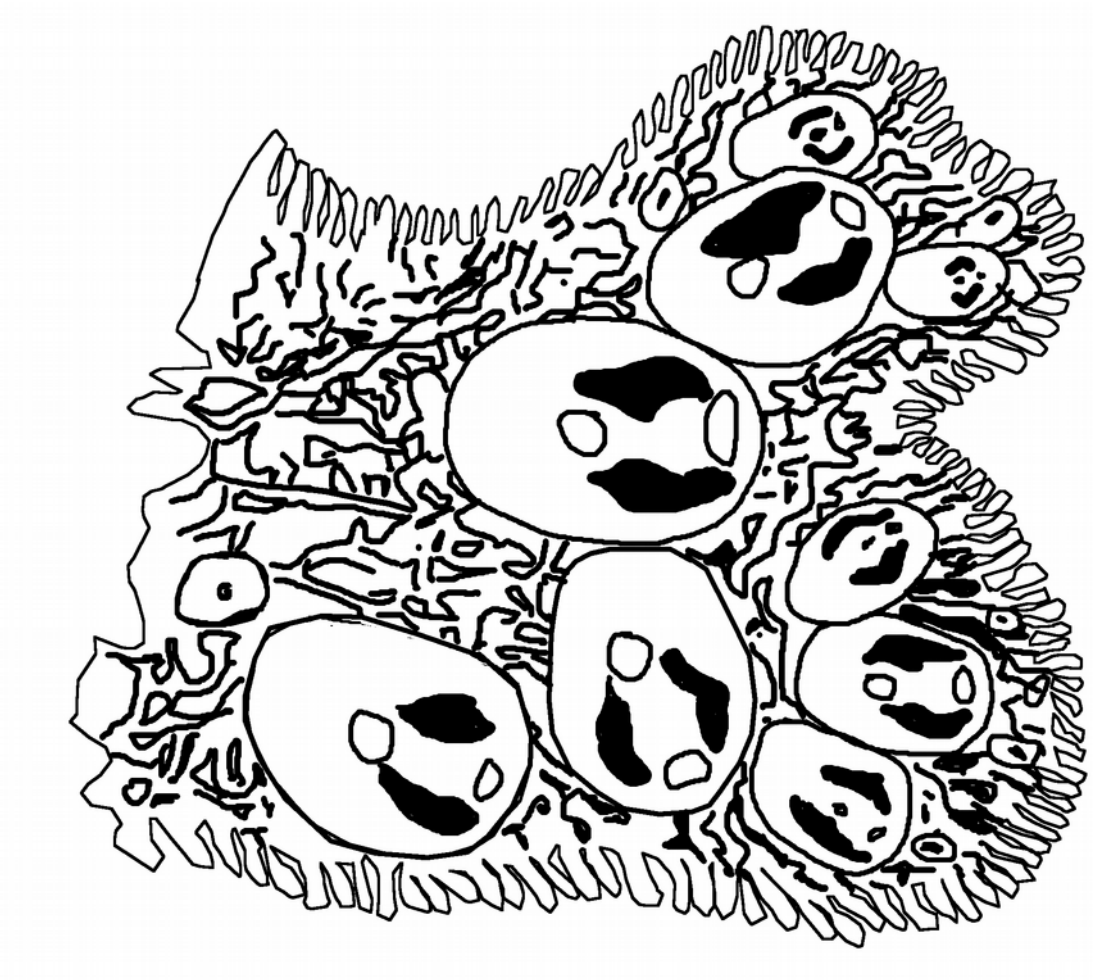

Developmental modularity in the colonial ascidian Symplegma: individual intercommunication, colonial hematopoiesis, and environmental factors involved in coloniality

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São Paulo

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Versão corrigida da tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Zoologia. A versão original encontra-se disponível no Instituto de Biociências da USP.

Orientador(a): Daniel J. G. Lahr

ERRATA

GUTIERREZ, Arianna Stefania Osorio. Desenvolvimento modular nas ascídias coloniais *Symplegma:* intercomunicação em indivíduos, hematopoiesis colonial, e fatores ambientais relacionados com a colonialidade. 2019. Tese (Doutorado)-Universidade de São Paulo.

Folha	Linha	Onde se lê	Leia-se
1	4	factores	fatores
2	4	factores	fatores
6	2	FAPESP	grant nº 2015/22650-2, São Paulo Research Foundation (FAPESP)

São Paulo

2019

Ficha Catalográfica

Gutierrez, Arianna Stefania Gutierrez Osorio
Desenvolvimento modular nas ascídias coloniais Symplegma: intercomunicação em indivíduos, hematopoiesis colonial, e factores ambientais relacionados com a colonialidade / Arianna Stefania Gutierrez Osorio Gutierrez; orientador Daniel J. G. Lahr . -- São Paulo, 2019.

126 f.

Tese (Doutorado) - Instituto de Biociências da Universidade de São Paulo, Departamento de Zoologia.

1. Coloniality. 2. Symplegma-tunicate. 3. Heterochrony. 4. modularity. 5. coordination. I. , Daniel J. G. Lahr, orient. II. Título.

Comissão Julgadora:

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a).

Prof. Dr. Daniel J. G. Lahr

Para Nelly y Anna Maria,

estoy aquí por su maravillosa herencia.

“In the beginning is the end;
But ends unfold, becoming strange.
Lives-- and generations-- suffer change.
The tested metabolic paths will tend
To last and shape the range
Of future evolution from the past.”

J.M Burns from *Biograffitti*.- Stephen Jay Gould
(Ontogeny and Phylogeny, 1977)

Acknowledgments

This research was supported by CAPES and the grant nº 2015/22650-2, São Paulo Research Foundation (FAPESP). I would like to thank to the members of EVODEVO laboratory Laurel, Juan, Denisse and Hans for their support and interesting discussion about colonial tunicates. I am also grateful to the technicians and administrative staff of Zoology Department, for their support with experimental procedures, reagents and patience with the bureaucracy. I am grateful with the professor Marie-Anne van Sluys and the members of her laboratory, specially the technician Tati, for all their help with the RNA extraction with my *Symplegma*, tunicates and cnidarians samples. I hope that this technique will be useful to another researches with marine invertebrates. I am grateful with my fellow students and professors from the embryology courses in the CEBIMar, specially Alvaro Migotto for his dedication to teach about marine animals and his comments about my manuscript.

I would like to thank the coordinator of the Graduate program in Zoology Daniel Lahr for his time, his advise provide me tools to continue my PhD and write this thesis. I am grateful also with the members of Prof. Lahr's laboratory for their technical support with laboratory equipment. Giulia and Alfredo for their comments to my thesis manuscript.

I would like to thank the professors and my fellow students from the embryology course at the MBL in Woods Hole, their talks and discussions inspired me to continue working in developmental biology research. Also my friend Laura, our discussions about science an life in Stony beach were fascinating.

I would like to thank to my Colombian friends, Jhon, Jimmy and Susi for supporting me and teaching me about cladistics, virology and science. I am grateful to my friend Rafa and her family, they adopt me and supporting me as a family.

I am very grateful with my life partner Eduard for supporting me spiritually during these PhD years. Eddy your encouragement and love in hard times, and your advises about science were essential to complete this thesis. I would like to thank to my family for their support throughout all my academic steps. My mom, brother and sister for their caring and loving support. I am grateful to my uncles to inspired me to think and be a scientist.

Finally, I would like to express my special thanks to Brazil, this country teach me about kindly and generosity. Brazilians are the most friendly people that I have ever met. The richness and potential of Brazil is immense.

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Abstract

Colonial animals are biological systems composed of discrete units (zooids) that are physiologically interconnected and require coordinated development for the colony to function properly. The origins of modular developmental mechanisms that facilitated the evolution of coloniality remain unclear. Genus Symplegma is a clade from the tunicate family Styelidae, in which coloniality evolved repeatedly from a solitary ancestor. My main objective is understand the process involved in evolution of colonial life history, studying the colonial strategy in Symplegma clade. Symplegma zooids are embedded in a common tunic and present a vascular system which interconnect zooids. Inside the blood vessels systems are in constant circulation. I classified S. brakenhielmi S.rubra blood cells by morphology using cytohistological assays and transmission electron microscopy. Eleven types of hemocytes were founded: (a) macrophage-like cells (MLCs) involved in phagocytosis and programmed cell death (PCD); (b) undifferentiated cells (UCs) involved in budding and regeneration; (c) morula cells (MC) involved in immune reactions; (d) nephrocytes or pigment cells involved in storage and excretion. Precursors cells changed their proportions during regeneration, suggesting continuous hematopoiesis, and hemocyte differentiation processes in the blood of the colonies. In vivo and histological observations suggest that the lifespan of zooids is controlled by endogenous mechanisms of the colony. As well that aging zooids are phagocyted and recycled for the colony. Colonial answer to external perturbations was tested by systemic bud or zooid removals. The vascular tissues of the colonies self-organize and rearrangement to regenerate zooids. As well the development of the buds accelerated when buds are absence after a systematical remotion of the buds. The results show that the zooids in Symplegma colonies are replacement components, which life span and development time are controlled by endogenous mechanisms related to colony necessities. Symplegma colonies are acting like a self-regulated system that can rearrange its components in case of particular perturbations. Therefore Symplegma colonies evolved communication between modules in the form of specialized migrating cells that transmit matter and information across the colony, coordinating the biological process of the zooids. The evolution of coloniality in this clade is mediated by novelties in the communication and cooperation between modules such as the PCD of old zooids and their resorption by the migration of MLC to recycle its tissues and use for the next generations of zooids.

Resumo

Os animais coloniais são sistemas biológicos compostos por unidades discretas (zooides) que são fisiologicamente interconectadas e requerem desenvolvimento coordenado para que a colônia funcione adequadamente. As origens dos mecanismos modulares de desenvolvimento que facilitaram a evolução da colonialidade ainda são desconhecidas. O género *Symplegma* é um clado colonial, da família de tunicados Styelidae, onde o tipo de vida colonial evoluiu em repetidamente de um ancestral solitário. Meu principal objetivo foi estudar a história de vida do clado Symplegma para entender melhor a evolução da colonialidade em animais. Os zoides de Symplegma estão embebidos em uma túnica comum e apresentam um sistema vascular, interconectando todos os zooides da colônia. Dentro do sistema vascular, células do sangue circulam constantemente. Classifiquei as células sanguíneas de *Symplegma brakenhielmi* e *S.rubra* morfologicamente usando ensaios cito-histológicos e microscopia eletrônica de transmissão. Onze tipos de hemócitos foram encontrados: (a) células semelhantes a macrófagos (MLCs) envolvidas em fagocitose e morte celular programada (PCD); (b) células indiferenciadas (UCs) envolvidas em brotamento e regeneração; c) células morula (MC) envolvidas em respostas imunes; (d) nefrócitos ou células pigmentares envolvidas no armazenamento e excreção. Algumas células precursoras alteraram suas proporções durante a regeneração, sugerindo uma contínua hematopoiese e diferenciação celular no sangue das colônias. Observações in vivo e histológicas sugerem que o tempo de vida dos zooides é controlado por mecanismos endógenos da colônia. Além disso, os zooides envelhecidos são fagocitados e reciclados pela colônia. A resposta colonial às perturbações externas foi testada por remoções sistêmicas de brotos ou zooides. Como resposta as perturbações, os tecidos vasculares das colônias se auto-organizam e se rearranjam para regenerar as partes perdidas. Também o desenvolvimento dos brotos acelerou quando os brotos foram removidos. Os resultados mostram que os zooides das colônias de Symplegma são componentes que podem ser substituídos, cujo tempo de vida e tempo de desenvolvimento são controlados por mecanismos endógenos relacionados às necessidades das colônias. As colônias de Symplegma estão agindo como um sistema auto-regulado que pode reorganizar seus componentes no caso de perturbações particulares. Minha proposta é que as colônias de Symplegma evoluíram a comunicação entre os zooides na forma de células migratórias especializadas que transmitem matéria e informação através da colônia, coordenando os processos biológicos dos zooides. A evolução da colonialidade neste clado é mediada por novidades na comunicação e cooperação entre zooides como a PCD dos antigos zoides e sua reabsorção pela migração do MLC para reciclar seus tecidos e utilizá-lo para as próximas gerações de zooides.

General Introduction

This thesis results from the study of the life history of the species *Symplegma brakenhielmi* and *Symplegm rubra*. These colonial tunicates described the last century are benthic organisms, abundant in tropical and subtropical benthic substrates, such as mangroves, rocky shores, and coral reefs. *Symplegma brakenhielmi* and *S.rubra* are characterized for the strong tunic pigmentation, S. brakenhielmi usually greenish with dark dots, and *S.rubra*, as was named, is red or dark orange. The biology and life history of these animals are mostly unknown, except for some studies fifty years ago in *Symplegma reptans* and S. viridae. *Symplegma* is a colonial genus, in a family (i.e Styelidae) in which, colonial life history evolved from a solitary ancestor. *Symplegma* has similar characters with solitary forms and with other colonial clades, being this genus an interesting clade to study the evolution of new life-forms.

Symplegma colonies are formed from a single larva after metamorphosis become in a oozoid which by budding forms the clonal zooids. Zooids are interconnected by blood vessels, with circulating blood cells involved in zooid communication. This colonial strategy involves, a way for communication and zooids coordination by migratory blood cells. Thus, hematopoiesis and precursor blood cells are modulated in relation to the colonial necessities. For example, in case of external damage increase the proportion of phagocytes and immune cells (i.e morula cells) and blood cells precursor. These colonies have the capacity of whole-body regeneration, recovering a complete colony from blood vessels tissues. *Symplegma* colony is a spectacular animal, a source of biological information, about evolutionary transitions, regeneration, cellular behaviors and regeneration, morphogenesis and aging. These topics were developed in the four chapters that composed this thesis. Chapters are written in the format of journal papers, for future publication.

Chapter 1 is a hypothesis paper, including theoretical and experimental information about the colonial life history. The objective is to propose the relevance of modularity, heterochrony, cooperation and biological information in evolutionary transitions.

Chapter 2 describes budding stages for *Symplegma brakenhielmi*, and blood cells by cytological and transmission electron microscopy. *S.brakenhielmi* discovers were contextualized in previous

information from Styelidae to provide more information about the evolution of coloniality in this family. This chapter is already published.

Chapter 3 is the study of a *Symplegma* colony formation, from a single larva to a young colony with clonal zooids. The description of blood cells in *S.rubra* and cellular behaviors of some precursor cells. This chapter is contextualized in the changes in complexity pattern in the evolution of new life histories.

Chapter 4 is a methodological workflow developed during the study of *Symplegma* colonies. This workflow includes useful technique and protocols, to collect, transport and culture of colonial tunicates. As well as the procedures to blood cell extraction, histological sections cytological dyes and high-quality RNA extraction.

Glossary:

- **Biological hierarchies:** or “levels of organizations” are biological units with the same size scale and spatial-temporal magnitudes. In this study the biological hierarchies are related with discrete units observed along the developmental process in animals (molecules for replication as DNA, cells, multicellular individuals, colonies composed of multicellular individuals).
- **Cooperation:** Process of mutual working between biological units in function of a communal benefit, sometimes reducing selfish benefits.
- **Individuality:** particular character or qualities which distinguishes a biological unit from another,
- **Module:** Functional part of a biological system. The modules are characterized by a constant communication with other parts by feedback loops to maintain the homeostasis between modules. In this study the module is a zooid, the functional part of the colonial system. Although, **modularity** refers as morphological modularity, the morphological modules that composed the colonies.
- **Nestedness in biological processes:** Biological processes that are related by a subordination in temporal order (i.e one processes precede the other); or by containing a subordinate element (e.g the budding in colonies is a nested process in the complete development of colonial tunicates).
- **Self-organization:** Property of a biological systems to maintain the homeostasis by the local interaction and communication between the modules without a organizer center.

Chapter 1

Colonial life history: a major evolutionary transition, involving modularization of multicellular individuals and heterochrony (miniaturization and adultation)

1. Introduction

Life in our planet has evolved to a great diversity of life forms, reaching an estimated of 2 billion species (Larsen et al., 2017). One way to organize such diversity of life forms, is in levels of biological organization. Size scale and spatio-temporal magnitudes are the main parameters used to define levels of biological organization (e.g molecular, cellular, multicellular, populations), classifying the specific type of biological unit in each level (Sadava et al., 2014; Wimsatt, 1980). Biological unit is the entity, which is systematically the target of natural selection (Wimsatt, 1980). For example at the cellular level, the biological unit is the cell; at the multicellular level, the multicellular individual is the biological unit. In biology we use these categories to understand life diversity, however life evolved in many spectacular ways, making difficult the generalizations. The integration and cooperation of biological unities results in the evolution of new biological systems (Grosber & Strathman, 2007; Wimsatt, 1980) Therefore, the biological unit becomes a component of a larger system, maintaining their own structural and functional identity (i.e. module). The resulting new system can be a new biological unit in another level of organization (Jablonka, 1994a; Laubichler, 2003; Wimsatt, 1980).

We are going to present four key concepts in evolution of life histories: cooperation, biological information, modularity and heterochrony. These concepts are contextualized and exemplified, studying the evolution of colonial life history in Styelidae (a family of benthic tunicates). Evolution of coloniality from a solitary ancestor is a process with common characteristics, to the major evolutionary transitions. We hypothesized that evolution of colonial life history is a major evolutionary transition.

1.1 Evolution involves innovation in the types of biological information and cooperation strategies

Functionality of biological systems requires the compatibility and harmony of their modules to form a cohesive whole. This cohesion is regulated by information exchange and cooperation between modules (Fig. 1) (Michod, 2006; Szathmáry, 2015). Novelties in the use and transmission of information, and cooperation between biological units were decisive in the evolution of new biological systems. Such as, in the major evolutionary transitions (MET) of (a) the auto-replication machinery of organic molecules RNA, DNA and proteins; (b) the compartmentalization of molecules and the use of DNA as the genetic code for cellular replication; (c) the consolidation of symbiotic cells to generate the eukaryotic cells with chloroplasts and mitochondrias; (d) the evolution of multicellular organisms from unicellular ancestors; and (e) the establishment of colonial animals composed by discrete multicellular clonal individuals (Grosberg & Strathmann, 2007; Jablonka & Lamb, 2006; Michod, 1996; Szathmáry, 2015).

In each of these cases, changes in the type of information and mechanisms of communication facilitated the origin of new biological systems. Known examples of this process are(a) the use of DNA, RNA and proteins as the fundamental elements of the genetic code and replication conditioned the origin of cells; (b) inter-cellular communication by feedback mechanisms using diffusible proteins (e.g. FGF, Hedgehog, TGF-β), and signal transduction pathways to establish tissues and multicellular organisms; (c) the mechanism of communication and coordination of the clonal individuals in animal colonies; (d) the use of symbolic and sound language and their transmission in the formation of animal societies (Gatenby & Frieden, 2007; Godfrey-Smith, 2000; Jablonka, 1994b; Maynard, 1999, 2000; Szathmáry, 2015). The transmission of biological information is fundamental in functionality, heredity and replication of biological systems. Thus, important shifts in biological organization are associated withchanges in the way to store and transmit information.

Cooperation happens when biological units integrate to form a collective, with units working together and becoming mutually dependent. These biological collectives can be formed by clonal propagation or by adhesion of units with different genotype (Godfrey-Smith, 2015). Cooperation involves an increase of fitness for the biological system as a whole, whereas individual fitness of

components may decrease. Thus altruist behaviors are required in the components to maintain the cohesion, and functionality of the whole system (Michod, 1998; Michod & Nedelcu, 2003). For example, in *Volvox* colonies cells with the same genotype exhibit cooperation by the division of labor: somatic cells are in charge of viability functions and germ cells are specialized in reproduction. Cellular differentiation has a cost at the individual fitness, however contribute in the benefit of the group (Herron, Hackett, Aylward, & Michod, 2009; Jablonka & Lamb, 2006; Michod, 2006).

Competition and conflicts need to be mitigated to maintain cooperation, therefore some may emerge to reduce conflicts. Examples are: (a) clonal replication to preserve same genotype in the unities of the system (Michod, 2006; Michod & Nedelcu, 2003); (b) DNA replication and the programmed cell death (PCD) to regulate cell number and prevent emergence of defector lineages (e.g. mutational process in cancer) (Ameisen, 1996; Blackstone & Kirkwood, 2003; Umansky, 1982) and; (c) kinship recognition systems to associate with relatives and prevent the invasions of foreigners genotypes (e.g parasitic infections) (Michod, 1996; Michod & Nedelcu, 2003). Cooperation is essential to maintain the cohesion of units and functionality of biological systems. Therefore, innovation in cooperation strategies is involved in evolution of new biological systems.

1.2 Heterochronic mechanisms orchestrated the development during the evolution of new life history

Heterochrony is the temporal displacement of characters in development, relative to the ancestor. The processes that produce heterochrony are acceleration and retardation in the somatic tissues development or sexual maturation.(Gould,1977; Smith, 2003). The main morphological results of heterochrony are recapitulation and paedomorphosis. Recapitulation is the appearing of features earlier in descendants. Whereas, paedomorphis is the later appearing of features in the lifetime of descendants in comparison with the ancestral ontogeny. Paedomorphis is usually resulting by retardation of somatic tissues development. This retardation generates the retention of juvenile characters (i.e neoteny), such as the axolotl (Fig. 2A). (Gould,1977; Mcnamara, 1986). Recapitulation can be result of retardation in sexual maturation. Retarded maturation with proportionated increase of growth, produces gigantism. Moreover, retarded maturation without body size increase, involves disproportionated increment in size of a body part (i.e positive allometry). This process is called

hypermorphosis, like the positive allometry of antlers in deers (Fig. 2B) (Gould,1977; McNamara, 2012).

Feature displacement by acceleration or retardation in somatic development or sexual maturation, produces huge morphological diversity. Heterochronic effects such as neoteny are hypothesized to be as a motor for evolutionary transitions: *"it (neoteny) supplies one of the very few Darwinian justifications for large and rapid evolutionary transitions, by permitting major changes in morphology without extensive genetic reorganization"* from Ontogeny and Phylogeny, pg 285, Gould, 1977. Moreover, neoteny may involves a mixture of juvenile and adult characters. As a result, neotenic descendants can enter in new adaptive zones, promoting ecological plasticity (Gould,1977). Retardation or acceleration of sexual maturation (i.e hypermorphosis and progenesis) are involved in the emergence of morphological diversity and evolution of new life histories. Retardation of sexual maturation is related with gigantism and positive allometry of body parts (Fig. 2B). The adaptive radiation of mammals is an example of morphological (e.g skulls, teeth and antlers) and life history (e.g carnivores, herbivores and frugivores) diversification, produced by heterochrony in sexual maturation and allometry (Klevezal, 2018; Kohler & Moya-Sola, 2009; Kolb et al., 2015).Acceleration of sexual maturation usually involves body size reduction. Precocious reproduction and smaller body size are related with the diversification of life histories in some insects groups, such as parthenogenetic aphids and mites (Gould,1977).Displacement in sexual maturation has ecological implications, for the increase in progeny, resources exploitations and competition. Therefore, heretochronic processes have been related with r-k ecological strategies. Acceleration in sexual maturation, reduction in body size, faster growth are related with r strategies. Whereas, retardation in sexual maturation, increase in body size, brooding are related with k strategies (Gould,1977; McNamara, 2012). Heterochronic mechanisms are essential in evolution, promoting ecological plasticity, morphological innovations, rapid evolutionary transitions and new life histories.

Modularity and cooperation are strategies involved in the functionality of biological systems. These strategies were associated with the appearance of novel "biological tools" in evolution. Specifically, new types of biological information and ways to communication evolved with modularity and cooperation strategies. Nevertheless heterochrony is the main developmental mechanism that

generates morphological variety an evolution. Modularity, cooperation, biological information and heterochrony are key concepts in the understanding of life evolution.

2. Colonial life history an evolutionary transition for benthic ecosystems

We consider that in colonial life history the multicellular individual transforms in a module of a new biological hierarchy. The multicellular modules are components of the colonial architecture maintaining physical cohesion (Jackson & Coates, 1986). These modules are formed by clonal replication maintaining identical genotype and a physiological cooperation (inclusive division of labor) (Hughes, 1989). Coloniality evolved by convergence in some lineages animals, involving huge diversification in life forms (Jackson & Coates, 1986; Wake, 2003) :

- In cnidarians coloniality evolved in scleractinean corals and octocorals (Hexacorallia and Alcyonaria). A pelagic larva settles and metamorphoses into a sessile polyp, forming a colony by asexual reproduction. These colonial animals are keystones species, forming coral reefs in marine ecosystems (Kaiser et al., 2010).Moreover in Hydrozoans coloniality state is alternate between polyp generation and solitary jellyfish, with an exceptional floating colony of polymorphic polyps in Siphonophores (e.g The Portuguese man of war) (Scrutton, 2015).
- In entoprocts, with a variety of asexual reproduction in colonial and solitary species. Colonies as we define them are formed by budding from the stalk base (Fuchs et al, 2010).
- Bryozoans compose a completely colonial phylum of filtering marine sessile animals. The colonies are polymorphic with a clear division of labors (i.e. Morphological differentiation and functional specialization between zooids) composed by zooids formed from bud primordia in adult zooids(Wood, 2014).
- Hemichordates a phylum of marine animals with a worm-like body shape that forms an external tubular structure in solitary or colonial clades. The colonies are composed by tubular chambers with clusters of zooids attached to basal discs, in which the buds are formed (Lester, 1985).
- Tunicates is a phylum with pelagic and benthic colonial animals. In class Thaliacea, the Doliolids are colonies with alternation of sexual and asexual generations in the life cycle. Colonies are composed by a buoyant zooid and zooids to feed the colony (i.e asexual

generation); otherwise colonies with the buoyant zooid and zooids to reproduction (i.e sexual generation) (Bone, 2003; Holland, 2016). The class ascidiacea is divided in three orders (Stolidobranchia, Applausobranchia and Phleobranchia) in which coloniality evolved at least once. Colonies in ascidians are not polymorphic but present a high diversity in asexual reproduction mechanisms (Holland, 2016; Kocot et al., 2018)

Colonial animals are mostly marine organisms and make up a considerable proportion of the diversity and biomass in the marine benthic and pelagic ecosystems (Jackson & Coates, 1986). Sessile colonial animals are essential components of benthic communities in tropical and subtropical regions (e.g coral reefs) (Jackson, 1977). There are several characteristics of colonial life history that may confer adaptive advantages for settlement and growth on marine substrata: (a) the compact size of zooids allows faster development and shorter generations; (b) increased survival rates after weather disturbance and predation by the dispersion of their genotype in the replaceable modules (i.e budding ans regeneration) and; (c) higher metabolic efficiency for the occurrence of different metabolic processes at the same time by functional specialization and division of labor between modules of the colonies (Coates & Jackson, 1985; Davidson et al., 2004; Greene et al, 1983).

2.2 The higher proportion of colonial species in tropical areas is the result of colonial animals resilience to environmental disturbance

Colonial animals are more predominant and diverse in tropical and subtropical benthos. This diversity decreases along the latitudinal increment. Whereas,solitary forms are dominant in the temperate marine substrata (Hiebert et al., 2019; Jackson, 1977).Tropical oceans are oligotrophic, therefore most of the biodiversity is concentrated in some ecosystems such as coral reefs, rocky substrate, mangroves and sea grasses (Kaiser et al., 2010). Competition and disturbances, such as predation or desiccation, are critical in these environments (Jackson & Hughes, 1985).

Colonial animals are conformed by multicellular modules (i.e zooids), which are replaceable. This characteristic confers them the capacity to colonize faster, and survive in case of environmental disturbance or predation (Jackson & Hughes, 1985). The resilience of colonial animals can be a factor

to explain their diversity and preponderance in tropics. Also this resilience related with colonial life history can be associated with the convergent of coloniality in tropical benthos.

3. Coloniality in styelids resulted from the evolution of complex characters through heterochrony

Styelidae is a tunicate family in which colonial life history, evolved by convergence (Fig. 3) (Alié et al., 2018). Morphological and molecular evidence suggests that the ancestor of this family was a solitary life-form. (Alié et al., 2018; Kott, 2005; Zeng et al., 2006). Although, in this family coloniality is derivate state (Fig. 3B). Evolution of coloniality in Styelids tunicates is correlated to (a) a reduction in the size of the modular units that compose the colonies (e.g. colonial zooids range in size from 2 mm to 20 mm), whereas solitary individuals range in size (from 15 mm to 10 cm); (b) the development of tissues and structures to maintain cohesion and communication between the colonial modules, such as extra-corporeal vascular systems or stolons; (c) the development of asexual reproduction strategies;; (d) the development of brooding organs for viviparous or ovoviviparus strategies inside mother colonies; (e) an increase of larval size and adultation in the larvae which corresponds to the development of adult structures during early larval stages; and (f) diversification of blood cell types to orchestrate the modular development of colonies (Alié et al., 2018; Loriano Ballarin et al., 2008; Gutierrez & Brown, 2017; Kott, 2005; Pérez-Portela, Bishop, Davis, & Turon, 2009; Zeng et al., 2006). These characters in colonial descendants (Fig. 3A), are the result of the develop in a more complex way of characters already present in solitary forms.

Heretochrony is observed in the evolution of coloniality in styelids (Berrill, 1935; Davidson et al., 2004). There is a retardation in the embryonic development, in colonial species (Fig. 4A). This retardation results in the increase of larval size and larval adultation (e.g larva in Botryllids hatched with an oozoid and buds primordiums). Retardation is related to the increase of egg size and brooding strategy in colonial styelids (Berrill, 1935). Moreover, there is an accelerated growth stage after metamorphosis (Fig. 4A) in colonial styelids. The growth stage in colonials is significantly faster than in solitary forms. Thus colonial oozoid has limited time to grow. This results in the miniaturization of individuals, the main characteristic of colonial animals. Heterochronic mechanisms (e.g adultation, miniaturization) are involved in evolution of colonial life-history in Styelidae. Although, changes in

developmental timing are mechanisms, related with evolution of morphological innovations and new life-histories.

3.1 Coloniality in Symplegma + Botryllids clade involves adultation and miniaturization by embryonic development retardation and growth stage acceleration

Colonial strategy in the clade *Symplegma+Botryllids* (Fig. 3B), involves embryonic development, larval settlement metamorphosis, and asexual development during budding (Berrill, 1940, 1941; Sabbadin, 1955a). Asexual development has analogous stages to ordinary development in solitaries, being the growth stage main different for the acceleration of this stage in zooids development (Fig. 4B). Nevertheless, retardation in the growth stage in *Botryllus*, is more related with the growth of the buds from the zooid at this stage. This retardation involves growth in biomass for buds development more than body size increase in the zooid (Fig. 4B) (Berrill, 1941; Sabbadin, 1955b).

Colonial characters such as extra-corporeal blood vessels, developmental modularity by circulatory blood cells, budding and regeneration, are observed in a simple state in solitary forms (Barros et al., 2007; Jimenez, 2018; Sawada et al., 1993). Juveniles of *Styela canopus* develops extra-corporeal blood vessels after metamorphosis, these vessels are shorter compared with vessels in colonies. However it has been hypothesized, that these structures, can be involved in gregarious strategy (Jimenez, 2018). Functional groups of blood cells (i.e phagocytosis, blood proliferation, allorecognition, storage) are conserve in solitary and colonial animals Ballarin & Cima, 2005; Barros et al., 2007; Sawada et al., 1993). We suggest that colonial life history characters developed in a complex way from simples characters in solitary ancestors. Heterochronic mechanism as retardation in larval development and acceleration of growth stage, are probably involved in the development of these complex characters in colonial descendants. Heterochrony during budding (e.g retardation in *Botryllus* growth stage for buds forming), suggests that heretochronic mechanisms are involved in developing new strategies.

4.The evolution of coloniality in Styelidae is mediated by novel systems of cooperation between multicellular modules

4.1 The trade-off between competitive exclusion and cooperation, may influence the evolution of life histories in benthic ecosystems.

The main limiting resource in the tropical benthic ecosystems the space (Barnes & Hughes, 1982; Coates & Jackson, 1985). Consequently, space competition for space is one of the critical environmental pressures in benthic ecosystems (Jackson, 1977). Benthonic organisms coexist with this pressure, developing strategies to maintain local coexistence or exclusion (Crowley et al., 2005).

Chimerism, philopatry and inbreeding are strategies to maintain coexistence, involving cooperation between organisms. Whereas, mechanical and chemical defenses (e.g spicules, strong tunicas, calcified skeletons, secondary metabolites); high rates of over-growth, young sexual maturity; dispersion by fragmentation are excluding strategies, involving competition (Barnes & Hughes, 1982; Kaiser et al., 2010). The benthos coexistence with competitive exclusion and cooperation, may promote the developing of communication ways between organisms. Evolution of new cooperation systems and ways of communication, are proposed mechanisms to be involved in major evolutionary transitions (MET) (Jablonka, 1994a; Szathmáry, 2015). Moreover, we hypothesize that coloniality is a major evolutionary transition, because the mechanisms for cooperation and evolution of new biological types of information have similar pattern to other MET.

4.2 Evolution of coloniality in the *Symplegma+ Botryllid*s clade resulting in an increase in cooperation, andreduction of individuality in multicellular modules

Colonial strategy in *Symplegma+Botryllids* clade is characterized by extra-corporeal blood vessels that interconnect zooids. Blood cells are in constant circulation inside the vessels.. Around the edge of the colony the vascular system forms bulbous projections, called ampullae, which exhibit contractile movements, facilitate growth and have a role in substrate recognition (Fig. 3A-5A,B) (Berrill, 1940, 1941; Mukai, Sugimoto, & Taneda, 1978; Sabbadin, 1955a).

The communication between modules of the colony and their environment is mediated by the transmission of blood cells inside the vessels and presumably unknown diffusible factors (Fig. 5C) (Ballarin et al., 2008; Franchi et al., 2016; Lauzon et al., 2002; Menin et al., 2005). Blood cell types are diverse in morphology and function. Some known functions of blood cells include: (a) phagocytosis and programmed cell death (PCD) by macrophage like cells (MLCs); (b) budding and regeneration by undifferentiated cells (UCs) with characteristics of stem cells; (c) immune reactions by morula cells (MCs); (d) storage and excretion by nephrocytes and pigment cells (Loriano Ballarin, Cima, & Sabbadin, 1995; Brown et al., 2009; Cima, Perin, Burighel, & Ballarin, 2001; Franchi et al., 2016; Gutierrez & Brown, 2017). Moreover, these migratory blood cells are related to the modulation of the non-embryonic developmental process characterized in *Symplegma+Botryllids* colonies. In these colonies while some buds are in morphogenesis, some zooids are in a programmed senescent process. UCs are more predominant at the stage of morphogenesis and MLCs and MCs are more predominant at the senescent stage (Fig. 5 C) (Ballarin et al., 2008; Franchi et al., 2016; Gutierrez, 2019).

The colonial strategy in the sister groups *Symplegma* and *Botryllids* is similar. However, Botryllids have a cyclic synchronous budding. In these colonial tunicates, three generations are developing simultaneously, parental zooid, primary bud and secondary bud (Fig. 5C). Moreover, these three generations are highly dependent: bud removal induces early senescent of zooids, and zooid removal reduce the size of zooids in the next generation (Lauzon et al., 2002; Lauzon et al., 2007). Blood cells in Botryllids have differential gene expressions during the budding to regulate the biological processes in each budding stage. UCs are more predominant and expressing anti-apoptotic factors during buds formation in Botrillyds (Fig. 5C-Stg.B). MLCs are more predominant and expressing pro-apoptotic factors during senescent process (Fig. 5C-Stg.D) (Franchi et al., 2016). Whereas, *Symplegma* colonies have asynchronous budding and more independence between zooids. Colonial development is unaltered with removals of individuals, with exception of bud removal, which induce and accelerated development of new buds. Blood cell proportions are maintained constantly in *Symplegma* colonies at normal conditions. The proportion of phagocytes and precursors cells increase in case of injuries, probably related with the regeneration of affected tissues (Gutierrez & Brown, 2017). These results suggest that the control of zooids development at the colonial level, is higher in more integrate colonies with higher interdependence between zooids (i.e *Botryllids*), than in the colonies with more independent zooids. Thus, individuality of units (i.e zooids) is reduced with the

increase of control by higher biological hierarchy (i.e colony). Though, coloniality such as other evolutionary transitions (e.g multicellularity), involve the cooperation and a reduction of individuality of modules that integrate the new biological hierarchy (Jablonka, 1994a; Michod, 2006).

5. Conclusions

Our proposal is that the colonial life history is a major evolutionary transition, in which the colony is a new biological hierarchy composed by multicellular modules and new communication ways evolved to maintain the cohesion and harmony in these biological systems.

We use as example of colonial life history the colonial strategy evolved in the tunicate clade *Botryllus+Symplegma,* from a family with a solitary ancestor (i.e Styelidae) (Alié et al., 2018; Kott,2005).Hypothesizing that *Botryllus+Symplegma* colonies evolved a cellular based communication system. In which signals are transmitted between modules by migratory cells, and as a result is able to coordinate biological processes across the colony. These cellular based communication system is supported by observations such as, the proliferation and differentiation of UCs in the blood to form buds, or PCD of zooids mediated by predetermined cycles of migration of MLCs to recycle resorbing tissues for the next asexual generations (Ballarin et al., 2008; Gutierrez & Brown, 2017; Lauzon et al., 2007).

Moreover, we propose that in more integrate colonies, the individuality of zooids reduce to increase the general benefit of the colony as a whole. By an inter-generational division of labor, where one generation is feeding (i.e. zooids), a second undergoing morphogenetic and inductive processes (i.e. buds), and a third undergoing phagocytosis (i.e. zooid during regression), increases the efficiency by dividing metabolic and physiological processes. Thus, colonies act as self-regulating or autonomous higher level systems that can respond to perturbations by altering the development of their individual modules.

Innovations in modularity and cooperation strategies; evolution of new types of biological information and communication; and heretochronic mechanisms, are essential in major evolutionary transitions. These strategies and mechanisms have been observed in evolutionary transitions such as multicellularity (Herron et al., 2009; Jablonka & Lamb, 2006; Michod, 2003; Szathmáry, 2015), and the evolution of animal colonies in which multicellular individual became a module of a more complex

biological system. Modularity, cooperation, biological information and heterochrony are useful concepts to understand biological innovations. Moreover, the relevance of developmental biology in the comprehension of life evolution.

6.References

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*(7), 1728–1743. https://doi.org/10.1093/molbev/msy068

Ameisen, J. C. (1996). The origin of programmed cell death. *Science*, *272*(5266), 1278–1279. https://doi.org/10.1126/science.272.5266.1278

Ballarin, L, & Cima, F. (2005). *Cytochemical properties of Botryllus schlosseri haemocytes: indications for morpho-functional characterisation*. *2009* (Vol. 49). Retrieved from http://ejh.pagepress.org/index.php/ejh/article/view/952

Ballarin, Loriano, Cima, F., & Sabbadin, A. (1995). Morula Cells and Histocompatibility in the Colonial Ascidian Botryllus schlosseri. *Zoolog Sci*, *12*(6), 757–764. https://doi.org/10.2108/zsj.12.757

Ballarin, Loriano, Menin, A., Tallandini, L., Matozzo, V., Burighel, P., Basso, G., … Cima, F. (2008). Haemocytes and blastogenetic cycle in the colonial ascidian Botryllus schlosseri: a matter of life and death. *Cell and Tissue Research*, *331*(2), 555–564. https://doi.org/10.1007/s00441-007-0513-4

Barnes, R. S. K., & Hughes, R. N. (1982). *An Introduction to Marine Ecology*. *An Introduction to Marine Ecology*. https://doi.org/10.1002/9781444313284

Berrill. (1940). The Development of a Colonial Organism: Symplegma viride. *Biological Bulletin*, *7*(2), 272–281. Retrieved from http://www.jstor.org/stable/1537822

Berrill, N. J. (1935). Studies in Tunicate Development. Part III. Differential Retardation and Acceleration. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *225*(525), 255–326. https://doi.org/10.1098/rstb.1935.0013

Berrill, N. J. (1941). The Development of the Bud in Botryllus. *Biological Bulletin*, *80*(2), 169–184. https://doi.org/10.2307/1537595

Blackstone, N. W., & Kirkwood, T. B. L. (2003). Mitochondria and Programmed Cell Death “Slave Revolt” or Community Homeostasis? In P. Hammerstein (Ed.), *Genetic and Cultural Evolution of Cooperation*. Massachusetts: MIT Press.

Bone, Q. (2003). The Biology of Pelagic Tunicates. Oxford: Oxford University Press.

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 (Cambridge, England)*, *136*(20), 3485–3494. https://doi.org/10.1242/dev.037754

Cima, F., Perin, A., Burighel, P., & Ballarin, L. (2001). Morpho-functional characterization of haemocytes of the compound ascidian Botrylloides leachi (Tunicata, Ascidiacea). *Acta Zoologica*, *82*(4), 261–274. https://doi.org/10.1046/j.1463-6395.2001.00087.x

Coates, a. G., & Jackson, J. B. C. (1985). Morphological Themes in the Evolution of Clonal and Aclonal Marine Invertebrates. In J. B. C. Jackson, L. W. Buss, & R. E. Cook (Eds.), *Population Biology and Evolution of Clonal Organisms*. Westford: Murray Printing Company.

Crowley, P. H., Davis, H. M., Ensminger, A. L., Fuselier, L. C., Jackson, J. K., & McLetchie, D. N. (2005). A general model of local competition for space. *Ecology Letters*, *8*(2), 176–188. https://doi.org/10.1111/j.1461-0248.2004.00709.x

Davidson, B., Jacobs, M., & Swalla, B. J. (2004). The individuals as a Module: Solitary-to-Colonial Transitions in Metazoan Evolution and Development. In G. Schlosser & G. Wagner (Eds.), *Modularity in Development and Evolution*. Chicago: The University of Chicago Press.

de Barros, C. M., Andrade, L. R., Allodi, S., Viskov, C., Mourier, P. A., Cavalcante, M. C. M., … Pavão, M. S. G. (2007). The Hemolymph of the ascidian Styela plicata (Chordata-Tunicata) contains heparin inside basophil-like cells and a unique sulfated galactoglucan in the plasma. *The Journal of Biological Chemistry*, *282*(3), 1615–1626. https://doi.org/10.1074/jbc.M604056200

Franchi, N., Ballin, F., Manni, L., Schiavon, F., Basso, G., & Ballarin, L. (2016). Recurrent phagocytosis-induced apoptosis in the cyclical generation change of the compound ascidian Botryllus schlosseri. *Developmental and Comparative Immunology*, *62*, 8–16. https://doi.org/10.1016/j.dci.2016.04.011

Fuchs, J., Iseto, T., Hirose, M., Sundberg, P., & Obst, M. (2010). The first internal molecular phylogeny of the animal phylum Entoprocta (Kamptozoa). *Molecular Phylogenetics and Evolution*, *56*(1), 370–379. https://doi.org/http://dx.doi.org/10.1016/j.ympev.2010.04.009

Gatenby, R. A., & Frieden, B. R. (2007). Information theory in living systems, methods, applications, and challenges. *Bulletin of Mathematical Biology*, *69*(2), 635–657. https://doi.org/10.1007/s11538-006-9141-5

Godfrey-Smith, P. (2000). On the Theoretical Role of "Genetic Coding." *Philosophy of Science*, *67*(1), 26. https://doi.org/10.1086/392760

Godfrey-Smith, P. (2015). Reproduction, symbiosis, and the eukaryotic cell. *Pnas*, *112*(33), 10120–10125. https://doi.org/10.1073/pnas.1421378112

Gould, Stephen, J. (1977). *Ontogeny and Phylogeny*. Harvard University Press.

Greene, C., Schoener, A., & Corets, E. (1983). Succession on marine hard substrata: the adaptive significance of solitary and colonial strategies in temperature fouling communities. *Marine Ecology Progress Series*, *13*(1977), 121–129. https://doi.org/10.3354/meps013121

Grosberg, R. K., & Strathmann, R. R. (2007). The Evolution of Multicellularity: A Minor Major Transition? *Annual Review of Ecology, Evolution, and Systematics*, *38*, 621–654. https://doi.org/10.2307/30033874

Gutierrez, S., & Brown, F. D. (2017). Vascular budding in Symplegma brakenhielmi and the evolution of coloniality in styelid ascidians. *Developmental Biology*, *423*(2). https://doi.org/10.1016/j.ydbio.2017.01.012

Herron, M. D., Hackett, J. D., Aylward, F. O., & Michod. (2009). Triassic origin and early radiation of multicellular volvocine algae. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(9), 3254–3258. https://doi.org/10.1073/pnas.0811205106

Hiebert, L. S., Vieira, E. A., Dias, G. M., Tiozzo, S., & Brown, F. D. (2019). Colonial ascidians strongly preyed upon, yet dominate the substrate in a subtropical fouling community. *BioRxiv*, 512699. https://doi.org/10.1101/512699

Holland, L. Z. (2016). Tunicates. *Current Biology*, *26*(4), R146–R152. https://doi.org/10.1016/j.cub.2015.12.024

Hughes, R. (1989). *A Functional Biology of Clonal Animals*. New York: Chapman and Hall.

Jablonka, E. (1994a). Inheritance systems and the evolution of new levels of individuality. *Journal of Theoretical Biology*. https://doi.org/10.1006/jtbi.1994.1191

Jablonka, E. (1994b). Inheritance Systems and the Evolution of New Levels of Individuality. *Journal of Theoretical Biology*, 301–309.

Jablonka, E., & Lamb, M. J. (2006). The evolution of information in the major transitions. *Journal of Theoretical Biology*, *239*(2), 236–246. https://doi.org/10.1016/j.jtbi.2005.08.038

Jackson, J. B. C., & Coates, a. G. (1986). Life Cycles and Evolution of Clonal (Modular) Animals. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *313*(1159), 7–22. https://doi.org/10.1098/rstb.1986.0022

Jackson, J B C. (1977). Competition on Marine Hard Substrata: The Adaptive Significance of Solitary and Colonial Strategies. *The American Naturalist*, *111*(980), 743–767. https://doi.org/10.2307/2460328

Jackson, Jeremy B C, & Hughes, T. P. (1985). Adaptive strategies of coral-reef invertebrates. *American Scientist*, *73*(3), 265–274.

Jimenez Merino, J. (2018). *Circulatory Stem Cells of Styela plicata (Lesueur, 1823) (Tunicata: Styelidae): An evolutionary Approach*. Universidade de São Paulo.

Kaiser, Michael, J., Attrill, M., & Jennings, S. (2010). *Marine Ecology. Process, Systems, and Impacts*. *Fish and Fisheries*. https://doi.org/10.1111/j.1467-2979.2006.00221.x

Klevezal, G. A. (2018). *Recording Structures of Mammals*. *Recording Structures of Mammals*. https://doi.org/10.1201/9780203741146

Kocot, K. M., Tassia, M. G., Halanych, K. M., & Swalla, B. J. (2018). *Phylogenomics offers resolution of major tunicate relationships*. *Molecular Phylogenetics and Evolution* (Vol. 121). https://doi.org/10.1016/j.ympev.2018.01.005

Kohler, M., & Moya-Sola, S. (2009). Physiological and life history strategies of a fossil large mammal in a resource-limited environment. *Proceedings of the National Academy of Sciences*, *106*(48), 20354–20358. https://doi.org/10.1073/pnas.0813385106

Kolb, C., Scheyer, T. M., Lister, A. M., Azorit, C., De Vos, J., Schlingemann, M. A. J., … Sánchez-Villagra, M. R. (2015). Growth in fossil and extant deer and implications for body size and life history evolution. *BMC Evolutionary Biology*, *15*(1), 1–15. https://doi.org/10.1186/s12862-015-0295-3

Kott, P. (2005). Novel Australian Polyzoinae (Styelidae, Tunicata). *Journal of Natural History*, *39*(32), 2997–3011. https://doi.org/10.1080/00222930500239702

Larsen, B. B., Miller, E. C., Rhodes, M. K., & Wiens, J. J. (2017). Inordinate Fondness Multiplied and Redistributed: the Number of Species on Earth and the New Pie of Life. *The Quarterly Review of Biology*, *92*(3), 229–265. https://doi.org/10.1086/693564

Laubichler, M. D. (2003). Selection: Units and Levels in Developing Systems. In *Keywords and Concepts in Evolutionary Developmental Biology*. Cambridge, Massachusett, London, England: Harvard University Press.

Lauzon, Ishizuka, K. J., & Weissman, I. L. (2002). Cyclical Generation and Degeneration of Organs in a Colonial Urochordate Involves Crosstalk between Old and New: A Model for Development and Regeneration. *Developmental Biology*, *249*(2), 333–348. https://doi.org/10.1006/dbio.2002.0772

Lauzon, Kidder, S. J., & Long, P. (2007). Suppression of programmed cell death regulates the cyclical degeneration of organs in a colonial urochordate. *Developmental Biology*, *301*(1), 92–105. https://doi.org/http://dx.doi.org/10.1016/j.ydbio.2006.08.055

Lester, S. M. (1985). Cephalodiscus sp. (Hemichordata: Pterobranchia): observations of functional morphology, behavior and occurrence in shallow water around Bermuda. *Marine Biology*, *85*(3), 263–268. https://doi.org/10.1007/BF00393246

Maynard, J. (1999). The idea of information in Biology. *The Quarterly Reviw of Biology*, *74*(4), 395–400. Retrieved from http://www.jstor.org/stable/2664718

Maynard, J. (2000). The concept of Information in Biology. *Phylosophy of Science*, *67*(2), 177–194. Retrieved from http://www.jstor.org/stable/188717

Mcnamara, K. J. (1986). Paleontological Society A Guide to the Nomenclature of Heterochrony Author (s): Kenneth J . McNamara Published by : Paleontological Society Stable URL : https://www.jstor.org/stable/1305091 REFERENCES Linked references are available on JSTOR for this ar, *60*(1), 4–13.

McNamara, K. J. (2012). Heterochrony: the Evolution of Development. *Evolution: Education and Outreach*, *5*(2), 203–218. https://doi.org/10.1007/s12052-012-0420-3

Menin, A., Favero, M., Cima, F., & Ballarin, L. (2005). Release of phagocytosis-stimulating factor(s) by morula cells in a colonial ascidian. *Marine Biology*, *148*(2), 225–230. https://doi.org/10.1007/s00227-005-0081-7

Michod. (1996). Cooperation and conflict in the evolution of individuality. II. Conflict mediation. *Proceedings: Biological Sciences*, *263*(1372), 813–822. https://doi.org/10.1016/S0303-2647(02)00133-8

Michod. (1998). Evolution of individuality. *Journal of Evolutionary Biology*, *11*(2), 225. https://doi.org/10.1007/s000360050081

Michod. (2003). Cooperation and Conflict Mediation duringthe Origin of Multicellularity BT - Genetic and Cultural Evolution of Cooperation. In P. Hammerstein (Ed.), *Genetic and Cultural Evolution of Cooperation* (pp. 291–307). MIT Press. Retrieved from papers2://publication/uuid/D1FF4A5E-CBE5-45A3-B7A6-EBB5531666EB

Michod. (2006). The group covariance effect and fitness trade-offs during evolutionary transitions in individuality. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(24), 9113–9117. https://doi.org/10.1073/pnas.0601080103

Michod, & Nedelcu, A. M. (2003). On the reorganization of fitness during evolutionary transitions in individuality. *Integrative and Comparative Biology*, *43*(1), 64–73. https://doi.org/10.1093/icb/43.1.64

Mukai, H., Sugimoto, K., & Taneda, Y. (1978). Comparative studies on the circulatory system of the compound ascidians, Botryllus, Botrylloides and Symplegma. *Journal of Morphology*, *157*(1), 49–77. https://doi.org/10.1002/jmor.1051570105

Pérez-Portela, R., Bishop, J. D. D., Davis, a R., & 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. https://doi.org/10.1016/j.ympev.2008.11.014

Pérez Portela, R., Bishop J,D., J., Davis R., A., & Turon, X. (2009). Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, *570*, 560–570. Retrieved from http://hdl.handle.net/10261/38798

Sabbadin, A. (1955a). Osservazioni sullo sviluppo, l’accrescimento e la riproduzione di Botryllus schlosseri (Pallas), in condizioni di laboratorio. *Boll. Zool.*, (22), 243–265.

Sabbadin, A. (1955b). Osservazioni sullo sviluppo, l’accrescimento e la riproduzione di Botryllus schlosseri (Pallas), in condizioni di laboratorio. *Bolletino Di Zoologia*, *22*(2), 243–263. https://doi.org/10.1080/11250005509439204

Sadava, D., Hillis, D. M., Heller, H. C., & Berenbaum, M. R. (2014). *Life The science of Biology* (Tenth). Sunderland, MA USA: Sinauer Associates, Inc.,.

Sawada, T., Zhang, J., & Cooper, E. L. (1993). Classification and Characterization of Hemocytes in Styela clava. *Biological Bulletin*, *184*(1), 87–96. https://doi.org/10.2307/1542382

Scrutton, C. T. (2015). Corals and Other Cnidaria. In *Reference Module in Earth Systems and Environmental Sciences*. https://doi.org/10.1016/B978-0-12-409548-9.09549-X

Smith, K. K. (2003). Time’s arrow: Heterochrony and the evolution of development. *International Journal of Developmental Biology*, *47*(7–8), 613–621.

Szathmáry, E. (2015). Toward major evolutionary transitions theory 2.0. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(33), 10104–10111. https://doi.org/10.1073/pnas.1421398112

Umansky, S. R. (1982). The genetic program of cell death. hypothesis and some applications: Transformation, carcinogenesis, ageing. *Journal of Theoretical Biology*, *97*(4), 591–602. https://doi.org/10.1016/0022-5193(82)90360-5

Wake, M. H. (2003). Life History Evolution. In B. K. Hall & W. Olson (Eds.), *Keywords and Concepts in Evolutionary Developmental Biology*. Cambridge, Massachusett, London, England: Harvard University Press.

Wimsatt, W. C. (1980). Reductionistic Research Strategies and Their Biases in the Units of Selection Controversy. In T. Nickles (Ed.), *Scientific Discovery: Case Studies* (pp. 213–259). Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-94-009-9015-9_13

Wood, T. S. (2014). *Phyla Ectoprocta and Entoprocta (Bryozoans)*. *Thorp and Covich's Freshwater Invertebrates: Ecology and General Biology: Fourth Edition* (Fourth Edi, Vol. 1). Elsevier. https://doi.org/10.1016/B978-0-12-385026-3.00016-4

Zeng, L., Jacobs, M. W., & Swalla, B. J. (2006). Coloniality has evolved once in Stolidobranch Ascidians. *Integrative and Comparative Biology*, *46*(3), 255–268. https://doi.org/10.1093/icb/icj035

7. Table 1. Categories of heterochronic mechanisms (Edited from Gould, 1977, pp229)

Timing			
Somatic features	Reproductive organs		Morphological result
Accelerated	--	Acceleration	Recapitulation (by acceleration)
--	Accelerated	Progenesis	Paedomorphosis (by truncation)
Retarded	--	Neoteny	Paedomorphosis (by retardation)
--	Retarded	Hypermorphosis	Recapitulation (by prolongation)

8. Figures

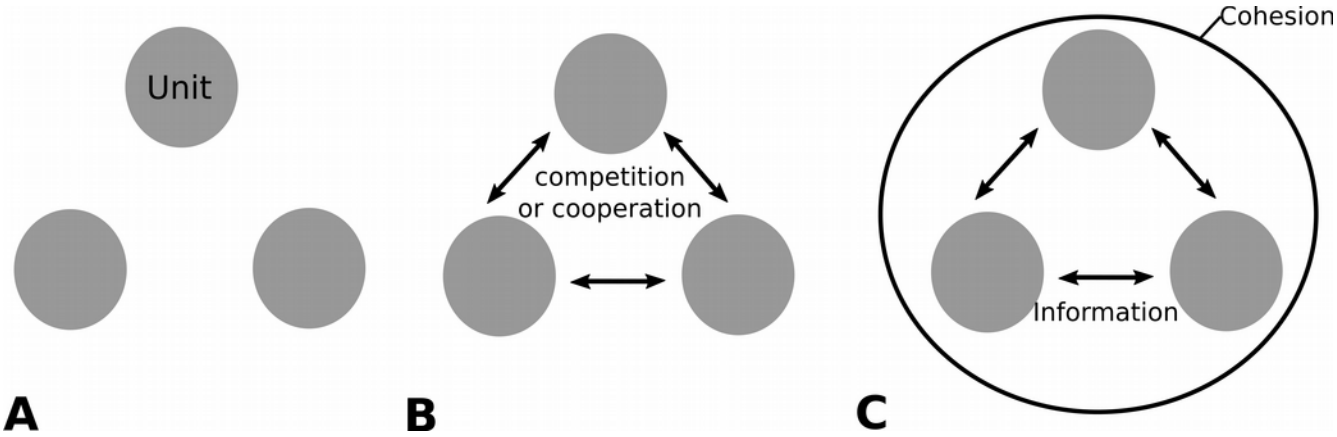

Figure. 1 Cooperation, modularity and biological information in evolutionary transitions. (A) Biological units coexist, sharing the same spatio-temporal situation. (B) Constant coexistence between biological units results in the development of interactions, such as competition or cooperation. (C) These interactions are maintained during time, consolidating new networks of interactions and new conglomerates that share information. The cohesion of biological systems is based in the harmonic interactions between the components, by constant transmission of information between these components.

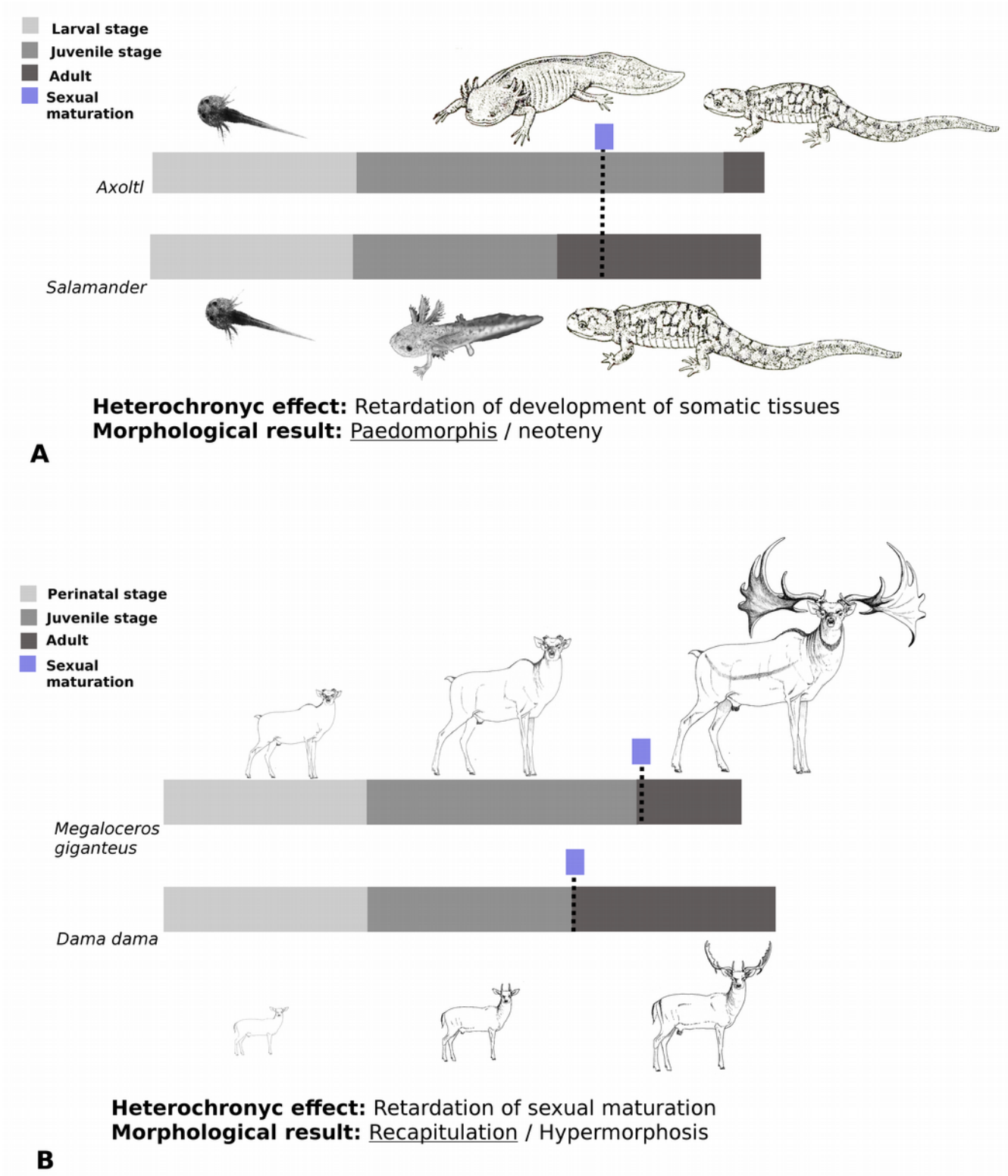

Figure. 2 Heterochronic mechanisms. Retardation and acceleration are the process of heterochrony. These heterochronic mechanisms can occurred during somatic development or sexual maturation, resulting in morphological alterations in descendants. (A) Retardation of somatic development in the

axolotl, resulting in a retention of juveniles characters or neoteny (Paedomorphosis). (B) Retardation of sexual maturation, resulting in the increase of growth stage and gigantism, like the giant deer (*Megaloceur giganteus*).

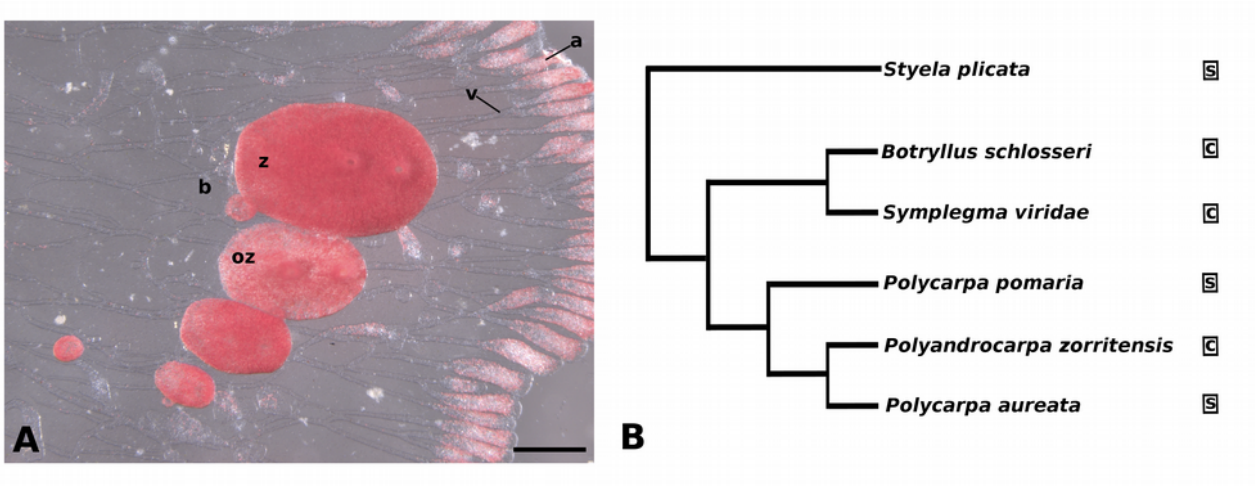

Figure. 3 Colonial life history in Styelids tunicates. (A) Colony of the genus *Symplegma*, the peripheral structures are the ampullae, the edges of blood vessels. Blood vessel systems that interconnect all the zooids (i.e buds, zooids and old zooids), containing circulatory blood cells. (B) Topology summarizing the phylogeny of the solitary and colonial species in Styelidae, based in (Alié et al., 2018; Hiebert et al., 2019; Zeng et al., 2006).*a:ampulla; b:bud; oz: old zooid; v: blood vessel; z: zooid.*

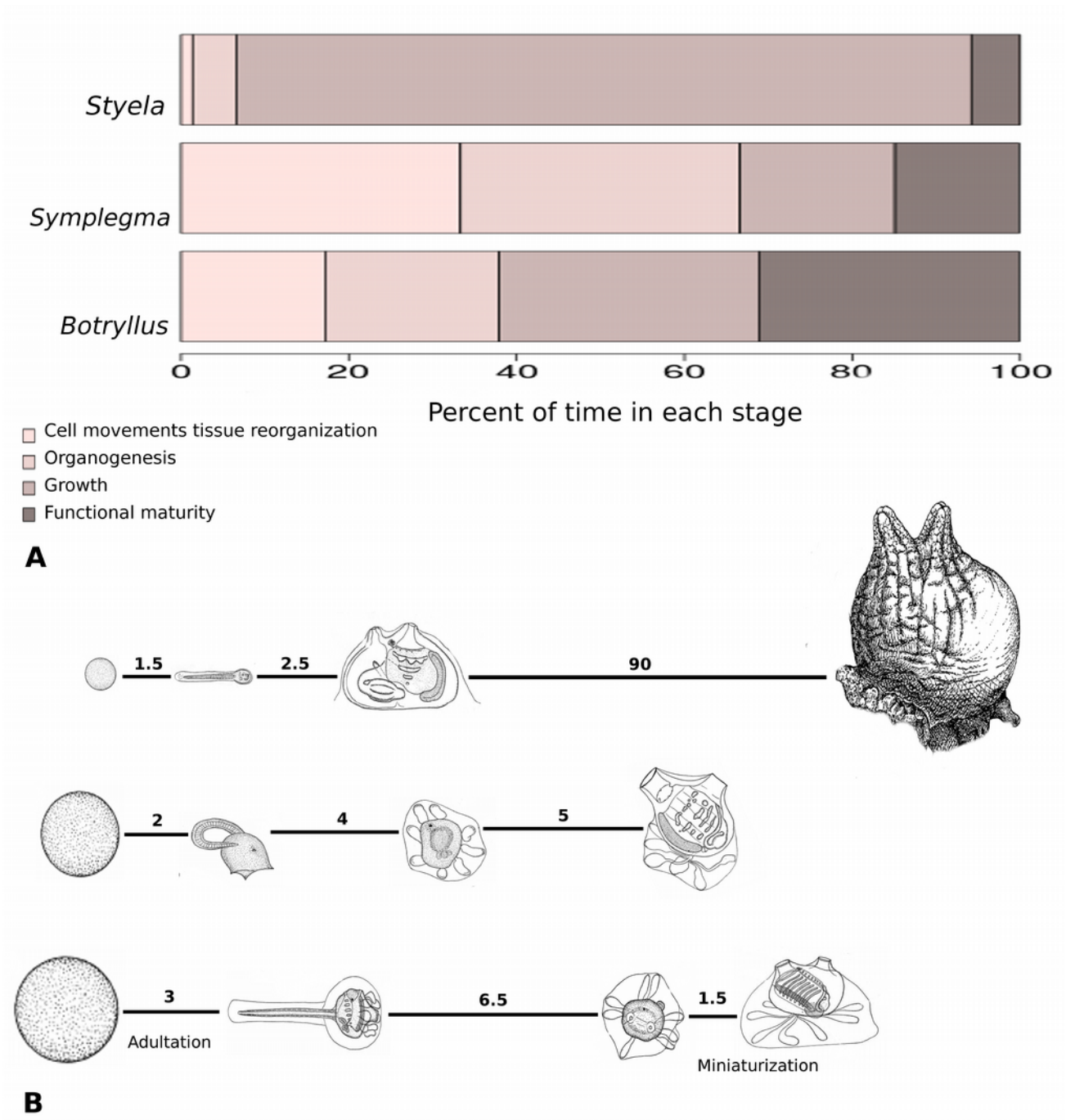

Figure. 4 Heterochrony in evolution of coloniality in Styelidae. (A) Average time in days of the embryogenesis, metamorphosis and zooid growth in *Styela plicata*, *Symplegma sp* and *Botryllus sp*, based in Berrill, 1935 and our data. (B)Comparison of analogous process in Styela plicata development and asexual reproduction in *Symplegma braknhielmi* and *Botryllus schlosseri*. Numbers are represent duration days between developmental stages: embryogenesis, larval development, metamorphosis, fully differentiated individual. Based in Gutierrez & Brown, 2017; Jimenez, 2018; and Berrill, 1945.

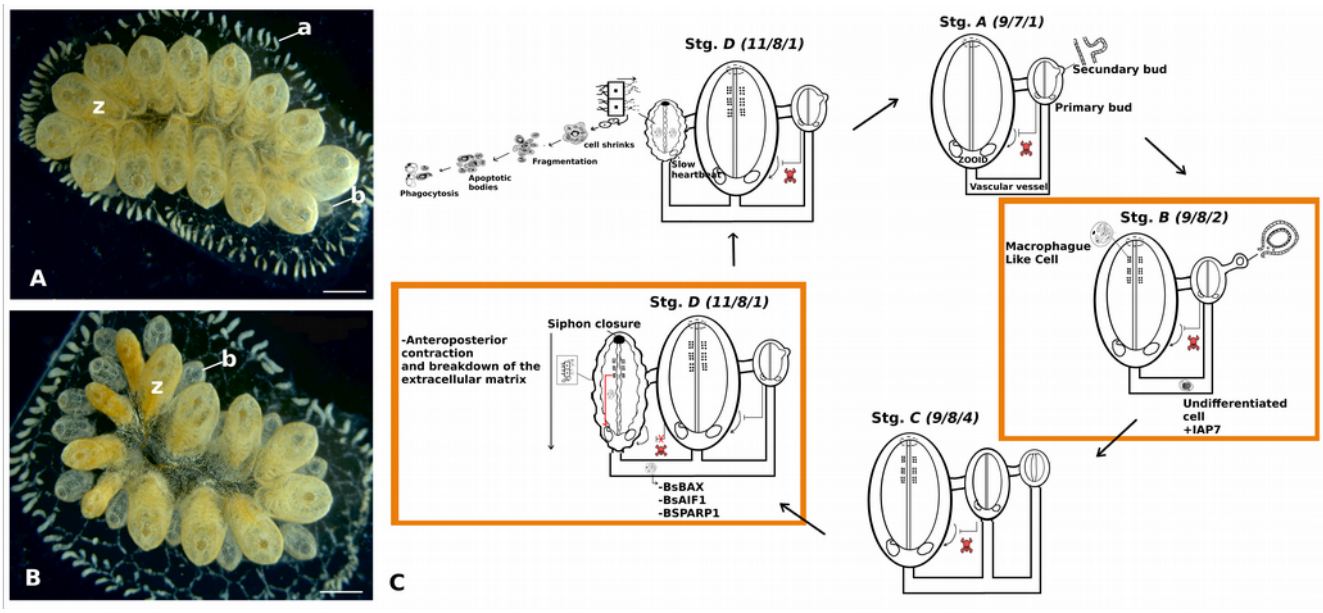

Figure. 5 *Botryllus* model of budding mechanisms. (A) *Botryllus tabori* colony at the middle of the budding cycle (Stage B- 9/8/2). Fully differentiated parental zooid, primary bud during organogenesis, secondary bud during morphogenesis. (B) *Botryllus tabori* colony at the take-over stage of the budding cycle (Stage D -11/8/1). Parental zooid enter in a programmed cell death process, the primary bud is a fully differentiated zooid and the secondary bud is in organogenesis. (C) The budding in *Botryllus* is a cycling process, buds are forming from zooid epitheliums. Budding is synchronized, all the buds are forming and developed at the same time. The stage nomenclature established by Sabbadin, 1955, uses a three numerical convention. For example the stage 11/8/1 represents: the aging zooid during programmed death process, after the apoptosis the zooid tissues are reabsorbed and recycled by phagocytes (stg. 9); the zooid finishing organogenesis (stg.8); and from the epithelium of the secondary bud is forming the primiordium of a new bud (stg. 1). A letter nomenclature was proposed to facilitate the budding stage identification. Stage A is the beginning of budding, ending at D. Stage B is the middle of the cycle (stg. 9/8/2) and stage D (11/8/1) is the final or take-over (Lauzon et al., 2002). During budding blood cell types are changing proportions. At the middle of the cycle (stg. B= 9/8/2) undifferentiated cell (UC) are more predominant. Otherwise at take-over stage (stg. D= 11/8/1) macrophage-like cells increase the proportion (Ballarin et al., 2008). Proportion in blood cell types at budding stages is also related with differences in gene expression. UCs express anti-apoptotic marker IAP7 at the middle of the cycle, MLCs express pro-apoptotic markes BsBAX, BSAIF1 and BsPARP1 at take-over (Franchi et al., 2016). This blood cell types modulation is probably related with, the

control of zooids lifespan. Development and presence of early buds in the colony arrests PCD of adult zooids.

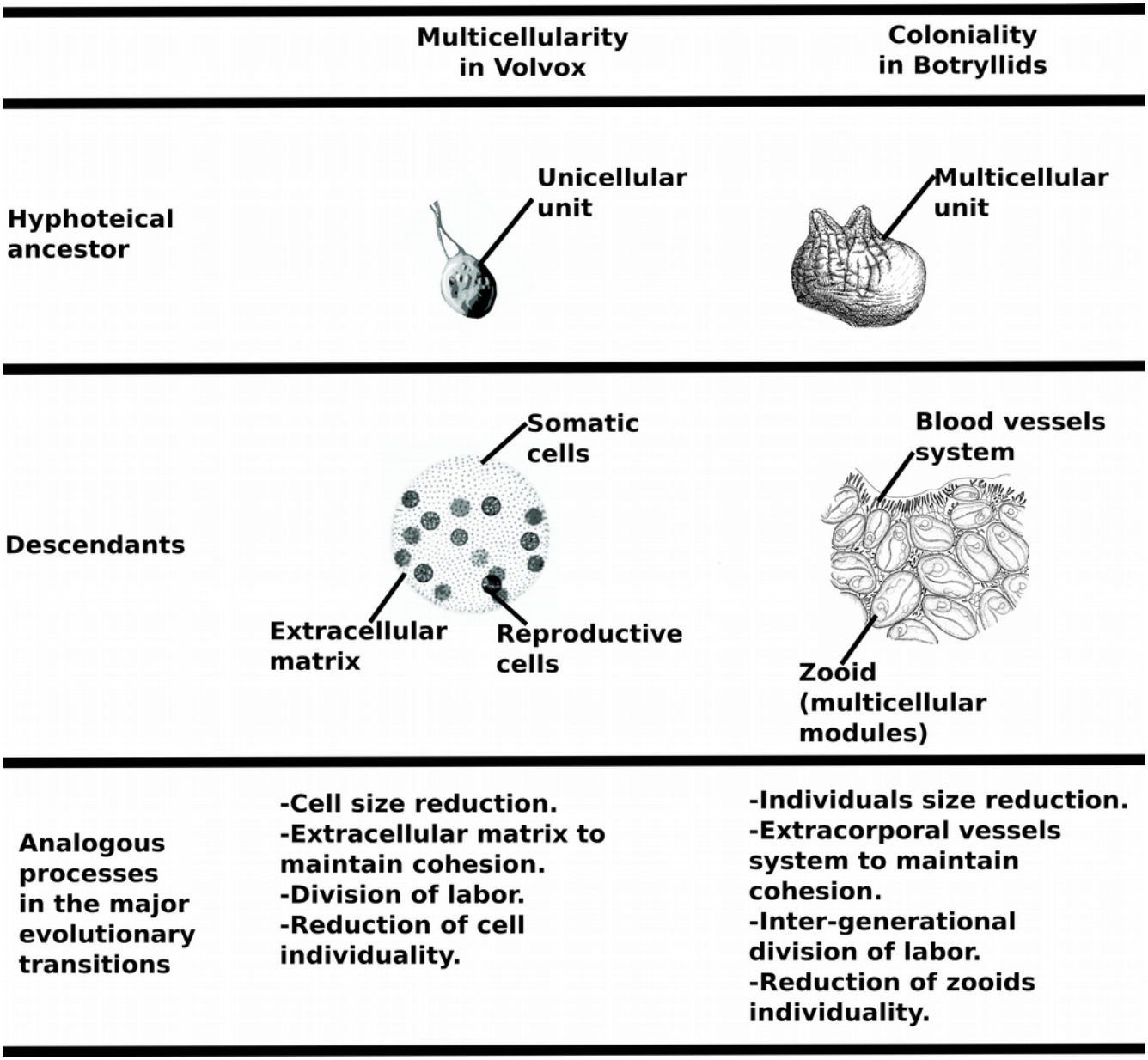

Figure. 6 Analogous processes in the major evolutionary transitions. Evolution of multicellularity in Volvox is related with transformation of cell wall into extracellular matrix; genetic control of cell number; and sterile somatic cells. Evolution of coloniality in Botryllids is related with extra-corporeal blood vessels; zooid size reduction; modularity of the multicellular individuals. Although, in evolution of major evolutionary transitions, such as multicellularity and coloniality, there are analogous processes. External tissues that interconnect and maintain the connection between units, reduction in size and individuality of units.

Chapter 2

Vascular budding in *Symplegma brakenhielmi* and the evolution of coloniality in styelid ascidians*

1. Introduction

Colonial organisms are composed of semi-dependent modules, which correspond to clonal individuals of asexual reproduction (e.g. zooids). In some animal group these modules are specialized in their function (Alberto and Brown, 2015; Ruppert et al., 2004) such as in hydrozoans pennatulaceans (sea pens), many bryozoans (i.e. fixed walled cyclostomate, ctenostomate, and cheilostomate bryozoans) (Ostrovsky, 2013), and the doliolids (pelagic tunicates) (Bone, 2003). In others, they remain as identical units, such as in many anthozoans (subclass Hexacorallia and Alcionaria), free-walled cyclostomate bryozoans, phoronids, entoprocts (Fuchs et al., 2010), colonial hemichordates (Lester, 1985), and most tunicates (i.e. ascidians, pyrosomes and salps) (Alberto and Brown, 2015; Bone, 2003).

How is modular development regulated in colonial animals? Studies in bryozoans (Bone and Keough, 2005) or ascidians (Lauzon et al., 2002) have shown that physical disturbance or direct removal of modular compartments affects the development of the whole colony. These results suggest that colony-wide homeostasis mechanisms maintain a tight control of the developmental processes of each module.

Tunicates provide an interesting case for the study of colonial evolution because many transitions occurred between solitary and colonial forms (Alberto and Brown, 2015). Repeated characteristics that evolved convergently in colonial ascidians include: (a) increased regenerative abilities, (b) reduction of body size and number of branchial folds in the zooids, (c) brooding and increased egg size, (d) shorter planktonic phase as a larva, and (e) early differentiation of adult structures in the larva (i.e. adultation) (Davidson et al., 2004; Hughes, 2005; Kott, 2005). Although it is

* This is a published chapter: Gutierrez, S., & Brown, F. D. (2017). "Vascular budding in Symplegma brakenhielmi and the evolution of coloniality in styelid ascidians" Developmental Biology, 423(2). https://doi.org/10.1016/j.ydbio.2017.01.012

not yet clear whether the ancestor of tunicates was solitary or colonial, distinct modes of budding across colonial groups suggest that the development of these processes arose independently. In the colonial aplousobranchs buds develop from pharyngeal or abdominal tissues of adult zooids (i.e. colony individuals); in the colonial phlebobranchs and stolidobranchs buds develop from filamentous extracorporeal structures (i.e. stolonial budding) (Monniot et al., 1991) ,from the lateral epidermis of adult zooids (i.e. palleal budding), or from vascular vessels that connect the zooids (i.e. vascular budding).

In the Styelidae (Stolidobranchia), solitary and colonial life histories occur. Most colonial genera in the family are represented by aggregated forms, in which individuals develop close to each other but within their own tunics. The phylogenetic relationships among these aggregated genera are not well understood, thus obscuring the number of times coloniality evolved in the group (Pérez Portela et al., 2009; Tsagkogeorga et al., 2009; Zeng et al., 2006). However, *Symplegma* and *Botrylloides+Botryllus* are represented by integrated colonies, in which all buds and zooids develop within a common tunic, and contain a common vascular system with a constant blood flow (Kott, 2005; Van Name G. et al., 1945). The latter form a clade of styelid colonial ascidians with the highest degree of colonial adaptations, including well studied mechanisms of allorecognition (Pérez Portela et al., 2009; Watanabe and Taneda, 1982). Therefore, *Symplegma* lies in an intermediate position between the highly integrated botryllids (*Botryllus* and *Botrylloides*) and the rest of styelids that are generally less integrated.

A semi-autonomous organization and development of individuals are observed in *Symplegma* than in botryllids. *Symplegma* zooids each contain their own atrial siphon, are distributed further apart from each other, and are arranged in a more disorganized and uncoordinated fashion than botryllids. Formation of buds and degeneration of zooids is a permanent and continuous process and occurs asynchronously. During morphogenesis of zooids and outgrowth of colony vasculature, *Symplegma reptans* individuals are constantly changing their position and orientation rapidly within the colony (Kawamura and Nakauchi, 1986). In contrast, a zooidal system arrangement is characteristic for the botryllids, i.e. zooids self-organize around a common cloacal aperture that is formed by multiple atrial openings of surrounding zooids (Berrill, 1941; Sabbadin, 1955a; Sugimoto and Nakauchi, 1974). In botryllids bud formation and zooid resorption are non-autonomous and occur synchronously in a very

coordinated fashion, which are regulated by colony-wide developmental processes (Ballarin et al., 2008; Lauzon et al., 2002, 1993, 1992). Thus, *Symplegma* buds develop “independently” and asynchronously, whereas botryllids show a high degree of crosstalk between developing individuals.

In *Symplegma* and botryllid budding, populations of hemocytes of the vascular system interact with epithelial tissues of zooids or colony vessels to form the initial stages of developing buds (Brown and Swalla, 2012, 2007; F D Brown et al., 2009). Four biological processes have been assigned to hemocytes of colonial styelid ascidians: (a) hyaline amebocytes (HAs) or macrophage like cells (MLCs) are related to immunity; (b) morula cells (MCs) produce cytotoxic compounds; (c) granulocytes and pigment cells serve in storage and excretion, and (d) hemoblasts (HE), lymphocytes or lymphocyte like cells are undifferentiated cells (UCs) involved in budding and regeneration (Ballarin and Kawamura, 2009; Ballarin et al., 1995; Cima et al., 2001; De Leo, 1992; Hirose et al., 2003; Sawada et al., 1993). Proportions of these cells in the circulatory system of *Botryllus schlosseri* oscillate in different stages of the asexual budding cycle, for example MLCs and UCs are more frequent during the brief period of takeover, i.e. when old zooids are resorbed and new buds continue to develop (Ballarin et al., 2008). MLCs phagocytize old tissues during takeover (Lauzon et al., 1993, 1992), whereas hemoblasts are blood stem cells known to occur in the earliest stages of budding and to function as precursors of new bud tissues (Brown and Swalla, 2007; F D Brown et al., 2009). How hemocytes operate during resorption or budding of the asynchronous development in *Symplegma* is unknown.

In this study we explored developmental processes involved in the evolution of colony integration in styelids. First, we established a common staging system to compare developmental stages among botryllids and *Symplegma* based on previously described stages of *Botryllus schlosseri* (Berrill, 1941; Sabbadin, 1955a). Next, we tested whether non-autonomous colony-wide processes of development are conserved in *Symplegma* by systemic removal experiments of zooids or buds. Finally, we characterized and studied the occurrence of hemocytes in *Symplegma* and compare them to hemocytes in other solitary and colonial ascidians, and discuss possible functional roles of these cells for the evolution of coloniality.

2. Materials and Methods

2.1. Collection of samples

S. brakenhielmi colonies were collected from mangrove roots and off docks at the Bocas Marina, Bocas Del Toro, Panamá (09°20’10’’N 82°14’59’’ W), at the Island Global Yachting (IGY) Marina, Santa Marta, Colombia (11º14’29.24’’N 74º12’57.37’’W), and at Yacht Club Ilhabela, SP, Brazil (23º46’20”S 45º21’20”W). Live colonies were tied on to glass slides (26mm x 75mm) and left on hanging racks for a two or three days. Settled colonies were transported to the Smithsonian Tropical Research Institute (Bocas del Toro, Panamá),the Universidad de los Andes (Bogotá, Colombia), or the Universidade de São Paulo (SP, Brazil) and maintained alive in circulating sea water tanks. Colonies from Panamá and Colombia were maintained at 28at28º C, whereas colonies from Brazil were maintained at 24º C following local temperature conditions. In Bocas del Toro colonies were fed daily with ten drops of MarineSnow Plankton diet, and in Bogotá and São Paulo colonies were fed daily with 150 ml of a phytoplankton mix containing *Isochrysis galbana*, *Tetraselmis maculata* and *Dunaliella tertiolecta* at a concentration of one million cells per millilitre. Algae strains were maintained in the laboratory during the experimental period using the protocol of (Stein, 1979) and were kindly provided by the Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM), San Pedro, Ecuador. Epibionts (mostly algae) that grew on slides were removed using razor blades and ascidian tunics were cleaned with a soft hairbrush three times a week.

2.2 Colonies observation and surgical ablations

We describe the blastogenic development in Panamanean and Brazilian *Symplegma brakenhielmi* by in vivo observations of colonies following the development of their individuals. Seven Panamanean and four Brazilian colonies of approximately 20 mm x 40 mm of distinct phenotypes were photographed daily for a two-month period in Panama and a one-month period in Brazil. To document a developmental series of budding until zooid regression, we used stereomicroscopes Nikon SMZ-745T and Leica M205 FA, as well as inverted microscope Leica DMi8. *S. brakenhielmi* buds were observed to emerge in the vasculature or from the peribranchial region of other buds. Vascular budding was the

preferred mode of budding in Panamanean colonies, whereas Brazilian colonies presented both vascular and palleal budding modes.. In addition, we did histological sections of buds and zooids to observe internal changes during development. We compared *S. brakenhielmi* stages to the *Botryllus schlosseri* numeric stages of development described by Berrill (1947) and Sabbadin (1955). We used indicative characters of each stage in *B. schlosseri* (Manni et al., 2007) to identify putatively homologous stages in *S. brakenhielmi.* (Table.1). This allowed us to generate a generic table of blastogenic development of *B. schlosseri* and *S. brakenhielmi*. We calculated the developmental time for each stage by carefully following 40 buds in seven Panamanean colonies and 20 buds in 4 Brazilian colonies. Average times and standard errors were calculated for each developmental stage.

We excised buds or zooids in *Symplegma brakenhielmi* colonies of similar size (4-8 functional zooids) in four treatments following previous colony manipulations reported for *Botryllus schlosseri* (Lauzon et al., 2002). First, we ablated all adult zooids (3-6 functional fully differentiated zooids) and left buds intact (4-10 early stage buds) in four colonies of distinct genotypes (n=4). We named this manipulation as ‘zooid extractions’. Second, we removed all observable buds (6-8 buds) except one that was left intact in three colonies of distinct genotypes (n=3) . We named this manipulation as ‘removal of all buds except one’. Third, we removed all individuals (3-6 functional zooids and 7-14 buds) and left only the vascular tissue in three colonies of distinct genotypes and four clonal replicates of the same genotype (n=7). We named this manipulation as ‘zooid and bud extractions’. Fourth, we ablated all buds (9-13 buds) present in four colonies of distinct genotypes and in four clonal replicates of the same genotype(n=8).We named this manipulation as ‘bud extractions’. Ablations were performed with scalpels and fine needles to prevent damage in vascular tissues and remnant structures. Colonies were maintained in sterile seawater, and were not fed one day before and three days after surgical procedures to prevent contamination.

2.3. Characterization of blood cells

2.3.1. Blood extractions and hemocyte staining

We followed protocols previously used for Botryllus schlosseri (Ballarin & Cima, 2005; Cima, 2010). Colonies were soaked in anticoagulant solution (10 mM L -cysteine and 0.38% sodium citrate)

for 5 min. Before collection of blood, anticoagulant was removed and colonies dried with a paper towel. For blood cell extraction, 4–8 prominent ampullae were cut and colonies were softly pressed with the fingertips to aid in the outflow of blood. Using a pipette, cells were pulled into a disposable plastic tip with a few drops of anticoagulant to avoid clotting. In a 1.5 ml centrifuge tube, blood and anticoagulant (approx. 100 µl) were collected and diluted again with 100 µl anticoagulant (1:1). Samples were centrifuged at 3000 rpm for 10 min, and cells were resuspended in a 1:3 solution of anticoagulant:filtered seawater (FSW). These resuspensions were transferred onto SuperFrost plus slides (Fisher, Waltham, MA, USA), and left to attach for 30 min. Hemocytes were stained with Neutral Red, Giemsa, or Sudan Black (Sigma Aldrich, St. Louis, USA). Neutral Red (8 mg/l in FSW) was added to the slides and cells were immediately prepared for observation. Giemsa stain (10% solution) was added to the slides for 5 min, fixed in 2.5% glutaraldehyde in FSW, washed with PBS and mounted with glycerol. For Sudan Black staining, hemocytes were fixed in 70% ethanol, stained in Sudan Black saturated solution (70%), washed once in ethanol (3 min), and once in distilled water (3 min) for observation. Stained hemocytes were observed in an inverted microscope AxioVert A1 (Zeiss, Oberkochen, Germany).

2.3.2. Hematoxylin and eosin staining on histological sections

Colonies attached on glass slides were fixed in 4% paraformaldehyde overnight. Tissues were dehydrated in ethanol series (30, 50, 70, 90, and 100%), followed by two xylol washes, kept for 30 minutes in a 1:1 Xylol:Paraffin solution, and embedded in paraffin blocks. Colonies were sectioned into 6µm slices with a Rotary Microtome (Leica Byosistems, Melbourne, Australia) and attached onto slides. We deparaffinized slides in two 10-minute washes on xylol, and rehydrated tissue sections with the above mentioned ethanol series and stained with hematoxylin and eosin.

We determined the proportion of hemocytes by counting cells in lacunae of the open circulation system among (25 µm) of the easily recognizable organs, such as the endostyle, stomach or oral siphon of excised colonies (zooid extractions and all buds except one) and in un-manipulated controls. We classified hemocytes by size, presence of granulocyte or vacuoles in the cytoplasm, nucleus-cytoplasm ratios, and differences in coloration. We classified blood cells in their respective categories and counted

total number of cells around each organ in all sections of four consecutive slides of two colony replicates. With this information, we calculated the frequency of each type of hemocyte, sizes, and standard errors. Tukey and Bonferroni tests were used to determine significant differences between the hemocyte frequencies of each cell type.

2.3.3. Phosphorylated histone 3 (pH3) immunocytochemistry

For mitotic cell identification by immunohistology, we used paraffin sections and Phospho-Histone 3 (pH3) as a marker (Brown et al.,2009). Sections were washed three times in PBS with 0.1% Tween(PBT) and blocked with 5% powder milk diluted in PBT. Samples were incubated overnight with a rabbit monoclonal anti-PH3 primary anti-body (Millipore, MA, US, Cat. No. MC463) at 1:500. Slides were washed three times for 10 min with PBT. As a secondary antibody we used anti-rabbit IgG-AP (Santa Cruz Biotechnology, TX, US, Cat. No.sc-2034) at 1:200 for four hours. Samples were washed three times with PBT for ten minutes. For the color reaction, we used NBT/BCIP (Promega, US) solution for twenty minutes. Samples were washed 3X with PBT and 1X in distilled water, and stained with eosin. Sections were dehydrated in ethanol series (100%, 90%, 70%, 50%, and 30%) and Xylol, and mounted on slides with Permount (Fisher, NJ, US) for observation and storage.

2.3.4. Transmission electron microscopy (TEM)

Colonies attached on glass slides were placed in a petri dish and fixed for 1 hour at 4°C in either a glutaraldehyde based fixative used previously in *Botryllus schlosseri* (1.5% glutarldehyde in 0.2M cacodylate buffer, 1% saccharose, 1.7 NaCl% adjusta at ph = 7.47.4) (Cima, 2010) or in Karnovsky modified solution for marine organisms (1.5% glutaraldehydeglutarldehyde, 1.2% paraformaldehyde in 0.2M cacodylate buffer, 1.5 % NaCl, 1% saccharose, adjust at ph = 7.4 (Karnovsky, 1965) . Two colonies were used as replicates for each fixative. After initial fixation, small pieces ($1mm^3$) of ampullae and vascular tissue were dissected and fixed for another 3 hours at 4°C. These vascular pieces of tissue were postfixed in 1% OsO_4 in ca 0.2M cacodylate buffer for 1 hour. Samples were dehydrated in ethanol series and embedded in Spurr's resin (Sigma-Aldrich, St. Louis, USA) and that polymerized at 58°C. Blocks were cut into sections using an ultramicrotome Leica Ultracut UCT.

Sections were collected on copper grids, contrasted with lead citrate, and observed in a Zeiss EM 900 Transmission Electronic Microscope operated at 80kV.

3. Results

3.1. Symplegma brakenhielmi budding

Laboratory grown colonies present two opposite regions of extension or regression respectively. Regions of extension were characterized by numerous, large, and protruding vascular termini at the edge of the colony, which were connected to each other by a highly vascularized network that contained many buds in all stages of development (Fig. 1A). Buds originate at distinct locations of the highly ramified vascular network and moved to other sites during development. Buds develop asynchronously and in disordered orientations (Fig. 1A). In between the regions of extension (red dotted line Fig. 1A) and regression (black dotted line Fig. 1A) lies a zone of predominantly fully differentiated zooids (yellow dotted line Fig. 1A). The region of regression presented less ramified vasculature, and mostly contained zooids in resorption, and resorbing ampullae (Fig. 1A). In contrast, studies in related botryllid species show that individuals develop in highly synchronous cycles, also known as blastogenic cycles (Ballarin et al., 2008). Therefore within a *Botryllus* colony, development of three consecutive generational stages of buds occurs simultaneously. Furthermore, other botryllid species present higher orders of colony organization, such as the flower shaped circular systems of *B. schlosseri*, in which individuals form well-organized assemblies around a common cloacal aperture (Fig.1B).

Symplegma brakenhielmi predominantly forms zooids by vascular budding (Fig.2) but also by palleal budding (Fig. 3). Interestingly, S. *brakenhielmi* colonies from Panamá only presented vascular budding, whereas S. *brakenhielmi* colonies from Brazil presented vascular budding predominantly, but also showed palleal budding. Differences in the modes of budding among Panamanean and Brazilian *S. brakenhielmi* colonies raise interesting questions about the factors generating blastogenic plasticity in these two distinct locations/populations.

3.2. Symplegma brakenhielmi developmental stages

To understand common developmental processes and facilitate comparative research across several species of colonial styelid ascidians we describe here a generic table of developmental stages (Table 1) based on the numerical staging system used for *Botryllus schlosseri* (stages 1-11; Berrill, 1941), and previously described blastogenic stages of *Symplegma reptans* (Kawamura and Nakauchi, 1986). The vascular budding reported in *Botryllus sp* was included to describe the differences observed between vascular and palleal in the earlier development between *Botryllus* and *Symplegma brankenhielmi*. These vascular stages followed also the numerical staging used by Berril, 1941 (Oka and Watanabe, 1957). Because in *B. schlosseri* three generations of zooids coexist at the same time, Sabbadin (1955) began to use a three numerical convention that describes the stage of each generation of individuals within the colony; for example, *B. schlosseri* stage 9/7/1 means the zooids of the colony are fully differentiated (st. 9), the primary buds present primordial stigmata in the branchial sac (st. 7), and the secondary buds have evaginated from the lateral wall of the primary buds (st.1). A three stage numerical convention continues to be used today for other botryllid species (Gasparini et al., 2015), but does not apply to *Symplegma spp* because the colonies show a continuum of individual development with no co-ocurring generations or synchronous development of individuals (Berrill, 1940; Kawamura and Nakauchi, 1986), observed in *B. schlosseri* colonies. However, if morphogenesis is analyzed in individual buds and zooids, *Symplegma spp*. and *Botryllus spp* show the same chronological organization of events during the development, i.e. siphon primordium formation, heart beating, stigma activation, and zooid resorption (Table 1). Thus, we categorized developmental stages of *Symplegma brankenhielmi* into the generic table of stages of botryllids based on in vivo and histological observations of morphogenesis.

We identified all eleven stages that occur in *B. schlosseri*, and included specific events in *S. brankenhielmi* (st. 9.2 Table 1). During the study period colonies were sexually immature so we could not describe the gonad maturation phase (st.10). The growth of an individual zooid during a blastogenic cycle is characterized by a sigmoid growth curve pattern followed by a sharp decline during the resorption phase. *S.brakenhielmi* blastogenesis grown at 24˚C lasts 12 days (Fig. 1C), whereas *B. schlosseri* blastogenesis grown at 26˚C takes 17 days (Berrill, 1941). The length of a blastogenic cycle

varied among species (Fig. 1C), or when grown at different temperatures. During early bud formation, size increased slowly (st.1-7), entered the log phase of growth after heart-activation (st. 8), and reached its fastest exponential growth at around the time stigmata were activated (st. 9.1; documented by in vivo observations of ciliary movement). Complete differentiation occurred at the stationary phase of the curve (st. 9.1-9.2), and concluded the blastogenic cycle by a drastic reduction of zooid size in the final resorption phase (st. 11) (Fig 1C-D).

Next, we describe each stage of *S. brakenhielmi* blastogenesis in detail (note that vascular or palleal buds have different origin and therefore specific description are provided for each of them in stage 1-4):

Stage $1_{vascular}$: The squamous epithelium of the vessel surface evaginated to form a vesicle. Small, round, and clear cells predominantly accumulated in the center of the vesicle, as well as few other maroon-colored cells with a conspicuous nucleolus that resembled H2 cells (see description of hemocytes below; Fig.2A; Table 2). Large pigmented cells were observed in the external part of the developing vesicle (Fig. 2A).

Stage $1_{palleal}$: The rudiment of the palleal bud (from now on budlet) forms by the evagination of the peribranchial epithelium of buds at stage 5 of blastogenesis, i.e. parental buds (Fig. 3A).

Stage $2_{vascular}$: Bud vesicle expanded to form a sphere. The sphere joined its proximal blood vessel by a stalk. Within the sphere, small cells with an irregular cytoplasm accumulated in the center, which resembled H1 cells (see description of hemocytes below; Fig.2B; Table 2). Large pigmented cells were often found in in the cell conglomerate (Fig.2B).

Stage $2_{palleal}$: The budlet grew and arched to form a vesicle or sphere, with two layers of tissue. The external layer is formed by the epidermis, and the internal layer is formed by an epithelium that originated from the peribranchial region of the parental bud (Fig. 3B).

Stage $3_{vascular}$: Internally, a simple cuboidal epithelium extended and closed forming an inner vesicle within the outer sphere. Large pigmented cells and small clear cells were located between these two epithelia. Within the subcuboidal epithelium of the inner vesicle, we observed round clear cells that resembled hemoblasts (HE), H1, and H2 cells (see description of hemocytes below; Fig.2C).

Stage $3_{palleal}$: An inner layer of tissue formed an enclosed sphere surrounded by an external epithelium, resembling the stereotypical double vesicle stage of botryllid blastogenesis. The budlet increased in size and moved away from the parental bud remaining attached to the parental bud only by

a thin vascular vessel extending from the basal part of the budlet (Fig. 3C-C').

Stage $4_{vascular}$: The internal simple cuboidal epithelium of the inner vesicle increased their size. Organogenesis began by the evagination of the inner vesicle to form the branchial and peribranchial cavities (Fig.2D). Large pigmented cells localized in the internal and external epithelia of the bud whereas round, small cells were observed within the forming cavities, in which the longitudanl axis was elongated.

Stage $4_{palleal}$: The budlet showed two clear vesicles: an external vesicle was formed by the epidermis, and an inner vesicle composed by a simple cuboidal epithelium that presented indications of morphogenetic folding (Fig. 3D-D'). By the end of stage 4, the budlet is completely separated from the parental bud (now at stage 5) and rapidly transitions into a freely moving stage bud within a proximal vascular vessel. The new bud (i.e. previous budlet) cannot be morphologically differentiated anymore from other vascular buds,. After stage 4 vascular and palleal buds converge in their appearance and continue through similar stages of blastogenesis (Table 1). During blastogenesis, buds moved actively through the highly ramified vascular network of the colony.

Stage 5: The bud maintained an oval shape but increased in size. The peribranchial cavity was clearly demarcated from the central cavity of the pharyngeal sac (i.e. seen on both sides in cross sections). A process of invagination formed the endostyle primordium along the midline, and epithelial folds around the pharynx began to differentiate into gut and organ primordia (Fig. 4A and B). In the dorsal part of the bud, the oral siphon primordium first appeared. Number of pigment cells increased and accumulated in the developing bud (Fig.2E).

Stage 6: The bud continued to elongate and the lateral peribranchial cavities fused posterior-dorsally. Inside the branchial cavity, cross sections of the endostyle showed a clear C-shaped structure ventrally, and a prominent dorsal lamina evaginated dorsally. Longitudinal vessels and stigma primordia began to form in the wall of the branchial sac. Stomach and gut continued to increase in size (Fig. 4D). The neural vesicle became evident in the dorsal part of bud posterior to the oral siphon primordium that presented a colored red pigmentation pattern around the siphon (Fig. 2F).

Stage 7: The bud continued to increase in size, the endostyle and dorsal lamina elongated and stigma rows of stigma became evident (Fig.4 E, F). The oral siphon primordium showed a clear round shape and a circular pattern of pigmentation (Fig. 2G). On the dorsal side of the bud, the atrial siphon primordium was first observed. An accumulation of pigments cells was observed on the dorsal side of the neural vesicle. The stomach and gut loop were evident was evident in the ventral part of the bud

and heartbeat was initiatedbegan the heart beating (Fig. 2G).

Stage 8: The bud began exponential growth. The endostyle, dorsal lamina, and longitudinal vessel continued to extend. Stigma perforated the branchial epithelia and rows of stigmata were clearly visible in the ventral side (Fig.2H). Stomach folds differentiated and gut length increased. Both oral and atrial siphons elevated on the dorsal side.

Stage 9.1: The bud reached functional differentiation (Fig. 1C). In general, organs and tissues showed apparent functionality. Both oral and atrial siphons were open. In the branchial sac, the endostyle continued to elongate, and stigmata showed abundant cilia thatand were evenly distributed in 8 rows (Fig. 4G and H). The stomach folds appeared fully differentiated and the internal lining of the gut contained microvilli (Fig. 2I).

Stage 9.24: Zooids reached their largest size. The pharynx completed differentiation following the formation of two additional rows of stigmata to the complete set of 11 rows (Fig. 2J), and abundant cilia lined the interior of the fully elongated stigmata. The stomach and gut loop reached their final size.

Stage 11: This represented the last stage of the blastogenic cycle of a zooid. It began with the closure of the siphons, followed by a rapid contraction and reduction of zooidal size. Branchial tissues and the digestive system began to reabsorbresorb from the anterior to the posterior end. The heart continued to pump actively throughout this stage, and was the final organ to complete resorption (Fig. 2K).

Heterochrony in organogenesis and accessory formation of stigmata rows represented the main differences between of *Symplegma brakenhielmi* and *Botryllus schlosseri* development. In *S. brakenhielmi*, the formation of the oral siphon primordium was one of the first events of organogenesis and occurred before the formation of the atrial siphon primordium. The oral siphon primordium formed as the first branchial and gut cavities became recognizable (stage 5), whereas the atrial siphon primordium only formed on later stages of organogenesis when stigma and stomach folds were recognizable (stage 9.1). In contrast, the oral and atrial siphon primordia in *B. schlosseri* form simultaneously after branchial cavities have already formed and the endostyle becomes recognizable (stage 6; Table 1) (Manni et al., 2007). Heart beating in *S. brakenhielmi* occurred in mid-organogenesis along with the formation of branchial cavities and endostyle differentiation (stage 7; Table 1), whereas in *B. schlosseri* heart beating is reported to begin by the end of organogenesis (stage 8; Table 1). Aperture of siphons in *S. brakenhielmi* occurred after the formation of eight rows of stigmata in the

branchial sac (stage 9.1). After both siphons opened *S. brakenhielmi* appended three additional rows of stigmata to its full complement of eleven rows (stage 9.2). This further development and growth of the pharynx after siphon aperture represents an additional stage of blastogenic development in *S. brakenhielmi* that is absent from *B. schlosseri* development (Fig. 1D; Table 1) (Berrill, 1941; Sabbadin, 1955b)

3.3. Complete removal of buds induces an accelerated formation of new buds in experimentally manipulated colonies

Individuals in *S. brakenhielmi* colonies were regularly regressing and being replaced by new zooids in repeated series of blastogenesis that showed no apparent cycles or coordinated periodicity; thus zooids in the colony appear to develop by completely independent and autonomous events of life and death. These observations contrasted dramatically to highly coordinated and synchronous botryllid blastogenic cycles. To test the apparent independence and autonomy in the development of individuals in *S. brakenhielmi* colonies, we carried systemic extraction experiments of buds or zooids. These experiments have been previously carried for *Botryllus schlosseri* (Lauzon et al., 2002). Although, in general we observed autonomous development of individuals after varied ablation experiments (i.e. *zooid extractions, removal of all buds except one*, and *complete zooid and bud extractions*), one ablation experiment (i.e. *extraction of all buds*) generated an unexpected modulation in the development of new buds. After *bud extraction*, we observed a rapid formation of new buds. Detailed descriptions of the results in the four surgical ablation experiments follow.

After zooid extractions, buds survived in three of the four colonies. Two days post ablation (dpa), the ampullar and vascular tissues of the colonies had retracted from the edges (supplementary Fig. 1A, B), but instead accumulated near the developing buds during three days. (Supplementary Fig. 1C). After five dpa buds continued to develop, the ampullae began to extend again, and vessels grew around developing buds (supplementary Fig. 1D). After 10 dpa buds developed into functional zooids, new vascular buds appeared, and the vascular system re-organized (supplementary Fig. 1E).

After removal of all buds except one, the only bud survived and continued development in all amputated colonies. Initially, ampullae of the amputated colonies showed a slight retraction

(supplementary Fig. 1F,G), and presented an accumulation of vessels at the sites of bud ablation (supplementary Fig. 1H), which lasted several days until the vascular system re-organized at around 5 dpa. After 2 dpa, the ampullae re-established extension (supplementary Fig. 1I) and the only bud continued blastogenic development. All other functional zooids continued their development as in the control colonies (supplementary Fig. 1J).

After extraction of all zooids and buds in the colony, the leftover vascular system regressed and formed an aggregate of blood tissue and vessels on the side of the colony, in which the growth zone was originally found, i.e. on the side with abundant and extended ampullae (supplementary Fig. 1K, L, and M). At 5 dpa, early buds were observed within this undifferentiated cellular mass (supplementary Fig. 1N). New functional zooids regenerated and a new vascular system formed by 10 dpa (supplementary Fig. 1O). After 12 dpa, ablated colonies were undistinguishable from unmanipulated controls; buds continued to develop in the vasculature and ampullae extended again in the growth zone.

After bud extractions (budectomy), we observed that new buds appeared, but these developed at least two times faster than in unmanipulated colonies. We observed stage 4 buds after two days post ablation (dpa) and stage 7 buds after 5 dpa (Fig. 5E, 5F). In contrast, control colonies required 2-3 days to form stage 5 buds (Fig. 5A, B, Fig. 2, Table I), and 6-7 days to develop stage 7 buds (Fig. 5C, Table. 1, Fig. 1). Accelerated early development of new buds after budectomy in *S. brakenhielmi* (Fig. 5H) was observed in several buds throughout the manipulated colonies. In summary, these results demonstrate that individuals of *Symplegma brakenhielmi* colonies develop in an almost complete autonomous manner. However, the observed modulatory effect in budectomy experiment serves as evidence for the existence of intercommunication mechanisms among individuals, or colony-wide modulatory mechanisms in this species.

3.4. Hemocyte proportions and proliferation patterns in the circulatory system

Symplegma spp., as well as all botryllid ascidians, have a vascular system that connects all individuals and developing buds of the colony. In *Symplegma brakenhielmi*, we found eleven types of hemocytes within the vessels (Fig. 6, Table. 2). Blood cell morphology and ultrastructure were

characterized by different histological stains (Fig. 6), and by transmission electron microscopy (Fig. 7).We used immunocytochemistry to characterize mitotically active cell types in the circulatory system. Four cell types were found mitotically active as assessed by phosphorilatedphosphorylated histone 3 expression, and two of these were newly reported for this species (H1 and H2; Table 2).

Hemoblasts (HE): Generally round and among the smallest blood cell types (3.6 μm), HE represented 10% of all hemocytes (Table 2). One out of five HEs (20%)presented mitotic activity of HEs as assessed by positive expression of phosphorilated histone 3 (pH3). The nucleus is characteristically large and centric. These cells show a high nuclear-cytoplasmic ratio of at least 1:2 in relation to overall cell diameter (Fig. 6A, 7A). The nucleolus presented a purple basophilic coloration in H&E stain (Fig. 6A), a light blue color using Giemsa (Fig. 6C), and a negative stain using NR (Fig. 6B). The nucleus contained dense granules of chromatin (Fig. 7A -B). The cytoplasm was relatively clear or showed a light pink coloration in H&E stain, or a blueish coloration in living cells (Fig. 6A-B). Orrganelles, including large mitochondria (Fig. 7B), and small dense granules could be observed only in living cells (Fig. 6B) or by TEM (Fig. 7A).

Hemocyte 1 (H1): Round hemocyte of about 5 μm that that occurred at a frequency of 13%. It presented a high mitotic activity, with positive pH3 cells reaching as high as 60% of all H1cells after buds were excised from from experimental colonies (Fig. 6AF-AG). H1 nuclei were generally observed to localize laterally within the cell and showed a strong basophilic purple coloration in H&E staining and a light blue coloration with Giemsa (Fig. 6D). The nucleus contained dense granules associated to chromatin and a nucleolus visible only in living cells (Fig. 6E) and in TEM (Fig. 7C). The cytoplasm was clear in H&E and Giemsa staining, small whereas granules and vesicles in the cytoplasm could be observed in living cells and in TEM (Fig. 7C). These cells also presented a high nuclar-cytoplasmic ratio.

Hemocyte 2 (H2): Round strong pigmented cells with a size of 6 μm that occurred at a frequency of 6% of all hemocytes. These cell types were the second most mitotically active hemocytes after H1swith 40% of all H2s showing positive expression of pH3. (Fig 6AF). H2s contained a dense chromatin and an acidophilic nucleus with a red-purple color in H&E staining, and a yellowish color in NR staining (Fig. 6G-H). The principal feature of these circular cells was a scarlet or maroon-colored cytoplasm visible with H&E staining, and an eosinophilic cytoplasm with a strong blueish coloration

using Giemsa (Fig. 6I). Living H2 (Fig. 6H) showed a yellowish cytoplasm with small granules, which were visualized as electron dense granules in the cytoplasm by TEM (Fig. 7D).

Hyaline amebocyte (HA): Ameboid hemocyte with a size of about 4.6 µm and that occurred at a frequency of 9% of all hemocytes. One out of five HAs (20%) also showed and positive pH3 signal. HAs contained a round nucleus with a purple basophilic pigmentation in H&E staining and a light blue color in Giemsa staining (Fig. 6J-L). The cytoplasm of HAs was pink in color after H&E staining and clear after Giemsa staining. In living cells some granules and vesicles were visible in the cytoplasm. A diverse array of granules with distinct shape and contents were observed in the cytoplasm by TEM (Fig. 7E -F).

Morula cell 1 (MC1): These cells reached a maximum size of 8.6 µm and were found at a frequency of 39%. MC1 cells have large characteristic vesicles, a small nucleus, and a strong reddish coloration in H&E staining (Fig. 6M), a strong purple coloration in living hemcoytes in NR staining, and a strong pink coloration using Giemsa probably due to eosinophilic structures in the cytoplasm. MC1 showed numerous vesicles with heterogeneous size (1-3µm) that are related with the amoeboid movement of the living cells (Fig. 6N). MC1 showed variation in vesicle colors after H&E staining (Fig. 6M) and SB staining (Fig. 6Q) suggesting the presence of lipidic contents. TEM showed heterogeneous contents inside the vesicles (Fig. 7G).

Morula cell 2 (MC2): These cells were approximately 6 µm in diameter, and were present at a frequency of 2%. MC2 contained between eight and twelve vesicles of regular size (2µm) in each cell, and presented orange or greenish cytoplasm with H&E staining, red-purple cytoplasm with NR staining, pink cytoplasm with Giemsa staining, or yellowish lipid vesicles with SB staining (Fig. 6M-Q). The homogeneity in density and contents of MC2 vesicles was corroborated by TEM (Fig. 7H). Different Morula subtypes observed may in fact reflect distinct maturation states or physiological rolesstates. A possible precursor of Morulas (Fig. 7I) has been suggested by Cima et al (2001) and Hirose et al. (2003) to contain bigger vesicles and a relatively larger nucleus than MC1 (Fig. 7G) or MC2 (Fig. 7H).

Macrophage-like cells (MLC): Large hemocytes of approximately 9 µm found at a frequency of 13%. MLC contained two or three large vacuoles (approx. 5µm each) and several small vesicles

(approx. 2µm) in the cytoplasm (Fig. 6V-Y). The color of the cytoplasm showed a characteristic strong orange coloration and red vesicles with H&E and NR suggesting the presence of eosinophilic components. These vesicles or phagosomes harbored heterogeneus contents, consistent with the presence of ingested materil. showed heterogeneus contents, related with the ingested material (Fig. 7J) . . After surgical interventions, i.e. zooid extractions or removal of all buds except one, we observed an increase in the proportion of MLCs throughout the circulatory system of S. *brakenhielmi* (Fig. 6AF), suggesting immune related functions to these cells.

Granular amebocyte (GA): Amoeboid hemocyte of about 10 µm with pseudopods and heterogeneous granular contents that varied in size and shape (Fig. 6Z). This blood cell type was observed only in living hemocytes stained with NR. TEM characterization of these cells showed a large nucleus with small dense granules, and small vesicles and larger electron dense granules in the cytoplasm of GAs (Fig. 7K).

Storage cells (SC): These cells contained large vacuoles with granules that showed Brownian movement. These cell type was only observed in living hemocyte preparations stained with NR. with active granular motion were observed only in living hemocytes stained with NR. We distinguished two subcategories of storage cells, Pigment Cells (PC) and Nephrocytes (N), following the classification of Ballarin & Cima (2005). PCs ranged from 11 to 19 µm, and showed a gray-blue coloration using NR staining. Most of the cell was occupied by a large vacuole filled with dark blue granules of heterogeneous size and ovoid shape (Fig. 6AB-AC). The nucleus was located in the peripheral part of the cell outside of the large vesicle (7L-nN). In contrast, Ns were larger (12- 20 µm) than PCs and contained one or two giant vacuoles that seem to displace the nucleus to the edge of the cell. These giant vacuoles contained electron dense contents (7L). Ns showed a yellowish or brownish color after neutral red stain (Fig. 6AA).

Granular cells (GC): Round cells with a medium sizes (7-8 µm) that were densely packed with small round granules of heterogeneous shapes and size (Fig. 6AD-AE). These granules showed a strong black or blue coloration after NR stain. TEM characterization of GCs showed large differences in nuclear size and granular size and density (7O-T). This large variation among GCs suggest that

these cells maybe has been suggestive to classify themas precursors of some other blood cell types (Cima et al. 2001).

4. Discussion

4.1 Modularity of blastogenesis in styelid ascidians

Perturbations to colonies activate response mechanisms in *Symplegma spp*. and botryllids for the maintenance of colony homeostasis. In *S. brakenhielmi* we observed a sequence of responses after colony disruptions : (1) retraction of ampullae and peripheral vessels, (2) accumulation of vascular tissue in a particular part of the colony, and (3) formation of new buds. These events deter the colony from further growth and expansion, but allow remaining cells and tissues to concentrate and form new buds as a strategy for survival. Similar responses have been observed to occur in botryllid species (Brown et al., 2009; Voskoboynik et al., 2007). Another colony response to perturbations observed in *S. brakenhielmi* was the acceleration of new buds to reach functional differentiation faster after budectomy, i.e. in the absence of buds, presumably before the remaining zooids of the colony reach their lifespan and reabsorb. This response reveals the occurrence of colony-wide sensorial mechanisms and retroactive communication among individuals of the colony,that enable individuals in the colony to communicate, and points to an unreported previously unreported mechanism of colony homeostasis that most likely also serves as a strategy for survival. Other effects on colony homeostasis known to occur after colony perturbations in *B. schlosseri*, include the reduction of size in buds following zooidectomy, or hyperplasia of one zooid (i.e. formation of a “super bud”) when only one bud is left to develop after all other zooids have been extirpated from the colony (Lauzon et al., 2002;2007).
Other effects on colony homeostasis known to occur after colony perturbations in *B. schlosseri*, include the reduction of size in buds following zooidectomy, or hyperplasia of one zooid (i.e. formation of a “super bud”) when only one bud is left to develop after all other zooids have been extirpated from the colony (Lauzon et al., 2002; 2007).

Effects on the size of next generation buds or zooids after colony disruptions reflects the highly integrated and modular nature of blastogenesis in *B. schlosseri*, as well as the importance of cell and tissue recycling of previous generations for normal development to continue. These results show that

an integrated response of modules in a colony likely elicits the evolution of unified , higher-level organismal interactions, i.e. above the level of the individual (Hughes, 2005; Simpson et al., 2007). Altogether, responses observed in *Symplegma* and botryllids indicate an early presence of colony-wide regulatory mechanisms of communication and signaling among buds in the ancestor, which evolved into highly coordinated processes of development and communication mechanisms within colonies of the botryllid lineage.

The intercommunication and coordination of individual development are crucial for homeostasis in colonial organisms (Lauzon et al., 2007; Simpson et al., 2007). Processes of communication between individuals of the colony probably maintain the coordinated development of cells, tissues and organs in each module (Lauzon et al., 2002; 2007; McShea and Anderson, 2005). Budectomy in *B. schlosseri* results in a progressive wave of zooid resorptions presumably generated by signaling gradients in the colony, and it has been suggested that the communication in *B. schlosseri* is via signaling molecules to maintain the timing of developmental events (Lauzon et al., 2007).

It is interesting to explore how widespread colony-wide sensorial systems occur among other styelid ascidians, as it may reveal important soluble factors and signaling systems that evolved during the integration of colonial life histories in the ascidians. Coloniality may have become highly specialized and complex during evolution of ascidians as long as modules and tissues remained intercommunicated within the colony. The evolution of signaling and response mechanisms that act at colony-wide levels to maintain homeostasis and unity of modules in the colony are essential to understand the concept of a superorganism (Grosberg and Strathmann, 2007; Marfenin, 1997; Simpson et al., 2007). In *Botryllus schlosseri,* the interconnection of individuals via the vascular system allows signals to spread rapidly (Lauzon et al., 2007, 2002), for the coordination of modular development, and for the activation of colony-wide responses to perturbations. These modulatory signals are therefore pivotal for bud development and for the maintenance of colony homeostasis in this species. Although an analogous vascular system that interconnects all individuals of the colony is also present in *Symplegma brakenhielmi*, the signals that regulate coordinated development of modules differ.

Symplegma brakenhielmi buds emerge continuously at different sites throughout the colony by either vascular budding or palleal budding. Vascular budding represents the predominant form of

budding in *Symplegma brakenhielmi,* however important differences were observed when comparing Caribbean vs. Brazilian populations. The exclusion of palleal budding from Caribbean colonies, or alternatively the prevalence of palleal budding in Brazilian colonies may reflect plastic responses due to environmental variations or genetic variations among these two sites. Alternative budding strategies have also been reported in several species of botryllid ascidians. *Botryllus schlosseri* and *Botrylloides violaceus* use palleal budding as the main budding strategy, whereas vascular buds can be induced to occur only after zooids and buds are surgically removed (Brown et al., 2009; Voskoboynik et al., 2007).

The natural co-occurrence of vascular and palleal budding has been reported in *Botryllus primigenus* that are known to use vascular budding as a strategy for fast growth and colony expansion, whereas palleal budding is used for generating sexually reproducing zooids, i.e. zooids that develop gonads (Oka & Watanabe, 1957). In *S. brakenhielmi* we did not observe any influence of surgical manipulations in regulation of alternative budding modes, and we could also rule out that palleal buds generate gonad producing zooids as none of our colonies showed sexual maturation during the course of this study, i.e. we did not find zooids with gonads. Thus, future studies are necessary to reveal other environmental factors that may be influencing the budding modes in *Symplegma*. It would be interesting to also test whether genetic influences can also affect the level of plasticity and proportion of budding modes in different *Symplegma brakenhielmi* populations.

Life and death of *S. brakenhielmi* zooids occurs in a disarticulated fashion, in contrast to the synchronous resorption of all zooids during the takeover stage in *B. schlosseri* that is generally activated by colony-wide signaling mechanisms. Zooid resorption in *S. brakenhielmi* occurs independently at individual modules after the lifespan of each zooid has been reached. These results indicate that the coordination of development in *S. brakenhielmi* modules functions more independently than in *B. schlosseri,* and that intrinsic signals of modules – not colony-wide signals of botryllids- can regulate the lifespan of zooids. Disruption of colony homeostasis in *B. schlosseri* is known to generate heterochronic shifts in the timing of bud development and is also known to affect programmed cell death (PCD) resulting in altered durations of zooid resorption and takeover (Ballarin et al., 2008; Brown et al., 2009; Lauzon et al., 2002). In contrast to the arrested development of buds after removal of zooids in *B. schlosseri* colonies (Lauzon et al., 2002), buds continued to develop normally after complete zooid removal in *S. brakenhielmi.* As a consequence, the takeover stage that is

essential for the development of a new generation of buds in *B. schlosseri* (Ballarin et al., 2008; Lauzon et al., 2002) is inexistent in *S. brakenhielmi*. Further investigation is required to determine the intrinsic mechanisms that establish the lifespan of *S. brakenhielmi* zooids.

4.2. Novel types of hemocytes in *Symplegma brakenhielmi* and their putative functions

Hemocytes found in solitary and colonial species of Styelidae show similarities in cell morphology and function (De Leo, 1992). One conserved cell type with stem cell characteristics that functions in the formation of new buds and regeneration of some styelids is the hemoblast (HE) (Brown and Swalla, 2007; Brown et al., 2009). We found a blood cell in *S. brakenhielmi* with similar morphological characteristics of hemoblasts in other botryllids. The small and round HE cell in *S.brakenhielmi* presented a high nuclear-cytoplasmic ratio, electron dense regions in the nucleus,large mitochondria, and mitotic activity. Altogether, these characteristics suggest a progenitor role of this cell type during blastogenesis or regeneration. In addition to HE, we found two other types of undifferentiated cells in *S. brakenhielmi*, that had not been reported in other ascidians, here referred to as hemocytes type 1 (H1) and hemocyte type 2 (H2). These cells also present electron dense granules in the nucleus, as well as mitotic activity, and may correspond to intermediate progenitors during the differentiation of specific cellular lineages. Hemocyte type 1 (H1) shows a relatively high mitotic activity in comparison to other undifferentiated cells, and its frequency in the blood decreased after buds or zooids of the colony were removed during the different regeneration assays. A depletion of H1 suggests that these progenitor cells may undergo differentiation processes necessary for the formation of new tissues during the regeneration of buds and zooids after colony manipulation. Molecular markers will be necessary to identify distinct populations of hemocytes and unambiguously identify their functions.

Larger hemocytes of animals are generally involved in recognition and activation of the immune system, but in colonial ascidians they are also involved in resorption of zooids and recycling of tissues (De Leo, 1992). Morula cells (MCs) function in recognition of foreign cells or molecules and synthesize and release cytotoxic compounds for allorejection (Ballarin et al., 1995). These cells are also responsible for the release of immuno-modulatory signals in the blood and to generate diffusion

gradients that activate cellular phagocytic responses by the macrophage-like cells (MLC) and the hyaline amebocytes (HA) (Menin et al., 2005). As also observed in other colonial styelid species, MCs and MLCs were the most abundant cell types in the blood of *S. brakienhelmi* (Ballarin and Kawamura, 2009; Hirose et al., 2003), thus indicating the importance of these cell types for colonial ascidian specific processes, likely in allorecognition during the formation of chimeras or in zooid resorption and tissue recycling. HAs are far less abundant than MCs or MLCs. We have shown that these cells are mitotically active and become depleted after zooid or bud ablation assays in *S. brakenhielmi*. The ultrastructural characterization of HAs showed variations of nuclear size and cytoplasmatic contents, suggesting that some of these may correspond to intermediate stages of differentiating cells. These cells are presumably precursors of MLCs and change shape into MLCs upon activation (Cima et al., 1996; Menin et al., 2005). In addition, we have also observed morphological variation among MCs. MC1 and MC2 showed differences in morphology and frequency, and differed greatly from another MC cell type that showed characteristics of a precursor cell by ultrastructural analyses (Cima et al., 2001).These results suggest that several hemocytes in the blood may in fact represent morpho-functional groups or precursor populations of other blood cells as has been previously suggested for the botryllids (Cima et al., 2001; Ballarin & Cima, 2005).

4.3 Coloniality in Styelidae

Individuals in a colony are part of a higher level of biological organization that requires a continuous crosstalk among individuals to coordinate colony-wide developmental processes. Convergent evolutionary trends observed in distinct groups of colonial animals show that a coordinated and modular expansion of individual units in the colony comes at a cost for individual growth, and as a result miniaturized forms develop by means of asexual reproduction of these modules (Davidson et al., 2004). The evolution of budding and asexual reproduction, consequently allow the evolution of highly regenerative capacities in these organisms, including the ability to regenerate whole bodies derived from adult cells or tissues. Hemocytes involved in phagocytosis and allorecognition of *Botryllus schlosseri* (Cima et al., 2001) were easily identified in *Symplegma brakenhielmi* suggesting that a common origin of at least some blood cell types may occur among these species.

In *S. brakenhielmi* blood we observed relatively high numbers of hemoblasts (10%) as well as two complementary undifferentiated cell types not previously reported in botryllids. In *Polyandrocarpa*

misakiensis, one type of progenitor blood cell was found at a relatively high frequency of occurrence (4.4%). Hemoblasts in the solitary *Styela plicata* also occur at a relatively high proportion (6.5%), and the species has been shown to regenerate neural tissues (Medina et al., 2015). However, these cells are substantially larger than those observed in any other ascidian (Radford et al., 1998), and thus raise questions about their true homology. Besides, a small lymphocyte-like cell type was also reported in *S. plicata* but it was shown to be involved in immune responses (Monteiro et al., 2009) raising some doubts about their homology to hemoblasts of colonial styelids, i.e. progenitor stem cells involved in regeneration or budding. Thus, undifferentiated blood cell types in solitary styelids are likely involved in tissue specific regeneration and other different functions compared to their colonial counterparts. Future cell sorting, gene expression, and functional studies experiments are needed to resolve whether hemoblasts contain distinct stem cell progenitor subpopulations.

Hierarchical levels of organization of animal colonies have led to the specialization in the morphology and function of individuals (Simpson et al., 2007) and to the evolution of sophisticated forms of communication among cells, tissues, and individual modules of the colony (Grosberg and Strathmann, 2007; Hughes, 2005). Polymorphic colonies have been documented in distantly related colonial animals (e.g. bryozoans or scyphozoan cnidarians), as well as in closely related pelagic tunicates (e.g. thaliaceans), but notably only ascidians present monomorphic individuals. The numerous evolutionary transitory shifts between solitary and colonial forms observed in ascidians may be a result of bypassing specialization of zooids. Identical individuals of colonies can develop from the aperiodic onset of the same developmental modules, thus resembling metamerism in other metazoans (Newman, 1993).

The modular nature of individual development allows the colony to survive and re-grow again after individuals are removed or the organization of the colony is disrupted. This is a relevant aspect in the success of sessile colonial marine invertebrates (Jackson, 1977). Patterns of re-growth and re-organization of ascidian colonies after individuals were removed were less severe in *S. brakenhielmi* than in *B. schlosseri* raising new questions about the levels of modulation and individuality of ascidian colonies. Whereas *B. schlosseri* shows that developmental processes are modulated at the level of the colonies by well-orchestrated systems of communication and coordination among individuals, our results in *S. brakenhielmi* show the presence of colony-wide communication signals but with lower

levels of developmental modulation. Our results indicate that the evolution of coloniality in Styelidae shows a trend from modular developmental regulatory mechanisms at the level of the individuals to highly integrated developmental processes that are modulated at the level of the colonies.

5. References

Alberto, S., Brown, F.D., 2015. Tunicata, in: Wanninger, A. (Ed.), Evolutionary Developmental Biology Invertebrates. Berlin.

Ballarin, L., Cima, F., 2005. Cytochemical properties of *Botryllus schlosseri* haemocytes: indications for morpho-functional characterization.

Ballarin, L., Cima, F., Sabbadin, A., 1995. Morula Cells and Histocompatibility in the Colonial Ascidian *Botryllus schlosseri*. Zool. Sci 12, 757–764. doi:10.2108/zsj.12.757

Ballarin, L., Kawamura, K., 2009. The hemocytes of *Polyandrocarpa mysakiensis*: morphology and immune-related activities. ISJ - Invertebr. Surviv. J. 6, 154–161.

Ballarin, L., Menin, A., Tallandini, L., Matozzo, V., Burighel, P., Basso, G., Fortunato, E., Cima, F., 2008. Haemocytes and blastogenetic cycle in the colonial ascidian Botryllus schlosseri: a matter of life and death. Cell Tissue Res. 331, 555–564. doi:10.1007/s00441-007-0513-4

Barros, C.M., Carvalho, D.R., Andrade, L.R., Pavão, M.S.G., Allodi, S., 2009. Nitric oxide production by hemocytes of the ascidian Styela plicata. Cell Tissue Res. 338, 117–28. doi:10.1007/s00441-009-0851-5

Berrill, N.J., 1941. The Development of the Bud in *Botryllus*. Biol. Bull. 80, 169–184. doi:10.2307/1537595

Berrill, N.J., 1940. The Development of a Colonial Organism*: Symplegma viride*. Biol. Bull. 79, 272–281. doi:10.2307/1537822

Bone, E.K., Keough, M.J., 2005. Responses to damage in an arborescent bryozoan: Effects of injury location. J. Exp. Mar. Bio. Ecol. 324, 127–140. doi:http://dx.doi.org/10.1016/j.jembe.2005.04.008

Bone, Q., 2003. The Biology of Pelagic Tunicates.

Brown, F.D., Keeling, E.L., Le, A.D., Swalla, B.J., 2009. Whole body regeneration in a colonial ascidian, *Botrylloides violaceus*. J Exp Zool B Mol Dev Evol 312, 885–900. doi:10.1002/jez.b.21303

Brown, F.D., Keeling, E.L., Le, A.D., Swalla, B.J., 2009. Whole body regeneration in a colonial ascidian, *Botrylloides violaceus*. J. Exp. Zool. B. Mol. Dev. Evol. 312, 885–900. doi:10.1002/jez.b.21303

Brown, F.D., Swalla, B.J., 2012. Evolution and development of budding by stem cells: Ascidian coloniality as a case study. Dev. Biol. 369, 151–162. doi:http://dx.doi.org/10.1016/j.ydbio.2012.05.038

Brown, F.D., Swalla, B.J., 2007. Vasa expression in a colonial ascidian, *Botrylloides violaceus*. Evol Dev 9, 165–177. doi:10.1111/j.1525-142X.2007.00147.x

Cima, F., Ballarin, L., Sabbadin, A., 1996. New data on phagocytes and phagocytosis in the compound ascidian *Botryllus schlosseri* (Tunicata, Ascidiacea). Ital. J. Zool. 63, 357–364. doi:10.1080/11250009609356159

Cima, F., Perin, A., Burighel, P., Ballarin, L., 2001. Morpho-functional characterization of haemocytes of the compound ascidian *Botrylloides leachi* (Tunicata, Ascidiacea). Acta Zool. 82, 261–274. doi:10.1046/j.1463-6395.2001.00087.x

Davidson, B., Jacobs, M., Swalla, B.J., 2004. The individuals as a Module: Solitary-to-Colonial Transitions in Metazoan Evolution and Development., in: Schlosser, G., Wagner, G. (Eds.), Modularity in Development and Evolution. The University of Chicago Press, Chicago.

De Leo, G., 1992. Ascidian hemocytes and their involvement in defence reactions. Bolletino di Zool. 59, 195–214. doi:10.1080/11250009209386669

Fuchs, J., Iseto, T., Hirose, M., Sundberg, P., Obst, M., 2010. The first internal molecular phylogeny of the animal phylum Entoprocta (Kamptozoa). Mol. Phylogenet. Evol. 56, 370–379. doi:http://dx.doi.org/10.1016/j.ympev.2010.04.009

Gasparini, F., Manni, L., Cima, F., Zaniolo, G., Burighel, P., Caicci, F., Franchi, N., Schiavon, F., Rigon, F., Campagna, D., Ballarin, L., 2015. Sexual and asexual reproduction in the colonial ascidian *Botryllus schlosseri*. Genesis 53, 105–120. doi:10.1002/dvg.22802

Grosberg, R.K., Strathmann, R.R., 2007. The Evolution of Multicellularity: A Minor Major Transition? Annu. Rev. Ecol. Evol. Syst. 38, 621–654. doi:10.2307/30033874

Hirose, E., Shirae, M., Saito, Y., 2003. Ultrastructures and Classification of Circulating Hemocytes in 9 Botryllid Ascidians (Chordata: Ascidiacea). Zool. Sci 20, 647–656. doi:10.2108/zsj.20.647

Hughes, R., 2005. Lessons in modularity: the evolutionary ecology of colonial invertebrates, 2005.

Jackson, J.B.C., 1977. Competition on Marine Hard Substrata: The Adaptive Significance of Solitary and Colonial Strategies. Am. Nat. 111, 743–767. doi:10.2307/2460328

Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell. Biol. 27, 137-8A.

Kawamura, K., Nakauchi, M., 1986. Development of spatial organization in palleal buds of the compound ascidian, Symplegma reptans. Biol.Bull. 171, 520–537. doi:10.2307/1541621

Kott, P., 2005. Novel Australian Polyzoinae (Styelidae, Tunicata). J. Nat. Hist. 39, 2997–3011. doi:10.1080/00222930500239702

Lauzon, R.J., Ishizuka, K.J., Weissman, I.L., 2002. Cyclical Generation and Degeneration of Organs in a Colonial Urochordate Involves Crosstalk between Old and New: A Model for Development and Regeneration. Dev. Biol. 249, 333–348. doi:10.1006/dbio.2002.0772

Lauzon, R.J., Ishizuka, K.J., Weissman, I.L., 1992. A cyclical, developmentally-regulated death phenomenon in a colonial urochordate. Dev Dyn 194, 71–83. doi:10.1002/aja.1001940109

Lauzon, R.J., Kidder, S.J., Long, P., 2007. Suppression of programmed cell death regulates the cyclical degeneration of organs in a colonial urochordate. Dev. Biol. 301, 92–105. doi:http://dx.doi.org/10.1016/j.ydbio.2006.08.055

Lauzon, R.J., Patton, C.W., Weissman, I.L., 1993. A morphological and immunohistochemical study of programmed cell death in *Botryllus schlosseri* (Tunicata, Ascidiacea). Cell Tissue Res 272, 115–127.

Lester, S.M., 1985. Cephalodiscus sp. (Hemichordata: Pterobranchia): observations of functional morphology, behavior and occurrence in shallow water around Bermuda. Mar. Biol. 85, 263–268. doi:10.1007/BF00393246

Manni, L., Zaniolo, G., Cima, F., Burighel, P., Ballarin, L., 2007. *Botryllus schlosseri*: A model ascidian for the study of asexual reproduction. Dev. Dyn. 236, 335–352. doi:10.1002/dvdy.21037

Marfenin, N.N., 1997. Adaptation capabilities of marine modular organisms. Hydrobiologia 355, 153–158. doi:10.1023/A:1003069831832

McShea W., D., Anderson, C., 2005. The Remodularizarion of the Organism, in: Callebaut, W., Rasskin-Gutman, D., Simon A., H. (Eds.), Modularity. Undersanding the Development and Evolution Os Natural Complex Systems. MIT Press, Cambridge, Massachusetts.

Medina, B., Santos de Abreu, I., Cavalcante, L., Silva, W., da Fonseca, R., Allodi, S., de Barros, C., 2015. 3-acetylpyridine-induced degeneration in the adult ascidian neural complex: Reactive and regenerative changes in glia and blood cells. Dev. Neurobiol. 75, 877–893. doi:10.1002/dneu.22255

Menin, A., Favero, M., Cima, F., Ballarin, L., 2005. Release of phagocytosis-stimulating factor(s) by morula cells in a colonial ascidian. Mar. Biol. 148, 225–230. doi:10.1007/s00227-005-0081-7

Monniot, C., Monniot, F., Laboute, P., 1991. Coral reef ascidians of New Caledonia, Collection Faune tropicale. Éditions de l'ORSTOM, Institut français de recherche scientifique pour le développement en coopération, Paris.

Monteiro, C., De Carvalho, D.R., Andrade, L.R., Pavão, M.S., Allodi, S., 2009. Nitric oxide production by hemocytes of the ascidian *Styela plicata*. Cell Tissue Res. 338, 117–28. doi:10.1007/s00441-009-0851-5

Newman, S.A., 1993. Problems and paradigms: Is segmentation generic? BioEssays 15, 277–283. doi:10.1002/bies.950150409

Oka, H., Watanabe, H., 1957. Vascular Budding, a New Type of Budding in Botryllus. Biol. Bull. 112, 225–240. doi:10.2307/1539200

Ostrovsky, A., 2013. Reproductive Patterns of Gymnolaemate Bryozoa: General Overview and Comparative Analysis, in: Evolution of Sexual Reproduction in Marine Invertebrates. Springer Netherlands, pp. 1–113. doi:10.1007/978-94-007-7146-8_1

Pérez Portela, R., Bishop J,D., J., Davis R., A., Turon, X., 2009. Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences. Mol. Phylogenet. Evol. 570, 560–570.

Radford, J., Hutchinson, A., Burandt, M., Raftos, D., 1998. A Hemocyte Classification Scheme for the Tunicate *Styela plicata*. Acta Zool. 79, 344–350. doi:10.1111/j.1463-6395.1998.tb01284.x

Ruppert, E., Fox, R., Barnes, R., 2004. Invertebrate Zoology: A Functional Evolutionary Approach., 7 edition. ed. Thomson-Brooks/Cole., Belmont, CA.

Sabbadin, A., 1955a. Osservazioni sullo sviluppo, l'accrescimento e la riproduzione di Botryllus schlosseri (Pallas), in condizioni di laboratorio. Bolletino di Zool. 22, 243–263. doi:10.1080/11250005509439204

Sabbadin, A., 1955b. Osservazioni sullo sviluppo, l'accrescimento e la riproduzione di *Botryllus schlosseri* (Pallas), in condizioni di laboratorio. Boll. Zool. 243– 265.

Sawada, T., Zhang, J., Cooper, E.L., 1993. Classification and Characterization of Hemocytes in *Styela clava*. Biol. Bull. 184, 87–96. doi:10.2307/1542382

Simpson, C., Fleming, L., Mcshea, D., 2007. Live together, die alone: Levels of selection in the transition to coloniality and sociality.

Stein, J., 1979. Handbook of Phycological Methods: Culture Methods and Growth Measurements. Cambridge University Press, New York.

Sugimoto, K., Nakauchi, M., 1974. Budding, Sexual Reproduction, and Degeneration in the Colonial Ascidian, *Symplegma reptans*. Biol. Bull. 147, 213–226.

Tsagkogeorga, G., Turon, X., Hopcroft, R., Tilak, M.-K., Feldstein, T., Shenkar, N., Loya, Y., Huchon, D., Douzery, E., Delsuc, F., 2009. An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. BMC Evol. Biol. 9, 187.

Van Name G., W., Trustees, O. or T., History, A.M. of N., 1945. The North and South Americans Ascidians. New York.

Voskoboynik, A., Simon-Blecher, N., Soen, Y., Rinkevich, B., De Tomaso, A.W., Ishizuka, K.J., Weissman, I.L., 2007. Striving for normality: whole body regeneration through a series of abnormal generations. FASEB J. 21 , 1335–1344. doi:10.1096/fj.06-7337com

Watanabe, H., Taneda, Y., 1982. Self or Non—self Recognition in Compound Ascidians. Am. Zool. 22, 775–782.

Zeng, L., Jacobs, M.W., Swalla, B.J., 2006. Coloniality has evolved once in Stolidobranch Ascidians. Integr. Comp. Biol. 46, 255–268. doi:10.1093/icb/icj035

6. Tables

Table 1. Generic table of developmental stages for botryllids (*Botryllus* spp. and *Botrylloides* spp.) and *Symplegma* spp.

Generic categories		Species specific descriptions			Bud or zooid size (mm)			Days (Temperature ºC)		
Stages	Descriptions	*Botryllus schlosseri* stages[1,2,3]	*Symplegma brakenhielmi* stages[4]	*Symplegma reptans stages*[5]	*B. schlosseri*[1]	*S. brakenhielmi*[4]	*S. reptans* (estimated sizes)[6]	*B. schlosseri* (26°)[1]	*S. brakenhielmi* (24°)[4]	S. reptans (18°)[5]
$1_{vasc/pal}$	Initial arching of bud primordium	1_{vasc}: Cells aggregate under evagination of ampullar/vascular epithelium	Initial evagination from vascular epithelium	-	0,02	0,05	-	2-3 hours	NA	-
		1_{pal}: Thickening of peribranchial epithelia, initial arching disc forms bud primordium	NA	0-1 Bud primordium evident	0,07	NA	0,25	0,7	NA	-
$2_{vasc/pal}$	Evagination of initial sphere	2_{vasc}: Vascular epithelia continues to extend around growing mass of cells	Evagination expands to form a sphere	-	0,04	0,17	-	4-5 hours	NA	-
		2_{pal}: Epidermis continues to extend and forms round sphere around a newly evaginating peribranchial tissue	Arching of the paleal bud primordium, formed by two layer of tissues	2: Arching of bud primordium	0,08	0,047	0,4	0,8	1	1,1
$3_{vasc/pal}$	Double vesicle, closure of internal epithelium	3_{vasc}: External epithelium forms a closed vesicle; internal cells form a smaller vesicle (double vesicle)	External epithelium extends and encloses a newly formed inner vesicle (double vesicle)	-	0,048	0,17	-	0,5	NA	-
		3_{pal}: Closure of disc (double vesicle), anteroposterior elongation	Double vesicle forms by inner and external closure of sphere discs; buds increase in size and begin to move away from parental zooid	3: Double vesicle with internal layer thickened on the right side of future zooid; 4: A secondary vascular vessel forms, active blood circulation	0,1	0,08	0.45-0.55	0,7	1	1,8
4	Organ primordia (folds)	4_{vasc}: Internal vesicle expands and begins to fold vertically	Size increases, internal tissue layer of vesicle folds	-		0,18	-	0,5	2	-
		4_{pal}: Formation of atrial folds and intestinal primordium	Bud attached to a vascular vessel, began formation of atrial folds.	5: Appearance of neural complex primordium; 6: Pharyngeal folds form by the inner vesicle	0,1	0,115	0.6-0.7	0,8	1	1,1
5	Pharynx primordium	Branchial, peribranchial chamber, gut, and neural complex rudiment become recognizable	Oral siphon and vascular ganglium primordia are evident	7: Bud primordium on the right anterior region of atrial epithelium; 8: Heart rudiment visible and pharyngeal folds extend	0,2	0,29	0.8-1.0	1,2	1.1	2,2
6	Endostyle	Endostyle is evident, expansion of branchial and peribranchial chambers	Circular coloring appears around oral siphon primordium, atrial siphon primordium is evident	9: Oral and atrial siphon primordia are evident; 10: Endostyle rudiment is evident	0,3	0,34	1.2-2.3	1,2	1.7 ±0.7	3,1
7	Stigmata primordia	Cerebral ganglion recognizable, primordial rows of	Heart beats begins, gut and pharyngeal sac are	Heart beats 11.1: Heart beat is irregular; 11.2:	0,3	0,63	2,3	1,2	1.5 ±0.5	2,8

		stigmata form	recognizable	Heart beat is regular, blood vessels of bud connect to colony vasculature						
8	Perforation of stigmata	Perforation of stigmata, gastric folds, heart beats. 8.1: Heart beats slowly; 8.2: heart beats normally	Perforation of stigmata, stomach folds are visible	12: Perforation of stigmata and growth of pharynx	1,5	1,22	3	1,2	0.8 ±0.1	1,3
9	Functional maturity	Stigmata are active, pharynx is functional; 9.1: Aperture of oral siphon; 9.2: Aperture of common cloaca	Stigmata are activate, aperture of oral and atrial siphons; 9.1: 8-9 stigmata rows in pharynx; 9.2: 11 rows of stigmata	13: Stigmata activation, zooids are fully functional.	3,4	9.1: 3.14 9.2: 4	NA	4,2	9.1: 0.9 ±0.1 9.2: 2.5 ±0.5	2,4
10	Gonad maturation	Gonad maturation	Gonad maturation (was not observed in our specimens)	14: Sexual maturity	3,9	NA	NA	1,3	NA	NA
11	Resorption	Resorption. 11.1: Siphons closed; 11.2; Reduction of zooid size; 11.3; Branchial degradation and resorption; 11.4 Heart beat stops	Resorption process. 11.1: Siphon close and zooid wrinkles; 11.2: heart stops beating, dramatic reduction of zooid size	15: degeneration	0,7	1,3	NA	0,7	1	NA

1 Berrill, 1941

2 Sabbadin, 1955

3 Manni et al., 2007

4 Oka & Watanabe, 1957

5 present study

6 Sugimoto and Nakauchi, 1974

7 Kawamura & Nakauchi, 1986

Table 2. Characterization of *Symplegma brakenhielmi* hemocytes in comparison to descriptions of other styelid species

Hemocyte type	Short description	*Symplegma brakenhielmi* (Present study)		*Botryllus schlosseri*[4]		Other botryllids[1,3]	*Polyandrocarpa mysakiensis*[4]		*Styela clava*[6]		*Styela plicata*[6]
		Size (µm)	Frequency (%)*	Size (µm)	Frequency (%)**	Size (µm)	Size (µm)	Frequency (%)***	Size (µm)	Frequency (%)**	Size (µm)
HE	Small round hemocyte, basophilic nucleus with a high nucleus-cytoplasmic ratio	3.65 ±0.3	10	4-6	2 mid-cycle, 4 take-over	3	4-6	4.4	4-6	14	3-5
H1	Round hemocyte with a strong basophilic coloration in nucleus, high nucleus-cytoplasmic ratio	5.28 ±0.2	13	-	-	-	-	-	-	-	
H2	Round hemocyte, maroon cytoplasm and basophilic nucleus	5.92 ±0.1	6	-	-	-	-	-	-	-	6
GA	Hemocyte with a irregular shape, few pseudopodia and some vacuoles in the cytoplasm	10†	-	7-10	2.5-5	3-5	4-6	15.3	-	-	
HA	Hemocyte with a irregular shape, and a high nucleus-cytoplasm ratio, strong bashophilic coloration in nucleus	4.65 ±0.2	9	6-12	23-42 mid-cycle, 12-25 take over	-	-	-	15	18	
MC	Heterogenous vesicles with acidophilic content occupied mostly of the cytoplasm	8.6 ±0.3	39	10	50 -60 mid-cycle, 70-75 take over	4-10	10-15	32.9	8-15	30	9-16
MLC	Hemocyte with few large vacuoles, inconspicuous nulecus	9.19 ±0.3	13	10-15	5 mid-cycle, 20 take-over	6-12	10-15	8.1	-	-	
SC	Larger hemocytes with large vacuoles with granular content	12-20†	-	10-15	3-10	10-15	10-15	1.2	-	-	5-13
GC	Round hemocyte, with small and large granules in cytoplasm	7-8†	-	-	-	7-12	-	-	-	-	4-5

[1] Ballarin and Cima (2005)
[2] Hirose, Shirae et al. (2003)
[3] Cima at al. (2001)
[4] Ballarin and Kawamura (2009)
[5] Sawada, Zhang et al. (1993)
[6] Barros et al. (2009)
* Frequencies calculated from Hematoxiline-Eosine stained histological sections
** Frequencies calculated from extracted & live hemocytes stained in Neutral Red
*** Frequencies calculated from extracted fixed hemocytes stained in Giemsa
† Corresponds to size of live cell
++ Hemocyte 1 (H1) described in the present study resembles the Hemoblast described by Barros et al. (2009)

7. Figures

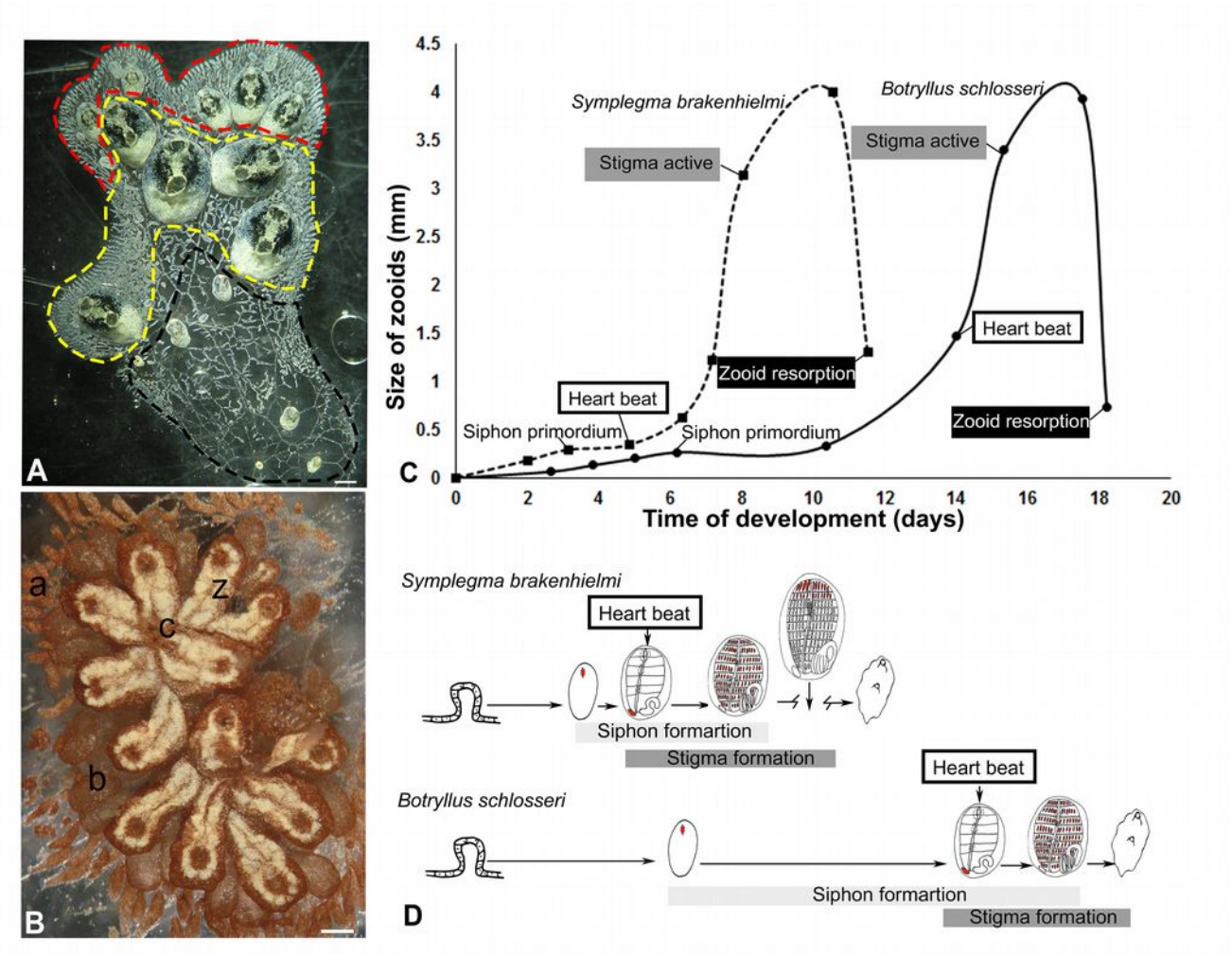

Figure 1. Comparison of budding in *Symplegma brakenhielmi* and *Botryllus schlosseri*.

(A) A laboratory grown *S. brakenhielmi* colony generally shows three zones: a bud containing extension zone (red dotted line), a zone of fully differentiated zooids (yellow dotted line), and the regression zone dominated by old zooids in the resorbtion phase (black dotted line). (B) *B. schlosseri* colony is recognized by the characteristic circular shape systems made up of zooids surrounding a common cloaca. (C) *S. brakenhielmi* and *B. schlosseri* growth curves the significant development events are shown. (D) Summary diagram of blastogenic development in S. *brakenhielmi* and *B. schlosseri;* note the rapid development and continued formation of stigma past oral siphon aperture in *S. brakenhielmi.* a: ampullae; b:bud; c: common cloaca; z: zooid. Scale bars, 1000 µm.

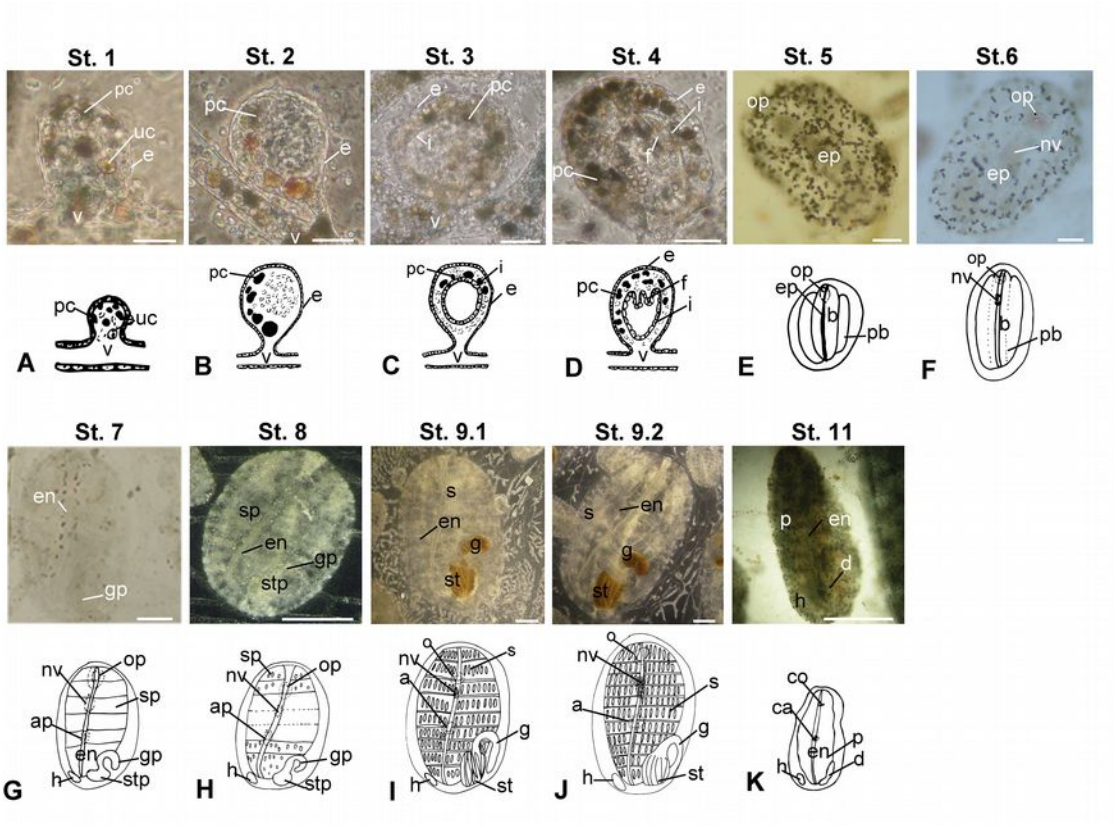

Figure 2. Developmental stages in *Symplegma brakenhielmi.* (A) Initial evagination of vessel epithelium to form a vesicle (stage 1). (B) Vesicle expands and forms a sphere (stage 2). (C) Double vesicle stage formed by two disc layers (stage 3). (D) Internal disc tissue layer begins to infold (stage 4). (E) Bud increases in size and becomes oval shaped; endostyle and siphons begin to form (stage 5). (F) Bud elongates, endostyle differentiates and siphons remain closed (stage 6). (G) Rudimentary gut and heart become recognizable (stage 7). (H) Internal organs are clearly formed (heart, stomach and gut), pharyngeal sac continues to differentiate (rows become visible and some irregular and short stigmata can be observed) (stage 8). (I) Nine rows of stigmata are visible, and all internal organs are completely formed; note the colored gut (stage 9.1) (J) Zooid reaches its largest size (4mm) and complete differentiation of branchial sac with eleven rows of stigmata (stage 9.2). (K) Size of zooid decreases, and tissues are rapidly resorbed from the anterior to the posterior. a: atrial siphon; ap: atrial siphon primordium; ca: closed atrial siphon; co: closed oral siphon; d: digestive tissues; e: external epithelium; en: endostyle; ep: endostyle primordium; f: folds; g: gut; gp: gut primordium; h: heart; i: internal epithelium; nv: neural vesicle; o: oral siphon; p: pharynx; pc: pigmented cells; ps: stigma primordium; s: stigma; sp: siphon primordium; st: stomach; stp: stomach primordium; uc: undifferentiated cells; v: vascular vessel. Scale bar is 20 µm in A, from B to G is 50 µm. from H to K is 500 µm.

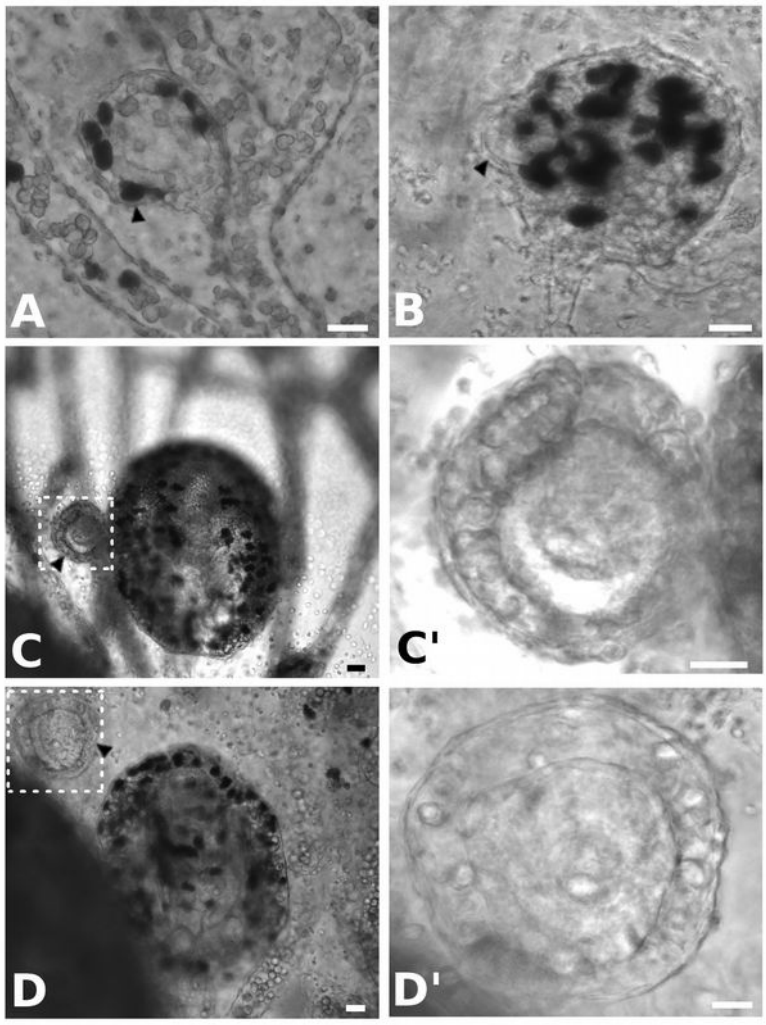

Figure 3. Palleal budding in *Symplegma brakenhielmi*. (A) Stage 4 vascular buds sometimes present a thickening on the right side of the peribranchial epithelium that corresponds to the site of origin of the stage $1_{palleal}$ bud or budlet (arrowhead). (B) Parental bud at early stage 5 shows an arching of the palleal bud. At this stage (stage $2_{palleal}$), the budlet is composed of an internal epithelium surrounded by the external epidermis at the right anterior region of the parental bud (arrowhead). (C) Parental bud at stage 5 with its budlet (arrowhead) presenting the characteristic double vesicle stage of blastogenesis (stage $3_{palleal}$) formed by the enclosure of an internal and external epithelia that remain attached to the parental bud. (C') Magnification of the two vesicles or spheres of the budlet in (C). (D) The palleal bud moves away from the parental bud (arrowhead) and attaches to the vascular system of the colony by the formation of a bridging vessel. (D') Magnification of the palleal derived bud shows the beginning of elongation of the longitudinal axis and the folding of internal layers(stage $4_{palleal}$). Following this stage palleal derived buds develop similarly to vascular derived buds (refer to Fig. 2).

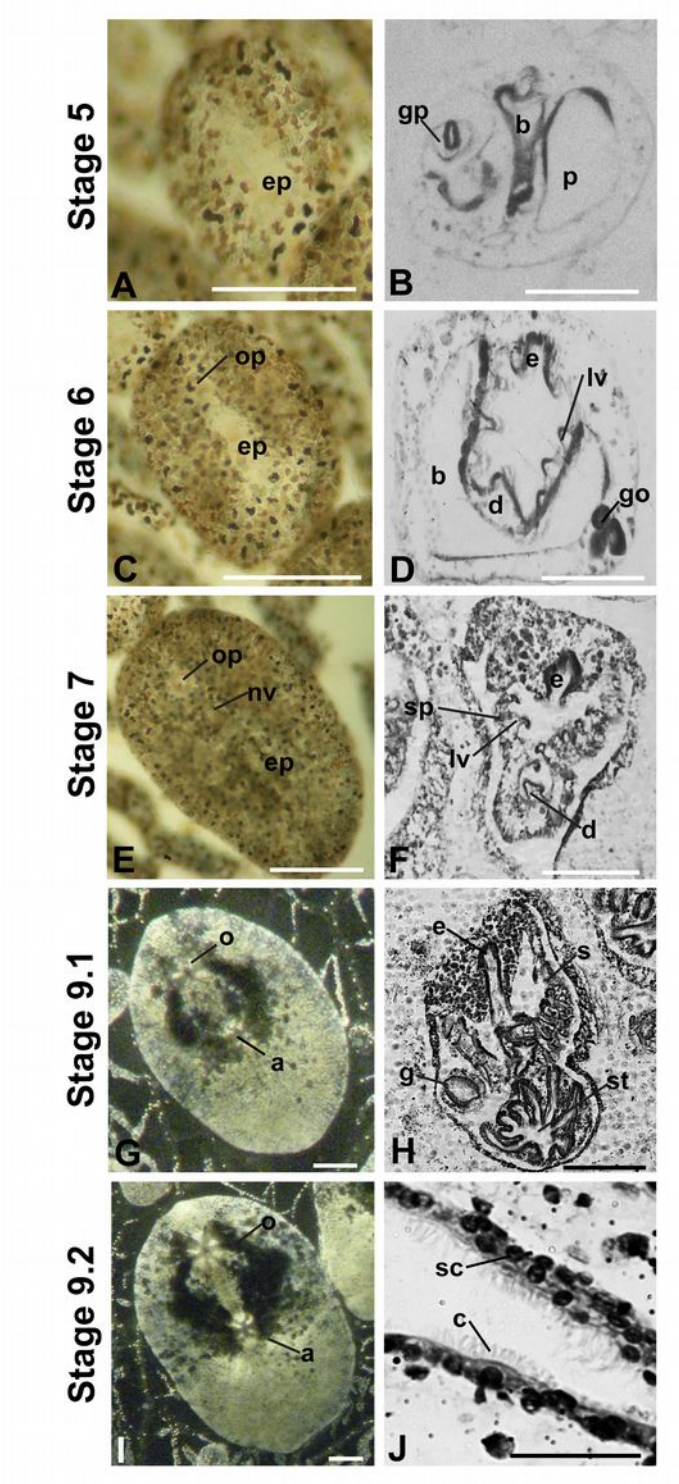

Figure 4. Characteristic internal features of development in *Symplegma brakenhielmi*. (A) Dorsal external view of a stage 5 bud with closed siphons. (B) Histological section of bud in stage 5, note the formation of the branchial chambers and gut. (C) Dorsal external view of a stage 6 bud with closed siphons and endostyle begins to elongate. (D) Histological section of bud in stage 6, note the differentiation of the gonads and tissues in the branchial chamber. (E) Dorsal external bud in stage 7, the oral siphon, and neural vesicle are recognizable in the dorsal side. (F) Histological section of bud in stage 7, note the development of the rudimentary endostyle in the pharynx. (G) Dorsal view of bud in stage 9, the oral and atrial siphon are open. (H) Histological section of bud in stage 9, note fully differentiated endostyle, stigmas, and ciliated gut. (I) Dorsal view of fully differentiated zooid in stage 9.1. (J) Histological section of the ciliated epithelia of a fully differentiated stigma of the branchial sac of a full grown zooid. a: atrial siphon; b: branchial chamber; c: ciliated structures; d: dorsal lamina; e: endostyle; ep: endostyle primordium; g: gut; go: gonad; gp: gut primordium; lv: longitudinal vessels; o: oral siphon; op: oral siphon primordium; p: peribranchial chamber; s: stigma; sc: stigma cells; sp: stigma primordium; st: stomach. Scale bar is 200 µm in A, C, E, G; B, D. F, H is 100 µm; I, is 500 µm; J is 50 µm.

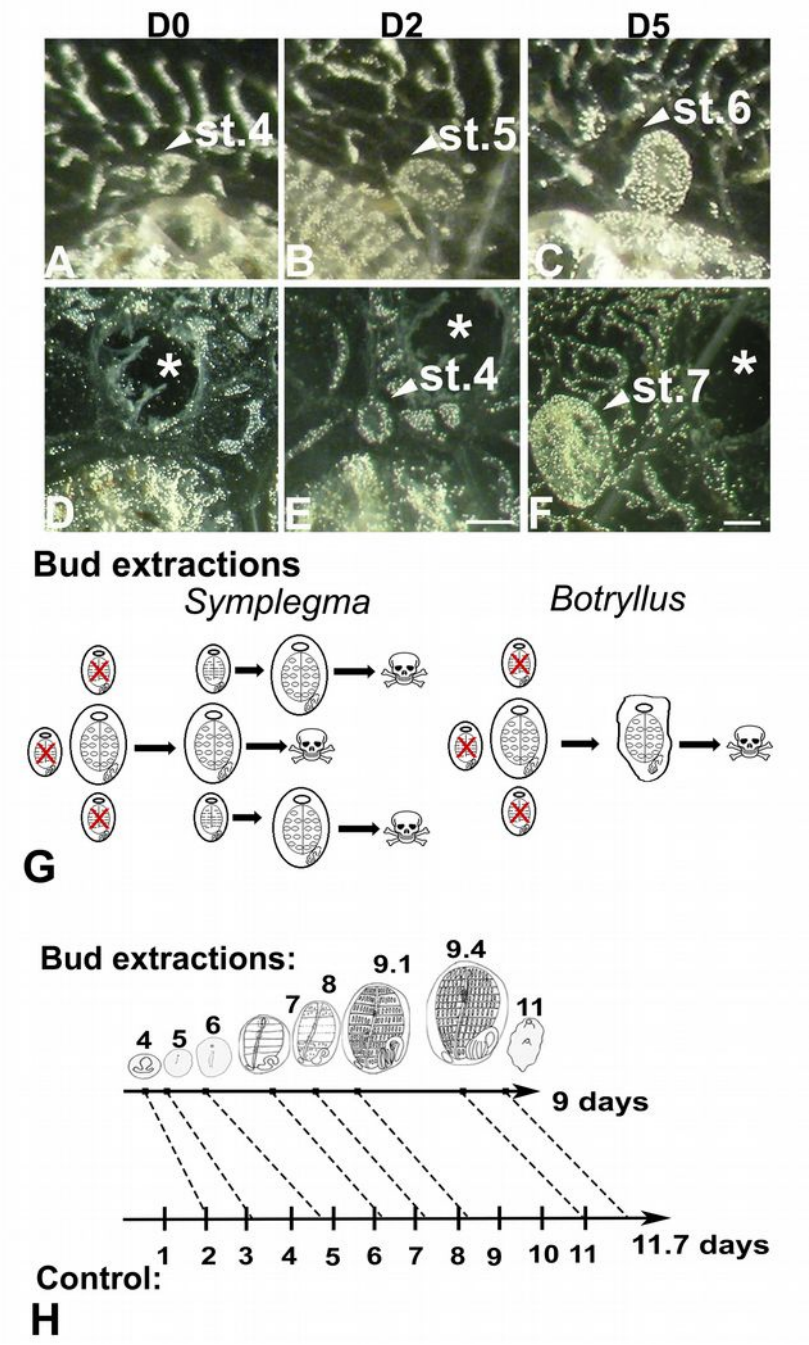

Figure 5. Accelerated development of buds after budectomy in *Symplegma brakenhielmi*. Vascular buds in *S. brakenhielmi* develop from stage 4 to stage 6 in 5 days in unmanipulated colonies (A-C), whereas budectomized colonies show an accelerated development of from stage 1 to stage 7 in the same period of time (D-F). (A) Vascular bud at stage 4 between zooid and peripheral vasculature (i.e. ampullae). (B) Oval shaped bud at stage 5 of development. (C) Elongated bud at stage 6 of development. (D) Leftover zooid (below) and vasculature (above) after budectomy, asterisk shows surgical scar from an earlier bud that was removed. (E) Bud at stage 4 is observed near the scar (asterisk) after 2 days of budectomy. (F) Same bud in (E) is at stage 7 after 5 days of budectomy, note the spatial reorganization of the bud in relation to the scar (asterisk). (G) Schematic representation of the effects of budectomy on the development of other buds in *S. brakenhielmi* and *B. schlosseri*, note that *S. brakenhielmi* undergoes rapid formation of new buds that continue to develop into functional zooids, whereas *B. schlosseri*, the rate of zooid regression is curtailed following *onset of zooid regression is arrested after* budectomy (see Lauzon, 2002). (H) Timeline representing the stages that become accelerated as a result of budectomy in *S. brakenhielmi* (above) compared to unmanipulated colonies (below). Note that rapid development only occurs on early stages of budding, and that total time of development is reduced by two days. Scale bars, 200 µm.

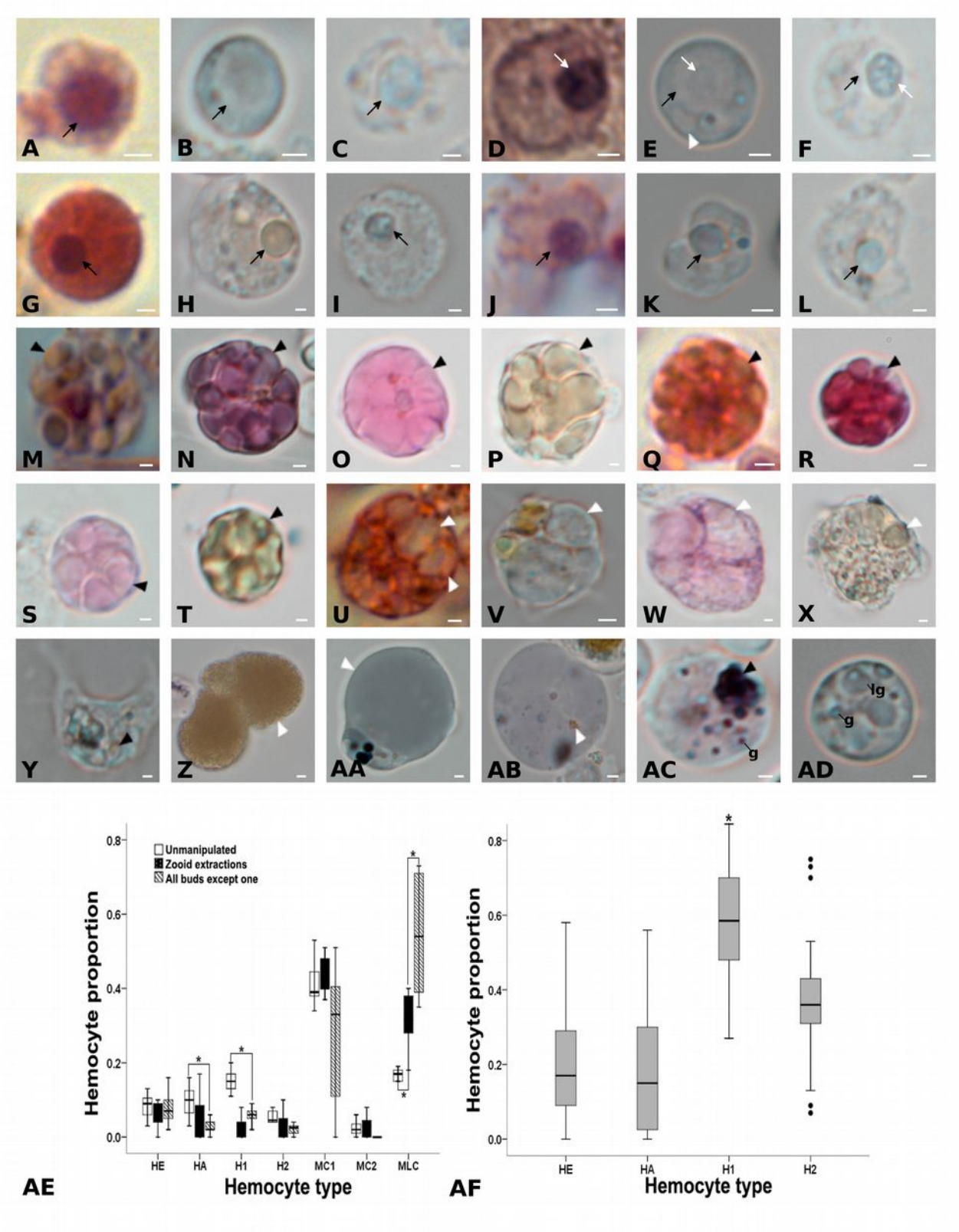

Figure 6. Characterization of hemocytes and fluctuating proportions patterns in *Symplegma brakenhielmi* Eleven hemocyte types were characterized in *S. brakenhielmi* by histological assays using

Hematoxylin and Eosin (H&E), Neutral Red (NR), Giemsa, and Sudan Black (SB) stains . (A) Hemoblast (HE) stained with H&E; the central nucleus shows a strong basophilic color (black arrow). (B) Living HE are NR negative (NR^-) to NR stain; the circular shape of the cell is clearly observed in living cells. (C) HE stained with Giemsa shows a light blue pigmentation. (D) Hemocyte 1 (H1) stained with H&E, shows a strong purple pigmentation on the nucleus and a clear cytoplasm. (E) Living H1 are $NR^{-\text{negative to NR stain}}$; note the centric nucleulus, small vacuoles, and granules in the cytoplasm. (F) H1 after giemsa stain shows a clear cytoplasm and a light blue nucleolus. (G) Hemocyte 2 (H2) stained with H&E shows a strong basophilic pigmentation on the nucleus, and a strong acidophilic color in the cytoplasm. (H) Living H2 stained with NR shows yellowish acidophilic pigmentation in the cytoplasm and in the nucleus, as well as in small granules in the cytoplasm. (I) H2 stained with giemsa shows a clear cytoplasm and light blue nucleus. (J) Hyaline amebocyte (HA) stained in H&E shows a purple basophilic nucleus and a pink irregular cytoplasm. (K) Living HA negative to NR stain; note the circular nucleus and a small granule in the cytoplasm. (L) H2 stained with giemsa shows a clear cytoplasm and light blue nucleus. (M) Morula cell 1 (MC1) stained with H&E shows a strong acidophilic color inside the vesicles, which present heterogeneus sizes and contents. (N) Living MC1 shows a purple color and heterogeneous sizes of vesicles that are related to ameboid movements of the cell. (O) MC1 stained with giemsa shows athe small inconspicuous nucleus. (P) MC1 stained with SB shows a yellowish pigmentation indicating the presence of lipids. (Q) Morula cell 2 stained with H&E shows a strong acidophilic pigmentation in the homogeneus sized- vesicles. (R) Living MC2 stained with NR shows a strong acidophilic red pigmentation. (S) MC2 stained with giemsa shows a pink pigmentation. (T) MC2 stained with SB shows the presence of lipids in the cytoplasm. (U) Macrophage-like cell (MLC) stained with H&E shows a strong red acidophilic pigmentation in the cytoplasm. (V) Living MLC stained with NR shows a yellowish acidophilic pirgmentation in some vacuoles. (W) MLC stained with giemsa shows a purple cytoplasm and clear large vesicles. (X) MLC stained with SB shows yellowish color of large vacuoles indicating the presence of lipids. (Y) Living granular amebocyte shows pseudopodia and an irregular cytoplasm filled with vesicles. (Z) Living nephrocyte (N) after NR stain shows a strong pigmentation of dense granules in large vacuoles. (AAB-AB) Living pigment cells (PC) positive to NR stain can show a large vacuole (arrowhead) as in (AA), or heterogeneous granular contents as in (AB). (AC-AD) Living granular cells (GC) have clear cytoplasm depicting heterogeny in granular forms; s and present heteregeneity in granular forms; these can be small with a strong dark pigmentation (g) or large with a blueish coloration (lg).(AE) Proportions of hemocyte types in unmanipulated colonies, colonies with zooid extractions, and colonies with all buds removed except one. Overall MLCs and MC1 were the most abundant hemocytes in the blood, and MC1s were more abundant than MC2s. After surgical manipulations HAs and H1s were reduced, and MLCs increased proportionately. in proportion. Asterisk depict significant differences using the Tukey and Bonferroni test. (AF) Mitotically active hemocytes as assessed by PH3 expression (HE, HA, H1 and H2). Note that H1s are were the hemocyte types with most actively cells in proliferating cells

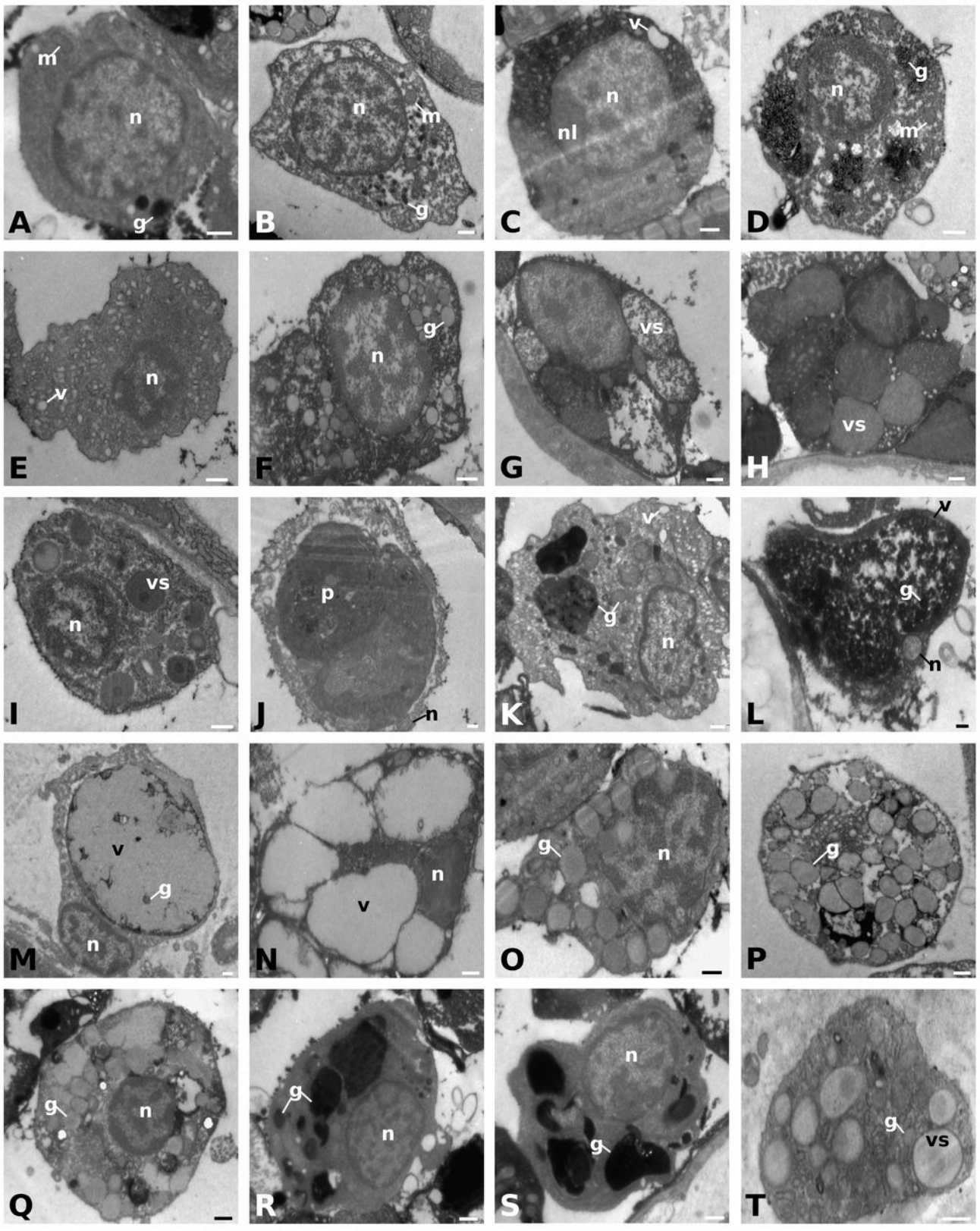
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Figure 7. Ultrastructural characterization of hemocytes in *Symplegma brakenhielmi* (A-B) Hemoblasts (HEs) present a high nuclear-cytoplasmic ratio; the nucleus contains chromatin dense regions, and mitochondria and granules can be seen in the cytoplasm; two HEs are shown to highlight variation of cell type. (C) Hemocyte 1 (H1) presents a lower nuclear-cytoplasmic ratio than HEs; the nucleolus and chromatin are visible within the nucleus; the cytoplasm presents small granules and vesicles. (D) Hemocyte 2 (H2) show large nuclei; the chromatin can be seen in the nucleus, and electron dense material and granules are observed in the cytoplasm. (E-F) Hyaline amebocytes (HAs) can present small vesicles (v) in their cytoplasm as in (E), or large and dense granules (g) as in (F); ; two HAs are shown to highlight variation of cell type. (G) Morula cell 1 (MC1) shows heterogeneity in vesicle size and contents. (H) Morula cell 2 (MC2) has more homogeneous vesicles in size and content. (I) Putative morula cell precursor shows a bigger nucleus, and vesicles with denser contents than MC1 or MC2. (J) Macrophage-like cell (MLC) with a phagosome (p) with heterogeneous material inside. (K) Granular amebocyte (GA) contains small and large dens granules in the cytoplasm. (L) Nephrocyte (N) has an inconspicuous nucleus at an eccentric position; a large vacuole with electron dense material surrounded by a thicker membrane can be observed. (M) Pigment cell (PC) show an eccentric nucleolus within the nucleus, and contain a large vesicle and small granules in the cytoplasm. (N) PC with large vesicles and small dense material. (O-T) Granular cells (GCs) are represented by a diversity of forms: for example (O-Q) contain dense homogeneus granules, (R, S)contain very dense granules that vary in size, (O, R, S) contain a large nucleus, and (P,T) show an inconspicuos nucleus.

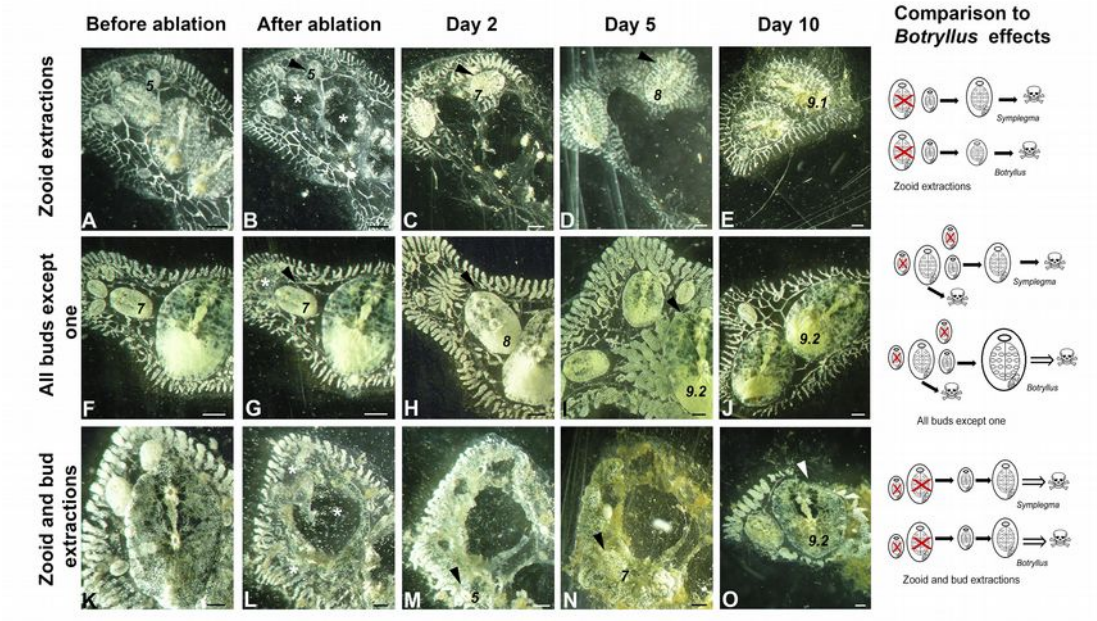


Figure S1. Independent developmental processes of individuals govern most regenerative processes in *Symplegma brakenhielmi* colonies. Micrographs show the 10-day regenerative process of colonies post-surgical ablations of zooids. Illustrations on the right show schematic representations of the regenerative processes post-ablation in *Symplegma brakenhielmi* and *Botryllus schlosseri*. Note that *Symplegma* individuals show no alterations in the development of individuals after “Zooid” or “All buds except one” extractions, whereas *Botryllus* bud development is impaired, resulting in smaller functional zooidsslows down development after “Zooid” removals and makes gigantic zooids when “All buds except one” are removed, as reported in Lauzon (2002); no alterations or differences were observed after “Zooid and bud extractions”. (A-E) First row shows “Zooid extractions” treatment: (A) Colony before ablation; a stage 5 bud is shown. (B) Colony immediately after ablation, all zooids removed are shown (asterisks) and one bud is marked (arrowhead) to follow its development in the series. (C) Day 2 after ablation, marked bud (arrowhead) is at stage 7. (D) Day 5 after ablation, marked bud (arrowhead) is at stage 8. (E) Day 10 after ablation, stage 9.1 zooid (arrowhead) has opened its siphons. (F-J) Second row shows “All buds except one”: (F) Colony before ablation, a stage 7 bud is shown. (G) Colony immediately after ablation, all buds were removed (asterisk) except the stage 7 bud that was followed during development (arrowhead). (H)Day 2 after ablation, marked bud is at stage 8. (I) Day 5 after ablation, zooid has reached stage 9.24 and is fully differentiated (arrowhead). (J) Day 10 after ablation, zooid from (I) has regressed but new zooids have now formed (st. 9.4). Third row shows “Zooid and bud extractions”: (K) Colony before ablation, a fully differentiated zooid is shown. (L) Colony immediately after ablation, zooid and buds were removed (asterisks) and only the vascular tissues remain. (M) Day 2 after ablation, vascular tissues have aggregated and new buds are observed at stage 5 (arrowhead); asterisk shows the scar of the surgery. (N) Day 5 after ablation, the vascular tissues form a mass and marked bud (arrowhead) continues to develop (stage 7). (O) Day 10 after ablation, the new zooid is fully differentiated (stage 9.2). Scale bar is 300 µm in A, B, C, D, F, G, H, I, M, N, and 500 µm.; in F, J, K, L, and O.

Chapter 3

Evolution of colonial life history in styelids tunicates involves changes in complexity patterns

1. Introduction

"Evolutionary change involves the increasing complexity of a feature already present in ancestors"

Stephen Jay Gould, from *Ontogeny and Phylogeny*, 1977

Gould (1977, pp 268-269) suggested that an increase in complexity is an inevitable condition for evolution by acceleration of developmental processes. Complexity in biology is used to refers number of cell types, body parts, or biological processes (McShea, 1996). Increase or reduction in number of these characters, results in changes of complexity patterns (McShea, 2017).

Complexity is a useful concept to understand how developmental mechanisms, environmental conditions and natural selection are acting in evolution of new life histories. Clarifying, this does not imply a directionality in evolution. On the contrary, the idea is that complexity is a useful concept to understand the evolution of new life histories- "We will be interested in the whole pattern of change, not just the increases … but also the decreases, the frequent retreats into simplicity"-as McShea (2017, pp 2) proposed. In biological systems complexity is represented by the number of modules (e.g. cell type, leg-pair type, zooids, polyps) that compose an organism, the type of interactions between these modules, new biological hierarchies or nestedness processes that describe these interactions (Adami, 2002; McShea, 1996, 2017), and also the capacity of self-organization in biological systems (Yaeger, 2009).

Evolution of colonial life history in animals is one example of the change in the complexity patterns. These changes are observed in the modularization of multicellular individuals, forming the colony as a new biological hierarchy (Davidson et al, 2004); or by new types of interactions of these

modules, such as the cellular migration and the molecules involved in communication between modules (Lauzon et al., 2007); or by self-organization, such as the rearrange of extracorporeal blood vessels to maintain homeostasis after a disturbance (Rodriguez et al., 2017).

1.2. Evolution of colonial life history in Styelidae from a solitary ancestor, imply increase of characters complexity in colonial descendants

Tunicates are useful to understand changes in patterns of complexity related to the colonial life history. Colonial tunicates evolved multiple times, in pelagic and benthic environments (Kocot et al., 2018). Such as, in the tunicate family Styelidae, colonial life history evolved by convergence two times from solitary ancestors (Alié et al., 2018). Evolution of colonial animals involved the increase in the number of modules, interactions, hierarchical processes and self-organization of the colonies (Fig.1) (Alié et al., 2018; Ballarin et al., 2008; Gutierrez & Brown, 2017). Although, evolution of colonial life history increased the complexity patterns in Styelids.

Genus *Symplegma* is one of the colonial animals in Styelidae. *Symplegma* colonies are composed by zooids and a extra-corporeal vessel system with specialized blood cells that circulate constantly(Mukai & Taneda, 1978). New buds appear from the evagination of vessels and adult zooids epithelia. Blood vessel system is very dynamic, buds move during development. Buds formed from adult zooids, move far away from parental zooid, by the formation of a new vessel to connect to the general vessels system of the colony. Vessels have the capacity to rearrange in case of different disturbance and external stimulus. Such as, in the whole-body regeneration, blood vessels pump blood by themselves, allowing cells circulation and regeneration (Gutierrez & Brown, 2017; Sugimoto & Nakauchi, 1974). In absence of young buds, by systematical removals, new buds are formed and developed faster. This suggest that the asexual development of zooids is modulate by mechanisms at the colony level (Gutierrez & Brown, 2017).

Solitary life form is consider the ancestral character in Styelidae. Thus, evolution of *Symplegma* colonial strategy, involved the develop of more complex characters in comparison with solitary forms. Modularization of multicellular individuals in colonies, imply modularization of developmental processes (e.g morphogenesis and aging). These developmental processes are occurring simultaneously,

one of the main innovation of the colonies in comparison with the solitary forms (Jackson & Coates, 1986; Jackson & Hughes, 1985). These processes are spatially and temporary impossible in solitary animals. Evolution of colonial life history is related with the increase of complexity in characters, such as extra-corporeal vessels; diversification of blood cells; and regulatory mechanisms to modules coordination.

The developmental processes are modularized in *Symplegma* zooids. Simultaneously a bud is forming, by a process analogous to a grastrulation; another bud is differentiated its organs; the fully differentiated zooid is filtering and feeding the colony; and an old zooid is aging and dying (Gutierrez & Brown, 2017; Kawamura & Nakauchi, 1986). The main question of this study is try to understand How is the regulation of the asexual developmental processes that occurred simultaneously in *Symplegma* colonies?

The premises to answer this question are: (a) all the modules of the colony are interconnected by a blood vessel system in which blood cells are in constant circulation (b) there are specific type of blood cells related with main biological processes of the colonies, such as phagocytosis, budding and regeneration, allorecognition and storage cells, (c) external disturbances as systematical remotion of modules (zooids or buds), cause changes in the proportion of the type blood cells, and alterations in the asexual development of the colony (Cima et al, 2001; Franchi et al., 2016; Gutierrez & Brown, 2017; Lauzon et al., 2007).

Although, the proposed hypothesis is that t*he regulation of the simultaneous developmental processes that occurred in Symplegma colonies are mediated by the modularization of these developmental processes. To coordinate these simultaneous processes new blood cells types evolved diversifying in relation with biological functions associated to specific developmental stages (e.g progenitor cells related to early buds, phagocytes related to old zooids). In Symplegma clade evolved a cellular based communication system where signals are transmitted between modules (i.e zooids) by migratory blood cells and molecules diffused in plasma.*

To test this hypothesis the development of *Symplegma rubra* and *S.brakenhielmi* was observed, from the oozoid to adult colonies, to understand the formation of a colony from a first module. The

colonial strategy in *S.rubra* was unknown, thus *S.rubra* budding and the blood cell types were characterized, comparing with the information reported before for *S.brakenhielmi*. Some blood cell types have an active behavior and fast movements. These cellular behaviors were observed and record in videos, to understand cellular behaviors associated with blood cell types. Blood cells were identified and counted, in the morphogenesis stages and aging stages. Comparing the types of blood cells at these these simultaneous stages. Finally the aging process of the zooids was describe to identify if is a programmed cell death, like in other colonies,or a senescent process.

2. Materials and Methods

2.1. colonies and budding characterization

Symplegma rubra and *S. brakenhielmi* colonies were collected from floating structures in the Yatch Club IlhaBela-YCI (Ilhabela, São Paulo, Brazil). Fragments of colonies were attached to microscope slides and kept in open cages. Cages were immersed in the water from floating docks for three weeks. Grown colonies were cleaned and transported to the laboratory at Universidade de São Paulo. Colonies were maintained at 25° C and fed with a mixture of living algae (*Isochrysis, Thalassiosira, Pavlona, Nanochlorpsis)* and commercial food. Colonies were observed under stereomicroscope Leica M205 FA. Colonies at the reproductive stage were transported to the Centro de Biologia Marinha da Universidade de São Paulo – *CEBIMar.* The larvae released from the colonies were obtained and transferred to microscope slides to observe them.

2.2. Blood cell characterization

For the blood cells extraction was followed a previously described protocol (Cima, 2010), with some changes to improved obtained results (See supplementary material 5.3 chapter 4). Colonies were immersed in anticoagulant for 5 minutes. Then ampullae were gently cut and blood was extracted. Anticoagulant was washed by centrifugation (10 minutes-3000 rpm), and the blood cells were resuspended in a solution of 1/3 anticoagulant-2/3 filtered sea water (FSW). The blood cells were attached to microscopy slides coated with Poly-L-Lysine. Afterwards the attached blood cells were stained using cytological techniques to observe the diversity and classify. Living blood cells were

stained with a neutral red solution (8mg/l in FSW) to observe acid cellular compartments. Blood cells were fixed with 4% paraformaldehide in FSW and stained with Hematoxylin and eosin (H&E) or Giemsa 10%, to identify the cellular morphology. Blood cells were observed and photographed using the inverted microscope Leica DMi8.

2.3. Cellular behavior

Blood cells were collected as mentioned above. The cells were added to coversilps coated with laminin (50 µg/mL) and filmed under a microscope Zeiss AxioVert A1, equipped with the Canon DSLR camera. Images were recorded every 3 seconds and processed in the photo editing free software Darktable.

2.4. Blood cells identification in the bud morphogenesis and aging zooids

Symplegma rubra and *S. brakenhielmi* colonies were fixed in paraformaldehide 4% in FSW overnight and afterwards washed with PBS. Fixed colonies were dehydrated by ethanol series (25%, 50%, 70%, 80%, 90%, 100%) and two xylol washes and embedded in paraffin to be sectioned. Serial sections of 5µm thickness, perpendicular to the longitudinal axis of the zooids were obtained using a microtome. Sections were mounted on glass slides, deparaffinized and rehydrated with the inverse ethanol series mentioned before. The tissue slides were stained with H&E, mounted with Entellan and examined under a Zeiss AxioVert A1 light microscope. The proportion of the types of blood cells was calculated by counting the cells in the early stages of budding (double vesicle, stage.5) and in the aging stages of the old zooids (stage 11). These buds and zooids were in the same colony, thus the analyzed stages were present simultaneously in the colonies. All the blood cells inside the zooids and buds were counted in four sections every 20 µm, in the smallest buds were counted two sections every 10 µm. Tukey and Bonferrni tests were used to determine significant differences between blood cells proportion in the budding stages.

2.5. Characterization of aging zooids

Old zooids were observed *in vivo* in colonies of *Symplegma rubra and* colonies of *S. brakenhielmi.* From these *in vivo* observations and the resorption stages reported in *Botryllus schlosseri* were established homologous stages in *Symplegma*. The morphology of these resorption stages was described by histological slides stained with H&E as mentioned above.

3. Results

3.1 Development of a *Symplegma* colony

Symplegma rubra and *S.brakenhielmi* are species with a similar strategy of coloniality. The colonies are formed by modules (zooids and buds) interconnected by a systems of blood vessels with circulating blood cells. The formation of buds is a constant process and the budding occurs asynchronously in contrast with other species, such as the botryllids. *Symplegma* colonies are characterized by the formation of extension and growth zones. The location of the extended ampullae and the buds (Fig. 2A-B). Growth area redirects the growth of the colony. Colonies were capable of small movements and the positions of the zooids change dynamically across the blood vessels system.

The colonies were reproductive during all the year, specially from December to February,the summer season at the south of Brazil. The reproduction is by internal fertilization and the larval development by brooding. Fully developed larva is approximately 2mm long, and has a circular head with sensorial papillae in the anterior part The tail has the notochord, the dorsal nerve and muscles. The beating of the tail propels the larva, which can swim for up to 12 hours before settlement (Fig. 2C). The metamorphosis starts with the resorption of the tail and the formation of the first zooid (i.e oozoid). At the beginning of the oozoid development some larval structures are remanent such as the ocellus and otolith (Fig. 2D). In *Symplegma brakenhielmi* and *S.rubra* the eight primordial ampullae form a remarkable symmetric pattern (Fig. 2D-C). Inside of these primordial ampullae, blood cells were circulating. Simultaneously with the formation of pharynx and internal organs of oozooid, the ampullae were growing and fusions forming the primordial blood vessels system (Fig. 2F). After a week of the

settlement the blood vessels system ramifies and the fully differentiated oozoid open the siphons to feed. One day after, the siphon apertures were observed on the first buds (Fig. 2G -H). The lifespan of the oozoid is approximately 20 days, then the zooids and buds are developing and the blood vessels are forming. It is the beginning of the formation of a colony.

The formation of the colonies was very similar between the two *Symplegma* species, however the budding in *S.rubra* is by palleal budding (i.e buds are formed by the evagination of the pharyngeal and external epitheliums of the parental zooid). In contrast to *S.rubra,* in which budding is exclusively palleal, *S.brakenhielmi* has simultaneously palleal and vascular budding.

Budding in *Symplegma rubra* was characterized by eleven stages. The stages 1 to 3 are the formation of the budlet from the parental bud. Stage 4 is the beginning of folding of internal epitheliums to form the organs, analogous to grastrulation. Organogenesis occurs during stages 5 to 8, with tissues differentiation and formation of all the internal organs. Finally in stage 9 the zooid is fully functional and starts to filter. Lastly, the zooid starts a senescent process and it is resorb by the colony. The stages were established following Berrill (1941) and Sabbadin (1955) nomenclature and compare with the homologous stages previously reported in *S. brakenhielmi* and botryllids.

3.2. Blood cells of *Symplegma rubra*

The blood cells in *Symplegma rubra* are composed by a variety of cellular populations, characterized by cytological morphology. Some of the populations are cells with characteristics of precursors. The other populations were characterized in three functional lineages: phagocytes, cells for allorecognition, and storage cells (Fig. 3AB).

Hemoblasts (HE): cells with a size between 3-4 µm. HEs have a round regular cytoplasm with a high nucleus-cytoplasmic ratio. The cytoplasm has a small number of organelles, for that HEs are negative to neutral red (this dye stains cellular acid compartments). The nucleolus is clearly visible with hematoxylin and eosin (H&E) stain. The nucleus is stained blue with Giemsa (Fig. 3A-C).

Hemocyte (H1): cells with a size between 5-6µm. H1s have a regular cytoplasm with a high

nucleus-cytoplasmic ratio and small number of organelles. The living H1s are negative to neutral red, and a strong basophilic stain in the nucleus with H&E. The nucleus and small vesicles are stained blue with Giemsa (Fig. 3D-F).

Hemocyte (H2): cells with a size between 6-7 µm. H2s are round shaped and have some granules in the cytoplasm. In living H2s the cytoplasm is stained with neutral red suggesting the content of acid compartments. The eccentric circular nucleus is characterized by a strong color in all the dyes (neutral red, H&E, and Giemsa) (Fig. 3G-I).

Hyaline amebocyte (HA): cells with a size between 4-6 µm. HAs have a irregular amoeboid cytoplasm with small number of organelles. HAs are negative to neutral red dye an the cytoplasm has a clear color in all dyes. HAs have a high nucleus-cytoplasmic ratio, nucleus has a strong basophilic color with H&E and blue color with Giemsa (Fig. 3J-L).

Morula cell (MC): cells with a size between 8-10 µm. MCs have a irregular cytoplasm full of homogeneous round vesicles. MCs are positive to neutral red dye, suggesting an acid content in their vesicles. Cytoplam has a strong brownish-dark red color in all the dyes. The nucleus is small stained purple with H&E and blue with Giemsa (Fig. 3M-O). MCs have an active movement (Video. 1).

Macrophage-like cell (MLC): cells with a size between 10-12 µm. MLCs have huge vacuoles and some heterogeneous vesicles. MCs are positive to neutral red, suggesting acid content in the vacuoles. MCs have strong yellowish-orange color with H&E and Giemsa. The nucleus is eccentric and small, visible with Giemsa (Fig. 3P-R).

Granular amebocyte (GA): cells with a size between 10-15 µm. GAs have a irregular amoeboid cytoplasm with pseudopods. The cytoplasm contains granules with light colors stained with H&E and negative to neutral red (Fig. 3S-T). GAs have an active movement (Video. 2).

Nephrocyte (N): cells with a size between 8-20 µm. Ns have a round or hourglass shape. The cytoplasm contains dense granules with Brownian movement. Ns are stained brownish-yellowish with neutral red, H&E and Giemsa, suggesting acid content in the cytoplasm (Fig. 3U-W).

Pigment cell (PC): cells with a size between 8-15 µm. PCs have a irregular cytoplasm with granules inside. PCs in *Symplegma rubra* are red in color, probably related with the characteristic color of this specie. Some granules are stained with neutral red (Fig. 3X-AA).

This diversity of blood cells in Symplegma colonies, is associated with specific cellular behaviors. Morula cells and amebocytes show dinamic movements, specially amebocytes with the pseudopods. These faster cellular movement can be involved in the biological processes and communication between zooids in colonies (Video 1.-2).

3.3. Buds morphogenesis

In *Symplegma rubra* the right side of the peribranchial epithelium of buds is thick. From this thickening a budlet starts to form in a parental bud stage 5. This budlet expand and growth forming a double vesicle (stage. 3) from the peribranchial epithelium and external epithelium of the parental bud (Fig. 4A,C). The bud separates from its parental zooid and a new blood vessel is forming from the bud to the colonial systems of vessels (Fig. 2B). The bud increase in size and the internal vesicle starts to fold, forming the pharynx and stomach primordium (Fig. 4D). Buds in *S. brakenhielmi* are formed by palleal budding and from vessels as was reported before (Gutierrez & Brown, 2017). The palleal body in *S.brakenhielmi* and *S. rubra* follow the same pattern.

Blood circulation is observable inside buds since double vesicle stage (St.3) and continues during all the asexual development. Blood cells with characteristics of precursors (i.e as hemoblasts and hyaline amebocytes) were observed in the morphogenesis of buds. During the bud development increase the number of macrophage-like cells (Fig. 4C,D,G,H). Hemoblasts, hyaline amebocytes and pigment cells were statistically more frequent in double vesicles than in old zooids for the two *Symplegma* species (Fig. 6).

3.4. Resorption of old zooids

Stage 11 is the final stage in *Symplegma* budding. The lifespan of the fully differentiated zooid is between 3 and 3.5 days in the two *Symplegma* species. The first step of the final stage 11.1 is the closure of siphons. When touched siphons did not react, as mentioned by Ballarin et al., (2008) for *Botryllus schlosseri* (Fig. 5A, D). After twelve hours the stage 11.2 started, with the longitudinal

antero-posterior contraction of the body. (Fig. 5 B, E). During this stage pharynx and stomach epithelia initiated a disintegration, by the separation of epithelial cells. Macrophage-like cells began to accumulate around pharynx and stomach (Fig. 5G). At stage 11.3 the size of the zooid reduced dramatically, and the heart beating was slower. The disintegration of the internal organs was clearly observable, and the body was inundate by macrophage-like cells. The blood that circulate around the old zooids was denser than around young zooids. These macrophage-like cells were seen moving outside from the old zooid, probably with the resorbed tissues. Finally mostly of the tissues of the old zooid were phagocyted and moved outside from the body and the heart beating stopped (Fig. 5C, F, H). Remanent of tunic and tissues stayed during two days before the resorption was complete. During the resorption stage the proportion of macrophage-like cells was significantly bigger than in young bud of the same colony. In *Symplegma rubra* the proportion of morulas was significantly bigger than in young bud (Fig. 6). The resorption process has similar pattern in the two *Symplegma* species.

4. Discussion

4.1. Modularity of a multicellular individual by the development of a colony of zooids

The colonies in *Symplegma* have defined areas, characterized by specific biological processes: extension area, with the younger buds in morphogenesis; area of fully differentiated zooids; and the regression area, with the aging zooids (Fig. 2A-B). The extension area is more evident in colonies that are growing, like the cultured colonies that were growing from their natural substratum to the glass slides. This suggests that the extension area redirect growth of the colony, probably related with the sensation of good environmental conditions received for ampullae. The oozoid in development and the primordial ampullae form a symmetric pattern with eight ampullae. This remarkable pattern was conserved in *S.rubra* and *S.brakenhielmi* oozoids (Fig. 2 D-E).

Simultaneously with the development of the oozoid, beginning the formation of blood vessels system by the fusion of primordial ampullae (Fig. 2F). The extra-corporeal system of vessels is an essential part in the colonial strategy in *Symplegma*. Blood vessels systems maintain cohesion and homeostasis of the colony. Vessels have the capacity to rearrange in case external disturbance

(Gutierrez & Brown, 2017); or by the dormancy at cold season, by the resorption of all zooids maintaining only the blood vessel system (Hyams et., 2017). The plasticity of the blood vessels system is decisive in the capacity of self-organization of the colonies. As a result *Symplegma* colonies are more resilient to external disturbances, in comparison with solitaries species.

4.2. Blood cell types distribution regulates the modularization of developmental processes in colonies

The blood cell types described in *S.rubra* (FIG.3) are very similar to *S.brakenhielmi* and botryllids, as well as other phylogenetically more distant colonial tunicates (Cima et al., 2001; Gutierrez & Brown, 2017; Hirose et al., 2003). These results sugges that this variety of blood cells are related with colonial life history.

One of the main characters of *Symplegma* coloniality is the simultaneous budding stages. The blood cells are continuously circulating and their proportions are constant all the time around the colony. In contrast to the botryllids, in which the blood cells proportions have fluctuating cycles in relation with the budding stage of the colony (Ballarin, Menin, et al., 2008). In botryllids all the zooids are in the same stage, because the asexual development of the zooids is synchronized (Lauzon et al., 2002).

In *Symplegma* double vesicle stage (st.3) hemoblasts (HE) and hyaline amebocytes (HA) were significantly more frequent, than in aging zooids (Fig. 4, Fig 6). In addition, macrophage-like cells (MLC) and morula cells (MC), were significantly more frequent in aging zooids. Suggesting a different location of the blood cells types related with the stage of the zooids and the specific developmental processes occurring in each stage. Thus, precursor cells (HE, HA) are located in buds during the morphogenesis. Probably these cells interact with the double vesicle epithelia in the cellular differentiation and migration before organogenesis (Brown et al., 2009). Phagocytes (MLCs) and cells for allorecongition (MCs) are located predominately in the aging zooids. The phagocytes have and active role in the resorption and recycling tissues of the aging zooids (Lauzon et al., 1993).
Dynamic cellular behaviors of amebocytes and morula cells, can be involved in the biological function of these cells (i.e buds morphogenesis and immune responses). These cells migrate in the blood cells

and inside zooids and buds, regulation budding, regeneration and immune responses. This faster cellular movements can be a factor related with plasticity of colonies to external disturbances. As well is a interesting source of unexplored biological information.

4.3. Aging in *Symplegma* is a regulate process involving programmed cell death and phagocytosis

The steps described in the stage 11 in *Symplegma* are very similar with the resorption stage described for botryllids (Ballarin, Burighel, et al., 2008). Beginning with the siphons closure, followed by the disintegration of epitheliums and an accumulation of MLCs around the pharynx and stomach (Fig. 5). Posteriorly, the epithelium cells and internal tissues start massive apoptosis. Finally these apoptotic bodies are reabsorb by phagocytes to move them outside from the aging zooids. Thus, the programmed aging of the old zooids and the resorption of these tissues by the colony are processes that evolve in the clade *Symplegma* + *Botryllids* (Ballarin, Menin, et al., 2008; Lauzon et al.,1992). Suggesting the develop of new and complex biological processes related to colonial life history. This programmed aging and the recycling of old zooids by this phagocytosis serie are innovations of colonies, suggesting an increase in the complexity pattern in the evolution of coloniality.

In conclusion this results support the hypothesis that the regulation of the simultaneous developmental processes in *Symplegma* is related by the distribution and modulation of this variety of blood cells types. Nevertheless, it is probable that the regulation of budding is also related with vascular and zooids epitheliums. By some mechanisms, regions of the epitheliums are committed to form the budlets, which interact with undifferentiated blood cells to start the asexual development.

4.4. Evolution of colonial life history in Styelidae a case of natural selection favoring increase in complexity?

The colonial life history evolved in Styelidae by different developmental mechanisms, during two independent events (Alié et al., 2018).However the concept of transform the unique individual in solitaries in a clonal module, it is convergent in the different colonial strategies. This colonial life history evolved by convergence in other marine pelagic animals. Such as, cnidarians, bryozoans and

hemichordates (Davidson et al., 2004).This support the idea that in some marine environments the coloniality can be a successful strategy.

Specifically in Styelidae the organisms are sessile filter-feeders, thus colonial strategy can give some advantages to survive. The colonies act as self-regulating systems that can rearrange it components (i.e zooids and blood vessels) to maintain the homeostasis in case of a disturbance. An example of this self-organization is the regeneration process when a portion of the colony is lost or in the whole-body regeneration. In which the remanent modules of the colony (i.e., zooids, buds and vascular tissues) rearrange themselves to replace the lost parts (Brown et al., 2009; Gutierrez & Brown, 2017).

Evolution of coloniality from a solitary ancestor involved an increase in complexity, such as Styelidae example. Though, in colonial animals increase number of modules, number of biological hierarchies (e.g colonial hierarchy) and nestedness processes (e.g sexual reproduction and budding to form colonies). In Styelidae example complexity increase with evolution of colonial animals, however coloniality evolved and probably disappear in tunicates (Kocot et al., 2018) and in cnidarians (Scrutton, 2015), these events involving changes in the complexity patterns. Life evolved without a directionality in a spectacular diversity of life forms, the study of complexity patterns can provide a useful way to understand the process of life evolution.

5. References

Adami, C. (2002). What is complexity ? *Bioessays*, *24*, 1085–1094. https://doi.org/10.1002/bies.10192

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*(7), 1728–1743. https://doi.org/10.1093/molbev/msy068

Ballarin, L., Burighel, P., & Cima, F. (2008). A tale of death and life: natural apoptosis in the colonial ascidian Botryllus schlosseri (Urochordata, Ascidiacea). *Current*

Pharmaceutical Design, *14*(2), 138–147. https://doi.org/10.2174/138161208783378798

Ballarin, L., Menin, A., Tallandini, L., Matozzo, V., Burighel, P., Basso, G., … Cima, F. (2008). Haemocytes and blastogenetic cycle in the colonial ascidian Botryllus schlosseri: a matter of life and death. *Cell and Tissue Research*, *331*(2), 555–564. https://doi.org/10.1007/s00441-007-0513-4

Berrill, N. J. (1941). The Development of the Bud in Botryllus. *Biological Bulletin*, *80*(2), 169–184. https://doi.org/10.2307/1537595

Brown, F. D., Keeling, E. L., Le, A. D., & Swalla, B. J. (2009). Whole body regeneration in a colonial ascidian, Botrylloides violaceus. *J Exp Zool B Mol Dev Evol*, *312*(8), 885–900. https://doi.org/10.1002/jez.b.21303

Cima, F., Perin, A., Burighel, P., & Ballarin, L. (2001). Morpho-functional characterization of haemocytes of the compound ascidian Botrylloides leachi (Tunicata, Ascidiacea). *Acta Zoologica*, *82*(4), 261–274. https://doi.org/10.1046/j.1463-6395.2001.00087.x

Cima, F., Peronato, A., & Ballarin, L. (2017). The haemocytes of the colonial aplousobranch ascidian Diplosoma listerianum: Structural, cytochemical and functional analyses. *Micron*, *102*(July), 51–64. https://doi.org/10.1016/j.micron.2017.08.007

Davidson, B., Jacobs, M., & Swalla, B. J. (2004). The individuals as a Module: Solitary-to-Colonial Transitions in Metazoan Evolution and Development. In G. Schlosser & G. Wagner (Eds.), *Modularity in Development and Evolution*. Chicago: The University of Chicago Press.

Franchi, N., Ballin, F., Manni, L., Schiavon, F., Basso, G., & Ballarin, L. (2016). Recurrent phagocytosis-induced apoptosis in the cyclical generation change of the compound ascidian Botryllus schlosseri. *Developmental and Comparative Immunology*, *62*, 8–16. https://doi.org/10.1016/j.dci.2016.04.011

Fujimoto, H., & Watanabe, H. (1976). *Studies on the asexual reproduction in the polystyelid ascidian,Polyzoa vesiculiphora Tokioka*. *Journal of Morphology* (Vol. 150). https://doi.org/10.1002/jmor.1051500302

Gasparini, F., Manni, L., Cima, F., Zaniolo, G., Burighel, P., Caicci, F., … Ballarin, L. (2015). Sexual and asexual reproduction in the colonial ascidian Botryllus schlosseri. *Genesis (New York, N.Y. : 2000)*, *53*(1), 105–120. https://doi.org/10.1002/dvg.22802

Gutierrez, S., & Brown, F. D. (2017). Vascular budding in *Symplegma* brakenhielmi and the evolution of coloniality in styelid ascidians. *Developmental Biology*, *423*(2). https://doi.org/10.1016/j.ydbio.2017.01.012

Hirose, E., Shirae, M., & Saito, Y. (2003). Ultrastructures and Classification of Circulating Hemocytes in 9 Botryllid Ascidians (Chordata: Ascidiacea). *Zoolog Sci*, *20*(5), 647–656. https://doi.org/10.2108/zsj.20.647

Hyams, Y., Paz, G., Rabinowitz, C., & Rinkevich, B. (2017). Insights into the unique torpor of Botrylloides leachi, a colonial urochordate. *Developmental Biology*, *428*(1), 101–117. https://doi.org/10.1016/j.ydbio.2017.05.020

Kott, P. (2005). Novel Australian Polyzoinae (Styelidae, Tunicata). *Journal of Natural History*, *39*(32), 2997–3011. https://doi.org/10.1080/00222930500239702

Lauzon, Ishizuka, K. J., & Weissman, I. L. (2002). Cyclical Generation and Degeneration of Organs in a Colonial Urochordate Involves Crosstalk between Old and New: A Model for Development and Regeneration. *Developmental Biology*, *249*(2), 333–348. https://doi.org/10.1006/dbio.2002.0772

Lauzon, Ishizuka, K., & Weissman, I. (1992). A cyclical, developmentally-regulated death phenomenon in a colonial urochordate. *Dev Dyn*, *194*(1), 71–83. https://doi.org/10.1002/aja.1001940109

Lauzon, Kidder, S. J., & Long, P. (2007). Suppression of programmed cell death regulates the cyclical degeneration of organs in a colonial urochordate. *Developmental Biology*, *301*(1), 92–105. https://doi.org/http://dx.doi.org/10.1016/j.ydbio.2006.08.055

Lauzon, Patton, C. W., & Weissman, I. L. (1993). A morphological and immunohistochemical study of programmed cell death in Botryllus schlosseri (Tunicata, Ascidiacea). *Cell Tissue Res*, *272*(1), 115–127.

McShea, D. W. (1996). Perspective metazoan complexity and evolution: is there a trend? *Evolution*, *50*, 447–492.

McShea, D. W. (2017). Evolution of Complexity. In L. Nuño de la Rosa & B. Muller, Gerard (Eds.), *Evolutionary Developmental Biology*. Springer. https://doi.org/10.1017/978-3-319-33038-9

Pérez-Portela, R., Bishop, J. D. D., Davis, a R., & 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. https://doi.org/10.1016/j.ympev.2008.11.014

Rodriguez, D., Braden, B. P., Boyer, S. W., Taketa, D. A., Setar, L., Calhoun, C., … De Tomaso, A. W. (2017). In vivo manipulation of the extracellular matrix induces vascular regression in a basal chordate. *Molecular Biology of the Cell*, *28*(14), 1883–1893. https://doi.org/10.1091/mbc.E17-01-0009

Sabbadin, A. (1955). Il Ciclo Biologico Di Botryllus Schlosseri (Pallas) [Ascidiacea] Nella Laguna di Veneza. *Estrato Dall'Archivo Di Oceanografia e Limnologia*, *10*, 219–231.

Sugimoto, K., & Nakauchi, M. (1974). Budding, Sexual Reproduction, and Degeneration in the Colonial Ascidian, *Symplegma* reptans. *Biological Bulletin*, *147*(1), 213–226. Retrieved from http://www.jstor.org/stable/1540579

Tsagkogeorga, G., Turon, X., Hopcroft, 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*(1), 187. https://doi.org/10.1186/1471-2148-9-187

Yaeger, L. S. (2009). How evolution guides complexity. *HFSP Journal*, *3*(5), 328–339. https://doi.org/10.2976/1.3233712

6. Figures

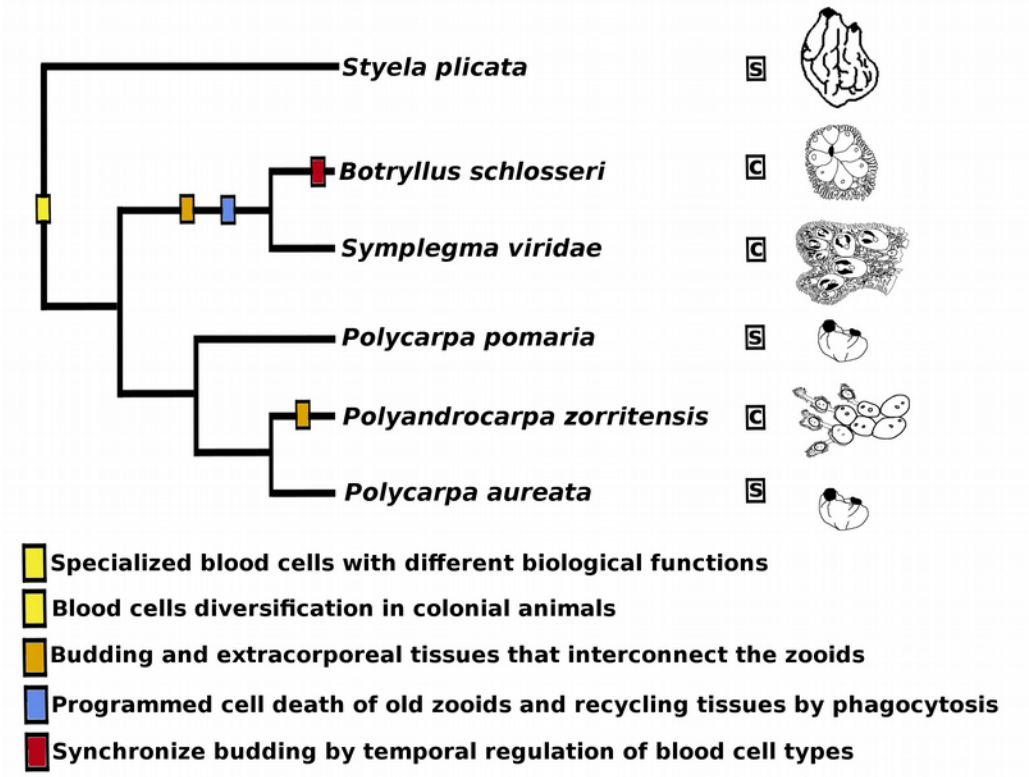


Figure.1 Phylogenetic summary of the characters related with colonial life history evolution in Styelidae. The proposed key characters for evolution of coloniality are: the specialization of blood cells; blood cells diversification to manage modules in colonies; budding and extra-corporeal tissues to interconnect zooids; programmed cells death of old zooids and their tissues recycling; the synchronized budding in botryllids.

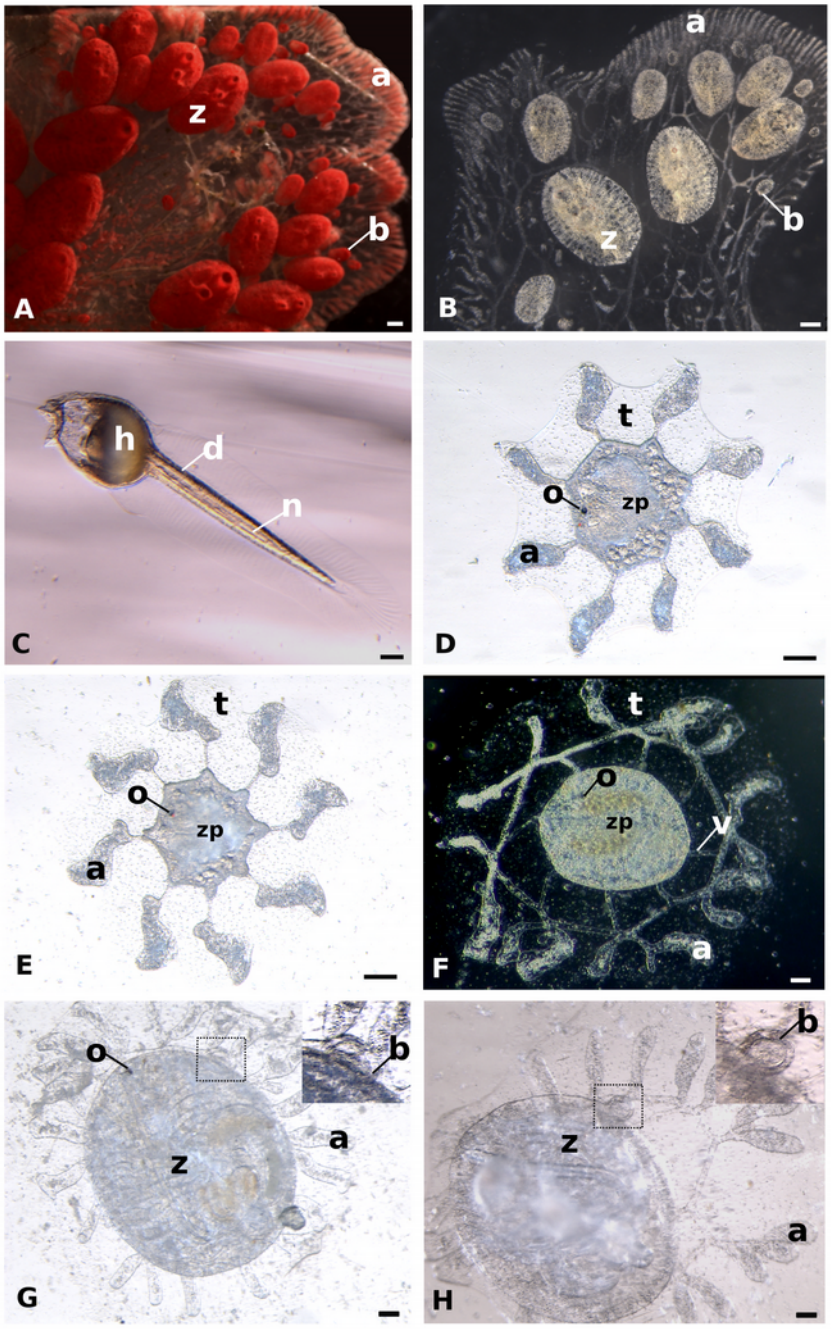


Figure.2 Coloniality in the genus *Symplegma*. (A) *Symplegma rubra* colony characterized by its red color. (B) Symplegma brakenhielmi colony characterize by its greenish color. (C) Larva of *S. rubra* after be released by the colony. (D) Larva of *S. rubra* after the settlement and metamorphosis (E) Larva of *S. brakenhielmi* after the settlement and metamorphosis. (F) Ozooid of *S. rubra* during the formation of the blood vessels syste*m.* (G) Formation of the new bud in *S. rubra.* (H) Formation of the new bud in *S. brakenhielmi. a: ampullae; b:bud; o: ocellus; t: tunic; v: blood vessel. Otholito; z: zooid; zp: zooid primordium.* Scale bar is 500 µm in A and B; 250 µm in C, D, E and F; 350 µm in G and F.

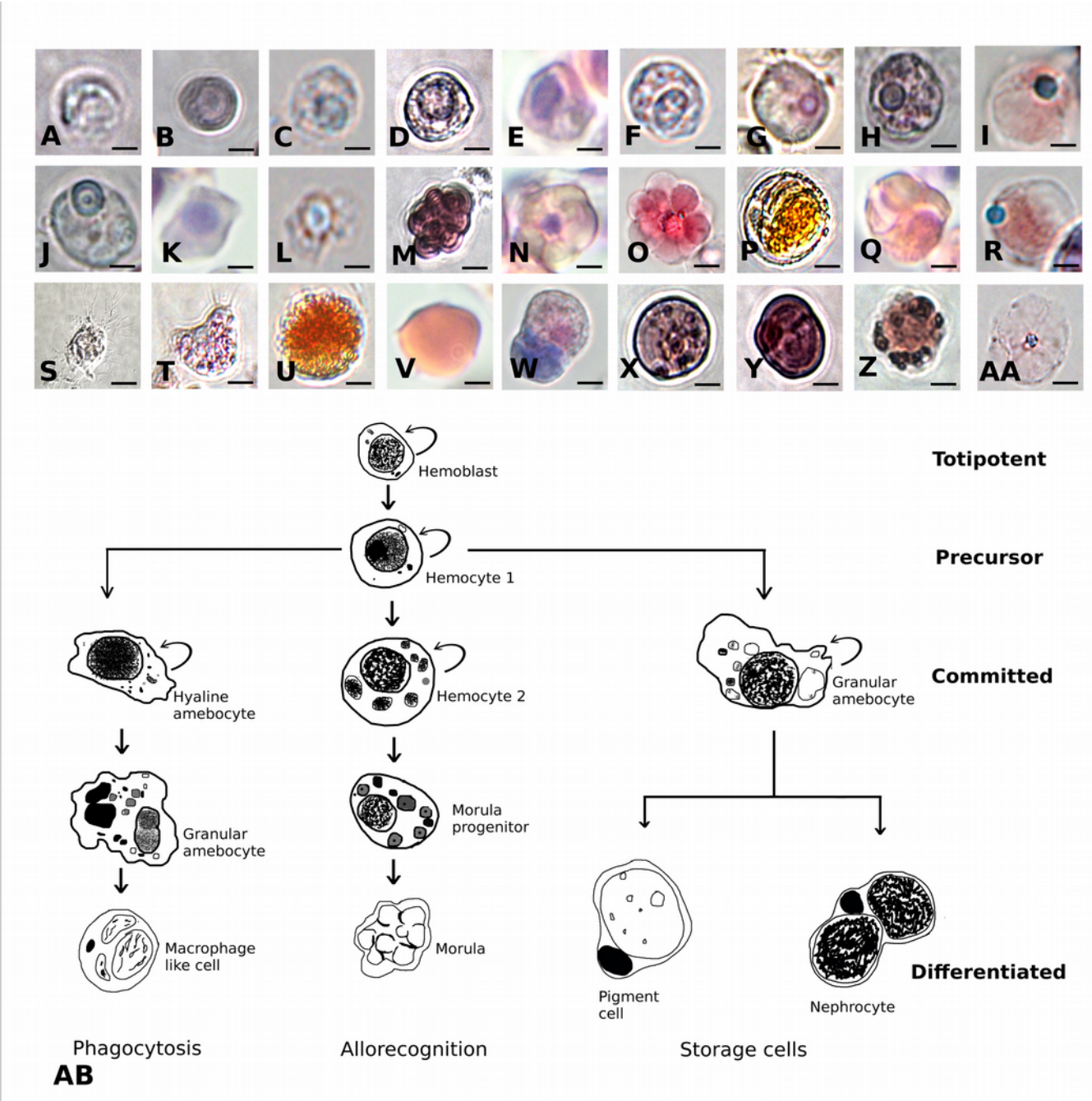


Figure. 3 Hypothetical model of hematopoiesis in *Symplegma.* In *Symplegma rubra* were observed blood cell types similar to *Symplegma brakenhielmi* blood cells and another colonial tunicates. Living hemoblast stained with neutral red (A). Fixed hemoblast stained with Hematoxylin and eosine (H&E) (B) and giemsa (C). Living hemocyte 1 stained with neutral red (D). Fixed hemocyte 1 stained with H&E (E) and giemsa (F). Living hemocyte 2 stained with neutral red (G). Fixed hemocyte 1 stained

with H&E (H) and giemsa (I). Living hyaline amebocyte stained with neutral red (J). Fixed hemocyte 1 stained with H&E (K) and giemsa (L). Living morula stained with neutral red (M). Fixed hemocyte 1 stained with H&E (N) and giemsa (O). Living macrophage-like cell stained with neutral red (P). Fixed macrophage-like cell stained with H&E (Q) and giemsa (R). Living granular amebocyte stained with neutral red (s). Fixed macrophage-like cell stained with H&E (T). Living nephrocyte stained with neutral red (U). Fixed nephrocyte stained with H&E (V) and giemsa (W). Living pigment cell stained with neutral red (X-Y). Fixed pigment cell stained with H&E (Z) and giemsa (AA). The hypothetical model of hematopoiesis is based in the cellular morphology of blood cells, frequency and proliferation observed in S. *brakenhielmi* and *S.rubra.* The hemoblast is proposed as the totipotent hematopoietic stem cell. The hemocyte 1 is propose as the precursor of the undifferentiated comamitted cells. These blood cells are the progenitors of the there genera blood cell lineages, phagocytes, allorecognition and storage cells. Scale bar is 1 µm in A and B; 2 µm in C and from X to AA; 3 µm from E to P, and from T to W; 4 µm in Q and R; 6 µm in S.

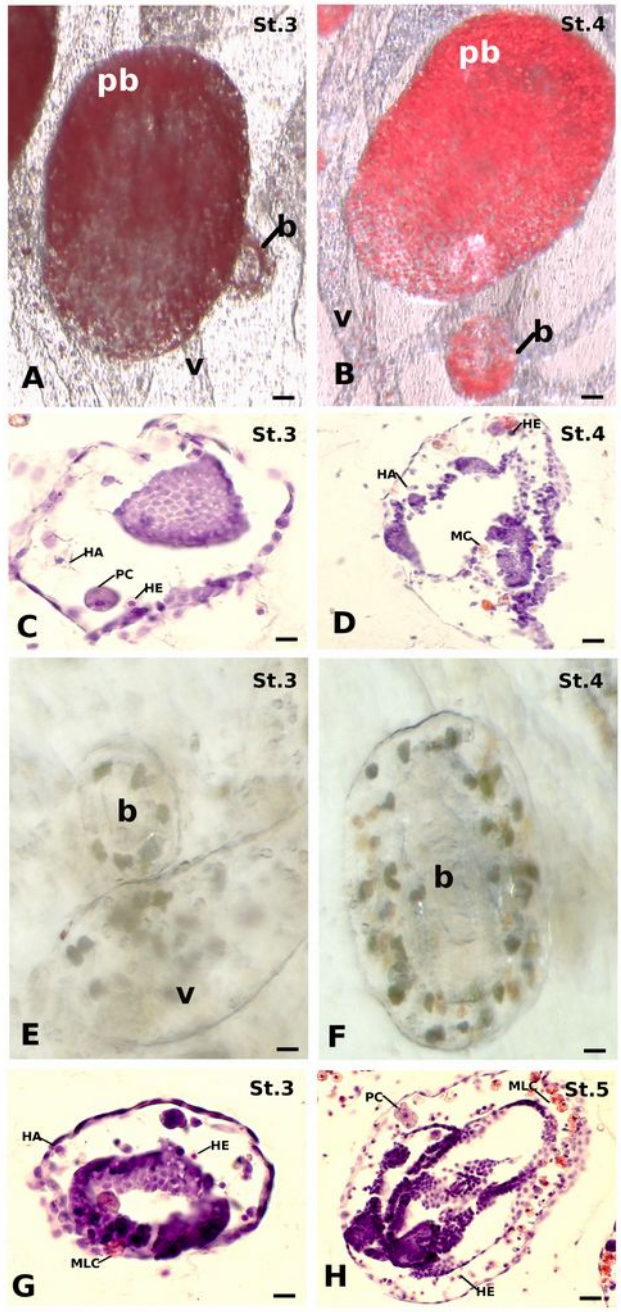


Figure.4 Buds morphogenesis in *Symplegma*. (A) The buds in *Symplegma rubra* are forming by paleal budding (i.e. buds are forming by the evagination of branchial and external epitheliums in the parental bud). Bud in the stage 3 or double vesicle is attached to its parental bud in stage 6. (B) The bud in stage 4 moved apart from the parental bud and it is attached to a near blood vessel. (C) Bud in stage 3 of *S. rubra*, the internal vesicle in in formation. Mostly of the cells observed inside the bud are hyaline amebocytes (HA), hemoblasts (HE) and a pigment cell (PC). (D) Bud in stage 4 of *S. rubra* HA, HE, MLC are between the external epithelium and the internal epithelium, at this stage there are morula cells (MC). (E) Buds in *Symplegma brakenhielmi* can be formed by paleal budding or vascular budding as is shown. (F) Vascular bud from a *S.brakenhielmi* colony. (G) Bud in stage 3 of *S. brakenhielmi.* Stage known as double vesicle has inside HA, PC and HE, also Macophage-like cells are entering in the bud. (H) Bud in stage 3 of *Symplegma brakenhielmi.* Stage known as double vesicle has inside HA, PC and HE, also Macophage-like cells are entering in the bud. *b:bud; pb: parental bud; v: blood vessel.* Scale bar is 50 µm in A and B; 10 µm in C; 20 µm from D to H.

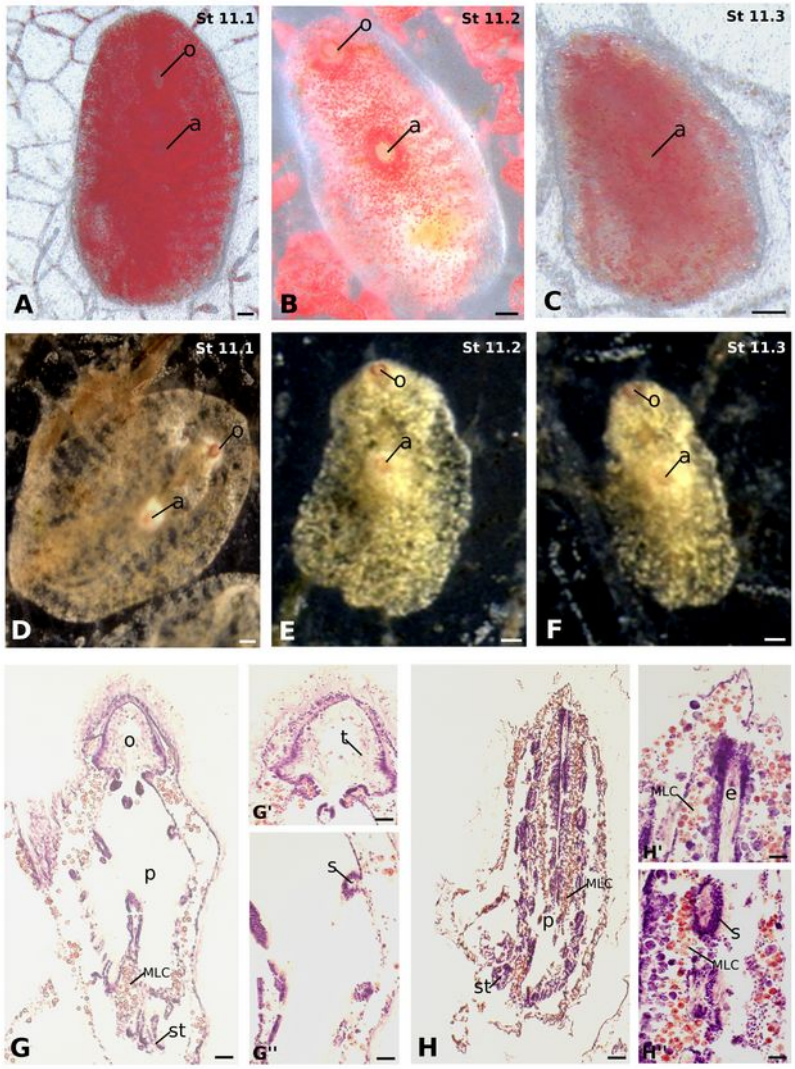


Figure.5 Resorption of old zooids in *Symplegma.* The resorption process of old zooids is similar in the species *Symplegma rubra* and *Symplegma brakenhielmi*. (A,D) Old zooid at the stage 11.1 (12 hours), the first step of resorption. Oral siphons closure and starting the antero-posterior contraction. (B,E) Old zooid at stage 11.2 (12 hours). The contraction of body continues and the zooid reduces size. It is possible to observe an empty tunicate, probably because many cell including pigment cells were phagocyted and moving outside from the old zooid. (C,F) Stage 11.3 the zooid continue reducing its size, the tunicate is more transparent and the hear beat is slower and stops progressively. (G) Old zooid starting the anterior-posterior contraction, the oral siphon is already close. Macrophage-like cells (MLC) are ingression and accumulation around the pharynx and the stomach. (G') Magnification of the closed oral siphon. (G'') Magnification of the pharynx, which start by the disintegration process of the epitheliums. (H) Old zooid in an advance resorption process. The body contraction is increasing. MLCs are increasing their proportion around the pharynx and the stomach. (H') Magnification of the endostile. (H'') Magnification of the stigmas. *a: atrial siphon; e: endostyle; o: oral siphon; MLC; macriphage-like cell; p: pharynx; s: stigma; st: stomach; t: tentacles.* Scale bar is 250 µm from A to D; 600 µm from E to F; 50 µm G and H; 30 µm in G' and G''; 20 µm in H' and H''.

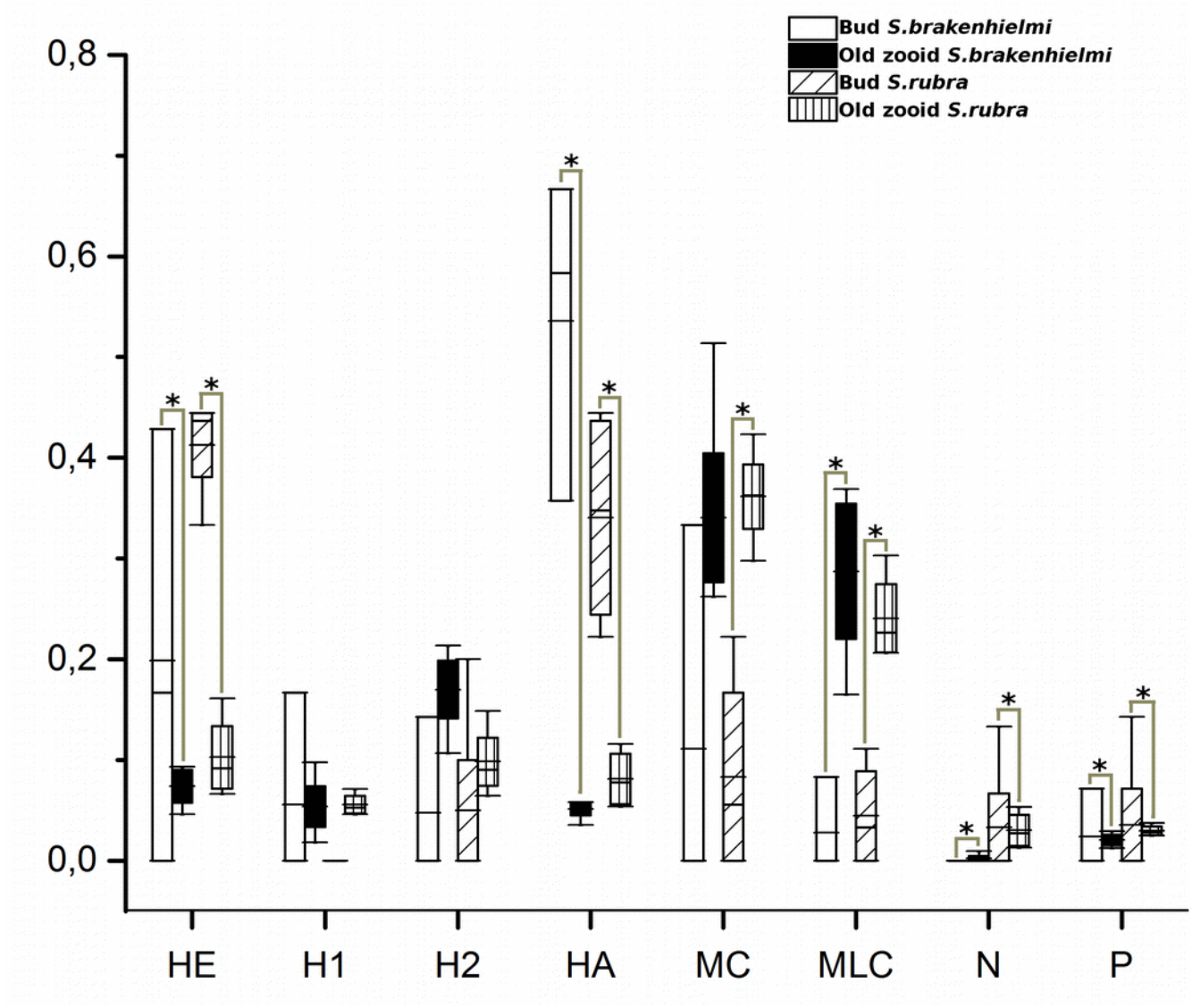


Figure.6 Proportion of blood cells in buds and in aging zooids. The proportion of blood cells is different between buds and aging zooids. Blood cells with undifferentiated characteristic as HE and HA are statistically more frequent in buds than in aging zooids. In contrast in aging zooids are more frequent MLC and MC. The proportion of storage cells (N,P) is variable. Pigment cells are statistically more frequent in buds, moreover nephrocytes have different results between *Symplegma* species. *S. brakenhielmi* has more Ns in aging zooids than in buds, the contrary to *S.rubra.*

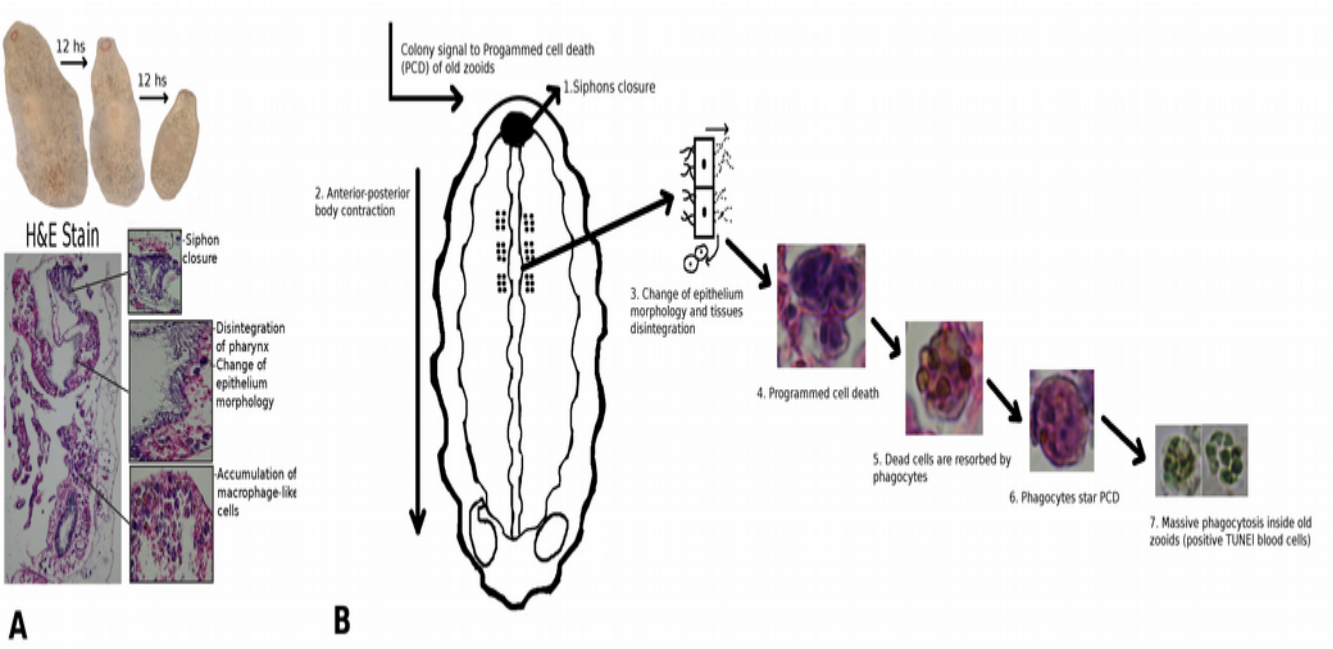


Figure.7 Hypothetical model of aging in *Symplegma*. (A) The aging in *Symplegma* colonies begins with the siphon closure followed by the anterior-posterior contraction around the longitudinal axis of the zooid. Macrophage-like cells (MLC) migrate to the zooid and the internal tissues start to disintegrate. (B) After the epithelial disintegration, these cells start apoptosis. The apoptotic bodies are phagocyted by the MLCs that migrated and increase the proportion MLCs in the aging zooid. Some types of blood cells show signals of DNA fragmentation by TUNEL, inside the aging zooid and the vessels. The hear-beating decrease and finally the internal tissues are phagocyted, and probably this phagocyted material is recycled by the colony.

Video. 2 Blood cells behavior from Symplegma rubra. Majority of the cells are morula cells, which a circular shape that change with the movements of the cells. Amebocyte located in the middle, has a dynamic movemnt, at the final of the video pseudopod are visible. Living cells are with natural colors, in the extracellular matrix laminin.

Video. 2 Amebocytes behaviors. Three amebocytes are showing dynamic movements, pseudopods are visible. Cellular movements are related with drasticall change in cytoplasm shape and pseudopod extensions. Living cells are with natural colors, in the extracellular matrix laminin. Link to watch the videos:https://drive.google.com/drive/folders/1rIvYPgon2QOw53CjMvVySlaWWmuza1fI?usp=sharing

Chapter 4

An efficient laboratory workflow for blood cell manipulations and RNA extractions in marine invertebrates with polysaccharides tunics and secondary metabolites

1. Introduction

The ocean is 70% of our planet surface. Life originated and diversified in the oceans, and a large proportion of biodiversity is concentrated in oceanic environments (Barnes & Hughes, 1982; Kaiser et al., 2010). Marine organisms are categorized in two main categories in relation to their location: pelagic organisms live in the water mass; and benthic organisms live in the bottom (Barnes & Hughes, 1982). Invertebrate animals are preponderant in pelagic and benthic ecosystems, being keystones species and a relevant proportion of marine biomass. It is possible to obtain massive biological information working with marine invertebrates in evolution, ecology, toxicology and developmental biology. Benthic invertebrates cephalochordates and hemichordates have been extensively studied regarding their evolutionay aspects (Swalla & Smith, 2008). Sea urchin and tunicate embryos are used in toxicity evaluation, of organic compounds, heavy metals and other pollutants (Bellas et al., 2005). In developmental biology sea urchin, tunicates, molluscs, polychates, are model organisms to study embryogenesis, gametogenesis, cell signaling and developmental processes in animals (Gilbert, 2003). Marine invertebrates have recently begun to be used as model lineages, regarding aspects such as research in new nanomaterials (cephalopods) (Phan et al., 2013);

regeneration (Özpolat et al, 2016) and genome evolution (Albertin et al., 2015) . Thus, marine invertebrates are an abundant source of biological knowledge.

Research with marine invertebrates can be restricted by the difficulty to replicate marine environment conditions, such as salinity, pH and temperature. Collection and transporting of samples, is another limiting factor in research. Some specimens are rare and hard to collect and transport in adequate conditions. Quantity and quality of samples affects laboratory procedures and nucleic acids extractions. Moreover, tissues from marine invertebrate have higher concentration of salts, calcareous or hard external tissues, pigments, secondary metabolites and polysaccharides. These marine invertebrates characteristics restrict extractions of nucleic acids, experimental procedures with living tissues and specimens, limiting research. As such, the objective of this laboratory workflow is to provide a useful and efficient methodologies to solve problems during extraction and manipulation of blood cells, as well as RNA extractions in marine invertebrates.

These procedures were developed during the study of the species *Symplema brakenhielmi* and *S.rubra*. Which are colonial tunicates with an elastic cellulose tunic, hardly breakable. The tunic is dense and the quantity of internal material is small. *Symplegma* species have high concentration of pigments and secondary metabolites, which interfere with nucleic acid extractions. RNA extractions were standardized to circumvent this difficult material, and was tested in other tunicates as *Styela plicata* and *Cassiopea sp* cnidarians. Blood cell extractions for cytological stains and electron microscopy, were standardized in Symplegma species .

2. Methodologies

2.1 Taxon sampling

Symplema brakenhielmi and *S.rubra* are benthic animals,living attached to hard marine substrates. Rocky shores, mangroves and Yatch port are locations with abundant colonial tunicates. Yatch ports are ideal places to collect, because these locations provide colonial animals in abundance and ease of colletion. Colonial tunicates are usually located in floating buoys, cords and pilots. Pieces of colonies between 1-2 cm^2 are carefully removed from the substrate, cleaned and placed in recipients with fresh seawater. Colony pieces are attached to microscope glass slides with a thread, and stored in microscope slide boxes with perforations for water circulation. Boxes are attached to floating structures in Yatch port during three weeks. The attached colonies are cleaned, to be subsequently used in experiments.

2.2 Sample transporting and culture

Transportation of alive colonies requires a thermal container with a oxygen source. Alive colonies need time to adapt from ocean conditions to culture system tanks. For the first twelve hours after transportation, new colonies need to be maintained with 50% of original seawater and system seawater, without food. A feeding regime with living phytoplankton is useful to maintain healthy colonies. The mixture of I*sochrysis*, *Thalassiosira*, *Pavlona*, *Nanochlorpsis* has nutrients for growing colonies. These transport and culture protocols allowed an approximate of 70% survival.

Tunicate survival is aproximately 70%, using this

Samples for nucleic extraction need to be cleaned to remove other animals. This activity is more efficient with the aid of a stereomicroscope. Specimens must be kept without food in filtered seawater (FSW) overnight before tissue extraction. Samples collected in remote areas, can be cleaned as best as possible and stored in 70 % ethilic alcohol for DNA extraction, or RNA later for RNA extraction. Tissues must be immersed in preserving solution, in case of hard tunic, cut in pieces to guarantee tissue preservation.

2.3 Blood cells characterization

Blood cells in *Symplegma* colonies are diverse, consisting of eleven types of cells. These blood cell populations have different sizes, cellular content (e.g. vesicles, vacuoles, dense granules) and cytoplasmic characteristics (e.g. amoeboid cytoplasm and pseudopods) (Gutierrez & Brown, 2017). Because blood cells present such diverse characteristics, different cells are observable with different stains and fixing methodologies. Therefore it is necessary to use diverse methodologies, to include all the blood cell populations.

The first approach to observe and characterize blood cells is cellular morphology. The cytology stains are made in histological sections and extracted blood cells. Histological sections are useful to identify internal morphology of zooids and buds, as well as the location of blood cell types within the colonies. However, amoeboid blood cells and cells with large vacuoles are excluded for abrasive steps (e.g. xylol and ethanol series of dehydration) in histological procedures. Cytology stains in extracted blood cells are useful to observe the complete cellular diversity in the blood.

2.3.1 Histology in Symplegma colonies

Tunicate colonies attached to slides must be cleaned with a soft brush and let without food overnight in FSW. Tissues colonies are relaxed in FSW with menthol crystals covering water surface for 15 minutes, to be fixed with 4% paraformaldehyde (diluted in FSW), at 4 °C overnight. After fixation colonies are washed three times for 15 minutes with PBS and distilled water. Tissues are dehydrated by ethanol series (25%, 50%, 70%, 80%, 90%, 100%) and two xylol washes for 30 minutes. Tissues are embedded in paraffin to cut in sections (6 um). Sections are rehydrated by xylol washes for 10 minutes and ethanol series (100%, 95%, 90%, 80% 70%). Finally sections are stained with hematoxylin and eosin, and mounted with Entellan.

2.3.2 Blood cell extraction

Blood cell extraction follows a previously described protocol (Cima, 2010),with some changes to improved obtained results. The main limitation to work with blood cells is the constant coagulation, and the preservation of blood cell integrity. Constant use of anticoagulant during all the blood extraction, is useful to reduce blood clots. Blood cell integrity is preserved by, maintaining the sample in ice and preparing reagents in FSW for osmoregulation.

Clean attached colonies are immersed in anticoagulant solution (10 mM L-cysteine and 0,38 % sodium citrate, diluted in FSW) during 5 minutes, then the colony is dried with a soft paper towel. Ampullae are gently cut, blood which comes out is collected with a micropipette, which is rinsed constantly with anticoagulant. After blood collection anticoagulant is washed by centrifuging 15 minutes at 3000 rpm. Hemocytes are resuspend in 500 µl of solution with 1/3 Anticoagulant and 2/3 FSW. This solution prevent the posterior coagulation, and maintain the integrity of the cells. Blood cell

solution is mixed gently with a micropipette. Drops from this blood solution are left in slides coated with Poly-L Lysine to attach the blood cells. Slides are maintained in a humid chamber to prevent cells desiccation. After blood cells attachment, liquid excess is discarded placing the slides vertically.

2.3.3 Cytological stains

The cytological stains, are used to observe the cellular size, content, and conspicuous organelles such as vacuoles, vesicles and pseudopods. The general cellular morphology characteristics (i.e. nucleus size, nucleus-cytoplasm ratio, acid and basic contents) are described with hematoxylin and eosin stain. Blood cell populations are characterized with Giemsa stain, used for blood cytology. The lipid content is described with Sudan Black stain. These stains can be abrasive for big vacuolated cells and cells with pseudopods. Also blood cells morphology change after fixative treatment. Finally, Neutral Red (vital stain) is used to observe, living cellular morphology and cellular behaviors.

Hematoxylin and eosin stain

Blood cells attached to slides are fixed for 15 minutes, with 4% Paraformaldehyde (diluted in FSW). Fixed cells are washed with PBS, and stained with Meyer hematoxylin for 10 minutes and eosine for 5 minutes. Blood cells are mounted using glycerin and sealed with a coverslip.

Giemsa stain

Blood cells attached to slides are fixed for 30 minutes at 4 °C, with the solution 1 g NaCl and 1 g sucrose in 1 % glutaraldehyde in FSW. Fixed cells are washed with PBS, and stained with 10% Giemsa for 5 minutes. Blood cells are mounted using glycerin and sealed with a coverslip.

Neutral Red for stain acid compartments

Neutral red solution (8m/L in FSW) is added to attached blood cells. Living hemocytes are observed directly with a coverslip.

Sudan Black for stain lipids

Blood cells attached are washed with PBS, and with 70% ethanol. Cells are stained with saturated solution of Sudan Black in 70% ethanol for 15 min at 70°C. Stain is washed with 70% ethanol and distilled water. Cells are mounted using glycerin.

2.3.4 Transmission electron microscopy

Transmission electron microscopy (TEM) is a useful technique to observe in details cellular morphology, and cellular organelles. TEM is a used technique for the blood cell types description and characterization (cima, 2010; Hirose, 2003). Maintain cellular morphology integrity is the vital step for TEM quality in colonial tunicates. Blood cell populations interacted differentially with fixatives. Thus a complete observation of blood cell diverstiy was possible using diverse fixatives. Fixative solution used previously by Cima (2010), (1.5% glutarldehyde in 0.2M cacodylate buffer, 1% saccharose, 1.7 NaCl% adjusta at ph = 7.47.4) is useful for smaller cells, precursors cells and cells with homogenous vesicles. Whereas, Karnovsky (1965) modified solution for marine organisms (1.5% glutaraldehydeglutarldehyde, 1.2% paraformaldehyde in 0.2M cacodylate buffer, 1.5 % NaCl, 1% saccharose, adjust at ph = 7.4) is useful for large vacuolated cells and ameboyd cells. Clean attached colonies are immersed in fixative solution for 1 hour at 4°C. Then pieces of blood vessels are dissected and fixed during 3 hours at 4°C. This step improve fixative penetration, limited by the cellulose tunic.

Tissues pieces are postfixed in n 1% OsO_4 in 0.2M cacodylate buffer for 1 hour. Samples are dehydrated in ethanol series, embedded in Spurr's resin and polymerized at 58°C. Tissues are sectioned and collected on copper grids. Sections are contrasted with lead citrate, and observed in a Zeiss EM 900 Transmission Electronic Microscope operated at 80kV.

2.4 RNA extractions

Nucleic acids contain useful biological information, thus DNA and RNA extraction are basic methodologies in biological studies. Biological information from RNA has (DNA transcription and expression) variable applications in developmental biology. Such as, transcriptomes, in situs hybridization, and quantitative RNA expression (Mehra, 1996).

Maintain RNA integrity require some cares, such as good samples quality, reduction of RNAases (enzymes that digest RNA), and cold temperatures. Main restrictions for RNA extraction in tunicates, are the elevate salt concentration and the hard cellulose tunic. Wash samples with ice-cold PBS before RNA extraction, reduces salt excess. Also, the addition of sodium compounds to isopropanol improves RNA precipitation. These steps are used in the modified general Tri-Reagent protocol as recommended (Sambrook & Russell, 2001). Dissection of muscular tissue, avoid the use of the cellulose tunic. This procedure is useful in solitary tunicates and in colonies with large zooids. Colonies with smaller zooids, require dissection of a large number of zooids to obtain enough tissue. Whereas, colonies with softer tunics, such as botryllids can be use complete, including tunic. Nevertheless in Symplegma colonies pigments and secondary metabolites degraded RNA quickly, making useless the dissections.

RNA extraction in Symplegma is difficult by the cellulose tunic, the secondary metabolites, and small quantity of internal tissues. Thus, the unique protocol that worked for RNA extraction in this colonial tunicate is a protocol with lithium chloride, after test general Tri-Reagent protocol, modified Tri-Reagent protocol and a variety of kits for animals and plants tissues.

2.4.1 Tri-Reagent modified protocol

For the Tri-Reagent protocol follows a previously described protocol (Sambrook & Russell, 2001). Fresh tissues, samples storage in RNA later and frozen in liquid nitrogen, are used for RNA extraction with satisfactory results. Samples are washed with ice-cold 1X PBS. Tissue homogenization is with liquid nitrogen and a ceramic mortar, or with a plastic homogenizer with TRIzol solution. After homogenization, solution is live during 5 minutes at room temperature, posterior steps are at 4℃ . The homogenate is centrifuge by 12,000 rpm for 10 minutes, to remove the insoluble material (extracellular membranes, polysaccharides). The supernant is transfer into a new tube, for organic separation with chloroform. Supernant and chloroform are mixed with a vortex shaker and stand for 5 minutes, then centrifuge at 12,000 rpm for 15 minutes. The organic separation is repeated, to reduce salt and proteins excess, and polysacharide residues from tunic. RNA is with isopropanol and a RNA precipitation solution (1.2M NaCl and 0.8M disssodiu citrate), during 10 minutes. Liquid excess is removed after 10 minutes of centrifugation at 12,000 rpm. Pellet is dried by air-drying for 5 minutes and washed with 75% ethanol. Ethanol is removed by 5 minutes of centrifugation at 12,000 rpm and evaporation of residues. Clean pellet is diluted in free RNAse water and storaged at -80℃.

2.4.2 RNA extraction using LiCl for precipitation

The principal steps for maintain RNA integration are: (i) maceration of samples after frozen with liquid nitrogen, with a Tissue Lyzer machine (40 seconds, 30 revolution per second); (ii) SDS detergent in the buffer extraction, to permeate cellular membranes and solubilize proteins; (iii) washes with LiCl to precipitate selectively the RNA, preventing polysaccharides precipitation; (iv) washes with sodium acetate to remove polysacharides from RNA pellet.

RNA protocol using LiCl was followed as recommended (Barlow & Gammack, 1963). Frozen powder is immersed and mixed in a solution 50 % of extraction buffer (0,2 M Tris-Hcl pH 7.5, 0,1 M LiCl, 5mM EDTA, 1/10 of the total volume of SDS 10%), 50 % of phenol:chloroform (1:1). Homogenate is centrifuge at 14000 rpm for 5 minutes at 4°C. A solution of 6M LiCl is added to the supernatant, mixture is storaged with ice at -20°C overnight. LiCl is washed by centrifugation at 14000 rpm for 10 minutes at 4°C. Pellet is washed with 3M LiCl by 30 minutes of vortex shaking, and centrifugation. Pellet is resuspended with free RNAse water and centrifugate for 1 minute, to remove the insoluble material (extracellular membranes, polysaccharides). Supernatant is mixed with solution of 1/3 of sodium acetate and 2/3 of 100% ethanol. After manually homogenization solution is storaged at -80°C for 15 minutes. Sodium acetate is washed with centrifugation for 15 minutes. Pellet is washed with 70% ethanol, then centrifugate for 5 minutes. Pellet is resuspended in free RNAse water and incubate for 15 minutes at 55°C, to dilute the RNA precipitate, then storage at -80°C.

3. Conclusions

The proposed worflow is efficient to characterize, the diversity of blood cells in marine colonies of genus *Symplegma*. Blood cell types show different characteristics, some methodologies work for specific cell types. Therefore observations using different methodologies, provided complementary information. A complete vision of blood cell types, allow the understanding of hematopoietic processes.

The two methods of RNA extractions, are useful to obtain high-quality RNA. The modified method of Trizol is simple. The modifications in the organic separation and precipitation of RNA, improves the obtained results, being this method a good option to extract RNA in marine organisms.

The method of extraction with lithium is useful to extract RNA from samples with pigments, secondary metabolites and polysaccharides. Although, this method can be useful in other organisms with the same characteristics. This method takes two days for the overnight precipitation with LiCL, however the results are very satisfactory, allowing high-quality RNA extraction from difficult samples. Finally, this RNA protocol uses common laboratory materials and procedures, making possible RNA extraction from diverse materials in widely available laboratory equipment.

4. References

Albertin, C. B., Simakov, O., Mitros, T., Wang, Z. Y., Pungor, J. R., Edsinger-Gonzales, E., … Rokhsar, D. S. (2015). The octopus genome and the evolution of cephalopod neural and morphological novelties. *Nature*, *524*(7564), 220–224. https://doi.org/10.1038/nature14668

Barlow, J., & Gammack, D. (1963). A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. *Biochemical and Biophysical Research Communications*, *13*(1), 61–66.

Barnes, R. S. K., & Hughes, R. N. (1982). *An Introduction to Marine Ecology*. *An Introduction to Marine Ecology*. https://doi.org/10.1002/9781444313284

Bellas, J., Beiras, R., Mariño-Balsa, J. C., & Fernández, N. (2005). Toxicity of Organic Compounds to Marine Invertebrate Embryos and Larvae: A Comparison Between the Sea Urchin Embryogenesis Bioassay and Alternative Test Species. *Ecotoxicology*, *14*(3), 337. https://doi.org/10.1007/s10646-004-6370-y

Cima, F. (2010). Microscopy methods for morpho-functional characterisation of marine invertebrate haemocytes. *Microscopy: Science, Technology, Applications and Education*, 1100–1107. Retrieved from http://www.formatex.info/microscopy4/1100-1107.pdf

Gilbert, S. (2003). *Developmental Biology* (Seventh Ed). Sunderland, MA: Sinauer Associates, Inc., .

Grøttum, J. A., Staurnes, M., & Sigholt, T. (1997). Effect of oxygenation, aeration and pH control on water quality and survival of turbot, Scophthalmus maximus (L.), kept at high densities during transport. *Aquaculture Research*, *28*(2), 159–164. https://doi.org/10.1111/j.1365-2109.1997.tb01028.x

Gutierrez, S., & Brown, F. D. (2017). Vascular budding in Symplegma brakenhielmi and the evolution of coloniality in styelid ascidians. *Developmental Biology*, *423*(2). https://doi.org/10.1016/j.ydbio.2017.01.012

Kaiser, Michael, J., Attrill, M., & Jennings, S. (2010). *Marine Ecology. Process, Systems, and Impacts*. *Fish and Fisheries*. https://doi.org/10.1111/j.1467-2979.2006.00221.x

Karnovsky, M. J. (1965). A Formaldehyde-Glutaraldehyde Fixative of High Osmolality for Use in Electron Microscopy. *The American Society for Cell Biology Source: The Journal of Cell Biology*, *27*(2), 1–149.

Losordo, T. M., Masser, M. P., & Rakocy, J. (1998). Recirculating Aquaculture Tank Production Systems: An Overview of Critical Considerations. *SRAC Publication*, *451*(451), 1–6. Retrieved from http://www.lssu.edu/faculty/gsteinhart/GBS-LSSU/BIOL372-Fish_Culture_files/Recirculating_culture.pdf

Marion, G. M., Millero, F. J., Camões, M. F., Spitzer, P., Feistel, R., & Chen, C. T. A. (2011). PH of seawater. *Marine Chemistry*, *126*(1–4), 89–96. https://doi.org/10.1016/j.marchem.2011.04.002

Mehra, M. (1996). RNA Isolation from cells and tissues. In P. Krieg (Ed.), *A laboratory Guide to RNA: Isolation, Analysis, and Synthesis* (p. 41). New York: Wiley-Liss.

Özpolat, B. D., Sloane, E. S., Zattara, E. E., & Bely, A. E. (2016). Plasticity and regeneration of gonads in the annelid Pristina leidyi. *EvoDevo*, *7*(1), 1–15. https://doi.org/10.1186/s13227-016-0059-1

Phan, L., Walkup IV, W. G., Ordinario, D. D., Karshalev, E., Jocson, J.-M., Burke, A. M., & Gorodetsky, A. A. (2013). Reconfigurable Infrared Camouflage Coatings from a Cephalopod Protein. *Advanced Materials*, *25*(39), 5621–5625. https://doi.org/doi:10.1002/adma.201301472

Sambrook, J., & Russell, D. (2001). *Molecular Cloning a Laboratory Manual* (Third edit). New York: Cold Spring Harbor Laboratory Press.

Swalla, B. J., & Smith, A. B. (2008). Deciphering deuterostome phylogeny: Molecular, morphological and palaeontological perspectives. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1496), 1557–1568. https://doi.org/10.1098/rstb.2007.2246

Wright, J. P. (2011). *pH Control in Recirculating Aquaculture Systems for Pāua (Haliotis iris)*. Victoria University of Wellington.

5. Figures

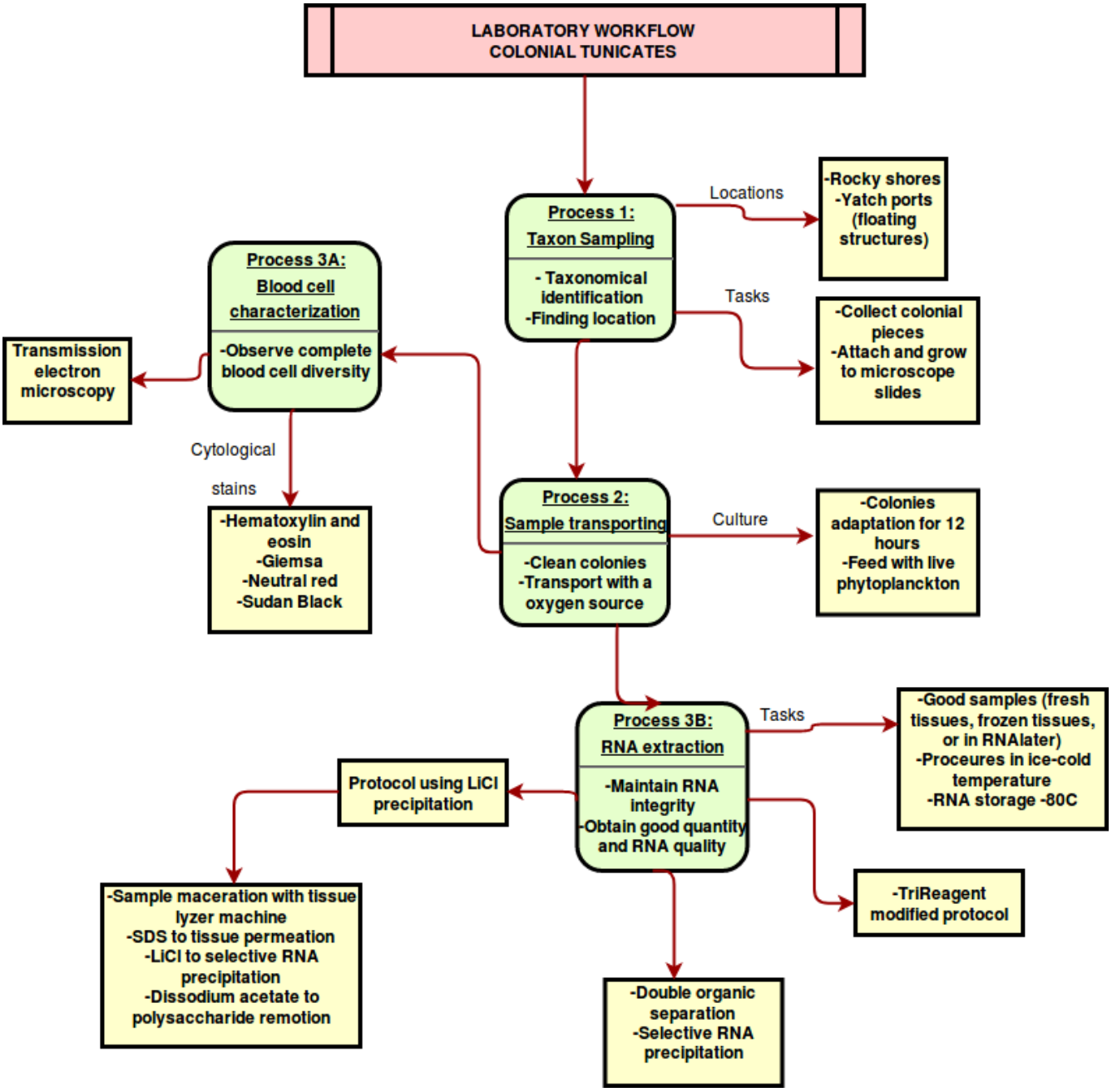


Figure. 1 Summary of the methodological workflow. The workflow is composed by taxon sampling (process 1), sample transporting (process 2), blood cell characterization (process 3A) and RNA extraction (process 3B).

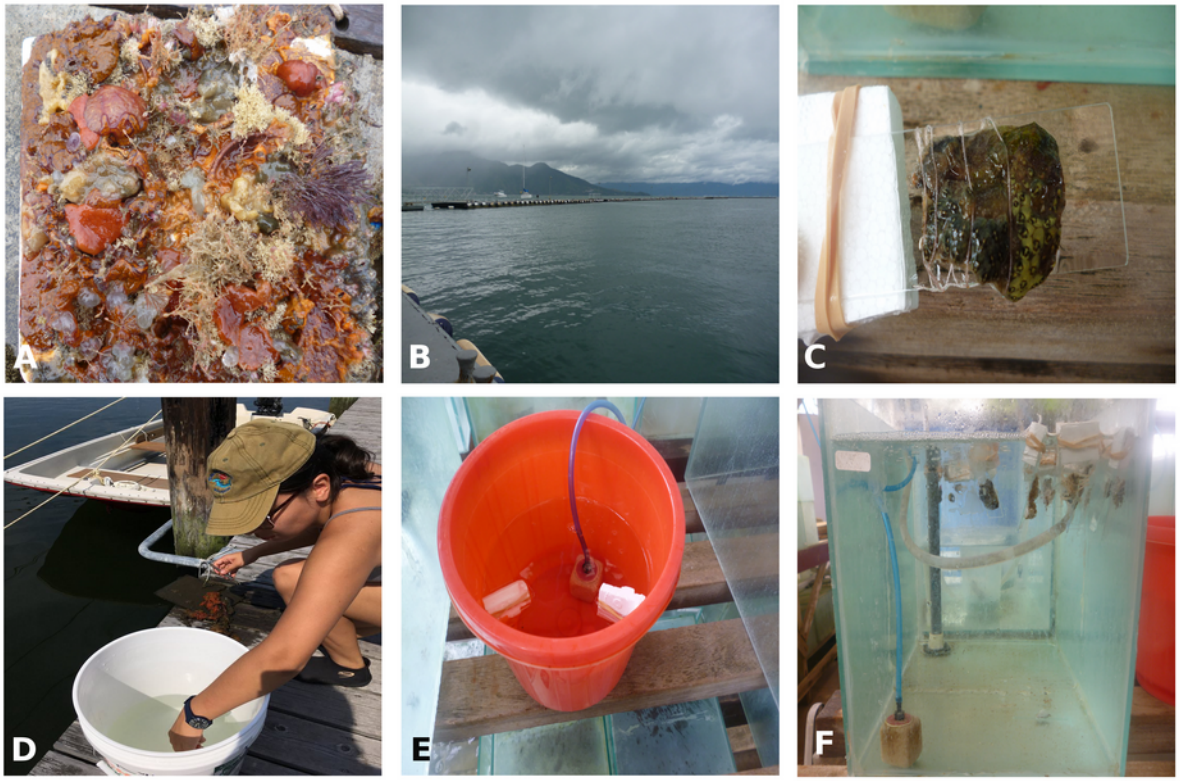


Figure. 2 Taxon sampling and sample transporting. (A) Benthic community in a buoyant structure, morphological characters (e.g. color, individual size, tunic texture and color) are used for taxonomic identification. (B) Floating yatch port structures, are useful to collect and grow attached tunicates. (C) *Symplegma brakenhielmi* colony, attached to a microscope slide with thread . (D) Pieces of collected colonies are maintained in recipients with fresh water, these pieces are attached to slides or transported to the laboratory. (E) Recipients for samples transportation have an air source, to maintain oxygen levels during transpor. (F) Attached colonies are maintained in culture systems with seawater and oxygenation. Colonies need to float or stay at higher level of aquarium bottom, to improve filtering and to prevent organic material contamination.

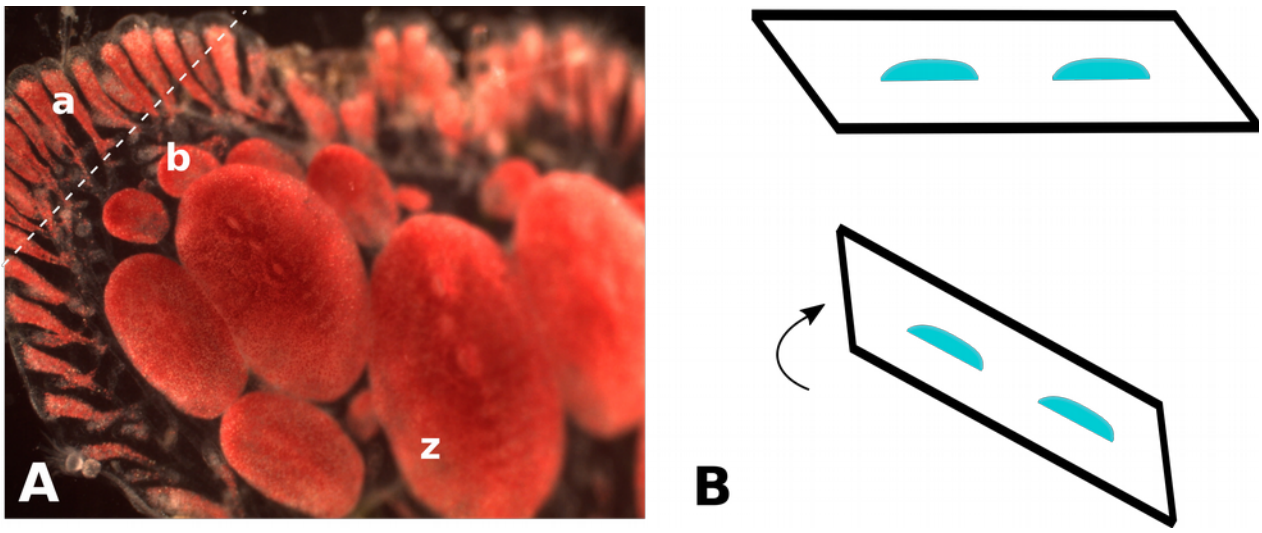


Figure. 3 Blood cells extraction. (A) Symplegma rubra colony after anticoagulant immersion, ampullae are cutting for extract the blood. (B) Blood cell solution is dropped in a slide, for cell adhesion, then slide is placing vertically to remove liquid excess.

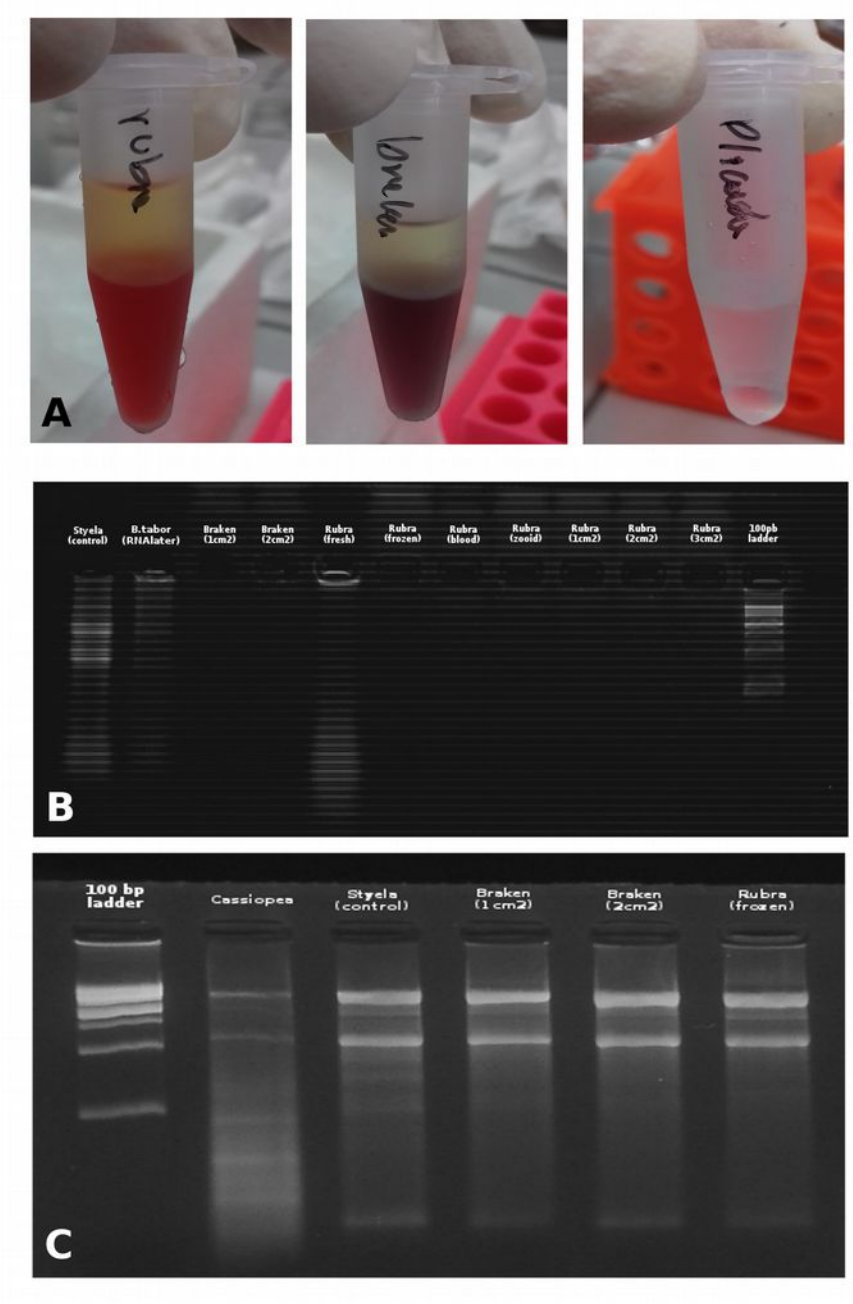


Figure. 5 RNA extraction. (A) *Symplegma rubra* and *S. brakenhielmi* samples contain pigments, which are extracted with the RNA, even in the organic separation. Whereas, *Styela plicata* (control) sample is colorless. (B) RNA extraction using the tri-reagent modified protocol is useful to extract high-quality RNA from tunicate samples (*Styela plicata*), and small tissue samples (*Botryllus tabori)*. However tri-reagent modified protocol is useless to extract RNA from *Symplegma* samples (frozen tissue, blood cells, dissected zooids, colonies of different sizes) or high-quality RNA (alive *S.rubra* colony). (C) RNA extraction using LiCl for precipitation is useful to extract high-quality RNA from *S.rubra and S. brakenhielmi* samples from frozen and fresh material, as well from *Styela plicata* and cnidarians such as *Cassiopea sp*.

6. Supplementary materials

6.1 Process 1: Taxon sampling

-Using knifes and spatulas collect the tunicates carefully to do not damage the organisms.

-Transport the samples in sea water to laboratory. The samples are classified by morphological characteristics, like color, form and shape. Each sample is labeled with a number and collect place.

-In colonial specimens sample is dividing for different analysis, in solitary specimens, more than one individual is collected. One sample fragment (or individual) is storage in 70% Ethanol to molecular analysis, other part of tissue is fix in 4% formol to morphological observations. The remaining tissue is using to settle in glass slides. For RNA analysis samples can be preserve in RNAlater, frozen in liquid nitrogen and storage at -80°C.

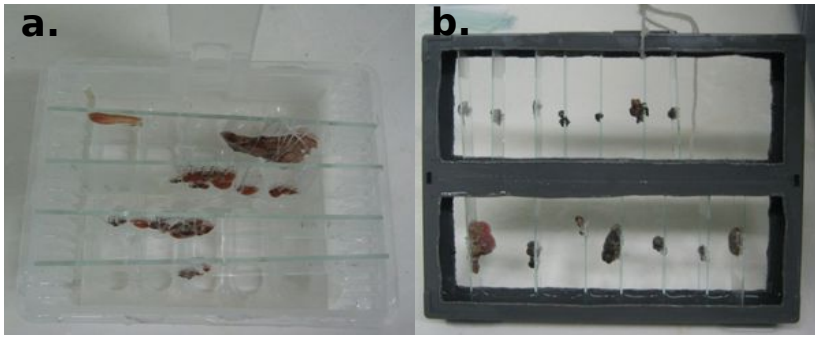
6.2 Process 2: Transporting and maintenance of living tunicates

6.2.1 Settled colonial tunicates

-Colonies are atached with thread on 5cm X 7.5cm glass slides.

Fig.1 Colonial ascidian on glass slide.

-During attachment process microscope slides are maintained in slide boxes with apertures to permit water circulation. Usually this process occurs in one or two weeks in the ocean (attach boxes to port structures) or two and three weeks in a culture system.



-When tunicate is attached to slide, the thread is remove with tweezers.It is important that the attached tunicates are floating or in a surface superior to tank bottom. This allows filtration and prevent contamination with organic wastes. Every day tunicates are feeding with living phytoplankton. The food mixture is prepare from ¼ of each living algae (*Isochrysis*, *Thalassiosira*, *Pavlona and Nanochlorpsi*s), in a approximated concentration of 170,000 cell/ml.

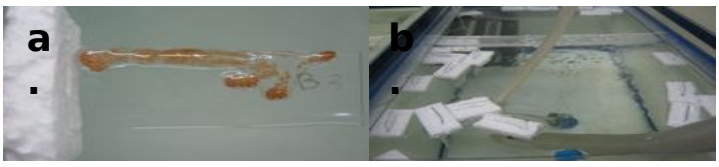


Figura.3 a. Botryllid ascidians settle on glass slide **b.** ascidians in sea water tank.

6.2.2 Precautions with culture system:

A. Ammonium and organic wastes:

One of the crucial aspect in recirculating systems is the accumulation of ammonium and other toxic compounds for animals. The first way to reduce the quantity of these compounds is to prevent the accumulation of organic material pieces. The rest of food and feces begin a process of decomposition affecting the quality of the water. The biofilter is the principal way to reduce the accumulation of ammonium, because the microorganisms in the biofilter processing the ammonium in a sequence of chemical reactions that decompose the ammonium in non toxic forms (Losordo et al.,1998; Wright, 2011) :

$$NH_3 \text{ (toxic)} \xrightarrow[N]{\textit{Nitrosomonas}} NO_2^- \text{ (toxic)} \xrightarrow[N]{\textit{Nitrobacter}} NO_3 \text{ (non-toxic)}$$

Consequently is necessary maintain the biofilter in good conditions controlling the variables of the system (pH, salinity and temperature). Also add probiotics to the biofilter to maintain system stability(Marion et al., 2011; Wright, 2011).

B. Oxygen levels

The levels of oxygen are usually a problem in recirculating systems, because the water is maintain in the system for a long period and it is necessary provide oxygen to the system. Also the process to reduce the ammonium in the biofilter consume oxygen, reducing the levels of oxygen in the system. Thus oxygen sources are critical to maintain healthy conditions to the animals. Specially in systems with a high density of animals, or with animals that require large food quantity and produce more organic wastes (e.g solitary tunicates) (Grøttum et al.,1997).

C. Rotten eggs smell = hydrogen sulfide presence

Rotten eggs smell in the seawater is a indicator of hydrogen sulfide presence. This compound is produced by anoxic bacterias, for the increment of organic decomposition. Resulting in anoxic areas in the culture system, usually in are with less water movement and less oxygen. A constant water movement and pipe cleaning, are strategies to control the increment of hydrogen sulfide (Grøttum et al., 1997; Losordo et al., 1998).

6.3 Process 3A: Blood cell characterization

6.3.1 Blood cell extraction follows a previously described protocol (Cima, 2010),with some changes to improved obtained results

-Anticoagulant solution:

-10 mM L-cysteine (which binds the thiols of plasmalemma proteins)

-0.38% sodium citrate (a calcium chelating agent)

-The solution is in filtered and sterilized sea water adjusted at pH 7.5.

**Note that, before blood collection, glass micropipette must be repeatedly rinsed with the anticoagulant solution, in order to prevent haemocytes from adhering to its glass walls.*

-Haemocyte collection solitary ascidians:

-The haemolymph containing haemocytes can be obtained by cutting the tunic and puncturing the heart.

-Haemocyte collection colonial ascidians:

1. Put the colony by 5 minutes at r.t in 10 cm petri dish containing 50 ml of an anticoagulant solution.

2. Dry the colony.

3. Cut gently some ampullae.

4. Collect the blood with a micropipette (If is necessary put anticoagulant on the slide to collect the blood).

6.3.2 Hemocytes manipulation and stain:

1. Wash away the anticoagulant by centrifuging for 15 minutes at 780g (3000rpm).
2. Resuspend the haemocytes by adding 500 µl of filtered sea water in a solution of 1/3 Anticoagulant and 2/3 FSW to prevent the posterior coagulation.
3. Gently stir the suspension with a micropipette. Do not use a mechanical stirrer.

-Prepare slides with hemocytes to stains

1. 6 drops of 20µl of isolated haemocytes were left for 30 min to attach on SuperFrost or Poly-L Lysine microscopy slides, in a humid chamber.
2.The drop was then discarded by placing the slide vertically.
3.The slides with hemocytes are ready to use by stains.

-Hematoxylin and eosin stain

1. Use the slides with the previously attached hemocytes.
2. Fix for 15 min at 4 °C in 4% Paraformaldehyde (In this case for the availability 4% formaldehyde with good quality)
3. Wash with PBS.
4. Stain using Meyer hematoxilyn for 10 minutes.
5. Distillate water for 5 minutes.
6. Eosine 5 for minutes.
7. Etanol 70% for 45 seconds.
8. Mounting using glycerin and sealed with a coverslip using nail polish.

-Giemsa stain to hemocytes

1. Use the slides with the previously attached hemocytes.
2. Fix for 30 min at 4 °C in ascidian fixative solution (1 g NaCl and 1 g sucrose in 1 % glutaraldehyde in FSW).
3. Wash the slides with 1X PBS
4. Stain using 10 % Giemsa stain by 5 minutes.
5. Mounting using glycerin and sealed with a coverslip using nail polish.

-Neutral red

1. After adhesion of hemocytes to slides, put 60 µl of Neutral Red (Merck) solution (8 mg/L) in FSW.
2. Living haemocytes were directly observed.
Note:This dye specifically stains acid compartments (e.g., lysosomes or acid vacuolate contents) of living cells (Mazzi, 1977).

-Sudan Black for lipids

1. After adhesion of hemocytes to slides

2. Wash the slides with 1X PBS
3. Wash the slides with 70% ethanol for 30 sec
4. Stain with a saturated solution of Sudan Black (Sigma) in 70% ethanol for 15 min at 70°C.
5. Wash with 70% ethanol and wash in distilled water.
6. Mounting using glycerin and sealed with a coverslip using nail polish.
Note: Black spots revealed the presence of lipids.

6.4 Process 3B: RNA extraction
6.4.1 For the Tri-Reagent protocol follows a previously described protocol (Sambrook & Russell, 2001).
Materials:
-PBS 1x cold.
-Trizol or TRI reagent.
-Chloroform.
-Free RNAse H2O
-*RNA precipitation solution:*
    -1.2 M NaCl
    -0.8M dissodium citrate 15 H2O.
    - No adjustment pH is required.
-Plastic pistil for homogenization.

1. Wash the sample with sterile ice-cold PBS.

2. Homogenize tissue samples in TRIzol (1ml per 50-100 mg of tissue) with a appropiate homogenizer.

3. Wait 5 minutes at room temperature.

4. Centrifuge the homogenate at 12,000 rpm for 10 minutes at 4°C to remove the insoluble material (extracellular membranes, polysaccharides).

5. Transfer the clear supernatan to a fresh tube.

6. Add 200 ul of chloroform per ml of TRIzol used.

7. Shake vigorously for 15 seconds and allow to stand for 5 minutes at 4°C.

8. Centrifuge the resulting mixture at 12,000 rpm for 15 minutes at 4°C.

9. Transfer the upper aqueous phase to a fresh tube.

10. Repeat the steps of organic separation (6-9 steps).

11. Precipitate the RNA: for each initial ml of TRIzol, add 250ul for isopropanol and 250 ul of *RNA precipitation solution.*

12. Mix and and wait for 10 minutes at 4°C.

13. Centrifuge the resulting mixture at 12,000 rpm for 10 minutes at 4°C.

14. Briefly dry the RNA pellet for 5 minutes by air-drying.

15. Wash the pellet twice with 75% ethanol and centrifuge at 12,000 rpm for 5 minutes at 4°C.

16. Remove any remaining ethanol, store the open tube on the bench for a few minutes to allow the ethanil evaporate. Do not allow pellet to dry completely.

17. Add 25 ul of free RNAse H2O, storage at -80°C.

6.4.2 RNA extraction using LiCl for precipitation following Barlow et al., (1963) protocol
Materials:
-Razer blades.
-Tweezers
-Spatula
-Scissors
-All these tool need to be autoclaved in the oven during 200° C by 4 hours.
-Metal beads for plants 5 mm.
-Eppendorf 2ml
-Tissue lysser machine
-Chloroform
-Free RNAse H2O
-Liquid Nitrogen

-*Extraction buffer:*
-0,2 M Tris-Hcl pH 7,5
-0,1 M LiCl
-5mM EDTA
-1/10 of the total volume of SDS 10%
-Autoclave the final solution

Tunicates samples
-For fresh material maintain the tunicate one day before in filtered sea water.
-For colonial tunicates clean the glass slide and the colony with paintbrush.
-For solitary tunicates remove the tunic and use muscle for the mantle avoiding the digestive structures.
Day 1
1. Put the tissues in the 2 ml eppendorf and freeze intermediately in liquid nitrogen.

2. Put one bead metal bead in the eppendor and shake the tube using a Tissues lysser machine during 40 seconds at a frequency of 30 revolution per second.

3. Add 0,5ml of extraction buffer.

4. Add 0,5ml of phenol:chloroform (1:1).

5. Dissolve the sample powder with vortex by 30 seconds.

6. Centrifuge the homogenate at 14000 rpm for 5 minutes at 4°C.

7. Transfer the upper aqueous phase to a fresh tube. Add the same volume 1X of 6M LiCl.

8. Storage the tubes in a recipient with ice and storage at -20°C overnight.

Day 2

9. Centrifuge the sample at 14000 rpm for 10 minutes at 4°C.

10. Discard the supernatant.

11. Resuspend the precipitate in 1ml of 3M LiCl.

12. Shake in vortex by 30 seconds.

13. Centrifuge the sample at 14000 rpm for 10 minutes at 4°C.

14. Discard the supernatant.

15. Resuspend the precipitate in 0,25ml of free RNAse H2O.

16. Centrifuge the homogenate at 14000 rpm for 1 minutes at 4°C to remove the insoluble material (extracellular membranes, polysaccharides).

17. Transfer the upper aqueous phase to a fresh tube. Add the same volume 0,1X of 3M Sodium acetate and 2x of ethanol 100%.

18. Homogenate the tube manually.

19. Storage at -80°C for15 minutes.

20. Centrifuge the sample at 14000 rpm for 15 minutes at 4°C.

21. Discard the supernate.

22. Add 1ml of ethanol 70%, homogenate manually.

23. Centrifuge the sample at 14000 rpm for 5 minutes at 4°C.

24. Discard the supernatant manually.

25. Store the open tube on the bench for a few minutes to allow the ethanol evaporate.

26. Resuspend the precipitate in 20 ul of free RNAse H2O.

27. Incubate the homogenate at 55°C for 15 minutes to dilute the RNA precipitate, then storage at -80°C.