consequence NED and coloniality probably arose at least twice independently in Styelidae in two branches: 1) the monophyletic PB clade, that is, Polyzoinae + Botryllinae; and 2) *P. zorritensis*. An alternative interpretation of NED evolution, considering its acquisition in deeper nodes, can only be explained with multiple independent reversions to solitary lifestyle (see examples of different scenarios in supplementary fig. 7*A*, Supplementary Material online). In general, our topology of major Stolidobranch clades is in accordance with previously reported phylogenies that retrieved paraphyletic pyurids (Zeng et al. 2006; Tsagkogeorga et al. 2009; Shenkar et al. 2016). Within styelids, our result contrasts with previous phylogenies based on ribosomal and mitochondrial genes showing either a single acquisition (Zeng et al. 2006; Tsagkogeorga et al. 2009; Shenkar et al. 2016) or at least five independent acquisitions of coloniality (Pérez-Portela et al. 2009). However, these studies either included a limited number of colonial styelids, notably lacking *P. zorritensis*, or their topologies received poor statistical support.

In the phylogenomic tree resulting from this study, only members of Botryllinae are known to perform vascular budding within the PB clade (for colony growth or regeneration), while in Polyzoinea only peribranchial budding has been reported (Selys-Longchamps 1917; Berrill 1950; Abbott 1953; Watanabe and Tokioka 1972; Kawamura and Watanabe 1981). Thus the species tree implies that vascular budding is a Botryllinae innovation, and secondarily acquired in an ancestor that already possessed the peribranchial budding. Our understanding of budding evolution could be further improved in the future by including more colonial styelids to our phylogenomic data set. For instance, adding species of the genera *Polyzoa* (peribranchial budding), *Metandrocarpa* (peribranchial budding), and *Symplegma* (vascular/peribranchial budding).

The fact that budding tissues are of a different nature between *P. zorritensis* and the PB clade species reinforces the hypothesis that NED has been acquired convergently. Brunetti and Mastrototaro (2004) provided a general description of *P. zorritensis* and defined NED in this species as a form of vascular budding. However, a detailed morphological description was not provided. In the Stolidobranchia, only peribranchial budding and vascular budding have been extensively studied. Both these NEDs pass through a double vesicle stage (fig. 1*G*, *H*), where the outer vesicle in peribranchial budding derives from the parental epidermis and gives rise to the epidermis of the new zooid, and an inner vesicle, which will give rise to most of the internal organs, derives either from a portion of peribranchial epithelia (fig. 1*G*) or from population(s) of circulating cells (fig. 1*H*), in peribranchial and vascular budding respectively. The histological analyses of *P. zorritensis* budding show that the new zooids originate from the blood vessel epithelia but follow completely different ontogenetic trajectories, in which both the inner and the outer vesicles derive from the same tissue, namely the vascular epidermis (fig. 4). To our knowledge, this mode of budding has never been described in any other species.

Each of these three events of NED acquisition involved nonhomologous tissues for the formation of the inner vesicle (vascular epidermis, peribranchial epithelium and hemocytes, respectively) but it remains unknown whether budding is triggered by orthologous genes. The example of *NK4* highlights the importance of having a robust phylogenetic framework to select relevant species for comparative studies but also to interpret gene expression patterns in an evolutionary perspective. *NK4* is a highly conserved transcription factor involved in heart specification during ascidian embryogenesis (Wang et al. 2013, Prünster et al. unpublished). It also shows localized expression patterns in the anterior endoderm of larve and juveniles in *Ciona intestinalis*, and in the ventral ectoderm of larvae in both *C. intestinalis* and *Molgula occidentalis* (Christiaen et al. 2010; Stolfi et al. 2014). In *B. schlosseri* and *P. zorritensis NK4* shows an additional domain of expression restricted to the tissues of the emerging bud, thus suggesting a putative new role in NED. By exploring the function of *NK4* and by adding the description of other bud-specific molecular markers it will be possible to establish whether

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

homologous programs are directing budding in different context and species, and to explore the plastic and modular nature of gene regulatory networks that are responsible for nonembryonic developmental processes (Ricci et al. 2016).

New Insights on Styelidae Systematics and Future Directions

Relationships of major Styelidae clades have been debated for more than a century. Berrill (1948) considered that all colonial species should be unified as a natural group because he assumed that they all perform peribranchial budding. In contrast Kott (1985), in her revision of Australian ascidians, suspected that budding modes may be more diverse than expected and proposed that "many species [of *Polyandrocarpa*] have a closer relationship with species of *Polycarpa* than they do with one another [. . .]" and that "Accurate resolution of their taxonomy must await information on the process of vegetative reproduction, which may indicate their true phylogeny." Our phylogenetic tree and the histological investigations on budding mode reconcile the views of Berrill and Kott. As Berrill suspected all peribranchial budding species form a monophyletic group, and as Kott suspected, the genus *Polyandrocarpa* is polyphyletic and *P. zorritensis* has an original budding mode.

The phylogenetic position of *P. zorritensis* is further supported by its gonad morphology, which is of the polycarpid type, that is, a large gonad containing numerous male and female lobules all together embedded in a common sac, also found in *Polycarpa* and *Dendrodoa* (Monniot and Monniot 1972; Monniot 2016). In contrast, other colonial styelids have either sex-separated gonads or small hermaphroditic gonads with no more than two testes (Monniot and Monniot 1972). It would be informative to incorporate into the existing phylogenomic matrix other *Polyandrocarpa* species having polycarpid gonad, e.g. *Polyandrocarpa arianae* (Monniot 2016), and to compare their budding mechanisms. Interestingly, our phylogenetic tree is also in agreement with the view that the acquisition of brooding predates the evolution of coloniality (Brown and Swalla 2012), and that internal brooding was secondarily lost in the oviparous *P. misakiensis* (see supplementary fig. 7*B*, Supplementary Material online for comparison between alternative scenarios). However, broader sampling of PB clade species would be necessary to better trace the evolution of this character.

Conclusions

Experimental approaches to study macroevolutionary transitions requires a priori definitions of homologous characters, but also the identification of the most suitable species in which to trace the evolution of such characters, as well as the knowledge of the phylogenetic relationships among these species. The phylogeny provided in this paper sets an initial framework to characterize the emergence and disappearance of other complex sets of characters, such as agametic asexual propagation and whole body regeneration. In this regard, the tools we generated already draw our attention to the plasticity of NED in an ascidian family and led to the discovery of an undescribed form of asexual development. Comparing the cellular and molecular mechanisms behind each form of NED can also lead to the discovery of conserved developmental modules that regulate regeneration and asexual development. The phylogenetic framework presented here needs further taxonomic sampling to fully understand the evolutionary transitions to NED, but we can already use these findings to trace and study other characters with known plastic evolution, such as larval photoreceptors (Sorrentino et al. 2001), adultation (Jeffery 2007) or brooding strategies.

Materials and Methods

Animal Collection and Identification

Sampling locations are provided in table 1. Brazilian collections were authorized by license No. 44082-1 of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio). Before relaxation and fixation in menthol and 10% formalin, animals were kept for ~12 h in running, grossly filtrated sea water, shortly after which they were fixed. Solitary species are not externally recognizable and need to be dissected, formaldehyde-fixed, and carefully examined. For this reason, a single individual cannot be used for both morphology-based identification and RNA extraction, since fresh tissues have to be frozen very rapidly to get good quality RNA. Therefore, we used the following strategy: when two individuals looked similar, one was dedicated to morphology-based identification and fixed in 4% formaldehyde, the other was used for RNA extraction and flash-frozen in liquid nitrogen. From both, a small piece of tissue was first removed and transferred in 80% ethanol in order to extract DNA and proceed to mitochondrial cytochrome oxidase subunit I (COI) and nuclear 18S rRNA barcoding. By this method, both individuals could be compared at the DNA level, allowing confirmation that the morphology-based identified specimen was of the same species as the frozen one, from which the transcriptome was to be sequenced. When COI and/or 18S rRNA sequences had been deposited in databases by others, morphology-based species determination was confirmed by sequence identity. Before flash-freezing specimens in liquid nitrogen, the tunic and digestive tract were removed in order to decrease the level of contaminating RNA. Concerning colonial species, three zooids or pieces of the same colony were fixed in ethanol, formaldehyde, and liquid nitrogen respectively. In some colonial species, the zooids were so small and so deeply embedded in the tunic that digestive tract and tunic could not be removed before fixation. In these cases, the animals were starved for 24 h and the tunic was cleaned by brush and forceps, as much as possible.

DNA Barcoding and Phylogenetic Analyses Based on COI and 18S rRNA

COI was amplified using primers modified from Folmer et al. (1994), Meyer (2003), and Schwendinger and Giribet (2005). Ribosomal 18S rRNA gene was amplified using 18S-TF and 18S-TR primers from Carreras-Carbonell et al. (2005). A list of primers used for each species is provided in supplementary table 5, Supplementary Material online. For Brazilian samples,

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

DNA was extracted with DNeasy Blood & Tissue kit (Qiagen) following manufacturer's instructions, and then quantified on a NanoDrop 2000 Spectrophotometer (Thermo Scientific). The PCR amplifications were carried out in 20 µl reactions with 0.15 µl GoTaq polymerase in supplied buffer (Promega), 10 mM dNTPs, and 10 mM of each primer. Cycling parameters were as follows: a 94 °C denaturation for 5 min, followed by 35 cycles of: 94 °C denaturation for 30 s, 45 °C annealing for COI or 66 °C for 18S for 50 s, 72 °C extension for 1 min, and a final extension at 72 °C for 5 min. The PCR products were labeled using BigDye Terminator ver. 3.1 Cycle Sequencing kit chemistry (Applied Biosystems), and sequenced on an Applied Biosystems 3730 DNA sequencer. For French samples, DNA was extracted as follows: tissues was first digested by 200 µg/ml Proteinase K at 37 °C for 30 min then mashed with a sterile tip; nucleic acids were purified by phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in sodium chloride 0.2 M and finally quantified on a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Cycling parameters were the same as in Brazil and France. Successful amplifications were sequenced on an ABI 3730 at Genewiz, Inc. (South Plainfield, NJ, USA). New COI and 18S rRNA sequences have were in GenBank (supplementary table 5, Supplementary Material online for accession numbers).

All sequences were aligned and trimmed using Geneious R7 (Biomatters LTD). We aligned the sequences with those from Pérez-Portela et al. (2009) and some available on Genbank (NCBI; see supplementary table 5, Supplementary Material online for accession numbers) using CLUSTALW (Higgins et al. 1996) and trimmed and confirmed by eye. The 18S rRNA alignment was 801 bp and the COI alignment was 679 bp. As in Pérez-Portela et al. (2009), we concatenated the COI and 18S rRNA sequences. Two partitions were specified, one for each gene. We conducted a Maximum Likelihood analysis using the program RAxML v.8 (Stamatakis 2014) with nucleotide substitution model GTR + G + I, and run 100 bootstrap replicates on our alignment of 100 sequences.

RNA Isolation and cDNA Sequencing

Information on tissue used for RNA extraction is provided in table 1. For the species collected in Roscoff, total RNA was extracted in the laboratory of S.Tiozzo (France), using and NucleoSpin RNA II kit (Macherey-Nagel), including a DNAase treatment, and following manufacturer's instructions. For *P. misakiensis*, RNA was extracted in the laboratory of K. Kawamura (Japan), using NucleoSpin RNA kit (Takara-Clontech). For species collected in Brazil, RNA was extracted in the laboratory of F. Brown (Brazil) using a NucleoSpin RNA Plus kit (Macherey-Nagel). Total RNA were sent to Beijing Genome Institute (BGI, China) where cDNA libraries were prepared before sequencing. For species collected in Banyuls, RNA was extracted in the laboratory of S. Darras (France) using Trizol (Life Technologies), followed by purification using the RNeasy kit (Qiagen) with a DNase treatment. cDNA preparation and sequencing were performed by Sistemas Genomicos (Spain). RNA-seq data were obtained using Illumina HiSeq 4000 technology. The exact number of reads per species is given in table 1.

De Novo Transcriptome Assembly

For all the species for which de novo transcriptomes were assembled, we applied the following eight-step pipeline: 1) Illumina reads were clipped and trimmed to eliminate low quality regions using Trimmomatic v. 0.35 (Bolger et al. 2014) with the following parameters: PE reads_1.fq reads_2.fq ILLUMINACLIP: adapters/TruSeq3-PE.fa: 2: 30: 10 LEADING: 36 TRAILING: 36 SLIDINGWINDOW: 4: 34 MINLEN: 100 and then sequence quality was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; last accessed April 2018); 2) reads were assembled with Trinity v. 2.4.0 (Grabherr et al. 2011) using default parameters; 3) open reading frames (ORF's) of a minimum of 90 amino acids were kept using Transdecoder v. 3.0.1 (https://github.com/TransDecoder/TransDecoder/wiki; last accessed April 2018); 4) very similar ORF's (e.g., putative isoforms or alleles) were collapsed using CD-HIT-EST v4.6 (Fu et al. 2012) with default parameters; 5) reads were then mapped back onto the remaining contigs using Kallisto v. 0.42.4 (Bray et al. 2016) with default parameters; 6) density plots were drawn with R, using hist() and density() functions. On the basis of these plots, contigs with low expression levels were eliminated. The threshold above which contigs were kept corresponds to the lower peak of the density plot; 7) exhaustiveness was assessed by running BUSCO (Simão et al. 2015) against metazoan database; 8) cross-contaminations were removed using CroCo (Simion et al. 2018). Number of contigs at each step for each species is given in supplementary table 1, Supplementary Material online.

Data Availability

The 18 de novo assembled transcriptomes can be downloaded from https://github.com/AlexAlie/styelidae; last accessed April 2018. Raw Illumina reads obtained in this study can be retrieved from NCBI BioProject PRJNA422120. Accession numbers or Illumina reads from *B. schlosseri* (Rodriguez et al. 2014) and *B. leachii* (Zondag et al. 2016) are provided in table 1.

H. roretzi and *H. aurantium* gene prediction are accessible via ANISEED (https://www.aniseed.cnrs.fr/aniseed/download/download_data; last accessed April 2018). *Molgula oculata* and *M. occidentalis* transcripts were retrieved from ANISEED. *C. robusta* data can be downloaded from http://ghost.zool.kyoto-u.ac.jp/download_kh.html, and *C. savignyi* data from Ensembl (ftp://ftp.ensembl.org/pub/release-91/fasta/ciona_savignyi/cdna/).

Construction of a New Set of Orthologous Alignments

We selected 15 chordate species for which genomic data were available (i.e., six tunicates, six vertebrates, one cephalochordate, one echinoderm, and one hemichordate, see supplementary table 3, Supplementary Material online), and we retained only the longest transcript for each gene of their corresponding proteomes. These proteomes were further dereplicated using CD-HIT (-c 1.0; Fu et al. 2012) and sequences <30 amino acids were removed. These 15 proteomes were clustered using OrthoMCL (-I 1.3, Li et al. 2003). We later added three new proteomes from tunicate species for which genomic data

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

became available (supplementary table 3, Supplementary Material online) into the clusters using Forty 2 (https://bitbucket.org/dbaurain/42/). The few sequences that were simultaneously added to different clusters were discarded, except in the cluster in which taxonomic diversity was the highest. We discarded all resulting clusters that did not possess at least: 1) two tunicate sequences or 2) one tunicate and one vertebrate sequence, retaining 14,552 clusters.

Gene Augmentation, Selection, and Concatenation

Transcriptomic data from 18 stolidobranchs including 16 styelids (table 1) were added to the 14,552 clusters of orthologous sequences using Forty 2. Clusters that contained apparent paralogy for at least one tunicate species among those that correspond to the genomic data set (i.e., species belonging to the *Ciona*, *Phallusia*, *Molgula*, and *Halocynthia* genera) or which contained <10 tunicate species or which contained >80 sequences were discarded. All nontunicate sequences were removed from the remaining 8,187 clusters in order to improve the accuracy of the subsequent aligning step, a. All clusters were aligned using MAFFT (–maxiterate 1000 –localpair, Katoh and Standley 2013). For every alignment, nonoverlapping sequences were fused as they most likely correspond to sequence fragments from the same transcript. We used an extended definition of a "nonoverlap" between two sequences: if actually overlapping, the total overlap is smaller than 200 amino acids and the two sequences differ by less than five amino acid positions. This allowed us to concatenate 64,340 fragmented transcripts (out of the initial 219,099 transcripts) into 28,188 longer ones. We then removed 4,631 low-quality transcripts (i.e., that had >70% missing data and were shorter than 100 amino acids). Alignments were then checked again for apparent paralogy, this time for all species, and we only kept the 4,908 alignments that showed no paralogy. We also selected alignments containing at least 24 species (out of the 26 total species) to create a smaller and more complete phylogenomic data set of 1,306 alignments. These alignments were concatenated into supermatrices using ScaFos (Roure et al. 2007), available on https://github.com/AlexAlie/styelidae; last accessed April 2018.

Functional annotation was done through the DAVID online service (Huang et al. 2009).

Phylogenetic Analyses

The assembled supermatrices as explained above were analyzed using several models of sequence evolution to assess the relative reliability of the different species to infer the phylogenies we might infer. The FULL supermatrix was analyzed under the Maximum Likelihood framework using the site-homogeneous model of sequence evolution LG + Γ4 + F implemented in IQ-TREE (Nguyen et al. 2015) and node support was estimated with 100 bootstrap replicates. The REDUCED supermatrix was analyzed with the exact same approach, as well as using the site-heterogeneous LG4X + R4 + F sequence evolution model (also in IQ-TREE). Lastly, we also analyzed the REDUCED supermatrix under a Bayesian framework using the site-heterogeneous CAT + F81+Γ4 model implemented in PhyloBayes (Lartillot et al. 2013). We ran two independent MCMC chains until the two topologies obtained perfectly converged (maxdiff = 0 with a 50% burnin).

In Situ Hybridization

In situ hybridization on *B. schlosseri* was performed as in Ricci et al. (2016). In situ hybridization on *P. zorritensis* was performed on paraffin sections as follows. Samples at the desired stage were fixed in paraformaldehyde (PFA) 4%, DMSO 1%, DEPC 0.2%, RNase inhibitor (Sigma, R7397-30ML) 1/500 (=thereafter 1XPR), in Phosphate Buffer Saline (PBS 1×), then washed twice in PBS 1× and then dehydrated by graded methanol bath of 15 min (from 25% to 100% in PBS + 1XPR), then samples were immediately processed or stored at -20 °C. For paraffin embedding, samples were washed 2 × 10 min in butanol 1, then 1 h in butanol 1/paraffin at 60 °C, then overnight in paraffin at 60 °C. Paraffin blocks were allowed to solidify 8 h at room temperature and then were stored at 4 °C no more than two days. Paraffin sections were performed on Leica Microtome, at a thickness of 15 µm and attached on SuperFrost glass slides and allowed to dry overnight at 37 °C. All tools were previously washed with RNAse decontamination solution. Then in situ hybridizations were performed as follows: Slides were dried at 55 °C for 30 min, followed by two baths of xylene of 10 min each, then rehydrated by graded ethanol baths and then washed 2 × 10 min in a solution PBST (PBS 1× + 0.1% Tween 20) + 1XPR, for 5 min. Slides were then treated with Proteinase K (2.5 ng/µl in PBST + RNAse inhibitor) for 10 min at 37 °C, and immediately rinsed in PBST + 1XPR, and bath for 2 min in PBST. Then they were postfixed in PFA 4% + glutaraldehyde 0.2% in PBST + 1XPR for 30 min at room temperature, and rinsed in in PBST + 1XPR 3 × 5 min. Slides were pre hybridized in buffer (Formamide deionized 65%; 5× SSC; 1× denhardt's solution; 0.1% tween 20; 5 mg/ml torula yeast RNA; 50 µg/ml heparine; 1% Dextran sulfate) for 1 h, after what probes at a final dilution of 1.5–1.7 ng/µl in 300 µl of hybridization buffer, for 44 or 68 h at 58 °C. Chambers of hybridization (FRAME-SEAL BIORAD SLF 3001) were applied to the slides who accepted 300 µl of probe dilution. The slides were rinsed in 2× SSC pH 4.5, 50% Formamide, 0.1% Tween 20 + 1XPR, 3 × 30 min at 58 °C, then rinsed in PBST + PR 2 × 5 min and in MABT (MAB 1× + 0.1% Tween 20) + RNase inhibitor 1× 2 × 5 min (each bath). Then the slides were blocked in MABT, 2% Blocking solution, 10% goat serum for 1 h at RT. 500 µl of antidig-AP (1/2,000 in MABT, 2% blocking solution, 10% goat serum) was deposited directly on the slides overnight at 4 °C. Slides were rinsed in MABT 2 × 5 min, then rinsed TMNT filtered (tris–HCl ph 9.5 100 mM, NaCl 100 mM, $MgCl_2$ 50 mM, Levamisole 10 mM, 0.1% Tween 20) 2 × 5 min. Then NBT/BCIP in TMNT was directly applied on the slides until the coloration developed. The reaction was stopped by 2 × 5 min rinse in PBST. Then slides were dehydrated in successive baths of ethanol (50%, 70%, 90%, 100%), 3 min each, and finally in xylene for 15 min and mounted in Permount medium.

P. zorritensis Husbandry and Histology

Colonies of *P. zorritensis* were collected in Taranto and La Spezia (Italy), fixed on glass slides and cultured in a circulating

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

sea water system at 24 °C. The developing buds were fixed in 4% PFA in phosphate-buffered saline (PBS, pH 7.4) over night at 4 °C, dehydrated in a graded series of ethanol and butanol and then included in paraffin wax. Transversal section (5–6 μm think) were cut using a hand operated microtome and stained with hematoxylin and eosin (H&E) following this protocol: stain for 3 min in Mayer's hematoxylin (Hematoxylin Solution, Mayer's; Sigma–Aldrich), wash for 10 min in running tap water, stain for 15 s in Eosin Y solution (1 g Eosin Y powder in 20 ml Mq water and 80 ml 95% EtOH), rinse in 95% EtOH, dehydrated and mounted.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

We would like to thank Philippe Dru and Faisal Bekkouche for help with bioinformatics resources, Laurent Gilletta, Loann Gissat, Regis Lasbleiz, Sophie Collet, and Camille Lejeune for setting up the aqua-culture systems. Françoise Monniot for advice on taxonomy and sample identification. Sophie Booker, Elodie Bourrigaud, Laurent Lévêque, Ronan Garnier, Francesco Mastrototaro, and the Service Mer of Banyuls-sur-mer, EMBRC-France for sample collection. We thank Martine Fioroni for providing literature and old manuscripts that were difficult to obtain. This work was supported by FAPESP Jovem Pesquisador grant (JP 2015/50164-5) to F.B., ANR grant (ANR-14-CE02-0019-01) to S.T., FAPESP postdoctoral fellowship (grant number 2015/14052-8) to L.H., ANR grant (ANR-13-BSV2-0011-01) to P.L. and C.D.; ISEM 2018-057-SUD to P.D. and E.J.P.D., ANR grant (ANR-11-JSV2-007-01) to S.D.

Author Contributions

A.A., L.H., F.B., and S.T. conceived and designed the study, collected and identified biological material and wrote the manuscript. A.A., L.H., and P.S. designed the assembly and clustering pipeline and performed sequence analyses. P.S. conducted orthology and phylogenomic analyses. A.A. and M.S. established *P. zorritensis* culture and conducted histological analyses. M.M.P. conducted *NK4* in situ hybridization on *B. schlosseri*. A.A. and L.H. conducted DNA barcoding and phylogenetic analysis. S.L. performed *NK4* in situ hybridization on *P. zorritensis*. E.D. and F.D. participated in the pipeline design and supported part of the transcriptome sequencing. P.L. and C.D. sequenced genomes and provided gene models. S.D. and K.K. provided RNA for several species and participated to species identification. All authors read and approved the final version of the manuscript.

References

Abbott D. 1953. Asexual reproduction in the colonial ascidian Metandrocarpa taylori. *Univ. Calif. publ. zool.* 61(1):1–78.

Agata K, Saito Y, Nakajima E. 2007. Unifying principles of regeneration I: epimorphosis versus morphallaxis. *Dev Growth Differ.* 49(2):73–78.

Alié A, Hayashi T, Sugimura I, Manuel M, Sugano W, Mano A, Satoh N, Agata K, Funayama N. 2015. The ancestral gene repertoire of animal stem cells. *Proc Natl Acad Sci U S A.* 112(51):E7093–E7100.

Ballenghien M, Faivre N, Galtier N. 2017. Patterns of cross-contamination in a multispecies population genomic project: detection, quantification, impact, and solutions. *BMC Biol.* 15(1):25.

Barbeitos MS, Romano SL, Lasker HR. 2010. Repeated loss of coloniality and symbiosis in scleractinian corals. *Proc Natl Acad Sci U S A.* 107(26):11877–11882.

Bell G. 1982. The masterpiece of nature: the evolution and genetics of sexuality. Berkeley: University of California Press.

Bely AE, Nyberg KG. 2010. Evolution of animal regeneration: re-emergence of a field. *Trends Ecol Evol.* 25(3):161–170.

Berrill NJ. 1935. Studies in Tunicate development Part IV – Asexual reproduction. *Proc Philos Trans Roy Soc B.* 225(526):327–379.

Berrill NJ. 1948. The gonads, larvae, and budding of the polystyelid ascidians *Stolonica* and *Distomus*. *J Mar Biol Assoc UK.* 27(03):633–650.

Berrill NJ. 1950. The Tunicata, with an account of the British species. Vol. 133. London: The Ray Society. p. 354.

Boardman RS, Cheetham AH, Oliver WA. 1973. Animal colonies. Development and function through time. Stroudsburg (PA): Dowden, Hutchinson & Ross.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15):2114–2120.

Bosch TCG. 2007. Why polyps regenerate and we don't: towards a cellular and molecular framework for Hydra regeneration. *Dev Biol.* 303(2):421–433.

Bray NL, Pimentel H, Melsted P, Pachter L. 2016. Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol.* 34(5):525–527.

Brown FD, Swalla BJ. 2012. Evolution and development of budding by stem cells: ascidian coloniality as a case study. *Dev Biol.* 369(2):151–162.

Brunetti R, Mastrototaro F. 2004. The non-indigenous stolidobranch ascidian *Polyandrocarpa zorritensis* in the Mediterranean: description, larval morphology and pattern of vascular budding. *Zootaxa* 528(1):1–8.

Brusca RC, Moore W, Shuster SM. 2016. Invertebrates. Sunderland (MA): Sinauer Associates, Inc., Publishers.

Burgess SC, Ryan WH, Blackstone NW, Edmunds PJ, Hoogenboom MO, Levitan DR, Wulff JL. 2017. Metabolic scaling in modular animals. *Invertebr Biol.* 136(4):456–472.

Cabau C, Escudié F, Djari A, Guiguen Y, Bobe J, Klopp C. 2017. Compacting and correcting Trinity and Oases RNA-Seq de novo assemblies. *PeerJ* 5:e2988.

Cahais V, Gayral P, Tsagkogeorga G, Melo-Ferreira J, Ballenghien M, Weinert L, Chiari Y, Belkhir K, Ranwez V, Galtier N. 2012. Reference-free transcriptome assembly in non-model animals from next-generation sequencing data. *Mol Ecol Resour.* 12(5):834–845.

Carlson BM. 2007. Principles of regenerative biology. Burlington (MA): Academic Press.

Carreras-Carbonell J, Macpherson E, Pascual M. 2005. Rapid radiation and cryptic speciation in Mediterranean triplefin blennies (Pisces: tripterygiidae) combining multiple genes. *Mol Phylogenet Evol.* 37(3):751–761.

Christiaen L, Stolfi A, Levine M. 2010. BMP signaling coordinates gene expression and cell migration during precardiac mesoderm development. *Dev Biol.* 340(2):179–187.

Davidson NM, Oshlack A. 2014. Corset: enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biol.* 15(7):410.

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein DM. 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* 298(5601):2157–2167.

Delsuc F, Brinkmann H, Chourrout D, Philippe H. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* 439(7079):965–968.

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

Delsuc F, Philippe H, Tsagkogeorga G, Simion P, Tilak MK, Turon X, et al. 2018. A phylogenomic framework and timescale for comparative studies of tunicates. *BMC Biol*. 16(1):39.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol*. 3(5):294–299.

Freeman G. 1964. The role of blood cells in the process of asexual reproduction in the tunicate *Perophora viridis*. *J Exp Zool*. 156:157–184.

Fu L, Niu B, Zhu Z, Wu S, Li W. 2012. CD-HIT: accelerated for clustering the next generation sequencing data. *Bioinformatics* 28(23):3150–3152.

Fujiwara S, Isozaki T, Mori K, Kawamura K. 2011. Expression and function of myc during asexual reproduction of the budding ascidian *Polyandrocarpa misakiensis*. *Dev Growth Differ*. 53(9):1004–1014.

Funayama N, Nakatsukasa M, Mohri K, Masuda Y, Agata K. 2010. Piwi expression in archeocytes and choanocytes in demosponges: insights into the stem cell system in demosponges. *Evol Dev*. 12(3):275–287.

Galliot B, Chera S. 2010. The Hydra model: disclosing an apoptosis-driven generator of Wnt-based regeneration. *Trends Cell Biol*. 20(9):514–523.

Geller JB, Fitzgerald LJ, King CE. 2005. Fission in sea anemones: integrative studies of life cycle evolution. *Integr Comp Biol*. 45(4):615–622.

Godeaux J, Bone Q, Braconnot JC. 1998. Anatomy of Thaliaceans. In: Bone Q, editor. The biology of pelagic tunicates. Oxford, New York, Tokyo (Japan): Oxford University Press. p. 1–24.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al. 2011. Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nat Biotechnol*. 29(7):644–652.

Gurley KA, Elliott SA, Simakov O, Schmidt HA, Holstein TW, Sánchez Alvarado A. 2010. Expression of secreted Wnt pathway components reveals unexpected complexity of the planarian amputation response. *Dev Biol*. 347(1):24–39.

Higgins DG, Thompson JD, Gibson TJ. 1996. Using CLUSTAL for multiple sequence alignments. *Methods Enzymol*. 266:383–402.

Huang W, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc*. 4(1):44–57.

Hughes RN. 1989. A functional biology of clonal animals. London, New York: Chapman and Hall. p. 331

Hughes RN, Cancino JM. 1985. An ecological overview of cloning in Metazoa. In: Jackson JBC, Buss LW, Cook RE, editors. Population biology and evolution of clonal organisms. New Haven (CT): Yale University Press. p. 153–186.

Jackson JBC, Coates AG. 1986. Life cycles and evolution of clonal (modular) animals. *Philos Trans Roy Soc B*. 313(1159):7–22.

Jackson JBC. 1977. Competition on marine hard substrata: the adaptive significance of solitary and colonial strategies. *Am Nat*. 111(980):743–767.

Jeffery WR. 2007. Chordate ancestry of the neural crest: new insights from ascidians. *Semin Cell Dev Biol*. 18(4):481–491.

Kassmer SH, Rodriguez D, Langenbacher AD, Bui C, De Tomaso AW. 2015. Migration of germline progenitor cells is directed by sphingosine-1-phosphate signalling in a basal chordate. *Nat Commun*. 6(1):8565.

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol*. 30(4):772–780.

Kawamura K, Sugino Y, Sunanaga T, Fujiwara S. 2007. Multipotent epithelial cells in the process of regeneration and asexual reproduction in colonial tunicates. *Dev Growth Differ*. 50(1):1–11.

Kawamura K, Sunanaga T. 2010. Hemoblasts in colonial tunicates: are they stem cells or tissue-restricted progenitor cells? *Dev Growth Differ*. 52(1):69–76.

Kawamura K, Watanabe H. 1981. Studies of Japanese compound styelid ascidians. III. A new, possibly asexual *Polyandrocarpa* from Shimoda Bay. *Publ Seto Mar Biol Lab*. XXVI(4/6):425–436.

Kocot KM, Tassia MG, Halanych KM, Swalla BJ. 2018. Phylogenomics offers resolution of major tunicate relationships. *Mol Phylogenet Evol*. 121:166–173.

Kott P. 1985. The Australian Ascidiacea. Part 1, Phlebobranchia and Stolidobranchia. *Mem Qd Mus*. 21:1–440.

Kürn U, Rendulic S, Tiozzo S, Lauzon RJ. 2011. Asexual propagation and regeneration in colonial ascidians. *Biol Bull*. 221(1):43–61.

Laird DJ, De Tomaso AW, Weissman IL. 2005. Stem cells are units of natural selection in a colonial ascidian. *Cell* 123(7):1351.

Lartillot N, Brinkmann H, Philippe H. 2007. Suppression of long-branch attraction artefacts in the animal phylogeny using a site-heterogeneous model. *BMC Evol Biol*. 7(Suppl 1):S4.

Lartillot N, Rodrigue N, Stubbs D, Richer J. 2013. PhyloBayes MPI: phylogenetic reconstruction with infinite mixtures of profiles in a parallel environment. *Syst Biol*. 62(4):611–615.

Li L, Stoeckert CJ, Roos DS. 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res*. 13(9):2178–2189.

Manni L, Gasparini F, Hotta K, Ishizuka KJ, Ricci L, Tiozzo S, Voskoboynik A, Dauga D. 2014. Ontology for the asexual development and anatomy of the colonial chordate *Botryllus schlosseri*. *PLoS ONE*. 9(5):e96434.

Martinez VG, Menger GJIII, Zoran MJ. 2005. Regeneration and asexual reproduction share common molecular changes: upregulation of a neural glycoepitope during morphallaxis in *Lumbriculus*. *Mech Dev*. 122(5):721–732.

Meyer CP. 2003. Molecular systematics of cowries (Gastropoda: cypraeidae) and diversification patterns in the tropics. *Biol J Linnean Soc*. 79(3):401–459.

Monniot F. 2016. A new species of Polyandrocarpa (Ascidiacea, Styelidae) in the Mediterranean Sea. *Zootaxa* 4132(1):87–096.

Monniot C, Monniot F. 1972. Clé mondiale des genres d'Ascidies. Key to Ascidian Genera of the world. *Ach Zool Exp Gén*. 113:311–367.

Nakauchi M. 1982. Asexual development of ascidians: its biological significance, diversity, and morphogenesis. *Am Zool*. 22(4): 753–763.

Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 32(1):268–274.

Nilsson Sköld H, Obst M, Sköld M, Åkesson B. 2009. Stem cells in asexual reproduction of marine invertebrates. In: Rinkevich B, Matranga V. editors. Stem cells in marine organisms. Dordrecht (the Netherlands): Springer. p. 105.

Nogueira AF, Costa CM, Lorena J, Moreira RN, Frota-Lima GN, Furtado C, Robinson M, Amemiya CT, Darnet S, Schneider I. 2016. Tetrapod limb and sarcopterygian fin regeneration share a core genetic programme. *Nat Commun*. 7:13364.

Pérez-Portela R, Bishop J, Davis A, Turon X. 2009. Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences. *Mol Phylogenet Evol*. 50(3):560–570.

Philippe H, Brinkmann H, Lavrov DV, Littlewood DT, Manuel M, Wörheide G, Baurain D. 2011. Resolving difficult phylogenetic questions: why more sequences are not enough. *PLoS Biol*. 9(3):e1000602.

Philippe H, Vienne de DM, Ranwez V, Roure B, Baurain D, Delsuc F. 2017. Pitfalls in supermatrix phylogenomics. *Eur J Tax*. 283:1–25.

Piette J, Lemaire P. 2015. Thaliaceans, the neglected pelagic relatives of ascidians: a developmental and evolutionary enigma. *Q Rev Biol*. 90(2):117–145.

Ricci L, Cabrera F, Lotito S, Tiozzo S. 2016. Redeployment of germ layers related TFs shows regionalized expression during two non-embryonic developments. *Dev Biol*. 416(1):235–248.

Rinkevich Y, Rosner A, Rabinowitz C, Lapidot Z, Moiseeva E, Rinkevich B. 2010. Piwi positive cells that line the vasculature epithelium, underlie whole body regeneration in a basal chordate. *Dev Biol*. 345(1):94–104.

Rodriguez D, Sanders EN, Farell K, Langenbacher AD, Taketa DA, Hopper MR, Kennedy M, Gracey A, De Tomaso AW. 2014. Analysis of the basal chordate *Botryllus schlosseri* reveals a set of genes associated with fertility. *BMC Genomics*. 15:1183.

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

Roure B, Rodriguez-Ezpeleta N, Philippe H. 2007. SCaFoS: a tool for selection, concatenation and fusion of sequences for phylogenomics. *BMC Evol Biol*. 7(Suppl 1):S2.

Sánchez Alvarado AS. 2000. Regeneration in the metazoans: why does it happen? *Bioessays* 22(6):578.

Sanchez-Alvarado A, Yamanaka S. 2014. Rethinking differentiation: stem cells, regeneration, and plasticity. *Cell* 157(1):110–119.

Schwendinger P, Giribet G. 2005. The systematics of south—east Asian genus Fangensis Rambla (Opiliones: cyphophthalmi: stylocellidae). *Invertebr Syst*. 19(4):297–323.

Selys-Longchamps MD. 1917. Sur le bourgeonnement des Polystiélinés *Stolonica* et *Heterocarpa* avec quelques notes sur l'anatomie de ces deux genres. *Bull Sci France Belg*, L(3): 170–276.

Shenkar N, Koplovitz G, Dray L, Gissi C, Huchon D. 2016. Back to solitude: solving the phylogenetic position of the Diazonidae using molecular and developmental characters. *Mol Phylogenet Evol*. 100:51–56.

Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31(19):3210–3212.

Simion P, Belkhir K, François C, Veyssier J, Rink JC, Manuel M, Philippe H, Telford MJ. 2018. A software tool 'CroCo' detects pervasive cross-species contamination in next generation sequencing data. *BMC Biol*. 16(1):28.

Sorrentino M, Manni L, Lane NJ, Burighel P. 2001. Evolution of cerebral vesicles and their sensory organs in an ascidian larva. *Acta Zool*. 81(3):243–258.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30(9):1312–1313.

Stolfi A, Lowe EK, Racioppi C, Ristoratore F, Brown CT, Swalla BJ, Christiaen L. 2014. Divergent mechanisms regulate conserved cardiopharyngeal development and gene expression in distantly related ascidians. *Elife* 3:1–28.

Tatián M, Lagger C, Demarchi M, Mattoni C. 2011. Molecular phylogeny endorses the relationship between carnivorous and filter-feeding tunicates (Tunicata, Ascidiacea). *Zool Scr*. 40(6):603–612.

Tiozzo S, Copley R. 2015. Reconsidering regeneration in metazoans: an evo-devo approach. *Front Ecol Evol*. 3. doi:10.3389/fevo.2015.00067.

Tsagkogeorga G, Turon X, Hopcroft RR, Tilak MK, Feldstein T, Shenkar N, Loya Y, Huchon D, Douzery EJP, Delsuc F. 2009. An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. *BMC Evol Biol*. 9:187.

Turon X, López-Legentil S. 2004. Ascidian molecular phylogeny inferred from mtDNA data with emphasis on the Aplousobranchiata. *Mol Phylogenet Evol*. 33(2):309–320.

Velandia-Huerto CA, Gittenberger AA, Brown FD, Stadler PF, Bermúdez-Santana CI. 2016. Automated detection of ncRNAs in the draft genome sequence of a colonial tunicate: the carpet sea squirt *Didemnum vexillum*. *BMC Genomics*. 17:691.

Voskoboynik A, Neff NF, Sahoo D, Newman AM, Pushkarev D, Koh W, Passarelli B, Fan HC, Mantalas GL, Palmeri KJ, et al. 2013. The genome sequence of the colonial chordate, *Botryllus schlosseri*. *Elife*. 2:00569.

Wang W, Razy-Krajka F, Siu E, Ketcham A, Christiaen L. 2013. NK4 antagonizes Tbx1/10 to promote cardiac versus pharyngeal muscle fate in the ascidian second heart field. *PLoS Biol*. 11(12):e1001725.

Watanabe H, Tokioka T. 1972. *Two new species* and one possibly new race of social styelids from *Sagami Bay* with remarks on their life history especially the mode of *budding*. *Publ Seto Mar Biol Lab*. 19(5):327–345.

West SA, Lively CM, Read AF. 1999. A pluralist approach to sex and recombination. *J Evol Biol*. 12(6):1003–1012.

White MJD. 1978. Modes of speciation. San Francisco (CA): W.H. Freeman.

WoRMS Editorial Board. 2017. World Register of Marine Species [accessed 2017 December 07]. Available from: http://www.marinespecies.org at VLIZ. doi:10.14284/170.

Zattara EE, Bely AE. 2016. Phylogenetic distribution of regeneration and asexual reproduction in Annelida: regeneration is ancestral and fission evolves in regenerative clades. *Invertebr Biol*. 135(4):400–415.

Zeng L, Jacobs MW, Swalla BJ. 2006. Coloniality has evolved once in Stolidobranch Ascidians. *Integr Comp Biol*. 46(3):255–268.

Zondag LE, Rutherford K, Gemmell NJ, Wilson MJ. 2016. Uncovering the pathways underlying whole body regeneration in a chordate model, *Botrylloides leachi* using de novo transcriptome analysis. *BMC Genomics*. 17(1):114.

Downloaded from https://academic.oup.com/mbe/article-abstract/35/7/1728/4969531 by UPMC user on 01 August 2020

Annex 3

Curriculum Vitae- Marta Scelzo

Education

2016-present PhD – Ecole Doctorale ED515 "Complexité du vivant", Sorbonne Université (France)

2013-2016 Master in Biology of marine productions- University of Napoli "Federico II" (Italy)

2009-2013 Bachelor in Biology of marine productions –University of Napoli "Federico II" (Italy)

2004-2009 High school "Liceo classico Plinio Seniore", Castellammare di Stabia (Italy)

Professional

2016-present UMR7009 Laboratoire de biologie du développement de Villefranche-sur-Mer – Institut de la Mer de Villefranche (IMEV) (France) – "Regeneration and pluripotency" Team: PhD student (supervisor Dr. Stefano Tiozzo)

July 2015- July 2016 Stazione zoological "Anton Dohrn", Napoli (Italy) – Ristoratore Team : Master student (supervisor Dr. Filomena Ristoratore). Project: Transcriptional control of *KLHL21/24/29*, a new player in *Ciona robusta* pigmentation.

2016- From January to February Stazione zoological "Anton Dohrn", Napoli (Italy) – D'Aniello Team : trainee student (supervisor Dr. D'Aniello Salvatore). Project: Expression pattern of *Rab* genes in *Branchiostoma lanceolatum.*

October 2012- May 2013 University of Napoli "Federico II" (Italy) : undergraduated student (supervisor Prof. Chiara Maria Motta). Project: Immunolocalization of IGF-1 receptor in the retina of *Danio rerio* exposed to stressors

Scientific communication

July 2019 10th International Tunicate Meeting (Villefranche sur Mer) Oral presentation: "Novel budding mode in *Polyandrocarpa zorritensis*: a model for comparative studies on asexual reproduction and whole body regeneration"

October 2018 GDR Stem cells meeting (Paris) Oral presentation: "*Polyandrocarpa zorritensis*: a model for comparative studies on budding and whole body regeneration"

July 2018 7th meeting of the European society for Evolutionary Developmental Biology (EuroEvoDevo) (Galway, Ireland) Poster presentation: "*Polyandrocarpa zorritensis*: a model for comparative studies on budding and whole body regeneration"

March 2018 9th meeting of André Picard Network (Paris) Oral presentation: "Novel form of asexual development in Styelid ascidians: vasal budding in *Polyandrocarpa zorritensis"*

September 2017 Young researchers' day - Ethic and Science (Villefranche-sur-Mer) Crisp-Cas9 session, oral presentation: "Transcriptional control of KLHL21/24/29, a new player in Ciona robusta pigmentation"

July 2017 9th International Tunicate Meeting (New York) Poster presentation: "*Polyandrocarpa zorritensis*: a new model for comparative studies on budding and whole body regeneration"

Pubblications

Alié A, Hiebert LS, Simion P, **Scelzo M**, Prünster MM, Lotito S, Delsuc F, Douzery EJP, Dantec C, Lemaire P, Darras S, Kawamura K, Brown FD, Tiozzo S. Convergent Acquisition of Non embryonic Development in Styelid Ascidians. *Mol Biol Evol. 2018 Jul1;35(7):1728-1743. doi: 10.1093/molbev/msy068.*

Coppola U, Caccavale F, **Scelzo M**, Holland ND, Ristoratore F, D'Aniello S. Ran GTPase, an eukaryotic gene novelty, is involved in amphioxus mitosis. *PLoS One. 2018 Oct 9;13(10):e0196930. doi:10.1371/journal.pone.0196930.*

Scelzo M, Alié A, Pagnotta S, Lejeune CS, Henry P, Gilletta L, Hiebert LS, Mastrototaro F, Tiozzo S. Novel budding mode in Polyandrocarpa zorritensis: a model for comparative studies on asexual development and whole body regeneration. *EvoDevo 10, 7 (2019). doi:10.1186/s13227-019-0121-x*

Alié A, Hiebert LS, **Scelzo M**, Tiozzo S. The eventful history of non-embryonic developments in tunicates. *J Exp Zool (Mol Dev Evol). 2020; 1-17 doi:10.1002/jez.b.22940*

Teaching and citizen science

5th February – 30th July 2018 : student supervision (Marie Deschler, Master 1 student, Université de Nice Sophia Antipolis - Master Science de la vie et de la santé) – Project : « Hematological analysis during the regeneration of the ascidian *Polyandrocarpa zorritensis* »

4th-6th October 2019: participation to « Fête de la science 2019 », Institut de la Mer de Villefranche sur Mer (IMEV) - stand «Le monde secret d'un laboratoire de biologie au bord de la mer»

13th- 19th December 2019: students supervision (DOMO Course Mini project – International program MU5BM200, Sorbonne Université)

Prizes

Best talk prize: July 2019 10th International Tunicate Meeting (Villefranche sur Mer), title of the presentation: "Novel budding mode in Polyandrocarpa zorritensis: a model for comparative studies on asexual reproduction and whole body regeneration"

Courses and workshops

SU Schmid training course: 06-17 March 2017 (60 hours)

EMBO MAMED course: 02-17 July 2017 (80 hours)

Photoshop- Niveau debutant: 20 July 2017 (3 hours)

ADOBE Illustrator- Niveau debutant: 27 July 2017: (3 hours)

Français langue étrangère – French classes: November 2017- May 2018 Délégation cote d'Azur du CNRS (38 hours)

Manipulation d'images avec le logiciel ImageJ : 11/06/2018 Formation DR20 CNRS (7 hours)

References

Adell, T., Cebrià, F., and Saló, E. (2010). Gradients in Planarian Regeneration and Homeostasis. Cold Spring Harb Perspect Biol *2*, a000505.

Akhmadieva, A.V., Shukalyuk, A.I., Aleksandrova, Ya.N., and Isaeva, V.V. (2007). Stem cells in asexual reproduction of the colonial ascidian Botryllus tubaratus (Tunicata: Ascidiacea). Russ J Mar Biol *33*, 181–186.

Albelda, S.M., and Buck, C.A. (1990). Integrins and other cell adhesion molecules. The FASEB Journal *4*, 2868–2880.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002a). Molecular Biology of the Cell (Garland Science).

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002b). Integrins. Molecular Biology of the Cell. 4th Edition.

Aldridge, R.J., Xian-Guang, H., Siveter, D.J., Siveter, D.J., and Gabbott, S.E. (2007). The Systematics and Phylogenetic Relationships of Vetulicolians. Palaeontology *50*, 131–168.

Alekseev, V., and Lampert, W. (2004). Maternal Effects of Photoperiod and Food Level on Life History Characteristics of the Cladoceran Daphnia Pulicaria Forbes. Hydrobiologia *526*, 225–230.

Alekseev, V., and Pinel-Alloul, B. (2019). Dormancy in Aquatic Organisms. Theory, Human Use and Modeling (Springer International Publishing).

Alekseev, V.R., and Vinogradova, E.B. (2019). Introduction to Dormancy in Aquatic Invertebrates: Mechanism of Induction and Termination, Hormonal and Molecular-Genetic Basis. In Dormancy in Aquatic Organisms. Theory, Human Use and Modeling, V.R. Alekseev, and B. Pinel-Alloul, eds. (Cham: Springer International Publishing), pp. 7–41.

Alié, A., Hayashi, T., Sugimura, I., Manuel, M., Sugano, W., Mano, A., Satoh, N., Agata, K., and Funayama, N. (2015). The ancestral gene repertoire of animal stem cells. PNAS *112*, E7093–E7100.

Alié, A., Hiebert, L.S., Simion, P., Scelzo, M., Prünster, M.M., Lotito, S., Delsuc, F., Douzery, E.J.P., Dantec, C., Lemaire, P., et al. (2018). Convergent Acquisition of Nonembryonic Development in Styelid Ascidians. Mol Biol Evol *35*, 1728–1743.

Alié, A., Hiebert, L.S., Scelzo, M., and Tiozzo, S. (2020). The eventful history of nonembryonic development in tunicates. J. Exp. Zool. (Mol. Dev. Evol.).

Alié, A., Hiebert, L.S., Scelzo, M., and Tiozzo, S. The eventful history of nonembryonic development in tunicates. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution *n/a*.

Alvarado, A.S. (2000). Regeneration in the metazoans: why does it happen? BioEssays *22*, 578–590.

Alvarado, A.S., and Tsonis, P.A. (2006). Bridging the regeneration gap: genetic insights from diverse animal models. Nature Reviews Genetics *7*, 873–884.

Amiel, A.R., Johnston, H.T., Nedoncelle, K., Warner, J.F., Ferreira, S., and Röttinger, E. (2015). Characterization of Morphological and Cellular Events Underlying Oral Regeneration in the Sea Anemone, Nematostella vectensis. International Journal of Molecular Sciences *16*, 28449–28471.

Amiel, A.R., Foucher, K., Ferreira, S., and Röttinger, E. (2019). Synergic coordination of stem cells is required to induce a regenerative response in anthozoan cnidarians. BioRxiv 2019.12.31.891804.

Anderson, V. (1998). Salp and pyrosomid blooms and their importance in biogeochemical cycles. The Biology of Pelagic Tunicates 125–137.

Andrews (2010). Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data.

Arai, M.N. (2009). The potential importance of podocysts to the formation of scyphozoan blooms: a review. In Jellyfish Blooms: Causes, Consequences, and Recent Advances: Proceedings of the Second International Jellyfish Blooms Symposium, Held at the Gold Coast, Queensland, Australia, 24–27 June, 2007, K.A. Pitt, and J.E. Purcell, eds. (Dordrecht: Springer Netherlands), pp. 241–246.

Baguñà, J. (2012). The planarian neoblast: the rambling history of its origin and some current black boxes. Int. J. Dev. Biol. *56*, 19–37.

Baguna, J., Salo, E., and Auladell, C. (1989). Regeneration and pattern formation in planarians. III. that neoblasts are totipotent stem cells and the cells. Development *107*, 77–86.

Balcer, L.J., and Black, R.E. (1991). Budding and strobilation inAurelia (Scyphozoa, Cnidaria): Functional requirement and spatial patterns of nucleic acid synthesis. Roux's Arch Dev Biol *200*, 45–50.

Ballarin, L. (2008). Immunobiology of compound ascidians, with particular reference to Botryllus schlosseri: state of art.

Ballarin, L., and Cima, F. (2005a). Cytochemical properties of Botryllus schlosseri haemocytes: indications for morpho-functional characterisation. European Journal of Histochemistry 255–264.

Ballarin, L., and Cima, F. (2005b). Cytochemical properties of Botryllus schlosseri haemocytes: indications for morpho-functional characterisation. European Journal of Histochemistry 255–264.

Ballarin, L., and Kawamura, K. (2009). The hemocytes of Polyandrocarpa mysakiensis: morphology and immune-related activities. Invertebrate Survival Journal *6*, 154–161.

Ballarin, L., Cima, F., and Sabbadin, A. (1993). Histoenzymatic staining and characterization of the colonial ascidian Botryllus schlösseri hemocytes. Bollettino Di Zoologia *60*, 19–24.

Ballarin, L., Cima, F., Floreani, M., and Sabbadin, A. (2002). Oxidative stress induces cytotoxicity during rejection reaction in the compound ascidian Botryllus schlosseri. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology *133*, 411–418.

Ballarin, L., Franchi, N., Schiavon, F., Tosatto, S.C.E., Mičetić, I., and Kawamura, K. (2012). Looking for putative phenoloxidases of compound ascidians: Haemocyanin-like proteins in Polyandrocarpa misakiensis and Botryllus schlosseri. Developmental & Comparative Immunology *38*, 232–242.

Barker, N. (2014). Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. Nature Reviews Molecular Cell Biology *15*, 19–33.

Barr, H.J. (1964). Regeneration and Natural Selection. The American Naturalist *98*, 183–186.

Barth, L.G., and Barth, L.J. (1966). A study of regression and budding in Perophora viridis. Journal of Morphology *118*, 451–459.

Barton, N.H., and Charlesworth, B. (1998). Why sex and recombination? Science *281*, 1986–1990.

Bates, W.R. (2005). Environmental factors affecting reproduction and development in ascidians and other protochordates. Can. J. Zool. *83*, 51–61.

Bates, W.R., and Mallett, J.E. (1991). Ultrastructural and histochemical study of anural development in the ascidian Molgula pacifica (Huntsman). Roux's Arch Dev Biol *200*, 193–201.

Beers, C.D. (1923). SOME POINTS IN THE BUD DEVELOPMENT OF A SIMPLE ASCIDIAN, ECTEINASCIDIA TURBINATA HERDMAN. Journal of the Elisha Mitchell Scientific Society *39*, 92–94.

Bell, G. (1982). The Masterpiece of Nature :: The Evolution and Genetics of Sexuality (CUP Archive).

Bely, A.E., and Nyberg, K.G. (2010). Evolution of animal regeneration: re-emergence of a field. Trends in Ecology & Evolution *25*, 161–170.

Belozerov, V.N. New aspects in investigations of diapause and non-diapause dormancy types in insects and other arthropods. *Entmol. Rev.* **89,** 127–136 (2009).

Berrill, N.J. (1931). Regeneration in Sabella pavonina (Sav.) and other sabellid worms. Journal of Experimental Zoology *58*, 495–523.

Berrill, N.J. (1940). THE DEVELOPMENT OF A COLONIAL ORGANISM: SYMPLEGMA VIRIDE. The Biological Bulletin *79*, 272–281.

Berrill, N.J. (1941). SIZE AND MORPHOGENESIS IN THE BUD OF BOTRYLLUS. The Biological Bulletin *80*, 185–193.

Berrill, N.J. (1947). The Structure, Tadpole and Budding of the Ascidian Pycnoclavella Aurilucens Garstang. Journal of the Marine Biological Association of the United Kingdom *27*, 245–251.

Berrill, N.J. (1948a). The Development, Morphology and Budding of the Ascidian Diazona. Journal of the Marine Biological Association of the United Kingdom *27*, 389–399.

Berrill, N.J. (1948b). Budding and the Reproductive Cycle of Distaplia. Journal of Cell Science *s3-89*, 253–289.

Berrill, N.J. (1948c). The Gonads, Larvae, and Budding of the Polystyelid Ascidians Stolonica and Distomus. Journal of the Marine Biological Association of the United Kingdom *27*, 633–650.

Berrill, N.J. (1950). The Tunicata with an Account of the British Species (Quaritch).

Berrill, N.J. (1951). Regeneration and Budding in Tunicates. Biological Reviews *26*, 456–475.

Berrill, N.J. (1952). Regeneraton and Budding in Worms. Biological Reviews *27*, 401–438.

Berrill, N.J., and Cohen, A. (1936). Regeneration in Clavelina lepadiformis. Journal of Experimental Biology *13*, 352–362.

Berrill, N.J., and Watson, D.M.S. (1935). IX — Studies in tunicate development Part IV—Asexual reproduction. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences *225*, 327–379.

Bertolani, R., Guidetti, R., Altiero, T., Nelson, D.R., and Rebecchi, L. (2019). Dormancy in Freshwater Tardigrades. In Dormancy in Aquatic Organisms. Theory, Human Use and Modeling, V.R. Alekseev, and B. Pinel-Alloul, eds. (Cham: Springer International Publishing), pp. 43–51.

Bickler, P.E., and Buck, L.T. (2007). Hypoxia Tolerance in Reptiles, Amphibians, and Fishes: Life with Variable Oxygen Availability. Annual Review of Physiology *69*, 145–170.

Bielefeld, K.A., Amini-Nik, S., and Alman, B.A. (2013). Cutaneous wound healing: recruiting developmental pathways for regeneration. Cell. Mol. Life Sci. *70*, 2059–2081.

Bilello, A.A., and Potswald, H.E. (1974). A cytological and quantitative study of neoblasts in the naidOphidonais serpentina (Oligochaeta). Wilhelm Roux Arch Entwickl Mech Org *174*, 234–249.

Bilton, D.T., Freeland, J.R., and Okamura, B. (2001). Dispersal in Freshwater Invertebrates. Annual Review of Ecology and Systematics *32*, 159–181.

Birnbaum, K.D., and Alvarado, A.S. (2008). Slicing across Kingdoms: Regeneration in Plants and Animals. Cell *132*, 697–710.

Black, R.E. (1981). Metabolism and ultrastructure of dormant podocysts of Chrysaora quinquecirrha (scyphozoa). Journal of Experimental Zoology *218*, 175–182.

Blanchoud, S., Zondag, L., Lamare, M.D., and Wilson, M.J. (2017). Hematological Analysis of the Ascidian Botrylloides leachii (Savigny, 1816) During Whole-Body Regeneration. The Biological Bulletin *232*, 143–157.

Bocharova, E. (2016). Reproduction of Sea Anemones and Other Hexacorals. In The Cnidaria, Past, Present and Future: The World of Medusa and Her Sisters, S. Goffredo, and Z. Dubinsky, eds. (Cham: Springer International Publishing), pp. 239–248.

Bode, H.R. (1996). The interstitial cell lineage of hydra: a stem cell system that arose early in evolution. J. Cell. Sci. *109 (Pt 6)*, 1155–1164.

Bode, H.R. (2003). Head regeneration in Hydra. Developmental Dynamics *226*, 225–236.

Bode, P.M., and Bode, H.R. (1984). Formation of pattern in regenerating tissue pieces of Hydra attenuata: II. Degree of proportion regulation is less in the hypostome and tentacle zone than in the tentacles and basal disc. Developmental Biology *103*, 304–312.

Bollner, T. (1989). Regeneration of the Central Nervous System of the Ascidian Ciona Intestinalis. In Recent Trends in Regeneration Research, V. Kiortsis, S. Koussoulakos, and H. Wallace, eds. (Boston, MA: Springer US), pp. 281–287.

Bollner, T., Beesley, P.W., and Thorndyke, M.C. (1992). Pattern of substance P- and cholecystokinin-like immunoreactivity during regeneration of the neural complex in the ascidian Ciona intestinalis. Journal of Comparative Neurology *325*, 572–580.

Borena, B.M., Martens, A., Broeckx, S.Y., Meyer, E., Chiers, K., Duchateau, L., and Spaas, J.H. (2015). Regenerative Skin Wound Healing in Mammals: State-of-the-Art on Growth Factor and Stem Cell Based Treatments. CPB *36*, 1–23.

Bosch, T.C.G. (2007). Why polyps regenerate and we don't: Towards a cellular and molecular framework for Hydra regeneration. Developmental Biology *303*, 421–433.

Bosch, T.C.G., and David, C.N. (1987). Stem cells of Hydra magnipapillata can differentiate into somatic cells and germ line cells. Developmental Biology *121*, 182–191.

Bossert, P.E., Dunn, M.P., and Thomsen, G.H. (2013). A staging system for the regeneration of a polyp from the aboral physa of the anthozoan Cnidarian Nematostella vectensis. Developmental Dynamics *242*, 1320–1331.

Braconnot, J.-C. (1970). Contribution à l'étude des stades successifs dans le cycle des tuniciers pélagiques doliolides: Les stades larvaire, oozoide, nourrice et gastrozoide (Editeur inconnu).

Braun, K., Leubner, F., and Stach, T. (2020). Phylogenetic analysis of phenotypic characters of Tunicata supports basal Appendicularia and monophyletic Ascidiacea. Cladistics *36*, 259–300.

Bray, N.L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. Nature Biotechnology *34*, 525–527.

Brewin, B.I. (1959). An Account of Larval Budding in the Compound Ascidian, Hypsistozoa fasmeriana. Journal of Cell Science *s3-100*, 575–589.

Brien, P. (1930). Contribution à l'étude de la régénération naturelle et expérimentale chez les Clavelinidae. Annales de la Société royale zoologique de Belgique, 61, 19– 112.

Brien, P. (1948). Embranchement des Tuniciers. In P. P. Grassé (Ed.), Traité de Zoologie, Anatomie, Systématique, Biologie (11, pp. 553– 930). Paris, France: Masson et Cie.

Brien, P., Reniers-Decoen, M. (1955): La signification des cellules interstitielles des hydros d'eau douce et le probleme de la reserve embryonnaire. Bull. biol. France Belg. 89, 258 325.

Brien, P., & Brien-Gavage, E. (1927). Contribution à l'étude de la blastogenèse des Tuniciers. III. Bourgeonnement de Clavelina lepadiformis Müller. Recueil de l'Institut zoologique Torley-Rousseau, 1, 31– 81.

Brien, P., & Brien-Gavage, E. (1928). Contribution à l'étude de la blastogenèse des Tuniciers. IV. Recherches sur le Bourgeonnement de Perophora Listeri Weigm. Origine mésoblastique du septum stolonial. Recueil de l'Institut zoologique Torley-Rousseau, 1, 123– 151.

Briski, E., Bailey, S.A., and MacIsaac, H.J. (2011). Invertebrates and their dormant eggs transported in ballast sediments of ships arriving to the Canadian coasts and the Laurentian Great Lakes. Limnology and Oceanography *56*, 1929–1939.

Brockes, J.P., and Kumar, A. (2008). Comparative Aspects of Animal Regeneration. Annual Review of Cell and Developmental Biology *24*, 525–549.

Brockes, J.P., Kumar, A., and Velloso, C.P. (2001). Regeneration as an evolutionary variable. The Journal of Anatomy *199*, 3–11.

Brookfield, M.E. (1988). A mid-Ordovician temperate carbonate shelf-the black river and trenton limestone groups of southern Ontario, Canada. Sedimentary Geology *60*, 137–153.

Brooks, W.K., and Metcalf, M.M. (1893). The Genus Salpa (Johns Hopkins Press).

Brown, F.D., and Swalla, B.J. (2007). Vasa expression in a colonial ascidian, Botrylloides violaceus. Evolution & Development *9*, 165–177.

Brown, F.D., and Swalla, B.J. (2012). Evolution and development of budding by stem cells: Ascidian coloniality as a case study. Developmental Biology *369*, 151–162.

Brown, F.D., Keeling, E.L., Le, A.D., and Swalla, B.J. (2009). Whole body regeneration in a colonial ascidian, Botrylloides violaceus. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution *312B*, 885–900.

Brunetti, R. (1978). POLYANDROCARPA ZORRITENSIS (VAN NAME, 1931) A COLONIAL ASCIDIAN NEW TO THE MEDITERRANEAN RECORD. POLYANDROCARPA ZORRITENSIS (VAN NAME, 1931) A COLONIAL ASCIDIAN NEW TO THE MEDITERRANEAN RECORD.

Brunetti, R., and Mastrototaro, F. (2004). The non-indigenous stolidobranch ascidian Polyandrocarpa zorritensis in the Mediterranean: description, larval morphology and pattern of vascular budding. Zootaxa *528*, 1–8.

Brusca, R.C., and Brusca, G.J. (2003). Invertebrates (Sinauer Associates).

Bryant, D.M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M.B., Payzin-Dogru, D., Lee, T.J., Leigh, N.D., Kuo, T.-H., Davis, F.G., et al. (2017). A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. Cell Reports *18*, 762–776.

Bryant, S.V., Endo, T., and Gardiner, D.M. (2004). Vertebrate limb regeneration and the origin of limb stem cells. Int. J. Dev. Biol. *46*, 887–896.

Burighel, P., and Milanesi, C. (1973). Fine structure of the gastric epithelium of the ascidian botryllus schlosseri vacuolated and zymogenic cells. Z.Zellforsch *145*, 541–555.

Burighel, P., and Milanesi, C. (1975). Fine structure of the gastric epithelium of the ascidian Botryllus schlosseri. Mucous, endocrine and plicated cells. Cell Tissue Res. *158*, 481–496.

Burighel, P., and Schiavinato, A. (1984). Degenerative regression of the digestive tract in the colonial ascidian Botryllus schlossen (Pallas). Cell Tissue Res. *235*, 309–318.

Burighel, P., Brunetti, R., and Zaniolo, G. (1976). Hibernation of the Colonial Ascidian *Botrylloides Leachi* (Savigny): Histological Observations. Bolletino Di Zoologia *43*, 293–301.

Burighel, P., Milanesi, C., and Sabbadin, A. (1983). Blood Cell Ultrastructure of the Ascidian Botryllus schlosseri L. II. Pigment Cells. Acta Zoologica *64*, 15–23.

Burton, P.M., and Finnerty, J.R. (2009). Conserved and novel gene expression between regeneration and asexual fission in Nematostella vectensis. Dev. Genes Evol. *219*, 79–87.

Bushnell, J.H., and Rao, K.S. (1974). Dormant or Quiescent Stages and Structures among the Ectoprocta: Physical and Chemical Factors Affecting Viability and Germination of Statoblasts. Transactions of the American Microscopical Society *93*, 524–543.

Buzgariu, W., Wenger, Y., Tcaciuc, N., Catunda-Lemos, A.-P., and Galliot, B. (2018). Impact of cycling cells and cell cycle regulation on Hydra regeneration. Developmental Biology *433*, 240–253.

Cáceres, C.E. (1997). Dormancy in Invertebrates. Invertebrate Biology *116*, 371–383.

Campbell, R.D. (1976). Elimination by Hydra interstitial and nerve cells by means of colchicine. Journal of Cell Science *21*, 1–13.

Caprioli, M., and Ricci, C. (2001). Recipes for successful anhydrobiosis in bdelloid rotifers. In Rotifera IX, L. Sanoamuang, H. Segers, R.J. Shiel, and R.D. Gulati, eds. (Dordrecht: Springer Netherlands), pp. 13–17.

Carlson, B.M. (2007). Principles of Regenerative Biology (Elsevier).

Carman, M., Bullard, S.G., Rocha, R.M. da, Lambert, G., Dijkstra, J.A., Roper, J.J., Goodwin, A.M., Carman, M.M., and Vail, E.M. (2011). Ascidians at the Pacific and Atlantic entrances to the Panama Canal. p.

Carmona, M.J., Dimas-Flores, N., García-Roger, E.M., and Serra, M. (2009). Selection of low investment in sex in a cyclically parthenogenetic rotifer. J. Evol. Biol. *22*, 1975–1983.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental Biology *46*, 326–342.

Cebrià, F., Adell, T., and Saló, E. (2018). Rebuilding a planarian: from early signaling to final shape. Int. J. Dev. Biol. *62*, 537–550.

Chapman (1968). Structure, histochemistry and formation of the podocyst and cuticle of Aurelia aurita | Journal of the Marine Biological Association of the United Kingdom | Cambridge Core.

Chen, J.-Y., Huang, D.-Y., Peng, Q.-Q., Chi, H.-M., Wang, X.-Q., and Feng, M. (2003). The first tunicate from the Early Cambrian of South China. PNAS *100*, 8314–8318.

Cima, F. (2010). Microscopy methods for morpho-functional characterisation of marine invertebrate haemocytes.

Cima, F., Ballarin, L., and Sabbadin, A. (1996). New data on phagocytes and phagocytosis in the compound ascidian *Botryllus schlosseri* (Tunicata, Ascidiacea). Italian Journal of Zoology *63*, 357–364.

Cima, F., Perin, A., Burighel, P., and Ballarin, L. (2001). Morpho-functional characterization of haemocytes of the compound ascidian Botrylloides leachi (Tunicata, Ascidiacea). Acta Zoologica *82*, 261–274.

Cima, F., Sabbadin, A., and Ballarin, L. (2004). Cellular aspects of allorecognition in the compound ascidian Botryllus schlosseri. Developmental & Comparative Immunology *28*, 881–889.

Cima, F., Caicci, F., and Sordino, P. (2014). The haemocytes of the salp Thalia democratica (Tunicata, Thaliacea): an ultrastructural and histochemical study in the oozoid. Acta Zoologica *95*, 375–391.

Cima, F., Franchi, N., and Ballarin, L. (2016). Chapter 2 - Origin and Functions of Tunicate Hemocytes. In The Evolution of the Immune System, D. Malagoli, ed. (Academic Press), pp. 29–49.

Cima, F., Peronato, A., and Ballarin, L. (2017). The haemocytes of the colonial aplousobranch ascidian Diplosoma listerianum: Structural, cytochemical and functional analyses. Micron *102*, 51–64.

Cima, F., Caicci, F., Nittoli, V., Marino, R., Crocetta, F., and Sordino, P. (2018). Immunological response to bacterial infection in a pelagic tunicate: Inflammation in the salp Thalia democratica. Journal of Invertebrate Pathology *159*, 28–40.

Civin, C.I., Trischmann, T., Kadan, N.S., Davis, J., Noga, S., Cohen, K., Duffy, B., Groenewegen, I., Wiley, J., Law, P., et al. (1996). Highly purified CD34-positive cells reconstitute hematopoiesis. JCO *14*, 2224–2233.

van Cleave, C.D. (1937). A Study of the Process of Fission in the Naid Pristina longiseta. Physiological Zoology *10*, 299–314.

Clegg, J.S. (2001). Cryptobiosis — a peculiar state of biological organization. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology *128*, 613–624.

Clevers, H., Loh, K.M., and Nusse, R. (2014). An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science *346*.

Cloney, R.A. (1982). Ascidian Larvae and the Events of Metamorphosis. Integr Comp Biol *22*, 817–826.

Conklin, E.G. (1905). Mosaic development in ascidian eggs. Journal of Experimental Zoology *2*, 145–223.

Conklin, E.G. (1906). Does half of an Ascidian egg give rise to a whole larva? Archiv Für Entwicklungsmechanik Der Organismen *21*, 727–753.

Corey, D.M., Rosental, B., Kowarsky, M., Sinha, R., Ishizuka, K.J., Palmeri, K.J., Quake, S.R., Voskoboynik, A., and Weissman, I.L. (2016). Developmental cell death programs license cytotoxic cells to eliminate histocompatible partners. PNAS *113*, 6520–6525.

Corliss, J.O. (2001). Protozoan Cysts and Spores. ELS.

Crow, J.F. (1992). An advantage of sexual reproduction in a rapidly changing environment. J. Hered. *83*, 169–173.

Crowe, J.H. (1971). Anhydrobiosis: An Unsolved Problem. The American Naturalist *105*, 563–573.

Crowe, J.H., Hoekstra, F.A., and Crowe, L.M. (1992). Anhydrobiosis. Annual Review of Physiology *54*, 579–599.

Cummings, S.G., and Bode, H.R. (1984). Head regeneration and polarity reversal inHydra attenuata can occur in the absence of DNA synthesis. Wilhelm Roux' Archiv *194*, 79–86.

Darling, J.A., Reitzel, A.R., Burton, P.M., Mazza, M.E., Ryan, J.F., Sullivan, J.C., and Finnerty, J.R. (2005). Rising starlet: the starlet sea anemone, Nematostella vectensis. BioEssays *27*, 211–221.

David, C.N., and Campbell, R.D. (1972). Cell Cycle Kinetics and Development of Hydra Attenuata : I. Epithelial Cells. Journal of Cell Science *11*, 557–568.

David, C.N., and Plotnick, I. (1980). Distribution of interstitial stem cells in Hydra. Developmental Biology *76*, 175–184.

Decaestecker, E., De Meester, L., and Mergeay, J. (2009). Cyclical Parthenogenesis in Daphnia: Sexual Versus Asexual Reproduction. In Lost Sex: The Evolutionary Biology of Parthenogenesis, I. Schön, K. Martens, and P. Dijk, eds. (Dordrecht: Springer Netherlands), pp. 295–316.

Deck, J.D., Hay, E.D., and Revel, J.-P. (1966). Fine structure and origin of the tunic of Perophora viridis. Journal of Morphology *120*, 267–280.

Della Valle, P. (1915). Studii sui rapporti fra differenziazione e rigenerazione - 3. Lo sviluppo di segmenti isolati di stolone di *Clavelina* di calibro eguale e di lunghezza diversa. *Bollettino della Società' di naturalisti in Napoli*, **27**, 195– 237.

Delsuc, F., Brinkmann, H., Chourrout, D., and Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature *439*, 965–968.

Delsuc, F., Philippe, H., Tsagkogeorga, G., Simion, P., Tilak, M.-K., Turon, X., López-Legentil, S., Piette, J., Lemaire, P., and Douzery, E.J.P. (2018). A phylogenomic framework and timescale for comparative studies of tunicates. BMC Biol *16*, 39.

Denekamp, N.Y., Thorne, M.A., Clark, M.S., Kube, M., Reinhardt, R., and Lubzens, E. (2009). Discovering genes associated with dormancy in the monogonont rotifer Brachionus plicatilis. BMC Genomics *10*, 108.

Denekamp, N.Y., Reinhardt, R., Albrecht, M.W., Drungowski, M., Kube, M., and Lubzens, E. (2011). The expression pattern of dormancy-associated genes in multiple life-history stages in the rotifer Brachionus plicatilis. Hydrobiologia *662*, 51–63.

Denlinger, D.L., Yocum, G.D., and Rinehart, J.P. (2012). 10 - Hormonal Control of Diapause. In Insect Endocrinology, L.I. Gilbert, ed. (San Diego: Academic Press), pp. 430–463.

Deviney, E.M. (1934). THE BEHAVIOR OF ISOLATED PIECES OF ASCIDIAN (PEROPHORA VIRIDIS) STOLON AS COMPARED WITH ORDINARY BUDDING. Journal of the Elisha Mitchell Scientific Society *49*, 185–224.

Dharani, G., and Altaff, K. (2004). Ultra structure of subitaneous and diapausing eggs of planktonic copepod Sinodiaptomus (Rhinediaptomus) indicus. Current Science *87*, 109–112.

Diehl, F.A., and Burnett, A.L. (1964). The role of interstitial cells in the maintenance of hydra. I. Specific destruction of interstitial cells in normal, asexual, non-budding animals. Journal of Experimental Zoology *155*, 253–259.

Dijkstra, J., Dutton, A., Westerman, E., and Harris, L. (2008). Heart rate reflects osmostic stress levels in two introduced colonial ascidians Botryllus schlosseri and Botrylloidesviolaceus. Mar Biol *154*, 805–811.

Docheva, D., Popov, C., Mutschler, W., and Schieker, M. (2007). Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system. Journal of Cellular and Molecular Medicine *11*, 21–38.

Domenici, L., and Gremigni, V. (1977). Fine structure and functional role of the coverings of the eggs inMesostoma ehrenbergii (Focke) (Turbellaria, Neorhabdocoela). Zoomorphologie *88*, 247–257.

Donaghy, L., Hong, H.-K., Park, K.-I., Nobuhisa, K., Youn, S.-H., Kang, C.-K., and Choi, K.-S. (2017). Flow cytometric characterization of hemocytes of the solitary ascidian, Halocynthia roretzi. Fish & Shellfish Immunology *66*, 289–299.

Driesch, H. (1902 a). Uber ein neues harmonisch - àquipotentielles System und iiber solche Systeme iiberhaupt: Arch. Entw. Mech. 14 Bd. p. 227-246, 7 fig.

Driesch, H. (1902 b). Studien iiber das Regulationsvermògen der Organismen. 6. Die Restitutionen von Clavelina lepadiformis : Arch. Entw. Mech. 14 Bd. p. 247-287, 6 fig.

DuBuc, T.Q., Traylor-Knowles, N., and Martindale, M.Q. (2014). Initiating a regenerative response; cellular and molecular features of wound healing in the cnidarian Nematostella vectensis. BMC Biology *12*, 24.

van Duyl, F.C., Bak, R.P.M., and Sybesma, J. (1981). The Ecology of the Tropical Compound Ascidian Tridemnum solidum. I. Reproductive Strategy and Larval Behaviour. Marine Ecology Progress Series *6*, 35–42.

Egger, B., Gschwentner, R., and Rieger, R. (2007). Free-living flatworms under the knife: past and present. Dev Genes Evol *217*, 89–104.

Ereskovsky, A.V., Tokina, D.B., Saidov, D.M., Baghdiguian, S., Goff, E.L., and Lavrov, A.I. (2020). Transdifferentiation and mesenchymal-to-epithelial transition during regeneration in Demospongiae (Porifera). Journal of Experimental Zoology Part B: Molecular and Developmental Evolution *334*, 37–58.

Ermak, T. (1976). The hematogenic tissues of tunicate.

Esposito, M. (2013). Weismann Versus Morgan Revisited: Clashing Interpretations on Animal Regeneration. J Hist Biol *46*, 511–541.

Fautin, D.G. (2002). Reproduction of Cnidaria. Can. J. Zool. *80*, 1735–1754.

Fedonkin, M.A., Vickers-Rich, P., Swalla, B.J., Trusler, P., and Hall, M. (2012). A new metazoan from the Vendian of the White Sea, Russia, with possible affinities to the ascidians. Paleontol. J. *46*, 1–11.

Fell, P.E. (1974). Diapause in the gemmules of the marine sponge, haliclona loosanoffi, with a note on the gemmules of haliclona oculata. The Biological Bulletin *147*, 333–351.

Fell PE (1994) Dormancy of the gemmules of *Eunapius fragilis* and *Ephydatia muelleri* in New England. In: van Soest RWM, van Kempen ThMG, Braekman JC (eds) Sponges in time and space. Balkema AA, Roterdam, pp 313–320

Ferreira-Cravo, M., Welker, A.F., and Hermes-Lima, M. (2010). The Connection Between Oxidative Stress and Estivation in Gastropods and Anurans. In Aestivation: Molecular and Physiological Aspects, C. Arturo Navas, and J.E. Carvalho, eds. (Berlin, Heidelberg: Springer), pp. 47–61.

Fierro-Constaín, L., Schenkelaars, Q., Gazave, E., Haguenauer, A., Rocher, C., Ereskovsky, A., Borchiellini, C., and Renard, E. (2017). The Conservation of the Germline Multipotency Program, from Sponges to Vertebrates: A Stepping Stone to Understanding the Somatic and Germline Origins. Genome Biol Evol *9*, 474–488.

Fincher, C.T., Wurtzel, O., Hoog, T. de, Kravarik, K.M., and Reddien, P.W. (2018). Cell type transcriptome atlas for the planarian Schmidtea mediterranea. Science *360*.

Flood, P.R., and Fiala-Medioni, A. (1981). Ultrastructure and Histochemistry of the Food Trapping Mucous Film in Benthic Filter-Feeders (Ascidians). Acta Zoologica *62*, 53–65.

Franchi, N., and Ballarin, L. (2017). Immunity in Protochordates: The Tunicate Perspective. Front. Immunol. *8*.

Franchi, N., Ballarin, L., and Cima, F. (2015). Insights on cytotoxic cells of the colonial ascidian Botryllus schlosseri. Invertebrate Survival Journal *12*, 109–117.

Francis, D.R. (2001). Bryozoan Statoblasts. In Tracking Environmental Change Using Lake Sediments: Volume 4: Zoological Indicators, J.P. Smol, H.J.B. Birks, and W.M. Last, eds. (Dordrecht: Springer Netherlands), pp. 105–123.

Frank, P., and Bleakney, J. (1978). ASEXUAL REPRODUCTION, DIET, AND ANOMALIES OF THE ANEMONE NEMATOSTELLA VECTENSIS IN NOVA SCOTIA. ASEXUAL REPRODUCTION, DIET, AND ANOMALIES OF THE ANEMONE NEMATOSTELLA VECTENSIS IN NOVA SCOTIA.

Frank, U., Plickert, G., and Müller, W.A. (2009). Cnidarian Interstitial Cells: The Dawn of Stem Cell Research. In Stem Cells in Marine Organisms, B. Rinkevich, and V. Matranga, eds. (Dordrecht: Springer Netherlands), pp. 33–59.

Freeman, G. (1964). The role of blood cells in the process of asexual reproduction in the tunicate Perophora viridis. Journal of Experimental Zoology *156*, 157–183.

Fryer, G. (1996). Diapause, a potent force in the evolution of freshwater crustaceans. Hydrobiologia *320*, 1–14.

Fujimoto, H., and Watanabe, H. (1976a). Studies on the asexual reproduction in the polystyelid ascidian, Polyzoa vesiculiphora Tokioka. Journal of Morphology *150*, 607–621.

Fujimoto, H., and Watanabe, H. (1976b). The characterization of granular amoebocytes and their possible roles in the asexual reproduction of the polystyelid ascidian, Polyzoa vesiculiphora. Journal of Morphology *150*, 623–637.

Fujita, H., and Nanba, H. (1971). Fine structure and its functional properties of the endostyle of Ascidians,Ciona intestinalis. Z.Zellforsch *121*, 455–469.

Fujiwara, S., and Kawamura, K. (1992a). Ascidian Budding as a Transdifferentiation-Like System: Multipotent Epithelium is not Undifferentiated. Development, Growth & Differentiation *34*, 463–472.

Fujiwara, S., and Kawamura, K. (1992b). Ascidian Budding as a Transdifferentiation-Like System: Multipotent Epithelium is not Undifferentiated. Development, Growth & Differentiation *34*, 463–472.

Fukumoto, M. (1971). Experimental Control of Budding and Stolon Elongation in Perophora Orientalis, a Compound Ascidian*. Development, Growth & Differentiation *13*, 73–88.

García-Roger, E.M., Lubzens, E., Fontaneto, D., and Serra, M. (2019). Facing Adversity: Dormant Embryos in Rotifers. The Biological Bulletin *237*, 119–144.

Gasparini, F., Longo, F., Manni, L., Burighel, P., and Zaniolo, G. (2007). Tubular sprouting as a mode of vascular formation in a colonial ascidian (tunicata). Developmental Dynamics *236*, 719–731.

Gasparini, F., Manni, L., Cima, F., Zaniolo, G., Burighel, P., Caicci, F., Franchi, N., Schiavon, F., Rigon, F., Campagna, D., et al. (2015). Sexual and asexual reproduction in the colonial ascidian Botryllus schlosseri. Genesis *53*, 105–120.

Gehrke, A.R., and Srivastava, M. (2016). Neoblasts and the evolution of whole-body regeneration. Current Opinion in Genetics & Development *40*, 131–137.

Geiser, F. (2010). Aestivation in Mammals and Birds. In Aestivation: Molecular and Physiological Aspects, C. Arturo Navas, and J.E. Carvalho, eds. (Berlin, Heidelberg: Springer), pp. 95–111.

Geiser, F. (2013). Hibernation. Current Biology *23*, R188–R193.

George, W.C. (1926). The histology of the blood of perophora viridis (ascidian). Journal of Morphology *41*, 311–331.

Gerber, N., and Kokko, H. (2018). Abandoning the ship using sex, dispersal or dormancy: multiple escape routes from challenging conditions. Philosophical Transactions of the Royal Society B: Biological Sciences *373*, 20170424.

Giangrande, A., and Licciano, M. (2014). Regeneration and clonality in Metazoa. The price to pay for evolving complexity. Invertebrate Reproduction & Development *58*, 1–8.

Giard, MMA., Caullery, M. (1896) On the hibernation of Clavelina lepadiformis, Müller, Annals and Magazine of Natural History, 18:108, 485-486.

Gierer, A., Berking, S., Bode, H., David, C.N., Flick, K., Hansmann, G., Schaller, H., and Trenkner, E. (1972). Regeneration of hydra from reaggregated cells. Nature New Biol. *239*, 98–101.

Giese, A. (2012). Reproduction of Marine Invertebrates V3: Annelids and Echiurans (Elsevier).

Gilbert, J.J. (1974). Dormancy in Rotifers. Transactions of the American Microscopical Society *93*, 490–513.

Gilbert, J.J. (1983). Control of sexuality in Asplanchna brightwelli: threshold levels of dietary tocopherol and modification of tocopherol response by exogenous and endogenous factors. In Biology of Rotifers, B. Pejler, R. Starkweather, and Th. Nogrady, eds. (Dordrecht: Springer Netherlands), pp. 167–173.

Gilbert, J.J. (2004). Females from resting eggs and parthenogenetic eggs in the rotifer Brachionus calyciflorus: lipid droplets, starvation resistance and reproduction. Freshwater Biology *49*, 1505–1515.

Gilbert, J.J., and Litton, J.R. (1978). Sexual reproduction in the rotifer Asplanchna girodi: Effects of tocopherol and population density. Journal of Experimental Zoology *204*, 113–121.

Gilbert, J.J., and Thompson, G.A. (1968). Alpha Tocopherol Control of Sexuality and Polymorphism in the Rotifer Asplanchna. Science *159*, 734–736.

Godeaux J. (1957). Contribution à la connaissance des thaliacés (pyrosome et doliolum): embryogénèse et blastogénèse du complexe neural, constitution et développement du stolon prolifère. Faculté des sciences: Université de Liège; 1957.

Goldin, A. (1948). REGENERATION IN PEROPHORA VIRIDIS. The Biological Bulletin *94*, 184–193.

Goodbody, I. (1961). Continuous Breeding in Three Species of Tropical Ascidian. Proceedings of the Zoological Society of London *136*, 403–409.

Goodbody, I. (1975). The Physiology of Ascidians. In Advances in Marine Biology, F.S. Russell, and M. Yonge, eds. (Academic Press), pp. 1–149.

Goss, R.J. (1969). Principles of Regeneration (Academic Press).

Goss, R.J. (1992). The evolution of regeneration: Adaptive or inherent? Journal of Theoretical Biology *159*, 241–260.

Goss, R.J. (2012). Deer Antlers: Regeneration, Function and Evolution (Academic Press).

Govindarajan, A.F., Bucklin, A., and Madin, L.P. (2011). A molecular phylogeny of the Thaliacea. J Plankton Res *33*, 843–853.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., et al. (2011). Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. Nat Biotechnol *29*, 644–652.

Graf, L., and Gierer, A. (1980). Size, shape and orientation of cells in budding hydra and regulation of regeneration in cell aggregates. Wilhelm Roux' Archiv *188*, 141–151.

Green, A.J., Sánchez, M.I., Amat, F., Figuerola, J., Hontoria, F., Ruiz, O., and Hortas, F. (2005). Dispersal of invasive and native brine shrimps Artemia (Anostraca) via waterbirds. Limnology and Oceanography *50*, 737–742.

Griffith, M., Walker, J.R., Spies, N.C., Ainscough, B.J., and Griffith, O.L. (2015). Informatics for RNA Sequencing: A Web Resource for Analysis on the Cloud. PLOS Computational Biology *11*, e1004393.

Guidetti, R., Boschini, D., Rebecchi, L., and Bertolani, R. (2006). Encystment Processes and the "Matrioshka-like Stage" in a Moss-dwelling and in a Limnic Species of Eutardigrades (Tardigrada). Hydrobiologia *558*, 9–21.

Guidetti, R., Altiero, T., and Rebecchi, L. (2011). On dormancy strategies in tardigrades. Journal of Insect Physiology *57*, 567–576.

Gurley, K.A., Elliott, S.A., Simakov, O., Schmidt, H.A., Holstein, T.W., and Alvarado, A.S. (2010). Expression of secreted Wnt pathway components reveals unexpected complexity of the planarian amputation response. Developmental Biology *347*, 24–39.

Gutierrez, S., and Brown, F.D. (2017). Vascular budding in Symplegma brakenhielmi and the evolution of coloniality in styelid ascidians. Developmental Biology *423*, 152–169.

Hager, G., and David, C.N. (1997). Pattern of differentiated nerve cells in hydra is determined by precursor migration. Development *124*, 569–576.

Hahn, D.A., and Denlinger, D.L. (2007). Meeting the energetic demands of insect diapause: Nutrient storage and utilization. Journal of Insect Physiology *53*, 760–773.

Hahn, D.A., and Denlinger, D.L. (2011). Energetics of Insect Diapause. Annual Review of Entomology *56*, 103–121.

Hand, S.C. (1991). Metabolic Dormancy in Aquatic Invertebrates. In Advances in Comparative and Environmental Physiology: Volume 8, M.A. Castellini, B. Fiévet, S.C. Hand, R. Motais, B. Pelster, and R.E. Weber, eds. (Berlin, Heidelberg: Springer), pp. 1–50.

Hand, C., and Uhlinger, K.R. (1995). Asexual Reproduction by Transverse Fission and Some Anomalies in the Sea Anemone Nematostella vectensis. Invertebrate Biology *114*, 9–18.

Harris, M., Moore, A., Lowen, B., and DiBacco, C. (2017). Seasonal reproduction of the non-native vase tunicate Ciona intestinalis (Linnaeus, 1767) in Nova Scotia, Canada, in relation to water temperature. AI *12*, 33–41.

Harrison, F. (2012). Aspects of Sponge Biology (Elsevier).

Hartenstein, V. (2006). Blood Cells and Blood Cell Development in the Animal Kingdom. Annual Review of Cell and Developmental Biology *22*, 677–712.

Harvell, C.D. (1991). Coloniality and Inducible Polymorphism. The American Naturalist *138*, 1–14.

Harvell, C.D. (1994). The Evolution of Polymorphism in Colonial Invertebrates and Social Insects. The Quarterly Review of Biology *69*, 155–185.

Haven, N.D. (1971). Temporal patterns of sexual and asexual reproduction in the colonial ascidian metandrocarpa taylori huntsman. The Biological Bulletin *140*, 400–415.

Hayward, S.A. (2014). Application of functional 'Omics' in environmental stress physiology: insights, limitations, and future challenges. Current Opinion in Insect Science *4*, 35–41.

Hellbach, A., Tiozzo, S., Ohn, J., Liebling, M., and Tomaso, A.W.D. (2011). Characterization of HCN and cardiac function in a colonial ascidian. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology *315A*, 476–486.

Hengherr, S., Heyer, A.G., Köhler, H.-R., and Schill, R.O. (2008). Trehalose and anhydrobiosis in tardigrades – evidence for divergence in responses to dehydration. The FEBS Journal *275*, 281–288.

Hengherr, S., Heyer, A.G., Brümmer, F., and Schill, R.O. (2011). Trehalose and Vitreous States: Desiccation Tolerance of Dormant Stages of the Crustaceans Triops and Daphnia. Physiological and Biochemical Zoology *84*, 147–153.

Henry, L.-A., and Hart, M. (2005). Regeneration from Injury and Resource Allocation in Sponges and Corals – a Review. International Review of Hydrobiology *90*, 125–158.

Hiebert, L.S., Simpson, C., and Tiozzo, S. Coloniality, clonality, and modularity in animals: The elephant in the room. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution *n/a*.

Hill, S.D. (1970). Origin of the Regeneration Blastema in Polychaete Annelids. Integr Comp Biol *10*, 101–112.

Hirose, E. (2009). Ascidian tunic cells: morphology and functional diversity of free cells outside the epidermis. Invertebrate Biology *128*, 83–96.

Hirose, E., and Mukai, H. (1992). An ultrastructural study on the origin of glomerulocytes, a type of blood cell in a styelid ascidian, Polyandrocarpa misakiensis. Journal of Morphology *211*, 269–273.

Hirose, E., Yoshida, T., Akiyama, T., Ito, S., and Iwanami, Y. (1998). Pigment Cells Representing Polychromatic Colony Color in Botrylloides simodensis (Ascidiacea, Urochordata): Cell Morphology and Pigment Substances. Jzoo *15*, 489–497.

Hirose, E., Kimura, S., Itoh, T., and Nishikawa, J. (1999). Tunic Morphology and Cellulosic Components of Pyrosomas, Doliolids, and Salps (Thaliacea, Urochordata). The Biological Bulletin *196*, 113–120.

Hirose, E., Shirae, M., and Saito, Y. (2003). Ultrastructures and Classification of Circulating Hemocytes in 9 Botryllid Ascidians (Chordata: Ascidiacea). Jzoo *20*, 647–656.

Hirose, M., Tochikubo, T., and Hirose, E. (2010). Taxonomic significance of tunic spicules in photosymbiotic ascidians: a quantitative and molecular evaluation. Journal of the Marine Biological Association of the United Kingdom *90*, 1065–1071.

Hobmayer, B., Jenewein, M., Eder, D., Eder, M.-K., Glasauer, S., Gufler, S., Hartl, M., and Salvenmoser, W. (2012). Stemness in Hydra - a current perspective. Int. J. Dev. Biol. *56*, 509–517.

Holland, L.Z. (2016). Tunicates. Current Biology *26*, R146–R152.

Holstein, T.W., Hobmayer, E., and David, C.N. (1991). Pattern of epithelial cell cycling in hydra. Developmental Biology 602–611.

Holstein, T.W., Hobmayer, E., and Technau, U. (2003). Cnidarians: An evolutionarily conserved model system for regeneration? Developmental Dynamics *226*, 257–267.

Holyoak, A.R. (1992). Morphogenetic Movements and Assembly of Strobilae into Zooidal Systems in Early Colony Development of the Compound Ascidian Polyclinum planum. The Biological Bulletin *183*, 432–439.

Horne, F.R. (1973). The utilization of foodstuffs and urea production by a land snail during estivation. The Biological Bulletin *144*, 321–330.

Huber, J.L., da Silva, K.B., Bates, W.R., and Swalla, B.J. (2000). The evolution of anural larvae in molgulid ascidians. Semin. Cell Dev. Biol. *11*, 419–426.

Huebner, E.A., and Strittmatter, S.M. (2009). Axon regeneration in the peripheral and central nervous systems. Results Probl Cell Differ *48*, 339–351.

Hughes, R.N. (1989). Functional Biology of Clonal Animals (Springer Science & Business Media).

Huxley, J. (1926). Studies in dedifferentiation, VI. Reduction phenomena in *Clavellina lepadiformis*. *Publication Stazione Zoologica Napoli*, *7*, 1–35.

Hyams, Y., Paz, G., Rabinowitz, C., and Rinkevich, B. (2017). Insights into the unique torpor of Botrylloides leachi, a colonial urochordate. Developmental Biology *428*, 101–117.

Ikeda, H., Ohtsu, K., and Uye, S.-I. (2011). Fine structure, Histochemistry, and Morphogenesis During Excystment of the Podocysts of the Giant Jellyfish Nemopilema nomurai (Scyphozoa, Rhizostomeae). The Biological Bulletin *221*, 248–260.

Imperadore, P., Uckermann, O., Galli, R., Steiner, G., Kirsch, M., and Fiorito, G. (2018). Nerve regeneration in the cephalopod mollusc Octopus vulgaris: label-free multiphoton microscopy as a tool for investigation. Journal of The Royal Society Interface *15*, 20170889.

Izzard, C.S. (1973). Development of polarity and bilateral asymmetry in the palleal bud of Botryllus schlosseri (Pallas). Journal of Morphology *139*, 1–25.

Jeffery, W.R. (2015). Closing the wounds: One hundred and twenty five years of regenerative biology in the ascidian Ciona intestinalis. Genesis *53*, 48–65.

Jiménez-Merino, J., Santos de Abreu, I., Hiebert, L.S., Allodi, S., Tiozzo, S., De Barros, C.M., and Brown, F.D. (2019). Putative stem cells in the hemolymph and in the intestinal submucosa of the solitary ascidian Styela plicata. EvoDevo *10*, 31.

Jones, S.E., and Lennon, J.T. (2010). Dormancy contributes to the maintenance of microbial diversity. PNAS *107*, 5881–5886.

Jönsson, K.I. (2005). The Evolution of Life Histories in Holo-anhydrobiotic Animals: A First Approach. Integr Comp Biol *45*, 764–770.

Jopling, C., Sleep, E., Raya, M., Martí, M., Raya, A., and Belmonte, J.C.I. (2010). Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature *464*, 606–609.

Jopling, C., Boue, S., and Belmonte, J.C.I. (2011). Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. Nature Reviews Molecular Cell Biology *12*, 79–89.

Juliano, C.E., Swartz, S.Z., and Wessel, G.M. (2010). A conserved germline multipotency program. Development *137*, 4113–4126.

Kamimura, M., Fujiwara, S., Kawamura, K., and Yubisui, T. (2000). Functional retinoid receptors in budding ascidians. Development, Growth & Differentiation *42*, 1–8.

Kaneko, N., Katsuyama, Y., Kawamura, K., and Fujiwara, S. (2010). Regeneration of the gut requires retinoic acid in the budding ascidian Polyandrocarpa misakiensis. Development, Growth & Differentiation *52*, 457–468.

Kassmer, S.H., Rodriguez, D., Langenbacher, A.D., Bui, C., and De Tomaso, A.W. (2015). Migration of germline progenitor cells is directed by sphingosine-1-phosphate signalling in a basal chordate. Nature Communications *6*, 8565.

Kassmer, S.H., Langenbacher, A., and Tomaso, A.W.D. (2019a). Integrin-alpha-6+ Stem Cells (ISCs) are responsible for whole body regeneration in an invertebrate chordate. BioRxiv 647578.

Kassmer, S.H., Nourizadeh, S., and De Tomaso, A.W. (2019b). Cellular and molecular mechanisms of regeneration in colonial and solitary Ascidians. Developmental Biology *448*, 271–278.

KAWAMURA, K. (1982). Localized morphogenetic activity in the palleal bud of a polystyelid ascidian, Polyandrocarpa misakiensis. I. A histological study. Mem. Fac. Sci. Kochi Univ. Ser. D *3*, 55–69.

Kawamura, K., and Fujiwara, S. (1995). Cellular and molecular characterization of transdifferentiation in the process of morphallaxis of budding tunicates. Seminars in Cell Biology *6*, 117–126.

Kawamura, K., and Nakauchi, M. (1986). Development of spatial organization in palleal buds of the compound ascidian, symplegma reptans. The Biological Bulletin *171*, 520–537.

KAWAMURA, K., and NAKAUCHI, M. (1991). Homeostatic Integration of Stem Cell dynamics during Palleal Budding of Ascidians. Zoological Science *8*, p11-22.

Kawamura, K., and Sugino, Y.M. (1999). Cell adhesion in the process of asexual reproduction of tunicates. Microscopy Research and Technique *44*, 269–278.

Kawamura, K., and Sunanaga, T. (2010). Hemoblasts in colonial tunicates: Are they stem cells or tissue-restricted progenitor cells? Development, Growth & Differentiation *52*, 69–76.

Kawamura, K., and Sunanaga, T. (2011). Role of Vasa, Piwi, and Myc-expressing coelomic cells in gonad regeneration of the colonial tunicate, Botryllus primigenus. Mechanisms of Development *128*, 457–470.

Kawamura, K., and Watanabe, H. (1982). Pattern development in palleal buds of the polystyelid ascidian, Polyandrocarpa misakiensis: Bud grafting induces bilateral asymmetry conversion through polarity reversal. Journal of Experimental Zoology *224*, 145–156.

Kawamura, K., Fujiwara, S., and Sugino, Y.M. (1991). Budding-specific lectin induced in epithelial cells is an extracellular matrix component for stem cell aggregation in tunicates. Development *113*, 995–1005.

Kawamura, K., Hara, K., and Fujiwara, S. (1993). Developmental role of endogenous retinoids in the determination of morphallactic field in budding tunicates. Development *117*, 835–845.

Kawamura, K., Hashimoto, K., and Nakauchi, M. (1995). Development of irradiated tunicate buds: Is cell division cycle required for morphallaxis? Development, Growth & Differentiation *37*, 487–496.

Kawamura, K., Sugino, Y., Sunanaga, T., and Fujiwara, S. (2008). Multipotent epithelial cells in the process of regeneration and asexual reproduction in colonial tunicates. Development, Growth & Differentiation *50*, 1–11.

Keilin, D. (1959). The Leeuwenhoek Lecture - The problem of anabiosis or latent life: history and current concept. Proceedings of the Royal Society of London. Series B - Biological Sciences *150*, 149–191.

Kerb, H. (1908). Biologische Beiträge zur Frage der Überwinterung der Ascidien. Archiv für Mikroskopische Anatomie, 72(1), 386– 414.

King, A.M., and MacRae, T.H. (2015). Insect Heat Shock Proteins During Stress and Diapause. Annual Review of Entomology *60*, 59–75.

Kocot, K.M., Tassia, M.G., Halanych, K.M., and Swalla, B.J. (2018). Phylogenomics offers resolution of major tunicate relationships. Molecular Phylogenetics and Evolution *121*, 166–173.

Koguchi, S. (1993). Dynamics of epithelial stem cell in the process of stolonial budding of the colonial ascidian Perophora japonica. Mem. Fac. Sci. Kochi Univ. Ser. D *14*, 7–14.

Korchelt, E. (1928). Regeneration und Transplantation. Nature *121*, 825–825.

Koštál, V. (2006). Eco-physiological phases of insect diapause. Journal of Insect Physiology *52*, 113–127.

Koštál, V., Pennacchio, F., and Denlinger, D.L. (2011). Special Issue: Dormancy and developmental arrest in invertebrates. Journal of Insect Physiology *57*, 537–680.

Koštál, V., Štětina, T., Poupardin, R., Korbelová, J., and Bruce, A.W. (2017). Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. PNAS *114*, 8532–8537.

Kowalewsky, A. (1874). Sur le bourgeonnement du Perophora Listeri . Journal of Morphology, 9(1), 473– 483. (Translated from russian by Giard, A.).

Kostyuchenko, R.P., Kozin, V.V., and Kupriashova, E.E. (2016). Regeneration and asexual reproduction in annelids: Cells, genes, and evolution. Biol Bull Russ Acad Sci *43*, 185–194.

Krebsbach, P.H., and Villa-Diaz, L.G. (2017). The Role of Integrin α6 (CD49f) in Stem Cells: More than a Conserved Biomarker. Stem Cells and Development *26*, 1090–1099.

Kriebel, M.E. (1968). Electrical Characteristics of Tunicate Heart Cell Membranes and Nexuses. J Gen Physiol *52*, 46–59.

Krishna, S., Palakodeti, D., and Solana, J. (2019). Post-transcriptional regulation in planarian stem cells. Seminars in Cell & Developmental Biology *87*, 69–78.

Kültz, D. (2003). Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. Journal of Experimental Biology *206*, 3119–3124.

Lacalli, T.C. (2002). Vetulicolians—are they deuterostomes? chordates? BioEssays *24*, 208–211.

Laflamme, M.A., and Murry, C.E. (2011). Heart regeneration. Nature *473*, 326–335.

Lai, A.G., and Aboobaker, A.A. (2018). EvoRegen in animals: Time to uncover deep conservation or convergence of adult stem cell evolution and regenerative processes. Developmental Biology *433*, 118–131.

Laird, D.J., and Weissman, I.L. (2004). Continuous development precludes radioprotection in a colonial ascidian. Dev. Comp. Immunol. *28*, 201–209.

Laird, D.J., De Tomaso, A.W., and Weissman, I.L. (2005). Stem Cells Are Units of Natural Selection in a Colonial Ascidian. Cell *123*, 1351–1360.

Lambert, C.C., and Lambert, G. (2003). Persistence and differential distribution of nonindigenous ascidians in harbors of the Southern California Bight. Marine Ecology Progress Series *259*, 145–161.

LaMontagne, J.M., and McCauley, E. (2001). Maternal effects in Daphnia: what mothers are telling their offspring and do they listen? Ecology Letters *4*, 64–71.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nature Methods *9*, 357–359.

Lauzon, R.J., Ishizuka, K.J., and Weissman, I.L. (1992). A cyclical, developmentally-regulated death phenomenon in a colonial urochordate. Developmental Dynamics *194*, 71–83.

Lauzon, R.J., Kidder, S.J., and Long, P. (2007). Suppression of programmed cell death regulates the cyclical degeneration of organs in a colonial urochordate. Developmental Biology *301*, 92–105.

Law, C.W., Chen, Y., Shi, W., and Smyth, G.K. (2014). voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol *15*, R29.

Lawrence, A.J., and Soame, J.M. (2004). The effects of climate change on the reproduction of coastal invertebrates. Ibis *146*, 29–39.

Lebrato, M., Mendes, P. de J., Steinberg, D.K., Cartes, J.E., Jones, B.M., Birsa, L.M., Benavides, R., and Oschlies, A. (2013). Jelly biomass sinking speed reveals a fast carbon export mechanism. Limnology and Oceanography *58*, 1113–1122.

Lefevre, G. (1898). Budding in Perophora. Journal of Morphology *14*, 367–424.

Lengfeld, T., Watanabe, H., Simakov, O., Lindgens, D., Gee, L., Law, L., Schmidt, H.A., Özbek, S., Bode, H., and Holstein, T.W. (2009). Multiple Wnts are involved in Hydra organizer formation and regeneration. Developmental Biology *330*, 186–199.

Li, B., Zheng, Y.-W., Sano, Y., and Taniguchi, H. (2011). Evidence for Mesenchymal–Epithelial Transition Associated with Mouse Hepatic Stem Cell Differentiation. PLOS ONE *6*, e17092.

Li, C., Kong, Y., Wang, H., Wang, S., Yu, H., Liu, X., Yang, L., Jiang, X., Li, L., and Li, L. (2009). Homing of bone marrow mesenchymal stem cells mediated by sphingosine 1-phosphate contributes to liver fibrosis. Journal of Hepatology *50*, 1174–1183.

Li, R., Liang, J., Ni, S., Zhou, T., Qing, X., Li, H., He, W., Chen, J., Li, F., Zhuang, Q., et al. (2010). A Mesenchymal-to-Epithelial Transition Initiates and Is Required for the Nuclear Reprogramming of Mouse Fibroblasts. Cell Stem Cell *7*, 51–63.

Lin, G., and Slack, J.M.W. (2008). Requirement for Wnt and FGF signaling in Xenopus tadpole tail regeneration. Developmental Biology *316*, 323–335.

Liu, S.-Y., Selck, C., Friedrich, B., Lutz, R., Vila-Farré, M., Dahl, A., Brandl, H., Lakshmanaperumal, N., Henry, I., and Rink, J.C. (2013). Reactivating head regrowth in a regeneration-deficient planarian species. Nature *500*, 81–84.

Loeb, V.J., and Santora, J.A. (2012). Population dynamics of Salpa thompsoni near the Antarctic Peninsula: Growth rates and interannual variations in reproductive activity (1993–2009). Progress in Oceanography *96*, 93–107.

Loomis, S.H. (2010). Diapause and Estivation in Sponges. In Aestivation: Molecular and Physiological Aspects, C. Arturo Navas, and J.E. Carvalho, eds. (Berlin, Heidelberg: Springer), pp. 231–243.

Lopez, L.C.S., Gonçalves, D.A., Mantovani, A., and Rios, R.I. (2002). Bromeliad ostracods pass through amphibian (Scinaxax perpusillus) and mammalian guts alive. Hydrobiologia *485*, 209–211.

Lucas, C.H., and Dawson, M.N. (2014). What Are Jellyfishes and Thaliaceans and Why Do They Bloom? In Jellyfish Blooms, K.A. Pitt, and C.H. Lucas, eds. (Dordrecht: Springer Netherlands), pp. 9–44.

Mackie, G.O., Harper, J.L., Rosen, B.R., and White, J. (1986). From aggregates to integrates: physiological aspects of modularity in colonial animals. Philosophical Transactions of the Royal Society of London. B, Biological Sciences *313*, 175–196.

MacRae, T.H. (2010). Gene expression, metabolic regulation and stress tolerance during diapause. Cell. Mol. Life Sci. *67*, 2405–2424.

Manconi, R., and Pronzato, R. (2008). Global diversity of sponges (Porifera: Spongillina) in freshwater. Hydrobiologia *595*, 27–33.

Manconi, R., and Pronzato, R. (2016). How to survive and persist in temporary freshwater? Adaptive traits of sponges (Porifera: Spongillida): A review. Hydrobiologia *782*, 11–22.

Manni, L., Zaniolo, G., and Burighel, P. (1993). Egg Envelope Cytodifferentiation in the Colonial Ascidian Botryllus schlosseri (Tunicata). Acta Zoologica *74*, 103–113.

Manni, L., Zaniolo, G., Cima, F., Burighel, P., and Ballarin, L. (2007). Botryllus schlosseri: A model ascidian for the study of asexual reproduction. Developmental Dynamics *236*, 335–352.

Mansingh, A. (1971). PHYSIOLOGICAL CLASSIFICATION OF DORMANCIES IN INSECTS. The Canadian Entomologist *103*, 983–1009.

Marcum, B.A., and Campbell, R.D. (1978). Development of Hydra lacking nerve and interstitial cells. Journal of Cell Science *29*, 17–33.

Marcus, N.H. (1996). Ecological and evolutionary significance of resting eggs in marine copepods: past, present, and future studies. Hydrobiologia *320*, 141–152.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal *17*, 10–12.

Martinez, V.G., Menger, G.J., and Zoran, M.J. (2005). Regeneration and asexual reproduction share common molecular changes: upregulation of a neural glycoepitope during morphallaxis in Lumbriculus. Mechanisms of Development *122*, 721–732.

Martinez, V.G., Reddy, P.K., and Zoran, M.J. (2006). Asexual reproduction and segmental regeneration, but not morphallaxis, are inhibited by boric acid in Lumbriculus variegatus (Annelida: Clitellata: Lumbriculidae). In Aquatic Oligochaete Biology IX: Selected Papers from the 9th Symposium on Aquatic Oligochaeta, 6–10 October 2003, Wageningen, The Netherlands, P.F.M. Verdonschot, H. Wang, A. Pinder, and R. Nijboer, eds. (Dordrecht: Springer Netherlands), pp. 73–86.

Martinucci, G.B., Dallai, R., and Burighel, P. (1987). A comparative study of ciliary differentiations in the branchial stigmata of ascidians. Tissue and Cell *19*, 251–263.

Mashanov, V.S., Dolmatov, I.Yu., and Heinzeller, T. (2005). Transdifferentiation in Holothurian Gut Regeneration. The Biological Bulletin *209*, 184–193.

Mashanov, V.S., Zueva, O.R., and García-Arrarás, J.E. (2015). Expression of pluripotency factors in echinoderm regeneration. Cell Tissue Res *359*, 521–536.

Massard, J.A., and Geimer, G. (2008). Global diversity of bryozoans (Bryozoa or Ectoprocta) in freshwater. In Freshwater Animal Diversity Assessment, E.V. Balian, C. Lévêque, H. Segers, and K. Martens, eds. (Dordrecht: Springer Netherlands), pp. 93–99.

Mastrototaro, F., D'Onghia, G., and Tursi, A. (2008). Spatial and seasonal distribution of ascidians in a semi-enclosed basin of the Mediterranean Sea. Journal of the Marine Biological Association of the United Kingdom *88*, 1053–1061.

Medina, B.N.S.P., Abreu, I.S. de, Cavalcante, L.A., Silva, W.A.B., Fonseca, R.N. da, Allodi, S., and Barros, C.M. de (2015). 3-acetylpyridine-induced degeneration in the adult ascidian neural complex: Reactive and regenerative changes in glia and blood cells. Developmental Neurobiology *75*, 877–893.

de Meeûs, T., Prugnolle, F., and Agnew, P. (2007). Asexual reproduction: Genetics and evolutionary aspects. Cell. Mol. Life Sci. *64*, 1355–1372.

Michibata, H., Yamaguchi, N., Uyama, T., and Ueki, T. (2003). Molecular biological approaches to the accumulation and reduction of vanadium by ascidians. Coordination Chemistry Reviews *237*, 41–51.

Milanesi, C., and Burighel, P. (1978). Blood Cell Ultrastructure of the Ascidian Botryllus schlosseri. Acta Zoologica *59*, 135–147.

Millar, R.H. (1958). The breeding season of some littoral ascidians in Scottish waters. Journal of the Marine Biological Association of the United Kingdom *37*, 649–652.

Millar, R.H. (1962). Budding in the ascidian Aplidium petrense Michaelsen. Annals and Magazine of Natural History *5*, 337–340.

Mochizuki, K., Nishimiya-Fujisawa, C., and Fujisawa, T. (2001). Universal occurrence of the vasa -related genes among metazoans and their germline expression in Hydra. Development Genes and Evolution *211*, 299–308.

Monniot, C., and Monniot, F. (1990). Relationships between deep-sea tunicate populations west and east of the Straits of Gibraltar. Progress in Oceanography *24*, 289–296.

Monniot, C., Monniot, F., and Millar, R.H. (1976). An account of six species of abyssal Styelidae (Ascidiacea), three of which are new species. Deep Sea Research and Oceanographic Abstracts *23*, 1187–1197.

Moraczewski, J. (1977). Asexual reproduction and regeneration of Catenula (Turbellaria, Archoophora). Zoomorphologie *88*, 65–80.

Moreno, T.R., and Rocha, R.M. (2008). Phylogeny of the Aplousobranchia (Tunicata: Ascidiacea). Revista Brasileira de Zoologia *25*, 269–298.

Morgan, T.H. (1898). Regeneration and Liability to Injury. Zoological Bulletin *1*, 287–300.

Morgan, T.H. (1901). Regeneration (Macmillan).

Morita, A., Soga, K., Hoson, T., Kamisaka, S., and Numata, H. (1999). Changes in mechanical properties of the cuticle and lipid accumulation in relation to adult diapause in the bean bug, Riptortus clavatus. Journal of Insect Physiology *45*, 241–247.

Morley, J.F., and Morimoto, R.I. (2003). Regulation of Longevity in Caenorhabditis elegans by Heat Shock Factor and Molecular Chaperones. MBoC *15*, 657–664.

Mukai, H., and Kobayashi, K. (1988). External observations on the formation of statoblasts in Plumatella emarginata (Bryozoa, Phylactolaemata). Journal of Morphology *196*, 205–216.

Mukai, H., and Watanabe, H. (1976). Studies on the formation of germ cells in a compound ascidian Botryllus, primigenus oka. Journal of Morphology *148*, 337–361.

Mukai, H., Koyama, H., and Watanabe, H. (1983a). Studies on the reproduction of three species of perophora (ascidiacea). The Biological Bulletin *164*, 251–266.

Mukai, H., Koyama, H., and Watanabe, H. (1983b). Studies on the reproduction of three species of perophora (ascidiacea). The Biological Bulletin *164*, 251–266.

Mukai, H., Saito, Y., and Watanabe, H. (1987). Viviparous development in Botrylloides (Compound Ascidians). Journal of Morphology *193*, 263–276.

Mukai, H., Hashimoto, K., and Watanabe, H. (1990). Tunic cords, glomerulocytes, and eosinophilic bodies in a styelid ascidian, Polyandrocarpa misakiensis. Journal of Morphology *206*, 197–210.

Müller, W.E.G. (2006). The stem cell concept in sponges (Porifera): Metazoan traits. Seminars in Cell & Developmental Biology *17*, 481–491.

Munro, A.D., Scott, A.P., and Lam, T.J. (1990). Reproductive Seasonality in Teleosts: Environmental Influences (CRC Press).

Munro, C., Vue, Z., Behringer, R.R., and Dunn, C.W. (2019). Morphology and development of the Portuguese man of war, Physalia physalis. Scientific Reports *9*, 15522.

Murawala, P., Tanaka, E.M., and Currie, J.D. (2012). Regeneration: The ultimate example of wound healing. Seminars in Cell & Developmental Biology *23*, 954–962.

Myohara, M. (2004). Differential tissue development during embryogenesis and regeneration in an annelid. Developmental Dynamics *231*, 349–358.

Myohara, M. (2012). What Role Do Annelid Neoblasts Play? A Comparison of the Regeneration Patterns in a Neoblast-Bearing and a Neoblast-Lacking Enchytraeid Oligochaete. PLOS ONE *7*, e37319.

Nakashima, K., Yamada, L., Satou, Y., Azuma, J.-I., and Satoh, N. (2004). The evolutionary origin of animal cellulose synthase. Dev. Genes Evol. *214*, 81–88.

Nakauchi, M. (1970). ASEXUAL REPRODUCTION IN AMAROUCIUM YAMAZII (A COLONIAL ASCIDIAN). Publ. SMBL *17*, 309–328.

Nakauchi, M. (1982). Asexual Development of Ascidians: Its Biological Significance, Diversity, and Morphogenesis. Integr Comp Biol *22*, 753–763.

Nakauchi, M. (1986). Oozooid development and budding in the polyclinid ascidian, Parascidia flemingii (Urochordata)*. Journal of Zoology *208*, 255–267.

Nakauchi, M., and Kawamura, K. (1978). Additional experiments on the behavior of buds in the ascidian, aplidium multiplicatum. The Biological Bulletin *154*, 453–462.

Nakayama-Ishimura, A., Chambon, J., Horie, T., Satoh, N., and Sasakura, Y. (2009). Delineating metamorphic pathways in the ascidian Ciona intestinalis. Developmental Biology *326*, 357–367.

van Name, G. (1921). Budding in compound ascidians and other invertebrates, and its bearing on the question of the early ancestry of the vertebrates. Bulletin of the AMNH ; v. 44, article 15.

van Name, W.G.V. (1945). The North and South American Ascidians (American Museum of Natural History).

Newmark, P.A., and Sánchez Alvarado, A. (2000). Bromodeoxyuridine Specifically Labels the Regenerative Stem Cells of Planarians. Developmental Biology *220*, 142–153.

Nicol, D., and Meinertzhagen, I.A. (1988a). Development of the central nervous system of the larva of the ascidian, Ciona intestinalis L: I. The early lineages of the neural plate. Developmental Biology *130*, 721–736.

Nicol, D., and Meinertzhagen, I.A. (1988b). Development of the central nervous system of the larva of the ascidian, Ciona intestinalis L: II. Neural plate morphogenesis and cell lineages during neurulation. Developmental Biology *130*, 737–766.

Nilsson Sköld, H., and Obst, M. (2011). Potential for clonal animals in longevity and ageing studies. Biogerontology *12*, 387–396.

Nishida, H. (2005). Specification of embryonic axis and mosaic development in ascidians. Developmental Dynamics *233*, 1177–1193.

Nishikawa, T. (1993). Probable introduction of Polyandrocarpa zorritensis (VAN NAME) to Kitakyushu and Kochi, Japan. Zoological Science *10*, 176.

Nomaguchi, T.A. (1974). Seasonal variations in life span of the ascidian, Ciona intestinalis. Experimental Gerontology *9*, 231–232.

Nomaguchi, T.A., Nishijima, C., Minowa, S., Hashimoto, M., Haraguchi, C., Amemiya, S., and Fujisawa, H. (1997). Embryonic Thermosensitivity of the Ascidian, Ciona savignyi. Jzoo *14*, 511–515.

Normark, B.B. (2013). Parthenogenesis. In Brenner's Encyclopedia of Genetics (Second Edition), S. Maloy, and K. Hughes, eds. (San Diego: Academic Press), pp. 233–235.

Nunzi, M.G., Burighel, P., and Schiaffino, S. (1979). Muscle cell differentiation in the ascidian heart. Developmental Biology *68*, 371–380.

Odland, G., and Ross, R. (1968). HUMAN WOUND REPAIR I. Epidermal Regeneration. J Cell Biol *39*, 135–151.

Ogasawara, M., Lauro, R.D., and Satoh, N. (1999). Ascidian homologs of mammalian thyroid peroxidase genes are expressed in the thyroid-equivalent region of the endostyle. Journal of Experimental Zoology *285*, 158–169.

Ogawa, A., and Brown, F. (2015). Dauer formation and dauer-specific behaviours in Pristionchus pacificus. *Pristionchus Pacificus* 257–299.

Ohashi, M., Kawamura, K., Fujii, N., Yubisui, T., and Fujiwara, S. (1999). A Retinoic Acid-Inducible Modular Protease in Budding Ascidians. Developmental Biology *214*, 38–45.

Oka, H., and Watanabe, H. (1957). Vascular Budding, a New Type of Budding in Botryllus. Biological Bulletin *112*, 225–240.

Ortolani, G. (1955). The presumptive territory of the mesoderm in the ascidian germ. Experientia *11*, 445–446.

Orton, J. H.. *Preliminary Account of a Contribution to an Evaluation of the Sea*. Journ. Mar. Biol. Assoc., Vol. X, No. 2, 1914, p. 312.

Orton, J.H. (1920). Sea-Temperature, Breeding and Distribution in Marine Animals. Journal of the Marine Biological Association of the United Kingdom *12*, 339–366.

ORTON, J. The Production of Living Clavellina Zooids in Winter by Experiment. *Nature***107,** 75 (1921)

Otto, J.J., and Campbell, R.D. (1977). Budding in Hydra attenuata: Bud stages and fate map. Journal of Experimental Zoology *200*, 417–428.

Otto, S.P., and Lenormand, T. (2002). Resolving the paradox of sex and recombination. Nature Reviews Genetics *3*, 252–261.

Overton, J. (1966). The fine structure of blood cells in the ascidian Perophora viridis. Journal of Morphology *119*, 305–326.

Oviedo, N.J., Nicolas, C.L., Adams, D.S., and Levin, M. (2008). Planarians: A Versatile and Powerful Model System for Molecular Studies of Regeneration, Adult Stem Cell Regulation, Aging, and Behavior. Cold Spring Harb Protoc *2008*, pdb.emo101.

Özpolat, B.D., and Bely, A.E. (2016). Developmental and molecular biology of annelid regeneration: a comparative review of recent studies. Current Opinion in Genetics & Development *40*, 144–153.

Palmberg, I., and Reuter, M. (1983). Asexual reproduction in Microstomum lineare (Turbellaria). I. An autoradiographic and ultrastructural study. International Journal of Invertebrate Reproduction *6*, 197–206.

Passamaneck, Y.J., and Martindale, M.Q. (2012). Cell proliferation is necessary for the regeneration of oral structures in the anthozoan cnidarian Nematostella vectensis. BMC Developmental Biology *12*, 34.

Pauwels, K., Stoks, R., Verbiest, A., and De Meester, L. (2007). Biochemical adaptation for dormancy in subitaneous and dormant eggs of Daphnia magna. Hydrobiologia *594*, 91–96.

Pearl, E.J., Li, J., and Green, J.B.A. (2017). Cellular systems for epithelial invagination. Philosophical Transactions of the Royal Society B: Biological Sciences *372*, 20150526.

Pei, D., Shu, X., Gassama-Diagne, A., and Thiery, J.P. (2019). Mesenchymal–epithelial transition in development and reprogramming. Nature Cell Biology *21*, 44–53.

Pellettieri, J. (2019). Regenerative tissue remodeling in planarians – The mysteries of morphallaxis. Seminars in Cell & Developmental Biology *87*, 13–21.

Penkov, S., Ogawa, A., Schmidt, U., Tate, D., Zagoriy, V., Boland, S., Gruner, M., Vorkel, D., Verbavatz, J.-M., Sommer, R.J., et al. (2014). A wax ester promotes collective host finding in the nematode Pristionchus pacificus. Nature Chemical Biology *10*, 281–285.

Petersen, J.K. (2007). Ascidian suspension feeding. Journal of Experimental Marine Biology and Ecology *342*, 127–137.

Petersen, C.P., and Reddien, P.W. (2009). Wnt Signaling and the Polarity of the Primary Body Axis. Cell *139*, 1056–1068.

Petersen, J., and Svane, I. (2002). Filtration rate in seven Scandinavian ascidians: implications of the morphology of the gill sac. Marine Biology *140*, 397–402.

Phillips, B., Kremer, P., and Madin, L.P. (2009). Defecation by Salpa thompsoni and its contribution to vertical flux in the Southern Ocean. Mar Biol *156*, 455–467.

Piette, J., and Lemaire, P. (2015). Thaliaceans, The Neglected Pelagic Relatives of Ascidians: A Developmental and Evolutionary Enigma. The Quarterly Review of Biology *90*, 117–145.

Pizon, A. (1905). L'évolution des diplosomes: (Ascidies composées). Archives de zoologie expérimentale et générale, ser, 4, 1– 68.

Planques, A., Malem, J., Parapar, J., Vervoort, M., and Gazave, E. (2019). Morphological, cellular and molecular characterization of posterior regeneration in the marine annelid Platynereis dumerilii. Developmental Biology *445*, 189–210.

Plass, M., Solana, J., Wolf, F.A., Ayoub, S., Misios, A., Glažar, P., Obermayer, B., Theis, F.J., Kocks, C., and Rajewsky, N. (2018). Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. Science *360*.

Poelchau, M.F., Reynolds, J.A., Elsik, C.G., Denlinger, D.L., and Armbruster, P.A. (2013). Deep sequencing reveals complex mechanisms of diapause preparation in the invasive mosquito, Aedes albopictus. Proceedings of the Royal Society B: Biological Sciences *280*, 20130143.

Pope, E.C., and Rowley, A.F. (2002). The heart of Ciona intestinalis: eicosanoid-generating capacity and the effects of precursor fatty acids and eicosanoids on heart rate. Journal of Experimental Biology *205*, 1577–1583.

Poss, K.D. (2010). Advances in understanding tissue regenerative capacity and mechanisms in animals. Nature Reviews Genetics *11*, 710–722.

Poupardin, R., Schöttner, K., Korbelová, J., Provazník, J., Doležel, D., Pavlinic, D., Beneš, V., and Koštál, V. (2015). Early transcriptional events linked to induction of diapause revealed by RNAseq in larvae of drosophilid fly, Chymomyza costata. BMC Genomics *16*, 720.

Prünster, M.M., Ricci, L., Brown, F.D., and Tiozzo, S. (2019a). De novo neurogenesis in a budding chordate: Co-option of larval anteroposterior patterning genes in a transitory neurogenic organ. Developmental Biology *448*, 342–352.

Prünster, M.M., Ricci, L., Brown, F.D., and Tiozzo, S. (2019b). Modular co-option of cardiopharyngeal genes during non-embryonic myogenesis. EvoDevo *10*, 3.

Qiu, Z., Tsoi, S.C.M., and MacRae, T.H. (2007). Gene expression in diapause-destined embryos of the crustacean, Artemia franciscana. Mechanisms of Development *124*, 856–867.

Rabeling, C., and Kronauer, D.J.C. (2013). Thelytokous Parthenogenesis in Eusocial Hymenoptera. Annual Review of Entomology *58*, 273–292.

Radzikowski, J. (2013). Resistance of dormant stages of planktonic invertebrates to adverse environmental conditions. J Plankton Res *35*, 707–723.

Ragland, G.J., and Keep, E. (2017). Comparative transcriptomics support evolutionary convergence of diapause responses across Insecta. Physiological Entomology *42*, 246–256.

Ragland, G.J., Denlinger, D.L., and Hahn, D.A. (2010). Mechanisms of suspended animation are revealed by transcript profiling of diapause in the flesh fly. PNAS *107*, 14909–14914.

Randolph, H. (1892). The regeneration of the tail in lumbriculus. Journal of Morphology *7*, 317–344.

Reichman, O.J. (1984). Evolution of Regeneration Capabilities. The American Naturalist *123*, 752–763.

Reitzel, A.M., Burton, P.M., Krone, C., and Finnerty, J.R. (2007). Comparison of developmental trajectories in the starlet sea anemone Nematostella vectensis: embryogenesis, regeneration, and two forms of asexual fission. Invertebrate Biology *126*, 99–112.

Reuter, M., and Kreshchenko, N. (2004). Flatworm asexual multiplication implicates stem cells and regeneration. Can. J. Zool. *82*, 334–356.

Rezende, G.L., Martins, A.J., Gentile, C., Farnesi, L.C., Pelajo-Machado, M., Peixoto, A.A., and Valle, D. (2008). Embryonic desiccation resistance in Aedes aegypti: presumptive role of the chitinized Serosal Cuticle. BMC Dev Biol *8*, 82.

Ricci, C. (1998). Anhydrobiotic capabilities of bdelloid rotifers. Hydrobiologia *387*, 321–326.

Ricci, C., and Fontaneto, D. (2009). The importance of being a bdelloid: Ecological and evolutionary consequences of dormancy. Italian Journal of Zoology *76*, 240–249.

Ricci, L., Cabrera, F., Lotito, S., and Tiozzo, S. (2016). Redeployment of germ layers related TFs shows regionalized expression during two non-embryonic developments. Developmental Biology *416*, 235–248.

Richards, J.G. (2010). Metabolic Rate Suppression as a Mechanism for Surviving Environmental Challenge in Fish. In Aestivation: Molecular and Physiological Aspects, C. Arturo Navas, and J.E. Carvalho, eds. (Berlin, Heidelberg: Springer), pp. 113–139.

Rinehart, J.P., Li, A., Yocum, G.D., Robich, R.M., Hayward, S.A.L., and Denlinger, D.L. (2007). Up-regulation of heat shock proteins is essential for cold survival during insect diapause. Proc Natl Acad Sci U S A *104*, 11130–11137.

Rink, J.C. (2013). Stem cell systems and regeneration in planaria. Dev. Genes Evol. *223*, 67–84.

Rinkevich, B., and Rabinowitz, C. (1993). In vitro Culture of Blood Cells from the Colonial Protochordate Botryllus schlosseri. In Vitro Cellular & Developmental Biology *29A*, 79–85.

Rinkevich, Y., Paz, G., Rinkevich, B., and Reshef, R. (2007). Systemic Bud Induction and Retinoic Acid Signaling Underlie Whole Body Regeneration in the Urochordate Botrylloides leachi. PLoS Biol *5*, e71.

Rinkevich, Y., Rosner, A., Rabinowitz, C., Lapidot, Z., Moiseeva, E., and Rinkevich, B. (2010). Piwi positive cells that line the vasculature epithelium, underlie whole body regeneration in a basal chordate. Developmental Biology *345*, 94–104.

Rinkevich, Y., Voskoboynik, A., Rosner, A., Rabinowitz, C., Paz, G., Oren, M., Douek, J., Alfassi, G., Moiseeva, E., Ishizuka, K.J., et al. (2013). Repeated, Long-Term Cycling of Putative Stem Cells between Niches in a Basal Chordate. Developmental Cell *24*, 76–88.

Rizzo, A.M., Negroni, M., Altiero, T., Montorfano, G., Corsetto, P., Berselli, P., Berra, B., Guidetti, R., and Rebecchi, L. (2010). Antioxidant defences in hydrated and desiccated states of the tardigrade Paramacrobiotus richtersi. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology *156*, 115–121.

Rodriguez, D., Nourizadeh, S., and De Tomaso, A.W. (2019). The biology of the extracorporeal vasculature of Botryllus schlosseri. Developmental Biology *448*, 309–319.

Romanov, V.N. 1989. "Colonial ascidians of the family Didemnidae from the seas of the USSR and adjacent waters". In *Fauna of USSR. New series, n.* ***138****. Tunicates* Vol. 1, Ascidians 225p. (In Russian with English summary and diagnoses)

Rosental, B., Kowarsky, M., Seita, J., Corey, D.M., Ishizuka, K.J., Palmeri, K.J., Chen, S.-Y., Sinha, R., Okamoto, J., Mantalas, G., et al. (2018). Complex mammalian-like haematopoietic system found in a colonial chordate. Nature *564*, 425–429.

Ruppert, E.E., and Barnes, R.D. (1994). Invertebrate Zoology (Saunders College Publishing).

Sabbadin, A. (1955). Osservazioni sullo sviluppo, l'accrescimento e la riproduzione di *Botryllus schlosseri* (Pallas), in condizioni di laboratorio. Bolletino di zoologia *22*, 243–263.

Sabbadin, A., and Zaniolo, G. (1979). Sexual differentiation and germ cell transfer in the colonial ascidian Botryllus schlosseri. Journal of Experimental Zoology *207*, 289–304.

Sacks, P.G., and Davis, L.E. (1979). Production of nerveless Hydra attenuata by hydroxyurea treatments. Journal of Cell Science *37*, 189–203.

Salfi, M. (1929) Sulla blastogenesi in Clavelina e su una nuova specie del genere. Pubblicazioni Stazione Zoologica di Napoli 9, 195–201.

Sánchez Alvarado, A. (2006). Planarian regeneration: its end is its beginning. Cell *124*, 241–245.

Scelzo, M., Alié, A., Pagnotta, S., Lejeune, C., Henry, P., Gilletta, L., Hiebert, L.S., Mastrototaro, F., and Tiozzo, S. (2019). Novel budding mode in Polyandrocarpa zorritensis: a model for comparative studies on asexual development and whole body regeneration. EvoDevo *10*.

Schack, C.R., Gordon, D.P., and Ryan, K.G. (2019). Modularity is the mother of invention: a review of polymorphism in bryozoans. Biological Reviews *94*, 773–809.

Schaffer, A.A., Bazarsky, M., Levy, K., Chalifa-Caspi, V., and Gat, U. (2016). A transcriptional time-course analysis of oral vs. aboral whole-body regeneration in the Sea anemone Nematostella vectensis. BMC Genomics *17*, 718.

Schlumpberger, J.M., Weissman, I.L., and Scofield, V.L. (1984). Separation and labeling of specific subpopulations of Botryllus blood cells. Journal of Experimental Zoology *229*, 401–411.

Secor, S.M., and Lignot, J.-H. (2010). Morphological Plasticity of Vertebrate Aestivation. In Aestivation: Molecular and Physiological Aspects, C. Arturo Navas, and J.E. Carvalho, eds. (Berlin, Heidelberg: Springer), pp. 183–208.

Seidman, L.A., and Larsen Jr., J.H. (1979). Ultrastructure of the envelopes of resistant and nonresistant Daphnia eggs. Can. J. Zool. *57*, 1773–1777.

Selys-Longchamps, M. de (1917). Sur le bourgeonnement des polystyélinés: Stolonica et Heterocarpa : avec quelques notes sur l'anatomie de ces deux genres (Lhomme).

Shenkar, N., and Swalla, B.J. (2011). Global Diversity of Ascidiacea. PLoS ONE *6*, e20657.

Shenkar, N., Koplovitz, G., Dray, L., Gissi, C., and Huchon, D. (2016). Back to solitude: Solving the phylogenetic position of the Diazonidae using molecular and developmental characters. Molecular Phylogenetics and Evolution *100*, 51–56.

Shibata, N., Umesono, Y., Orii, H., Sakurai, T., Watanabe, K., and Agata, K. (1999). Expression ofvasa(vas)-Related Genes in Germline Cells and Totipotent Somatic Stem Cells of Planarians. Developmental Biology *206*, 73–87.

Shimizu, H., Sawada, Y., and Sugiyama, T. (1993). Minimum Tissue Size Required for Hydra Regeneration. Developmental Biology *155*, 287–296.

Shirae, M., and Saito, Y. (2000). A Comparison of Hemocytes and Their Phenoloxidase Activity among Botryllid Ascidians. ZOOLOGICAL SCIENCE *17*, 881–891.

Shirae, M., Ballarin, L., Frizzo, A., Saito, Y., and Hirose, E. (2002). Involvement of quinones and phenoloxidase in the allorejection reaction in a colonial ascidian, Botrylloides simodensis: histochemical and immunohistochemical study. Marine Biology *141*, 659–665.

Shostak, S., Bisbee, J.W., Ashkin, C., and Tammariello, R.V. (1968). Budding in Hydra viridis. Journal of Experimental Zoology *169*, 423–429.

Shu, D.-G., Morris, S.C., Han, J., Chen, L., Zhang, X.-L., Zhang, Z.-F., Liu, H.-Q., Li, Y., and Liu, J.-N. (2001). Primitive deuterostomes from the Chengjiang Lagerstätte (Lower Cambrian, China). Nature *414*, 419–424.

Shumway, S.E. (1978). Respiration, pumping activity and heart rate in Ciona intestinalis exposed to fluctuating salinities. Mar. Biol. *48*, 235–242.

Sibly, R., and Calow, P. (1982). Asexual reproduction in protozoa and invertebrates. Journal of Theoretical Biology *96*, 401–424.

Sikes, J.M., and Newmark, P.A. (2013). Restoration of anterior regeneration in a planarian with limited regenerative ability. Nature *500*, 77–80.

da Silva, P.G.C., Abreu, I.S. de, Cavalcante, L.A., Barros, C.M.D., and Allodi, S. (2015). Role of hemocytes in invertebrate adult neurogenesis and brain repair. Invertebrate Survival Journal *12*, 142–154.

Simion, P., Narayan, J., Houtain, A., Derzelle, A., Baudry, L., Nicolas, E., Cariou, M., Guiglielmoni, N., Kozlowski, D.K., Gaudray, F.R., et al. (2020). Homologous chromosomes in asexual rotifer Adineta vaga suggest automixis. BioRxiv 2020.06.16.155473.

Simpson, T.L. (2012). The Cell Biology of Sponges (Springer Science & Business Media).

Simpson, T.L., and Fell, P.E. (1974). Dormancy among the Porifera: Gemmule Formation and Germination in Fresh-Water and Marine Sponges. Transactions of the American Microscopical Society *93*, 544–577.

Simpson, T.L., and Rodan, G.A. (1976). Role of cAMP in the release from dormancy of freshwater sponge gemmules. Developmental Biology *49*, 544–547.

Sköld, H.N., Stach, T., Bishop, J.D.D., Herbst, E., and Thorndyke, M.C. (2011). Pattern of Cell Proliferation During Budding in the Colonial Ascidian Diplosoma listerianum. The Biological Bulletin *221*, 126–136.

Slack, J.M. (2017). Animal regeneration: ancestral character or evolutionary novelty? EMBO Reports *18*, 1497–1508.

Smith, M.J. (1970). The blood cells and tunic of the ascidian halocynthia aurantium (pallas). i. hematology, tunic morphology, and partition of cells between blood and tunic. The Biological Bulletin *138*, 354–378.

Smith, L.D., and Hughes, T.P. (1999). An experimental assessment of survival, re-attachment and fecundity of coral fragments. Journal of Experimental Marine Biology and Ecology *235*, 147–164.

Solana, J. (2013). Closing the circle of germline and stem cells: the Primordial Stem Cell hypothesis. EvoDevo *4*, 2.

Song, L., and Florea, L. (2015). Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. Gigascience *4*.

Sonneborn, T.M. (1930). Genetic studies on stenostomum incaudatum (Nov. spec.). I. The nature and origin of differences among individuals formed during vegetative reproduction. Journal of Experimental Zoology *57*, 57–108.

Stabili, L., Licciano, M., Longo, C., Lezzi, M., and Giangrande, A. (2015). The Mediterranean non-indigenous ascidian Polyandrocarpa zorritensis: Microbiological accumulation capability and environmental implications. Marine Pollution Bulletin *101*, 146–152.

Storey, K.B. (1997). Organic Solutes in Freezing Tolerance. Comparative Biochemistry and Physiology Part A: Physiology *117*, 319–326.

Storey, K.B., and Storey, J.M. (1988). Freeze tolerance in animals. Physiological Reviews *68*, 27–84.

Storey, K.B., and Storey, J.M. (2010). Metabolic Regulation and Gene Expression During Aestivation. In Aestivation: Molecular and Physiological Aspects, C. Arturo Navas, and J.E. Carvalho, eds. (Berlin, Heidelberg: Springer), pp. 25–45.

Strachan, S.R., Chester, E.T., and Robson, B.J. (2015). Freshwater Invertebrate Life History Strategies for Surviving Desiccation. Springer Science Reviews *3*, 57–75.

Stross, R.G., and Hill, J.C. (1968). Photoperiod control of winter diapause in the fresh-water crustacean, daphnia. The Biological Bulletin *134*, 176–198.

Subramoniam, T. (2018). Mode of Reproduction: Invertebrate Animals. In Encyclopedia of Reproduction, (Elsevier), pp. 32–40.

Sugino, Y.M. (1993). An ultrastructural study of blood cells in the ascidian, Polyandrocarpa misakiensis: Their classification and behavioral characteristics. Mem. Fac. Sci. Kochi Univ. Ser. D *14*, 33–41.

Sugino, Y.M., Matsumura, M., and Kawamura, K. (2007). Body Muscle-Cell Differentiation from Coelomic Stem Cells in Colonial Tunicates. Jzoo *24*, 542–546.

Sumida, P.Y.G., Güth, A.Z., Mies, M., Sumida, P.Y.G., Güth, A.Z., and Mies, M. (2015). Pressure tolerance of tadpole larvae of the Atlantic ascidian Polyandrocarpa zorritensis: potential for deep-sea invasion. Brazilian Journal of Oceanography *63*, 515–520.

Sunanaga, T., Saito, Y., and Kawamura, K. (2006). Postembryonic epigenesis of Vasa-positive germ cells from aggregated hemoblasts in the colonial ascidian, Botryllus primigenus. Development, Growth & Differentiation *48*, 87–100.

Sunanaga, T., Inubushi, H., and Kawamura, K. (2010). Piwi-expressing hemoblasts serve as germline stem cells during postembryonic germ cell specification in colonial ascidian, Botryllus primigenus. Development, Growth & Differentiation *52*, 603–614.

Suomalainen, E. (1950). Parthenogenesis in Animals**Received for publication March 11, 1948. In Advances in Genetics, M. Demerec, ed. (Academic Press), pp. 193–253.

Sutherland, K.R., Madin, L.P., and Stocker, R. (2010). Filtration of submicrometer particles by pelagic tunicates. PNAS *107*, 15129–15134.

Suzuki, M., Yakushiji, N., Nakada, Y., Satoh, A., Ide, H., and Tamura, K. (2006). Limb Regeneration in *Xenopus laevis* Froglet. The Scientific World JOURNAL *6*, 26–37.

Suzuki, T., Takagi, T., Furukohri, T., Kawamura, K., and Nakauchi, M. (1990). A calcium-dependent galactose-binding lectin from the tunicate Polyandrocarpa misakiensis. Isolation, characterization, and amino acid sequence. J. Biol. Chem. *265*, 1274–1281.

Swalla, B.J., and Jeffery, W.R. (1990). Interspecific hybridization between an anural and urodele ascidian: Differential expression of urodele features suggests multiple mechanisms control anural development. Developmental Biology *142*, 319–334.

Swaminathan, G., and Cartwright, C.A. (2012). Rack1 promotes epithelial cell–cell adhesion by regulating E-cadherin endocytosis. Oncogene *31*, 376–389.

Sztalryd, C., and Brasaemle, D.L. (2017). The perilipin family of lipid droplet proteins: Gatekeepers of intracellular lipolysis. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids *1862*, 1221–1232.

Tadokoro, R., Sugio, M., Kutsuna, J., Tochinai, S., and Takahashi, Y. (2006). Early Segregation of Germ and Somatic Lineages during Gonadal Regeneration in the Annelid Enchytraeus japonensis. Current Biology *16*, 1012–1017.

Takahashi, K., Ichikawa, T., Fukugama, C., Yamane, M., Kakehi, S., Okazaki, Y., Kubota, H., and Furuya, K. (2015). In situ observations of a doliolid bloom in a warm water filament using a video plankton recorder: Bloom development, fate, and effect on biogeochemical cycles and planktonic food webs. Limnology and Oceanography *60*, 1763–1780.

Taneda, Y. (1985). Size regulation of regenerated organs in the compound ascidian, Polyandrocarpa misakiensis. Journal of Experimental Zoology *233*, 331–334.

Tanguay, J.A., Reyes, R.C., and Clegg, J.S. (2004). Habitat diversity and adaptation to environmental stress in encysted embryos of the crustacean Artemia. J. Biosci. *29*, 489–501.

Tatián, M., Lagger, C., Demarchi, M., and Mattoni, C. (2011). Molecular phylogeny endorses the relationship between carnivorous and filter-feeding tunicates (Tunicata, Ascidiacea): Phylogeny of carnivorous tunicates. Zoologica Scripta *40*, 603–612.

Tatzuke, Y., Sunanaga, T., Fujiwara, S., and Kawamura, K. (2012). RACK1 regulates mesenchymal cell recruitment during sexual and asexual reproduction of budding tunicates. Developmental Biology *368*, 393–403.

Technau, U., Laue, C.C. von, Rentzsch, F., Luft, S., Hobmayer, B., Bode, H.R., and Holstein, T.W. (2000). Parameters of self-organization in Hydra aggregates. PNAS *97*, 12127–12131.

Terakado, K., and Mukai, H. (1978). Ultrastructural studies on the formation of yolk granules in the statoblast of a fresh-water bryozoan, Pectinatella gelatinosa. Journal of Morphology *156*, 317–337.

Thorndyke, M.C. (1977). Observations on the gastric epithelium of ascidians with special reference to Styela clava. Cell Tissue Res. *184*, 539–550.

Tiozzo, S., and Copley, R.R. (2015). Reconsidering regeneration in metazoans: an evo-devo approach. Front. Ecol. Evol. *3*.

Tiozzo, S., Ballarin, L., Burighel, P., and Zaniolo, G. (2006). Programmed cell death in vegetative development: Apoptosis during the colonial life cycle of the ascidian Botryllus schlosseri. Tissue and Cell *38*, 193–201.

Tiozzo, S., Brown, F.D., and De Tomaso, A.W. (2008). Regeneration and Stem Cells in Ascidians. In Stem Cells: From Hydra to Man, T.C.G. Bosch, ed. (Dordrecht: Springer Netherlands), pp. 95–112.

Trivers, R. (1983). The Evolution of Sex. The Quarterly Review of Biology *58*, 62–67.

Trudgill, D.L., Honek, A., Li, D., and Straalen, N.M.V. (2005). Thermal time – concepts and utility. Annals of Applied Biology *146*, 1–14.

Turon, X. (1992). Periods of non-feeding in Polysyncraton lacazei (Ascidiacea: Didemnidae): a rejuvenative process? Marine Biology *112*, 647–655.

Turon, X., and Becerro, M.A. (1992). Growth and survival of several ascidian species from the northwestern Mediterranean. Marine Ecology Progress Series *82*, 235–247.

Tweeten, K.A., and Anderson, A. (2008). Analysis of cell proliferation and migration during regeneration in Lumbriculus variegatus (Clitellata: Lumbriculidae). Bios *79*, 183–190.

Umesono, Y., Tasaki, J., Nishimura, Y., Hrouda, M., Kawaguchi, E., Yazawa, S., Nishimura, O., Hosoda, K., Inoue, T., and Agata, K. (2013). The molecular logic for planarian regeneration along the anterior–posterior axis. Nature *500*, 73–76.

Van Name, W.G. (1931). New North and South American ascidians. Bulletin of the AMNH ; v. 61, article 6.

van Wolfswinkel, J.C., Wagner, D.E., and Reddien, P.W. (2014). Single-Cell Analysis Reveals Functionally Distinct Classes within the Planarian Stem Cell Compartment. Cell Stem Cell *15*, 326–339.

Vázquez, E., and Young, C. (1996). Responses of compound ascidian larvae to haloclines. Marine Ecology Progress Series *133*, 179–190.

Vázquez, E., and Young, C.M. (1998). Ontogenetic changes in phototaxis during larval life of the Ascidian Polyandrocarpa zorritensis (Van Name, 1931). Journal of Experimental Marine Biology and Ecology *231*, 267–277.

Vervoort, M. (2011). Regeneration and Development in Animals. Biol Theory *6*, 25–35.

Viard, F., Roby, C., Turon, X., Bouchemousse, S., and Bishop, J. (2019). Cryptic Diversity and Database Errors Challenge Non-indigenous Species Surveys: An Illustration With Botrylloides spp. in the English Channel and Mediterranean Sea. Front. Mar. Sci. *6*.

Viemont, J.D., and Crabbe, J. (2000). Dormancy in Plants: From Whole Plant Behaviour to Cellular Control (CABI).

Voitkevich, A.A. (1966). The feathers and plumage of birds. The Feathers and Plumage of Birds.

Voskoboynik, A., Simon-Blecher, N., Soen, Y., Rinkevich, B., Tomaso, A.W.D., Ishizuka, K.J., and Weissman, I.L. (2007). Striving for normality: whole body regeneration through a series of abnormal generations. The FASEB Journal *21*, 1335–1344.

Voskoboynik, A., Soen, Y., Rinkevich, Y., Rosner, A., Ueno, H., Reshef, R., Ishizuka, K.J., Palmeri, K.J., Moiseeva, E., Rinkevich, B., et al. (2008). Identification of the Endostyle as a Stem Cell Niche in a Colonial Chordate. Cell Stem Cell *3*, 456–464.

Wagner, D.E., Wang, I.E., and Reddien, P.W. (2011). Clonogenic Neoblasts Are Pluripotent Adult Stem Cells That Underlie Planarian Regeneration. Science *332*, 811–816.

Wahl, M., and Lafargue, F. (1990). Marine epibiosis. Oecologia *82*, 275–282.

Wang, J., and Kim, S.K. (2003). Global analysis of dauer gene expression in Caenorhabditis elegans. Development *130*, 1621–1634.

Wang, Y., Ezemaduka, A.N., Tang, Y., and Chang, Z. (2009). Understanding the mechanism of the dormant dauer formation of C. elegans: From genetics to biochemistry. IUBMB Life *61*, 607–612.

Warner, J.F., Guerlais, V., Amiel, A.R., Johnston, H., Nedoncelle, K., and Röttinger, E. (2018). NvERTx: a gene expression database to compare embryogenesis and regeneration in the sea anemone Nematostella vectensis. Development *145*.

Warner, J.F., Amiel, A.R., Johnston, H., and Röttinger, E. (2019). Regeneration is a partial redeployment of the embryonic gene network. BioRxiv 658930.

Watanabe, H., and Tokioka, T. (1973). ON A NEW SPECIES OF CLAVELINA FROM JAPAN, WITH REMARKS ON ITS MODE OF BUDDING. PUBLICATIONS OF THE SETO MARINE BIOLOGICAL LABORATORY *21*, 99–107.

Watanabe, H., Hoang, V.T., Mättner, R., and Holstein, T.W. (2009). Immortality and the base of multicellular life: Lessons from cnidarian stem cells. Seminars in Cell & Developmental Biology *20*, 1114–1125.

Waterhouse, R.M., Seppey, M., Simão, F.A., Manni, M., Ioannidis, P., Klioutchnikov, G., Kriventseva, E.V., and Zdobnov, E.M. (2018). BUSCO Applications from Quality Assessments to Gene Prediction and Phylogenomics. Mol Biol Evol *35*, 543–548.

Waterman, A.J. (1943). Further Study of the Action of Drugs on the Heart of the Compound Ascidian, Perophora viridis. Physiological Zoology *16*, 388–405.

Wehner, D., and Weidinger, G. (2015). Signaling networks organizing regenerative growth of the zebrafish fin. Trends in Genetics *31*, 336–343.

Wenemoser, D., and Reddien, P.W. (2010). Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. Developmental Biology *344*, 979–991.

Williams, G.C. (1975). Sex and Evolution (Princeton University Press).

Withers, P.C., and Cooper, C.E. (2010). Metabolic Depression: A Historical Perspective. In Aestivation: Molecular and Physiological Aspects, C. Arturo Navas, and J.E. Carvalho, eds. (Berlin, Heidelberg: Springer), pp. 1–23.

Wittlieb, J., Khalturin, K., Lohmann, J.U., Anton-Erxleben, F., and Bosch, T.C.G. (2006). Transgenic Hydra allow in vivo tracking of individual stem cells during morphogenesis. Proc Natl Acad Sci USA *103*, 6208.

Wolff, E., and Lender, T. (1962). THE NEOBLAST AND PHENOMENA OF INDUCTION AND INHIBITION IN REGENERATION OF PLANARIA. Annee Biologique (France) *Vol: 38*.

Wolkers, W.F., Tablin, F., and Crowe, J.H. (2002). From anhydrobiosis to freeze-drying of eukaryotic cells. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology *131*, 535–543.

Wood, T.S. (2001). 14 - BRYOZOANS. In Ecology and Classification of North American Freshwater Invertebrates (Second Edition), J.H. Thorp, and A.P. Covich, eds. (San Diego: Academic Press), pp. 505–525.

Wright, J.C. (1989). Desiccation Tolerance and Water-Retentive Mechanisms in Tardigrades. Journal of Experimental Biology *142*, 267–292.

Wright, R. (1981). Urochordata. Invertebrate Blood Cells 565–626.

Wulff, J.L. (1991). Asexual fragmentation, genotype success, and population dynamics of erect branching sponges. Journal of Experimental Marine Biology and Ecology *149*, 227–247.

Yamaguchi, M. (1975). Growth and reproductive cycles of the marine fouling ascidians Ciona intestinalis, Styela plicata, Botrylloides violaceus, and Leptoclinum mitsukurii at Aburatsubo-Moroiso Inlet (central Japan). Marine Biology *29*, 253–259.

Yamaguchi, N., Amakawa, Y., Yamada, H., Ueki, T., and Michibata, H. (2006). Localization of Vanabins, Vanadium-Binding Proteins, in the Blood Cells of the Vanadium-Rich Ascidian, Ascidia sydneiensis samea. Jzoo *23*, 909–915.

Yang, X., Dunning, K.R., Wu, L.L.-Y., Hickey, T.E., Norman, R.J., Russell, D.L., Liang, X., and Robker, R.L. (2010). Identification of Perilipin-2 as a lipid droplet protein regulated in oocytes during maturation. Reprod. Fertil. Dev. *22*, 1262–1271.

Yocum, G.D. (2001). Differential expression of two HSP70 transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. Journal of Insect Physiology *47*, 1139–1145.

Yocum, G.D., Rinehart, J.P., Horvath, D.P., Kemp, W.P., Bosch, J., Alroobi, R., and Salem, S. (2015). Key molecular processes of the diapause to post-diapause quiescence transition in the alfalfa leafcutting bee Megachile rotundata identified by comparative transcriptome analysis. Physiological Entomology *40*, 103–112.

Zattara, E.E., and Bely, A.E. (2016). Phylogenetic distribution of regeneration and asexual reproduction in Annelida: regeneration is ancestral and fission evolves in regenerative clades. Invertebrate Biology *135*, 400–414.

Zeng, A., Li, H., Guo, L., Gao, X., McKinney, S., Wang, Y., Yu, Z., Park, J., Semerad, C., Ross, E., et al. (2018). Prospectively Isolated Tetraspanin+ Neoblasts Are Adult Pluripotent Stem Cells Underlying Planaria Regeneration. Cell *173*, 1593-1608.e20.

Zondag, L.E., Rutherford, K., Gemmell, N.J., and Wilson, M.J. (2016). Uncovering the pathways underlying whole body regeneration in a chordate model, Botrylloides leachi using de novo transcriptome analysis. BMC Genomics *17*, 114.