Juan Jiménez Merino

Células tronco circulatórias em *Styela plicata* (Lesueur, 1823) (Tunicata: Styelidae): Uma abordagem evolutiva

Circulatory Stem Cells of *Styela plicata (*Lesueur, 1823) (Tunicata: Styelidae): An Evolutionary Approach

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A Rita e Nicolás

“We need a dream-world in order to discover
the features of the real world we think we inhabit.”

Paul Karl Feyerabend
(Against Method: Outline of an Anarchistic Theory of Knowledge)

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Índice

General Introduction

Regeneration, growth, and development of most animals rely on cell populations that are capable of replacing lost differentiated cells during their lifetime (COOPER, 2000). These cells are known as stem cells (SANCHEZ-ALVARADO & YAMANAKA, 2014). Stem cells are universally defined by their simultaneous capacity to give rise to daughter cells that remain in a similar state (self-renew) and to undergo cellular differentiation (HALL & WATT, 1989). Many metazoan adult tissues maintain a subpopulation of stem cells which continually replace aging or damaged tissues (NOMBELA-ARRIETA, 2012). These renewing populations serve a variety of functions in the body. For example, they may form a protective barrier of cells against the external environment, such as in the digestive tract or skin, or they may provide a constant supply of cell types involved in developmental or physiological functions, such as the blood or epithelial cells (LI & XIE, 2005; SCADDEN, 2006).

Although most stem cells are restricted to producing somatic cell lineages in metazoans, stem cells that retain the potential to give rise to both soma and the germline in the adult have been reported in acoels, flatworms, cnidarians, sponges, and urochordates (reviewed in SOLANA, 2013). Furthermore, a conserved gene repertoire has been documented to give identity to this type of stem cell across metazoans, from sponges to mammals (ALIÉ et al., 2015). This commonality among distant metazoans suggest that stem cells may have originated from a single ancestral type of animal stem cell.

In many metazoans, stem cells that belong to the hematopoietic lineage are responsible for the biogenesis of blood. Comparisons of molecular profiles indicate that at least one type of blood cell already existed in the last common bilaterian ancestor (ARENDT, 2008; HARTENSTEIN, 2006). Furthermore, all hemocytes participate in similar functions: blood coagulation, immunological defense, and oxygen transport. Phagocytosis, aggregation, encapsulation, and graft rejection have also been reported among the immunological functions of these cell types (MUÑOZ-CHÁPULI et al., 2005; WRIGHT, 1981)

Some tunicates possess blood stem cells with a capacity to give origin to the germline and allow whole-body regeneration (BROWN et al., 2009; KAWAMURA & FUJIWARA, 1995; TIOZZO et al., 2008; WEISSMAN, 2015). This has prompted many studies of the circulatory cells in this group (KASSMER et al., 2016; VOSKOBOYNIK et al., 2008). For instance,

circulatory stem cells in colonial ascidians are fundamental for developmental processes that are related to asexual reproduction. Adult stem cells have been studied in several species that retain the potential to produce both the germline and several somatic cell types. LAIRD et al. (2005) further demonstrated precursor-progeny relationships between adult circulatory cells and germline parasite lineages, proving that these cells are responsible for stable long term chimerism and parasitism in *Botryllus schlosseri.*

Botryllids constitute a well-studied and highly derived taxon of styelids (KASSMER et al., 2016). Many observations indicate that in these colonial ascidians, circulating stem cells participate in non-embryonic development throughout the complete life cycle of the animal. For instance, circulating hemoblasts mediate whole-body regeneration in *Botryllus schlosseri* (RINKEVICH et al., 2010). This process generally takes 10-14 days to develop a fully functional adult after complete tissue ablation, but leaving the extracorporeal vasculature behind (RINKEVICH et al., 2010). We find the most remarkable evidence of circulating hemoblast involvement during vascular regeneration in *Botryllus primigenus* (KAWAMURA & SUGINO, 1999). Here, circulating hemoblasts aggregate in ridges of the vasculature at the basal region of the colony forming a solid cell mass. Next, a hollow vesicle is formed and begins to undergo morphogenesis, giving rise to the pharynx, digestive tract, the atrial epithelium, and the neural complex. Finally, the developing bud gives origin to a new individual of the colony (KAWAMURA & SUGINO, 1999).

Blood-related stem cells have also been documented accomplishing regenerative functions in solitary ascidians. For instance, regeneration research in *Ciona intestinalis* –a solitary phlebobranch– has been focused on the potential of adult stem cells to replace injured tissues and organs in the adult. After amputation of the oral siphon, *C. intestinalis* is capable or regenerating this organ through two distinct processes: One process involves blastema formation by the migration of progenitor cells produced from branchial sac stem cells (JEFFERY, 2014), and the other involves the replacement of oral siphon pigment organs without new cell division by direct differentiation of locally deployed cell precursors or by stem cells that migrate from lymph nodes at the branchial sac. Within the branchial sac, the nodules that appeared to be the source of regenerative stem cells are also related to blood cell renewal (ERMAK, 1975; JEFFERY, 2014).

The ascidian blood circulates through a semi-open system and is diverse in both composition and function. The circulatory system consists of a tubular heart enclosed in a pericardium. Blood is pumped through the system by means of peristaltic contraction originating

at one end of the heart. Due to the presence of two pacemakers, one at each end of the heart, the direction of peristalsis and blood flow reverses periodically. The heart contains no valves. A single blood vessel exits from each end of the heart, distributing blood throughout the animal (WRIGHT, 1981). Most of the blood vessels lack an endothelial lining and, as a result, the blood cells are free to migrate out of the vascular system into the surrounding tissues (ROWLEY, 1984). The blood channels in the tunic are often a conspicuous feature and in colonial forms these end in swollen epidermal projections called ampullae. Ascidian hemolymph is analogous to vertebrate blood, as it possesses circulating cells known as hemocytes that circulate in a plasm (BARNES, 1987; WRIGHT, 1981). Ascidian hemocytes however, not only participate in storage and transportation of nutrients, excretion, or defense reactions, but also in reproduction, regeneration, formation of the tunic and accumulation of heavy metals (DE LEO, 1992).

The embryological origin of blood cells has been investigated in ascidians, with the solitary *Halocynthia roretzi* and *Ciona intestinalis* as models. In *H. roretzi*, hemocytes belong to a cellular lineage that begins as a subgroup of the trunk lateral cells (TLCs). TLCs conform a group of not well-defined embryonic cells, which arise from the A7.6 blastomeres of a 64-cell embryo (CONKLIN, 1905; HIRANO & NISHIDA, 1997). Through the reaction of a specific monoclonal antibody, the fate of the TLCs was traced by Nishide et al. (1989). On this assay, antigenicity appeared on a pair of TLC-clusters situated lateral to the brain stem of the bilaterally symmetrical embryo. After hatching, stained cells were found in the entire trunk region of the swimming larva. Finally, after metamorphosis, cells in *H. roretzi* that expressed the antigen were present within the coelom and within the tunic layer of the juvenile. In addition, the antibody stained adult basophilic blood cells (NISHIDE et al., 1989). Further analyses of cell lineages from the embryonic stage to juvenile (adult) stage have confirmed this notion, namely that TLCs are the only source of coelomic cells (blood cells) of juveniles. TLCs also give rise to adult muscle cells of the oral siphon and the longitudinal muscle (HIRANO & NISHIDA, 1997).

Various tissues have been identified as putative hematopoietic niches in ascidians. In mammals, hematopoietic stem cells (HSCs) are regulated by their physical association with a discrete cellular microenvironment (i.e. the bone marrow) (PARK et al., 2012; SCHOFIELD, 1978). In ascidians, several tissues have been proposed to serve as the niche for HSCs, similarly to the microenvironments found in mammals (ERMAK, 1982; WRIGHT, 1981). These tissues may be diffuse or organized into clearly distinct assemblies. Examples of putative hematopoietic microenvironments in various ascidian species were reported by Ermak (1976). These include the

transverse vessels of the branchial sac in *C. intestinalis*, where the hematopoietic cells are arranged in small diffuse clusters adjacent to the pharyngeal epithelium. Similar clusters were observed to be associated with the connective tissue around the gut in *Chelysoma productum* and *Ascidia ceratodes*. These nodules are associated with the gonads and the connective tissue and blood vessels, but do not come in contact with the intestine epithelia (ERMAK, 1976). The colonial *Polyclinum planum* and *Euherdmania claviformis* have many distinct nodules with patches of hematopoietic cells in the connective tissue and in the blood vessels around the gut loop. Few nodules have also been identified around the post pharyngeal gut or esophagus (ERMAK, 1976).

The hematopoietic sites of *Styela clava* have also been characterized through ultrastructural assays and cell tracking (ERMAK, 1982, 1975; WRIGHT et al., 1976). The putative niches of this species are represented by clusters of hemoblasts, surrounded by maturing blood cells in various stages of differentiation. Hemoblasts are in direct contact to one another, giving them an angular shape appearance. They contain a large spherical nucleus with one or two nucleoli and diffuse chromatin. As differentiation occurs in cells surrounding these hemoblast clusters, cells lose their nucleolus and the chromatin becomes more discernible in the nucleus. Meanwhile, electron-dense granules begin to appear in the cytoplasm of some cell types. Further away from the putative niche, maturing blood cells increase in size, their granules become larger and more numerous. Cellular differentiation is also marked by the loss of polyribosomes, more numerous and elongate mitochondria, a larger Golgi apparatus, and longer cisternae of rough endoplasmic reticulum. Only a few of the peripheral cells in the putative niche appear fully mature, with complete differentiation only occurring in the circulating blood (WRIGHT et al., 1976). The proliferation rates at these putative niches have been determined using tritiated thymidine and autoradiography. Following exposure, blood cells engaged in premitotic DNA synthesis were labeled near the putative niches and in cells of the blood in surrounding vessels; in particular, the hemoblasts, lymphocytes, and leucocytes were labelled. By day 20 of tritiated thymidine exposure, most labelled cells remained in the periphery of the putative niche and presumably had differentiated into mature blood cells, whereas cells in the interior were no longer labelled. Few lymphocytes were labelled in the blood, most of the labelled cells in the nodule were unlabeled. Therefore, it appears that blood cells in this species have a relatively rapid turnover rate of several weeks.

More recent studies have suggested the intestine and the endostyle as stem cell niches. In the solitary *Styela plicata*, experimental evidence points towards the intestine as a hematopoietic niche (SANTOS DE ABREU, 2014). In this species, cell cultures of intestinal epithelia resulted in a population of proliferative cells. These cells were labelled by an anti-CD34 antibody, which is a common HSC marker. CD34 remains to be found in tunicate genomes and its presence has been recently questioned (BRADEN et al., 2014; unpublished work from our lab). Cultured cells from the intestine were induced to differentiate into putative neuroblasts (SANTOS DE ABREU, 2014). In contrast, in the colonial *Botryllus schlosseri*, the endostyle was suggested to serve as a putative niche because cell islands along the endostyle express stem cell markers, and transplants of these cells indicated somatic and germline fate potentials of differentiation (RINKEVICH et al., 2014, 2013). However, populations of cells within these cell islands include mixed populations of cell types, including phagocytes and other cell types that transiently remain there without evidence of cell homing or proliferation (LAUZON et al., 2013).

Comparative studies of development in the Styelidae offer the opportunity to explore the relationship between stem cell evolution and coloniality. This family encompasses 535 described species, half of which are colonial (SHENKAR & SWALLA, 2011). Whereas solitary ascidians reproduce strictly sexually, colonial styelids reproduce both sexually and asexually via diverse forms of budding. Colonial styelids show structural adaptations for propagation that allow them to successfully establish in a wide variety of environments. In each colonial lineage, morphologically distinct types of budding processes occur (HIEBERT et al., 2018; KOTT, 1981). In this family, transitions to asexual propagation represent a derived condition in response to selective pressures for coloniality rather than an ancestral condition (KOTT, 1981). Furthermore, the most recent phylogeny with Pyuridae and Molgulidae as outgroups suggests early-diverging solitary sister styelid lineages, including *Styela* and *Asterocarpa*, and at least two independent lineages that evolved budding: 1) a clade that encompasses Polyzoinae, plus all botryllids; and 2) *Polyandrocarpa zorritensis* (HIEBERT et al., 2018).

Despite what we know about the development of colonial styelids, the factors that facilitated the emergence of the highly derived developmental processes of asexual development are still unclear. Therefore, during this research, I have focused in the study of *Styela*, a genus that is closely related to a colonial clade, to contribute to fill the gap of knowledge necessary to understand the ancestral mechanisms of stem cell function.

Styela is a genus of early-branching solitary styelids that is readily available on the coast of São Paulo, and can be easily maintained in the laboratory. Two species were chosen in this study, *Styela plicata* and *Styela canopus*. Both species are cosmopolitan and very abundant in harbors along the Atlantic coastline of South and North America. Juveniles grow rapidly and can attain a maximum size of 8 cm in approximately 6 months (LOCKE et al., 2009; ROCHA et al., 2012). The lifecycle of these two species are similar. The larval period lasts approximately 1 to 2 days before the animal settles in a definitive substrate. Adults grow to an average size of 4 cm in length in *S. plicata* and 1 cm in *S. canopus*, and can live up to 6 months. Both species are protandric hermaphrodites with a seasonal preference to reproduce in the summer (YAMAGUCHI, 1975). In summary, the close phylogenetic position to colonial clades, extensive availability, and easy culture, make *Styela* a good representative genus to study the evolutionary transition to coloniality.

Objectives

The general objective of this dissertation is to investigate the characteristics of solitary ascidians of the genus *Styela* that are presumably involved with the evolution of colonial traits of colonial species within the Styelidae *Styela* is used to understand possible mechanisms of development that may have changed to facilitate the transition to colonial life forms from a solitary ancestor. Specifically, this research addresses the following three goals:

• To analyze the hemocyte populations, seeking to characterize and recognize cell types; in particular, undifferentiated cells that are possibly involved in regeneration.

• To analyze the morphology and histology of the distinct organs and tissues in *Styela* to distinguish putative hematopoietic niches.

• To develop a method for isolating putative circulatory stem cells in *Styela*.

Thesis organization

Apart from the General Introduction and Final Considerations, this dissertation is organized in two chapters: The first one compiles data that was generated in relation to the biology of two species of Styela, and contributes to the central theme of the thesis, whereas the second follows the format of a scientific article. The corresponding references, tables and figures are presented independently at the end of each chapter.

The first chapter describes the development and compares key blood cell types in two species of *Styela*: *S. plicata* and *S. canopus.* After a carefully analysis of these two solitary species, I discuss in this chapter relevant aspects about the putative ancestral features of colonial styelid ascidians. I highlight similarities and differences of these two species with what has been published in their colonial counterparts.

The second chapter is entitled "*Styela plicata* hemoblasts and the origin of circulatory stem cells in styelid ascidians". I characterize the blood populations by imaging flow cytometry analyses, and propose cellular characteristics to identify the hemocyte progenitors and circulatory stem cells in *Styela plicata*. In addition, I collaborated with Isadora Santos de Abreu, a doctoral student supervised by Cintia Monteiro and Silvana Allodi at the UFRJ, to describe the structure of a putative hematopoietic niche in the intestine of this species.

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Chapter 1

Comparison of hemocytes and the development of two solitary styelid species

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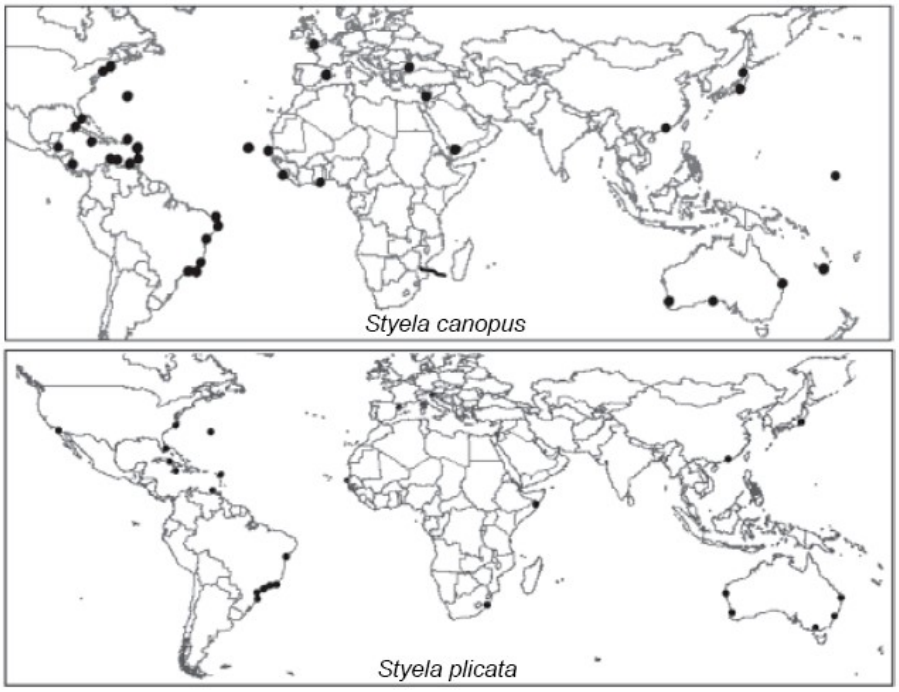
Abstract

Due to their diverse developmental modes, styelid ascidians are a fascinating group to study major evolutionary novelties. *Styela plicata* and *Styela canopus* are two species of solitary styelids with similar distributions in Brazil and good availability for laboratory experimentation. Here, I have conducted a characterization of the early development and metamorphosis of these two species. In addition, I compare the blood cell populations of the adults. Despite their conserved embryonic development, the studied species present differences at the juvenile stage. Soon after metamorphosis, *Styela canopus* briefly develops a network of extracorporeal vessels with numerous terminal ampullae. These characters are usually associated to colonial ascidians, and were not found in *Styela plicata*. With respect to the hemocyte populations, similar morphotypes were present in both species. However, *Styela canopus* shows a lower frequency of vacuolated cells, which may be due to a reduced level of cytotoxicity in the tunic relative to *S. plicata.* These differences observed between *Styela canopus* and *Styela plicata* may be related to differences in the degrees of gregariousness or body size among the two species. The relationship of the differences in characters to their proximity to the colonial groups of Styelidae should be further studied to reconstruct the evolutionary transitions to coloniality.

Key Words – cell type, evolution, hemolymph, *Styela canopus*, *Styela plicata*

Introduction

Among ascidians, the styelid body organization shows the greatest variation, and includes both solitary and colonial species with a range of intermediate morphologies (Monniot et al., 1991). The most recent phylogeny of Styelidae using the transcriptomes of 16 species shows an early diverging clade of solitary species (Hiebert et al., 2018). *Styela plicata* and *Styela canopus* are both members of this clade. Both of these species are widely distributed in temperate and tropical coastal waters (i.e. Europe, Africa, North America, the Caribbean, Brazil, Japan, Australia, and the tropical Indo-Pacific) are often overlapping in their distributions (Fofonoff et al., 2018; Locke et al., 2009; Moreno et al., 2014; Rocha et al., 2011; Rocha and Kremer, 2005). They are found on ship hulls, floating structures, piers, docks, mangroves and coral reefs (Fofonoff et al., 2018).



Global distributions of *Styela canopus* and *Styela plicata*. Reported occurrences are marked with black dots. Adapted from Rocha and Kremer, 2005.

Despite both species presenting a resemblance to the typical solitary ascidian body plan, *S. canopus* and *S. plicata* possess contrasting adult morphologies. *Styela canopus* adult body shape varies from globular to elongate depending on whether it's growing as a single individual or in a dense cluster. The adult diameter ranges from 1 to 3 cm. When growing individually, *S.*

canopus remains attached to the substrate by much of its ventral surface, with the oral siphon slightly posterior to the most anterior tip of the body. When overcrowded, the body remains attached by the ventral surface, with the oral siphon at the most anterior tip of the body, and the atrial siphon slightly posterior to it (Fofonoff et al., 2018). In the case of *S. plicata*, the body is upright and oval, covered with a tough and leathery tunic, with numerous rounded warts and pleated grooves (Carlton and Ruckelshaus, 1997). Adults of these species have been observed of up to 8 cm of diameter.

Styela plicata single specimen (right) and *Styela canopus aggregation (left).*
Collected at the Ilhabela Yacht Club.

In this chapter, I will present several observations of the biology of *S. plicata* and *S. canopus*, focusing mainly in two aspects: early development and blood cell populations. I observe sets of characters of these two species and try to relate them to the ecological niche and life cycle of each species. Comparisons among these two species allow me to address several questions about the evolutionary significance of the observed characters.

Ascidian development

The ascidian life cycle may be separated into three important developmental stages: embryogenesis (i.e., the formation of the larva), larval development (i.e., settlement and

metamorphosis), and maturation (i.e., transition from a juvenile to a sexually mature adult) (Stolfi and Brown, 2015). Embryonic development in ascidians is characterized by a highly conserved bilateral holoblastic cleavage. Embryogenesis proceeds rapidly and involves a relatively small number of cells compared to other animal models (Stolfi and Brown, 2015). After fertilization, the egg undergoes three series of synchronous cleavages and two series of asynchronous cleavages. At this point the embryo reaches the 110-cell stage and gastrulation begins. During gastrulation, a single layer of endoderm and mesoderm cells in the vegetal hemisphere invaginates into the interior as the ectoderm layer migrates toward the vegetal pole to form a surface around the embryo. The spatial relationship between the cells derived from the vegetal hemisphere is dramatically changed during morphogenetic cell movements (Kumano and Nishida, 2007).

Ascidian neurulation begins soon after gastrulation is completed. As in vertebrates, the ascidian neural plate is curled up dorsally to form a tube-like structure known as the neural tube. The formation of the neural tube progresses from posterior to anterior (Kumano and Nishida, 2007). Once it is completely closed, the larval tail is clearly identifiable. During this stage, commonly known as the tailbud, the tail continues to elongate until the embryo is ready to hatch. The notochord cells converge and extend from neurulation until an early tailbud stage (Munro and Odell, 2002b). After the notochord cells are forming a single-cell row, they further stretch along the anterior–posterior axis. This movement provides a driving force for tail elongation (Munro and Odell, 2002a).

The mature ascidian larva is similar to a tadpole. It possesses a notochord flanked by blocks of muscle and paralleled by a dorsal neural tube. The main tissues that constitute this larva are the epidermis, nervous system, notochord, muscle, mesenchyme, trunk lateral cells, trunk ventral cells (TLCs and TVCs), and endoderm (Kumano and Nishida, 2007). Ascidian embryonic development is conserved to the point of being nearly indistinguishable between some distantly related species (Stolfi and Brown, 2015).

Despite the high morphological conservation of embryonic development in ascidians, there are greater phenotypic differences in later stages. Considerable interspecific variation is found in egg/embryo size, reproductive strategies, and adult zooidal size (Stolfi and Brown, 2015). In addition, the relative timing of the different stages of the ascidian life cycle also varies substantially between species. This diversity of the ascidian adult development becomes apparent during metamorphosis. Tissue-remodeling processes during animal metamorphosis are known to occur mainly through the activation of programmed cell death in larval tissues and the

differentiation of juvenile and adult tissues (Stolfi and Brown, 2015). In the ascidians, metamorphosis into a sessile filter feeder involves an extensive reorganization of the body plan (Karaiskou et al., 2015). Metamorphosis involves a radical modification of the larval body and behavior to occupy a distinct ecological niche. This process can be divided into two phases: a rapid adhesion reaction, followed by a prolonged period of juvenile differentiation (Karaiskou et al., 2015). This transition involves numerous rapid morphogenetic movements and physiological changes, including secretion of adhesives, tail regression, sensory vesicle retraction, loss of larval tunic, phagocytosis of visceral ganglion and sensory organs, papillae eversion and retraction, emigration of blood and pigmented cells, rotation of the body axis, development of adult organs, and development of ampullae (Davidson and Swalla, 2002; Karaiskou et al., 2015).

One of the most radical novelties within ascidian taxa, is the formation of asexually-derived colonies. Colonial ascidians carry out a fourth developmental stage as a part of their life cycle: asexual blastogenesis. During adulthood, colonial ascidians develop bud-derived bodies that derive mainly from a pool of circulatory stem cells not unlike embryonic stem cells (Stoner et al., 1999). Such stem cells are required for asexual regeneration throughout the lifespan of the organism, as zooids derive from cycles of bud development (Brown and Swalla, 2012).

The ascidian blood cell classification

Many investigators have studied the blood cells of ascidians with a focus on their various functions, including immune response (Fuke, 1979; Raftos et al., 1987), accumulation of heavy metals from sea water (De Caralt et al., 2002; Pisut and Pawlik, 2002), tunic synthesis (Endean, 1954; Izzard, 1974; Sabbadin et al., 1992; Smith, 1948), and development (Freeman, 1964; Gutierrez and Brown, 2017; Medina et al., 2014). There is also a considerable amount of literature on ascidian blood cells (George, 1939; Goodbody, 1975; Rowley et al., 1984). Many classification systems have been used to classify hemocytes of different species of tunicates based on a variety of microscopical techniques, including bright field, phase contrast, fluorescence, as well as transmission and scanning electron microscopy (Fuke and Fukumoto, 1993; George, 1939; Wright, 1981).

Radford et al. (1998), Wright (1981), and De Leo (1992) have proposed generalized ascidian hemocyte classifications. Based on these, blood cells can be classified into two main categories depending on their morphological characters:

(1) agranular hemocytes, which include hemoblasts, lymphocytes-like cells, and hyaline amoebocytes;
(2) granular hemocytes, which include granulocytes with small granules, granulocytes with large granules, signet ring cells that present a single vacuole with granules, and morula cells that assume a berry like appearance (Arizza and Parrinello, 2009; de Leo, 1992).

In this study, I describe the development of *S. plicata* and *S. canopus* from their fertilization onward until the end of the metamorphosis. In addition, I compare the blood cell populations of these species, and discuss the possible causes of evolutionary similarities and differences between them.

Materials and methods

Animals

Styela plicata and *Styela canopus* adult individuals were collected at the Ilhabela Yacht Club, Ilhabela, SP. Animals were maintained in an aerated aquarium containing filtered seawater at the local temperature conditions, ranging between 21ºC and 24ºC. Laboratory animals were fed with a phytoplankton mix containing *Isochrysis galbana*, *Tetraselmis maculata* and *Dunaliella tertiolecta* at a concentration of one million cells per milliliter every two days.

Fertilization and rearing in the laboratory

I carried out a modified protocol based on Swalla, 2004. From 1 to 2 days after collection, sets of mature adults (4 in *Styela plicata*, 12 in *Styela canopus*) were separated and each specimen was bisected on the sagittal plane. Gonads were separated and removed from both halves with forceps, and minced over a mesh (250 um pore size) suspended over a beaker with 50 ml of filtered seawater (FSW). The resulting gamete suspension was kept still for 15 minutes, after which all oocytes settled on the bottom of the beaker. Supernatant containing suspended sperm was decanted in 15 mL tubes for cross-fertilization. The remaining oocyte medium was washed through reverse filtration with a 50 µm mesh and resuspension with FSW.

Sperm from each individuals was pooled to cross-fertilize each oocyte set. Gametes were mixed for 15 minutes before removal of excess sperm. Further washes were performed through

resuspension on FSW every 30 minutes for the first 2 hours after fertilization, and three times more every hour.

At 12 hours post-fertilization, developed larvae were left in the dark to settle on glass dishes. At around 48 hours post fertilization, after tail resorption, young metamorphs were carefully removed from the glass with a tungsten needle and relocated to microscopy slides using a glass pipette. During settlement, adhesive papillae fully differentiated, which allowed the animals to adhere to the slide surface. Development was documented with a stereomicroscope Leica M205 FA, and an inverted microscope Leica DMi8.

Observations on hemocyte populations

Histological analyses

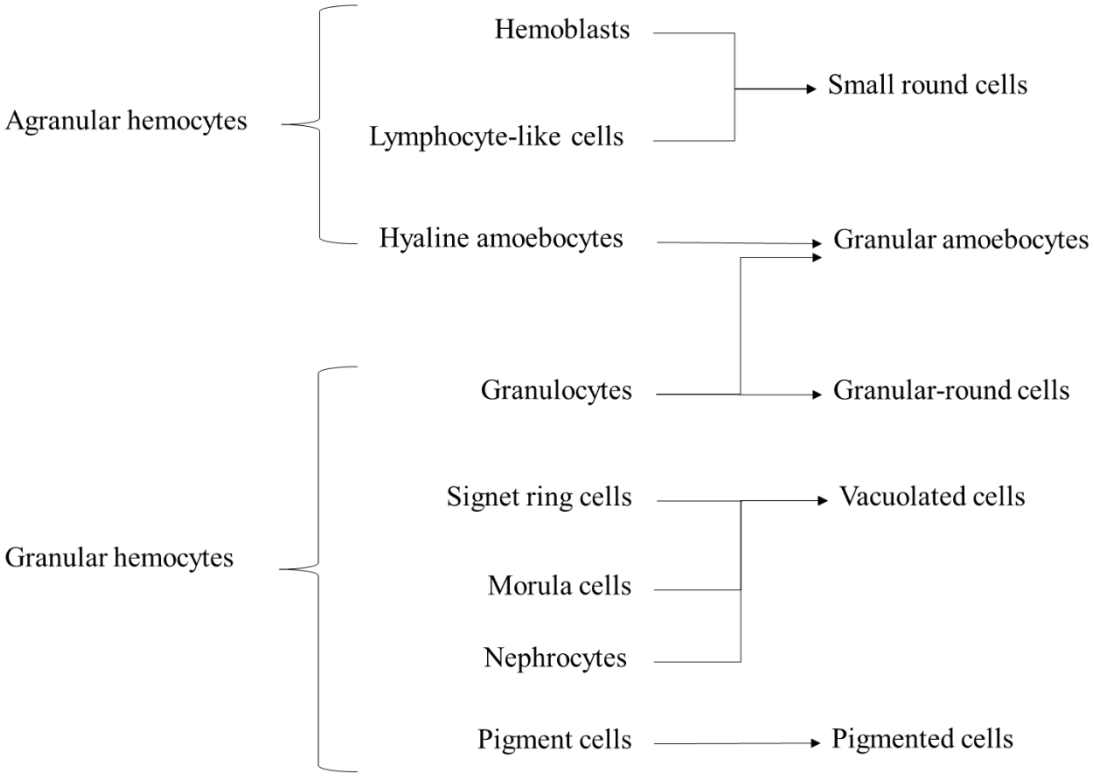
Animals were fixed in 4% paraformaldehyde (PFA) in FSW, and embedded in paraffin. Sagittal sections of 6 µm were performed in whole individuals using a Leica RM2255 microtome (Wetzlar, Germany). I used hematoxylin and eosin staining following the method of Gurr (1973). Mallory-Cason trichrome and Gomori trichrome stains were applied following the protocol by Humason (1972). Hemocytes within tissues and body cavities were identified and photographed.

Blood extraction and analysis

Blood cells were obtained by cutting the oral siphon and decanting the hemolymph into a 1,5 mL tube half-full with *Botryllus* buffer (25 mM HEPES, 10 mM cysteine, 50 mM EDTA in filtered sea water). The hemocyte suspension was centrifuged at 780 g for 10 min, and cells were resuspended in *Botryllus* buffer. Drops (approx. 60 µl) of the resuspension were transferred onto SuperFrost Plus slides (Fisher, Waltham, MA, USA). Hemocyte morphotype counts were performed on slides immediately after sample transfer, and on slides where hemocytes were left to settle for 30 min. The frequencies of the distinct cell types were calculated on 5 individuals by capturing images from successive view fields, and performing counts of main cellular morphotypes. I counted 300 cells per individual.

Classification of hemocytes was performed based on characters that could be identifiable on bright field microscopy (i.e. nucleus/cytoplasm ratio, refractile vacuoles, granules, pigment and shape). I defined 5 morphotypes as follows: small round cells, granular amoebocytes,

granular-round cells, vacuolated cells, and pigmented cells. The categories were selected because they allowed me to easily designate a category to every cell in each blood sample, which included a significant amount of cells in ambiguous or intermediate states. Moreover, I could categorize the cells with microscopical information alone through this approach. Thus, the categories used for this study joined cell types that were defined by structures that appeared on a continuum (e.g., granules), or excluded cell types that could not be identified with certainty using bright field microscopy.



Classification of hemocytes used in this study. Ascidian hemocyte types described in the literature (de Leo, 1992; Rowley et al., 1984; Wright, 1981) with the corresponding categories used in this study (far right).

Results

Field observations

At the Ilhabela Yacht club, *S. plicata* occurred in a scattered fashion. Individuals rarely settled immediately adjacent to each other. This species was most often found growing on submerged ropes or on the submerged side of floating buoys. In contrast, *S. canopus* was often

found as aggregated individuals, in which the individual tunics were often adhered to each other. The tunic of *S. canopus* was leathery, and aggregates were usually overgrown by fouling organisms. Generally multiple members of each cluster were sexually mature. In some locations of the Brazilian coastline, such as Guanabara Bay (RJ) and Santos (SP), *S. plicata* was abundant. However, even in crowded conditions, the tunic of this species remained separate (except for its base) and was rarely covered by fouling organisms.

Styela plicata and Styela canopus early development

The egg size of these two species is similar. There is an amber pigmentation at the vegetal pole of the egg. *Styela* cleavage and early development follow the conserved patterns described for the tunicates. The mature egg is defined by various layers: The follicle-cell layer, the chorion, and the space where the test cells reside. Follicle cells are conical in shape, and cover the exterior of the egg. The chorion is separated from the egg plasma membrane by a wide space filled with test cells (Fig. 1A). According to Conklin (1905), test cells originate from follicle cells that invade the egg during the development of the oocyte. The follicle and test cells in *Styela* also contain orange pigment granules gathered close around the nucleus.

The first cleavage of the fertilized egg occurs along the anteroposterior axis (Fig. 1B), followed by two perpendicular cleavages that result in a bilaterally symmetric eight-cell embryo (Fig 1C, D). Starting from each of these octants, cell lineages follow the fates corresponding to the conserved ascidian body plan (Fig. 1E-Q). Gastrulation begins during the seventh cleavage with the invagination of the large endodermal cells at the vegetal pole of the embryo (Fig. 1F). The neural plate appears as a thickening of the epidermis on the dorsal side of the larva during the ninth cleavage; then the neural folds are formed and close, generating the neural tube (Fig. 1G, H).

Following neurulation, the tail continues to elongate as the neural tube and notochord cells converge at the midline of the embryo (Fig 1H). The tail continues to extend until the embryo is ready to hatch during the tailbud stage, which lasts approx. 6 hours (Fig 1I). A fully formed larva hatches around 12h after fertilization. The larval brain, at the anterior-most part of the trunk, contains a central otolith and a reduced ocellus towards the upper anterior side (Fig 1 J, K) (For detailed time lapse of the early development, please see the supplementary videos. Vid. 1 *S. plicata*; Vid 2. *S. canopus*).

Settling and Metamorphosis

The free-swimming stage of the larva lasted between 1 and 3 days, after which the larvae undergo metamorphosis. Prior to initiating metamorphosis, the larval head changed from a streamlined teardrop shape to an oval shape (Fig. 1J, K). In addition, the anterior epithelium of the head formed three papillae (Fig 1K). The papillae secreted adhesives that facilitated the settlement of the larva to a definitive substrate up to eight hours after tail reabsorption initiated (Fig. 1K, L). Adherence to a substrate was not a prerequisite for the larva to begin metamorphosis. However, developed papillae facilitated adhesion. The beginning of the tail reabsorption was defined by the reshaping of the notochordal and muscle cells from the tail. These cells switched from their stacked configuration to globular clusters of cells (Fig 1L). These aggregates were filled with pigment and migrated towards the head, accompanying a shortening of the tail epithelium. The end of this process was marked by a total reabsorption of the larval tail. The globular mesodermal cells that migrated from posterior structures surrounded the visceral primordium, located at the anterior segment of the head (Fig 1M).

After around 8 hours from the beginning of metamorphosis, amoeboid cells embedded in the tunic of the juvenile became active, and the tunic expanded. Additionally, the epithelium of the animal started to generate four ventral protrusions (Fig. 1N). During the following two to five hours, these protrusions extended to form tube-like epidermal projections called ampullae. Ampullae extend on the underlying surface or remain extended outwards in the absence of a substrate (Fig. 1 O, P). These organs showed circulation of cells and contractile wave motions that lasted around one minute per wave (Fig 1, P). Ampullae were reabsorbed a week after settlement. This reabsorption is accompanied by an increase of the tunic thickness, and the opening of the siphons. Simultaneously, the cilia in the gill slits of the branchial basket started beating (Fig. 1Q). Heartbeats started shortly after these events. By the first week after fertilization, the circulatory system was clearly functional. Hemoblast-like cells were observed circulating through the heart and the peripheral tissues. These cells accumulated in the region surrounding the intestine and the endostyle (Fig. 2A, B).

Differences between S. plicata and S. canopus early development

The development of *Styela plicata* and *S. canopus* up until the metamorphosis follows the conserved developmental pattern of a solitary ascidian. However, variation in the hue of egg pigments in *S. canopus* was more noticeable than the variation of egg color in *S. plicata*. In the latter, egg pigment maintained a similar tone of amber, while in *S. canopus* the pigment varied from a pale yellow to an intense orange. A more conspicuous feature that was only observed in *S. canopus* took place four weeks after metamorphosis, i.e. numerous, transient ampullae extended radially from the ventral basal region of the juvenile (Fig. 2C). These ampullae were structurally and morphologically different from those that originated at metamorphosis. The terminal region of these ampullae possessed a broadened, oval terminal region, which was delimited on an external epithelium composed by thickened columnar cells (Fig. 2D) (For video of ampulla contractile wave and circulation of cells, please see supplementary material. Vid. 3). The heart was fully formed at this stage, allowing the circulation throughout the outer network of vessels. As a consequence, I observed the characteristic bidirectional circulation of ascidian blood within these vascular structures (see supplementary material. Vid. 4).

Hemocyte populations

Hemocyte populations of *Styela* showed similar morphological profiles, with differences between the relative frequencies between the studied species. In both *S. plicata* and *S. canopus*, the biggest fraction of hemocytes was composed of granular cells with various amounts of cytoplasmic contents. Cell shapes in this category ranged from round to amoeboid. Small-round cell abundance varied between individuals. (For more detailed description of the morphotypes determined in this study, please see Chapter 2). The effect of letting hemocytes settle on the slide for 30 minutes was found not significant for the morphotype relative frequencies (Fig. 3A). Even though both species showed similar cell types (i.e. hemoblasts, lymphocyte-like cells, vacuolated cells, granulocytes, and pigmented cells), the proportion of morphotypes varied interspecifically (Table 2, Fig. 3B). *S. canopus* presented a lower frequency of vacuolated cells (0,32 vs 0,06), a bigger proportion of small-round cells (0,29 vs 0,12), and a smaller proportion of granular amoeboid cells (0,06 vs 0,12) in comparison to *S. plicata*. In the case of whole-body histological sections, some characteristic cell types were recognizable (shown in Fig. 4). However, not all the features that are present in live cells could be identified in section, for which this treatment wasn't incorporated into the cell counts. Finally, most granular cells showed a strong reaction towards the presence of *E. coli*. The incubation was immediately followed by amoeboid

movements from cells with a granular profile. Reactive cells stretched their cytoplasm with a crawling motion towards the location of bacteria clusters (Fig. 5).

Discussion

In this study, I report differences between *Styela plicata* and *Styela canopus* in the development at the maturation stage, and in the proportions of cell type in the blood. I hypothesize that these differences are related to settlement strategies, which are influenced by environmental pressures on an evolutionary scale. Both species are obligate sexual reproducers. However, I argue that *S. canopus* has a preference for aggregative settlement, while *S. plicata* most commonly settles in a scattered fashion. If so, the presence of ampullae in *S. canopus* may be consistent with a developed recognition in relation to *S. plicata*.

Due to functions in immunity and development, ascidians hemocyte populations are highly variable. For instance, there are various examples that show a relationship between non-embryonic development and circulating cell populations. Macrophage-like cells (MLCs) and undifferentiated cells (UCs) in *Botryllus schlosseri* are more frequent during the brief period of takeover, i.e. when old zooids are reabsorbed and new buds continue to develop (Ballarin et al., 2008). MLCs phagocytize old tissues during takeover (Lauzon et al., 1993), whereas undifferentiated cells function as precursors of new bud tissues (Brown and Swalla, 2007). In *Symplegma brankenhielmi*, faster bud development is observed after buds are excised from experimental colonies, presumably due to higher mitotic activity of UCs, and MLCs become more abundant in the blood after surgical ablation of all buds or all zooids of the colony (Gutierrez and Brown, 2017). In the case of lymphocyte-like cells were found to be abundant in response to immune reactions in *Styela* (Raftos, 1987; de Barros et al., 2009) and morula cells belong to a cytotoxic lineage that is involved in the tunic defense to pathogens (Ballarin et al., 1995). Thus, differences in the frequencies of cell types is generally expected not only across different species, but also between individuals over time. The observed lower relative frequency of vacuolated cells in *S. canopus* could be related to the thickness of the tunic. It is worth pointing out that *S. plicata* are rarely covered by epibionts in the field, while the opposite was true for *S. canopus*. *S. canopus* have a thinner tunic and are highly aggregated. Occasionally I observed individuals that were not separated by any layer of tunic, but were only divided by individual mantles.

Various functions have been suggested for the ampullae in ascidians, including synthesis of tunic materials (Katow et al., 1978) and respiratory functions (Torrence et al., 1981). However, the most investigated role of the ampullae has been their involvement in reproduction (e.g. budding) and allorecognition of colonial ascidians (Bates, 1991; Franchi and Ballarin, 2017). For instance, ampullae have been found to participate in palleal budding in *Metandrocarpa taylori* (Watanabe et al., 1976), *Polyandrocarpa misakiensis* (Kawamura et al., 1984), *Metandrocarpa uedai*, *P. stolonifera* (Kawamura et al., 1982); and in vascular budding in *Botryllus schlosseri* (Sabbadin et al., 1975). In several colonial species, vascular ampullae are also known to participate in self and non-self recognition between colonies (Weissman et al., 1988; Oka and Watanabe, 1957). In *Botryllus*, a single gene complex comprised of approximately 30 alleles is responsible for self and non-self recognition (McKitrick and De Tomaso, 2013). If two adjacent colonies share the same alleles at this locus, their vascular systems will fuse together. The transitory vasculature observed in juveniles of *S. canopus* resembles in morphology the ampullae of a colonial styelid ascidian. To my knowledge, no reports of such structures have been reported for a solitary species. This finding supports a function of vascular ampullae in solitary ascidians that is related to processes that are not intrinsic to budding or coloniality. Therefore, the original and ancestral function of ampullae may be substrate adhesion or recognition, whereas a reproductive role in budding and allorecognition among colonies may represent a derived function of colonial ampullae in the Styelidae.

Jackson (1978) argues that vertical growth (escape in size) and aggregation favor survival of certain solitary animals. Aggregative living may be more advantageous in colonial styelids that have modular growth. For instance, modular growth is an advantageous strategy in sedentary animals for a number of reasons (Hughes, 1989): It allows organisms to grow large while preserving critical dimensions of organ systems that require limited size for optimal function. In addition, it enables greater plasticity in the form of growth, helps contain damage, and imparts regenerative capacity (Hughes, 1989). In fact, slow-growing, sheet-forming, and mound-forming animals are competitively superior in stable habitats, encroaching space and surviving for longer periods of time in one spot (Jackson and Coates, 1986). While lacking asexual reproduction, aggregative settlement in the smaller *S. canopus* may be adaptive through two mechanisms: (i) securing the growth of siblings in the adjacent space and (ii) accelerating the rate of reproduction at the expense of individual size. If advantageous, selection may act on these aggregative life forms, which may eventually lead to developmental changes that enable the evolution of

coloniality The emergence of budding could be partially a result of such evolutionary processes. However, these are hypothesis yet to undergo careful testing.

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TABLES

Table 1. Developmental times in *Styela plicata* and *Styela canopus* (in minutes)

	Styela plicata	*Styela canopus*
Fertilization	0	0
2-cell stage	20	20
4-cell stage	31	28,5
8-cell stage	46	51
16-cell stage	67	75,5
32-cell stage	107	95,5
Morula stage	128	134,5
Early gastrula stage	142	150
Mid gastrula stage	158	180,5
Late gastrula stage	179	204
Early tailbud stage	255	239,5
Mid tailbud stage	311	329,4
Late tailbud	484	405
Tadpole stage (Hatch)	525	520
Metamorphosis beginning	840-1020	840-900
Feeding beginning	2880	2880

Table 2. Observed differences in blood cell populations and development of *Styela plicata* and *Styela canopus*

	Styela plicata	*Styela canopus*
Small-round cells	0,12±0,09	0,29±0,14
Granular amoeboid cells	0,12±0,06	0,06±0,04
Large granulocytes	0,41±0,1	0,56±0,14
Vacuolated cells	0,32±0,09	0,06±0,03
Pigmented cell	0,03±0,03	0,03±0,01

FIGURES

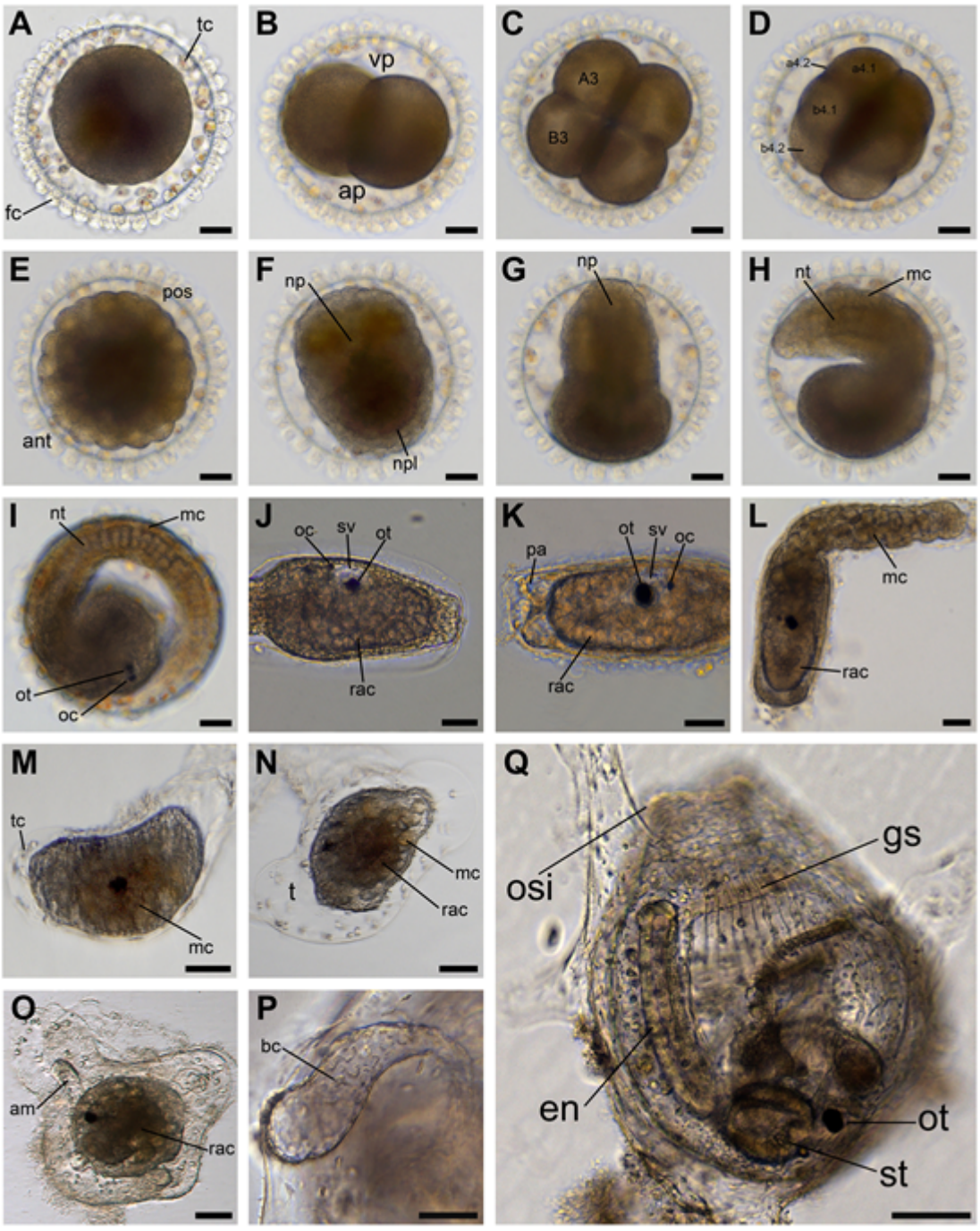
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Figure 1 - The developmental stages of *Styela plicata*

(A) Unfertilized egg; (B) Two cell stage, 30 minutes post fertilization (pf); (C) Four cell stage, 48 mins. pf. characteristic blastomeres of ascidian development can be distinguished (Hirano and Nishida, 1997); (D) Eight cell embryo 1 hour pf; (E) Blastula stage, 2h10 pf; (F) Gastrula stage, 4 hpf; (G) Tailbud stage 5 hpf; (H) Late tailbud stage, 5h30 pf; (I) Pre-hatching larva, 10 hpf; (J) Hatched larva head 12h pf; (K) Competent larva head, 14 hpf; (L) Larva during tail regression, 15h pf; (M) Early metamorphosis stage, 17h30 pf; (N) Median metamorphosis stage, 20h30 pf; (O) Late metamorphosis stage, 25h15 pf ; (P) Post-metamorphic ampulla, 48h pf; (Q) Young ascidian, 48h pf. Follicle cell (fc), test cell (tc), animal pole (ap), vegetal pole (vp), anterior region (ant), posterior region (pos), neural pore (np), neural plate (npl), notochord (nt), muscle cell (mc), otolith (ot), ocellus (oc), sensory vesicle (sv), rudiment of the alimentary cannal, tunic cell (tc), tunic (t), ampulla (am), blood cell (bc), oral siphon (osi), endostyle (en), gill slit epithelium (gs), stomach (st). Scale bars = 100µm.

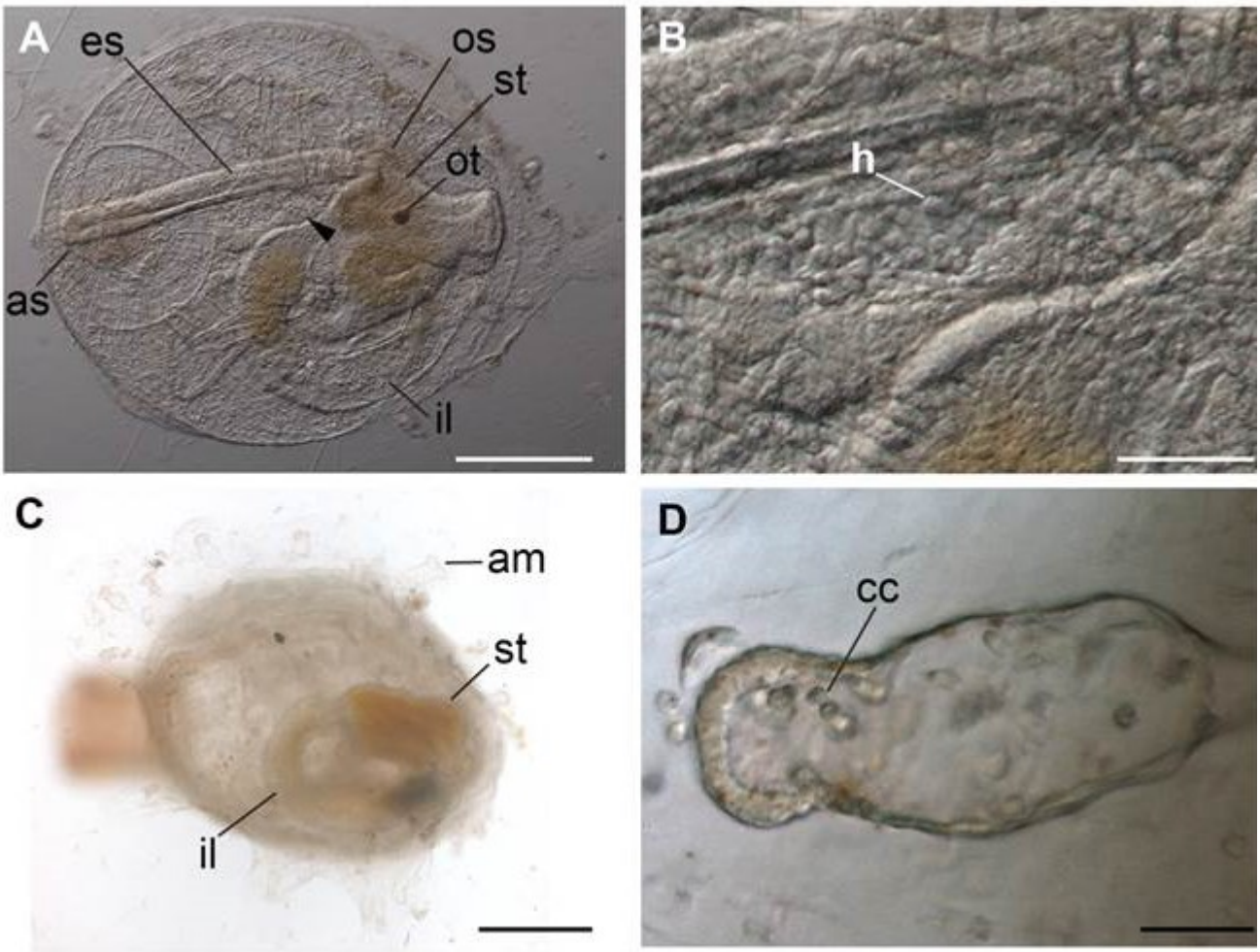

Figure 2 – *S. canopus* juveniles develop a set of ampullae after the end of metamorphosis (A) Juvenile, one week post fertilization (pf). Arrow shows hemoblast agglomeration. (B) Augmentation of hemoblast island (C) Juvenile, four weeks pf. Ampulla augmentation is shown in (D).

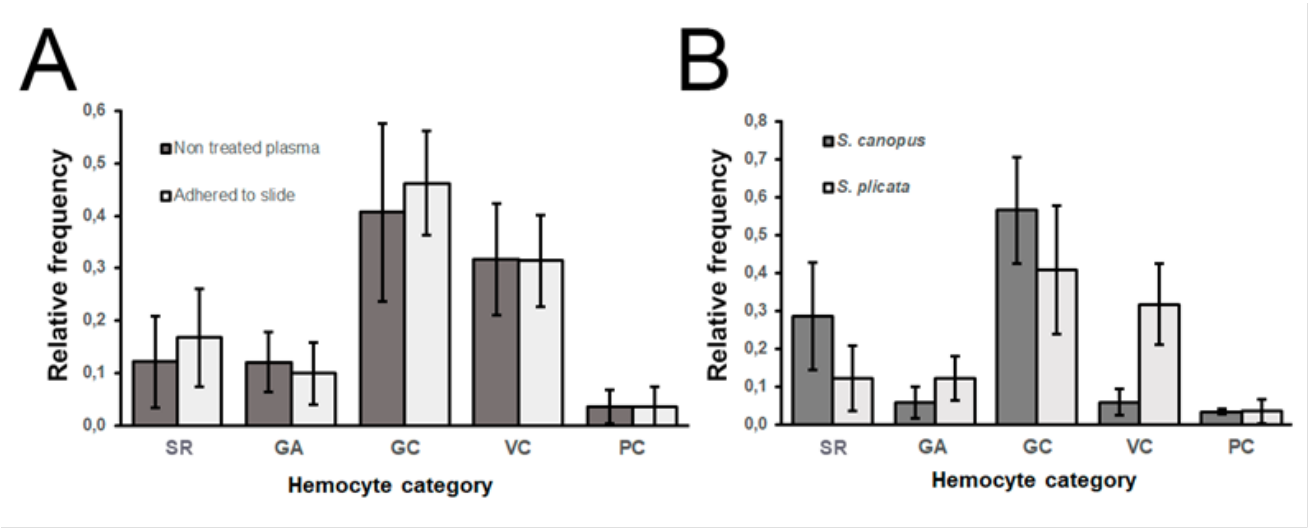

Figure 3. Cell counts on blood samples from *S. canopus* and *S. plicata* (A) Test between samples left over slide against samples analyzed immediately after extraction (B) Morphotypes counted on adhered samples, separated by species. (SR) Small-round cells (GA) Granular-amoeboid cells (GC) Differentiated granulocytes (VC) Vacuolated cells (PC) Pigmented cells

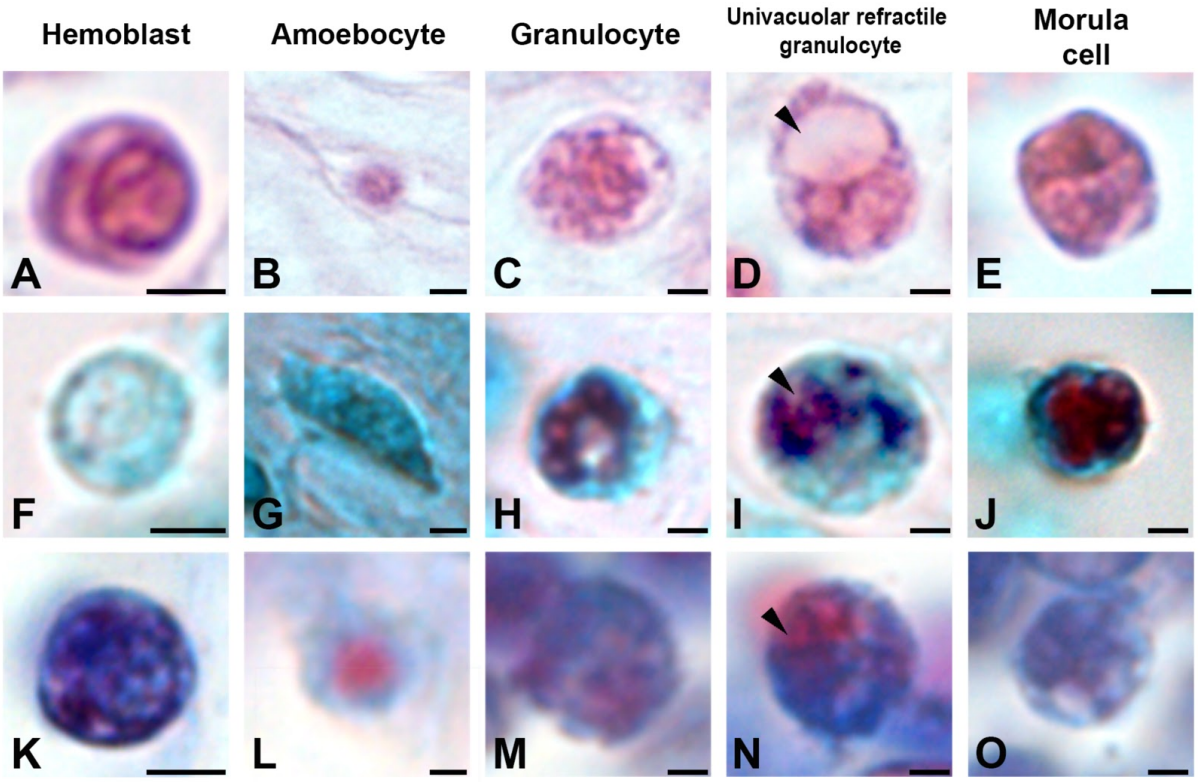

Figure 4 – Blood cell types as found in histological sections of *Styela plicata*

(A, F, K) Hemoblast-like cells; (B, G, L) Amoeboid cells (C, H, M) Granulocytes; (D, I, N) Large round granulocytes (possibly refractile) arrow points; (E, J, O) Multivacuolated cells. Microscopy techniques used: (A-E) Eosin-hematoxylin, (F-J) Gomori trichrome, (K-O) Cason trichrome. Hemocyte types (shown above) are based on de Barros (2009, 2014). Scale bars = 2µm.

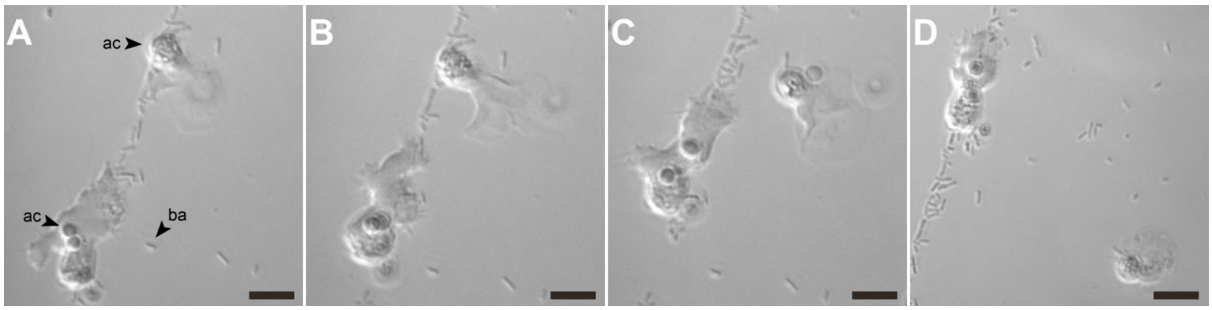

Figure 5 - Amoeboid cells from *Styela plicata* show taxis towards *E. coli* bacteria

(A) 2 minutes after exposition (B) 2,5 minutes after exposition (C) 3 minutes after exposition (B) 3,5 minutes after exposition. (ac) amoeboid cell (ba) E. coli bacteria. Scale bars = 10µm.

Chapter 2

Styela plicata hemoblasts and the origin of circulatory stem cells in styelid ascidians

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Abstract

Mesenchymal progenitor cells are present in all metazoans. In various ascidian species, circulatory putative stem cells (CPSCs) have been suggested to be involved in asexual reproduction and whole-body regeneration. However, studies of these cell population/s are restricted to colonial species. Here, we investigate the solitary *Styela plicata*. This species belongs to the sister clade to a group with two independent origins of coloniality in Styelidae. We used imaging flow cytometry to characterize CPSCs in *S. plicata* through morphological measurements and by measuring aldehyde dehydrogenase (ALDH) activity. The correlation between these parameters allowed to determine 2 gates enriched with particular cell types. We found a significant difference on the ALDH+ population within a gate of cells with low granularity, suggesting the presence of cells among circulatory hemocytes. To scrutinize the

biogenesis of CPSCs in *S. plicata*, we performed an exhaustive histological survey for hemoblast-like cells, complemented with immunohistochemistry with stem cell (piwi) and proliferation (pHH3) markers. We found morphological and expression profiles that support the intestinal submucosa (IS) as a hematopoietic niche. At this region, we found aggregations of hemoblasts, corroborated by ultrastructural analysis. Furthermore, the IS holds high cellular proliferation and frequency of piwi+ cells.

Key Words – Blood cells, cell progenitors, Evolution and Development, hematopoiesis, hemocytes, intestine, proliferation

Introduction

Most of the metazoans possess freely moving cells within their body, referred in coelomate animals as coelomocytes and/or hemocytes (Hartenstein, 2006). The diversity of structure and function among these cells is wide. However, they accomplish similar functions among taxa, namely, blood coagulation, immunological defense, oxygen transport and tissue repair (Glynn, 1974; Muñoz-Chápuli et al., 2005). It is widely accepted that animal hemocyte lineages derive from an evolutionarily conserved cell type, the blood stem cell (Lebestky et al., 2000; Wright, 1981, Hartenstein, 2006). In a comparative review, Hartenstein (2006) linked conserved molecular pathways to the development and function of blood cells. Among the homologous cell types that were identified, the hemocytoblast, or blood stem cell, is likely to be the lineage that gave rise to other hemocyte types.

Two distinct life forms are distinguished among ascidian species: Solitary and colonial. While solitary ascidians possess a unitary adult body plan, colonial species follow a modular layout supported by the asexual replication of clonal zooids (Wanninger, 2015). Both solitary and colonial ascidians possess circulating progenitor cells, referred to as hemoblasts (Wright, 1981). In colonial ascidians, hemoblasts have been suggested to be involved in key developmental processes that are linked to the organism life history, i.e. whole-body regeneration and budding. The cellular mechanisms that underlie this relationship have been partially elucidated. Freeman (1964) contributed to this knowledge on his seminal study of *Perophora viridis*. In this colonial species, whole-body regeneration and budding occur as a consequence of stem cells circulating in the blood (Freeman, 1964).

The origin and function of circulatory putative stem cells (CPSCs) has been well described in botryllids. In this group, multipotent cells are progenitors of the blood cell line

(Sabbadin, 1955) and germline (Sabbadin and Zaniolo, 1979). Botryllid CPSCs seems to play a fundamental role in the biogenesis of new zooids (Brown and Swalla, 2012; Laird et al., 2005; Rinkevich et al., 2010), and in the transmission of germline and somatic cell lineages (Brown et al., 2009a). In addition, the differentiation potential of hemoblasts has been experimentally proven in colonies surgically reduced to the peripheral tunic with circulatory vessels containing blood, moved by the contractile ampullae (Brown et al., 2009b). In these conditions, hemoblasts adhere to vessel walls and give rise to budlets, which mature into functional zooids, thus restoring the colony to its original phenotype (Manni et al., 2007; Sabbadin et al., 1975). As in *P. viridis*, regenerative potential in *Botryllus schlosseri* appears to be mediated by a specific population of self-renewing circulating cells (Laird et al., 2005). Both, *Perophora* and *Botryllus*, maintain circulatory progenitors in the blood. *Perophora* progenitors were proven to be pluripotent (Freeman, 1964), whereas circulatory *Botryllus* progenitors are considered multi or unipotent (Kawamura and Sunanaga, 2010).

Based on these observations, we hypothesize that in ascidians, the origin of novel lineages of circulatory putative stem cells is likely linked to major developmental transitions. Therefore, the evolutionary events that gave rise to botryllids and other colonial styelids could be understood by investigating the ancestral character state of hemoblasts in the group. This has been made possible by a recent understanding of the phylogenetic context in which colonial styelids evolved. A recent transcriptome-based phylogeny has shown at least two independent origins of coloniality in Styelidae (Hiebert et al., 2018). According to this phylogeny, solitary styelids, including *Styela* and *Asterocarpa*, form a sister clade to another lineage, in which two events of coloniality occurred. One of these events gave rise to the colonial lineage of *Polyandrocarpa zorritensis*, while the other comprises the rest of colonial styelids (Hiebert et al., 2018).

Therefore, we study the solitary *Styela plicata* as an outgroup species to approximate the ancestral character state of hemoblast populations in the styelid ascidians. *S. plicata* is a cosmopolitan species, abundant in harbors of the Atlantic coastline. Juveniles grow rapidly and can attain its maximum size of 8 cm in six months (Locke et al., 2009; Rocha et al., 2012). In Brazil, this species is found along the southeastern coastline, including the harbors of Rio de Janeiro, São Sebastião, and Santos (Locke et al., 2009). The life cycle encompasses a brief (1 to 2 days) larval period before the animal settles on a definitive substrate.

The two main classification studies on *S. plicata* hemocytes (Radford et al., 1998; de Barros et al., 2009) revealed that this species presents five main cell type in its hemolymph: Granulocytes, lymphocyte-like cells, morula cells, pigment cells and hemoblasts. Lymphocyte-like cells are 3,1

to 4,8 in diameter, and present small vesicles that resemble the rough endoplasmic reticulum, and mitochondria.

In *S. plicata*, hemoblasts are also presumably involved in regeneration and share similar morphological characteristics to the colonial circulatory stem cells (Medina et al., 2014). After experimentally induced neurodegeneration, recruitment of a non-neural cell type to the site of injury was observed in *S. plicata* adults. These cells showed morphological and molecular characteristics of undifferentiated cells (Medina et al., 2014). A somatic stem cell role has been proposed for this specific subpopulation of hemocytes. Thus, we hypothesize that solitary and colonial circulatory stem cells derive from a common ancestral stem cell lineage, constituting a symplesiomorphic character of styelids.

In addition to hemoblasts, lymphocyte-like cells have been identified in *S. plicata* as a distinct blood cell type. This hemocyte presents similarities to the hemoblast, such as a small size and a large nucleus. However, *S. plicata* lymphocyte-like cells lack a nucleolus and are related to inflammatory reactions. This could be a source of confusion in nomenclature, since *Botryllus* hemoblasts have been frequently denominated "lymphocyte-like cells".

Here, we performed a complementary analysis of circulating hemocytes applying imaging flow cytometry using morphometric parameters and a stem cell marker. We provide a characterization of the progenitor of hemocyte population of the solitary styelid *S. plicata* using light and electron microscopy techniques. We identified sites of hemoblast aggregation that suggest the presence of hematopoietic niches in ascidians (Sawada et al., 1994). Finally, we explore the possibility of functional divergence of the ancestral stem cell type between *S. plicata* and other colonial styelids.

Methodology

Animal collection

Adult individuals were collected from the Ilhabela Yacht Club, São Sebastião (23°46'20'' S, 45°21'20'' W), the Ponte Edgard Perdigão dock, Santos, (23°59'30.60'' S, 46°18'10.27'' W) and Praia da Urca harbor (22°56'43" S, 043°09'48" W), Rio de Janeiro, Brazil. Animals were maintained in aerated aquaria containing seawater and following local temperature conditions that ranged between 20 and 24˚C, pH between 8 and 8.5 and salinity 35 g/kg.

Hemocyte description

For hemocytes harvest, five adult *S. plicata* were cut through the oral siphon and hemolymph was decanted into a 1.5 mL tube half-filled with *Botryllus* buffer (25 mM HEPES, 10 mM cysteine, 50 mM ethylenediaminetetraacetic acid in seawater, pH 7.5). The hemocyte suspension was centrifuged at 780 X g for 10 min, and cells were resuspended in 60 µL *Botryllus* buffer. Drops of this suspension were transferred onto SuperFrost plus slides (Fisher, Waltham, MA, USA), and left to settle for 30 min. To distinguish intracellular compartments, hemocytes were stained with Neutral Red, for acid vesicles, or Sudan Black for lipids. Neutral Red (Merck, Darmstadt, Germany) saturated solution on FSW was added on equal volume to the slides and cells were immediately prepared for observation. For Sudan Black staining, hemocytes were fixed in 70% ethanol, immersed in a Sudan Black (Sigma Aldrich, St. Louis, USA) saturated solution (70%), washed once in ethanol (3 min), and once in distilled water (3 min). Coverslips were mounted with glycerol and sealed with nail polish.

S. plicata hemocytes have been characterized (Radford et al., 1998; de Barros et al., 2009). Five cell types comprise the blood cell population in this species: Hemoblasts, lymphocyte-like cells, granulocytes, morula cells and pigment cells (Table 2). Hemocyte types were identified in live cell pools and key structures were determined for further classification of cells in ambiguous stages of differentiation. To be able to classify and measure relative frequencies from all observed cells, including intermediate states, we defined and described general morphotypes that can be identified with light microscopy. The relative frequency of morphotypes was estimated by counting Neutral Red-stained cells (two replicates for each individual). Relative frequencies were evaluated after counting 10 fields for each glass slide, corresponding to at least 400 cells, from contiguous fields of view of the cell suspension at 63x. We used four adult individuals of similar size (approximately 4 cm) as replicates for our analyses.

Flow cytometry

Twelve animals with a wide range of sizes (1-4.5 cm length), in order to capture variation between body mass, were selected for analyses. Water ejection was induced through light pressure and weight was measured. Blood was extracted from each individual as previously

described. Cell concentrations were calculated using a hemocytometer. All samples obtained had a cell concentration above 3 x 10^6 cells/mL. We used 50 µL samples of hemocyte suspensions at a concentration of approximately 1x10^6 cells/mL, which were acquired by centrifugation at 790 g for 10 min and resuspension in *Botryllus* buffer.

To further identify putative CSC populations in the blood, we used the viable stem cell, BODIPY-amino acetaldehyde (BAAA)-based kit, Aldefluor (STEMCELL Technologies) to test for the presence of undifferentiated cells. Aldefluor has been extensively used to detect HSCs and progenitors in humans and mice (Fallon and Gentry, 2003; Jones et al., 1995; Storms et al., 1999). Additionally, ALDH is present both in mammalian HSCs (Jones et al., 1996) and in the growing asexual buds of a colonial ascidian, *Polyandrocarpa misakiensis* (Kawamura and Fujiwara, 1995, 1994) as well as somatic and germline circulating stem cells in *B. schlosseri* (Kassmer et al., 2016; Laird et al., 2005). Cells were stained in 1–2 mM BAAA solution in *Botryllus* buffer at 37°C for 20 min. N, N-diethylaminobenzaldehyde (DEAB), an inhibitor of ALDH, was used at 200 mM in the staining solution to generate negative controls. Cells were washed twice, resuspended in *Botryllus* buffer and maintained on ice until analysis.

Sample acquisition was performed in an image flow cytometer (FlowSight, Amnis-Merck Millipore) from the Central de Aquisição de Imagens e Microscopia of the Instituto de Biociências (CAIMI-IB). Acquisition speed was set to low and the highest resolution. About 20,000 cells were acquired based on area and the aspect ratio, defined as the value of minor axis divided by major cell axis on channel 1. The focused cells were gated using root mean squared gradient (RMS gradient) based in channel 1. Channels 1, 2 and 6 were used to analyze bright field, Aldefluor labeling and side scatter (SSC) parameters respectively, using the IDEAS software (Amnis-Merck Millipore). Cell debris and doublets were gated out based on area and aspect ratio features. The singlets were gated in ALDH+ cells and subpopulations of hemocytes were determined based in channel 2 and channel 6 intensities. Data acquisition was performed using the IDEAS package (Amnis, Seattle, USA). To detect significant increases in ALDH+ event abundances using the Aldefluor test, we performed Wilcoxon matched-pairs signed rank tests between tests and controls (as defined by the ALDH inhibitor DEAB).

Whole-body histological analyses

Whole animals were fixed in 4% paraformaldehyde (PFA) in FSW, and embedded in paraffin. Sagittal sections of 6 µm thick of whole individuals were performed using a Leica RM2255

microtome (Wetzlar, Germany). For observing general tissue morphology and organization, we used hematoxylin and eosin staining following the method of Gurr (1973). Mallory-Cason trichrome and Gomori trichrome stains were applied following Humason protocol (1972).

Immunofluorescence

The intestines of five ascidians were dissected and fixed with 4% PFA in artificial seawater for 24 h. Tissues were dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, 100%) for 30 min on each concentration. They were cleared with xylene for 20 min, and embedded in Paraplast (Sigma, St. Louis, MO, EUA). Histological sections of 5 µm were obtained on a rotating microtome (American Optical). Tissues were dewaxed with xylene and rehydrated in decreasing concentrations of ethanol and water.

PIWI labeling

Histological sections obtained as described above were incubated with 10 mM of citrate buffer (with 0,05% Tween 20, pH 6) at 90°C for 30 min for antigen retrieval, and cooled for 20 min in a 0,1% Tween (PBT) solution in PBS, followed by a 5-min wash with PBT. Sections were incubated with PIWI primary antibody (1:100; Abcam, Cambridge, UK) in PBT for 48 h. Then the sections were washed in PBS, incubated with anti-rabbit biotin (K0679, DAKO; 1:100) for 1 h, subsequently incubated in Cy3-associated streptavidin (Sigma-S6402; 1:500) for 1 h. After, the sections were washed in PBS, the nuclei were labeled with DAPI for 10 min and the sections washed and mounted with Fluoromount (Sigma-F4680). Control samples skipped the primary antibody incubation.

pHH3 labeling

Sections obtained as explained above were kept in 2M HCl at 50°C for 30 min, then transferred to 10 mM citrate buffer pH6 at 90°C for 30 min, chilled in PBT for 20 min and incubated in anti pHH3 antibody (Santa Cruz, sc8656-R; 1:50) for 4 days in the refrigerator. The final PBS washes, incubation with secondary antibody, DAPI staining and mounting were performed as described above.

Raw data were collected from a series of independent assays of three animals of each group. For the control group, the immunofluorescence reaction was performed in the mantle, for the PH3 group, in the intestine. Analysis of fluorescence/area of images to phospho-histone-H3 immunofluorescence were then compared using Student's t-test. Results are expressed as the mean standard error, and differences were considered statistically significant at $p<0.05$ (*). The analyses were done using GraphPad Prism version 6.00 from GraphPad Software, Inc.

Transmission electron microscopy

Ascidian intestines were dissected and fixed with a 2.5% glutaraldehyde solution and 0.1 M sodium cacodylate in artificial sea water for 24 h, washed in 0.1 M cacodylate buffer and post fixed in OsO_4 for 40 min. Following this, the material was pre-contrasted with 1% uranyl acetate diluted in artificial sea water for 24 h. The intestines were washed again in 0.1 M cacodylate buffer, dehydrated in increasing concentrations of acetone (30% to 100%) and infiltrated in Embed 812 resin (Electron Microscopy Sciences). Ultrathin sections (60 nm) were obtained in an RMC ultramicrotome, collected on copper grids (300 mesh), contrasted with 2% uranyl acetate for 20 min and with 1% lead citrate for 3 min. The sections were observed under a JEOL 1011 transmission electron microscope, operated at 80 kV, in the Rudolf Barth Electron Microscopy Platform from Instituto Oswaldo Cruz/FIOCRUZ (Rio de Janeiro).

Results

Frequency and characterization of hemocyte morphotypes in Styela plicata

We established hemocyte categories based on morphological features. We found 8 cell types that were previously described in *S. plicata*, as well as in other ascidians (De Barros et al., 2014, 2009, 2007; de Leo, 1992; Wright, 1981): Lymphocyte-like cells, hemoblasts, univacuolar refractile granulocytes, amoeboid granulocytes, compartment cells, univacuolar cells, morula cells, and pigment cells (Fig. 1A-X). Roughly 50% of blood cells were not easily classifiable into any of the previous cell types or lead to ambiguous classification (data not shown). For simplicity, we decided to combine some of the blood cell categories above into the following five hemocyte morphotypes (Table 1):

1. **Small-round cells:** These represent the smallest cell types (2.8 – 6 μm in diameter), which a represent 1/6 of all cells in the blood (16.7 ± 9.3%, n= 4). Circular cells with a high nucleus/cytoplasm ratio with few or no granules in the cytoplasm.
2. **Granular amoeboid cells:** Small amoeboid cells (4 - 8 μm) with granular cytoplasm that represent 1/10 of all cell types (9.9 ± 5.9%, n=4).
3. **Fully differentiated granulocytes**: Rounded cells with intermediate size (5 – 8.6 μm) that represent nearly 1/2 of all blood cell types (46.2 ± 9.9%, n=4). These cells contain numerous granular structures in the cytoplasm, which are most likely inside a large refractile vacuole.
4. **Vacuolated cells:** These cells represent ⅓ of all cells (31.4 ± 8.8%, n=4), and vary in size (5 – 20 μm). One or more prominent basophilic vacuoles are present in the cytoplasm.
5. **Pigment cells:** These large cells (8 - 13 μm) are rare (3.5 ± 3.9%, n=4), and are saturated with cytoplasmic compartments filled with amber pigment.

Overall, granulocytes represented the most common morphotype, followed by vacuolated cells, small-round cells, and pigmented cells (Fig. 1Y; Table 1). Hemocyte populations presented fluctuating frequencies of blood cell morphotypes among individuals analyzed.

Hemocyte populations with high aldehyde dehydrogenase activity —typical of stem cells— are enriched with hemoblast-like cells

To test whether stem cells are present in the blood of *Styela plicata*, we used imaging flow cytometry to characterize morphological features and the enzymatic activity of cell populations. We analyzed the overall composition of blood cells in individuals of different size (1 - 6.5g), and used a combined strategy that evaluates light refraction and enzymatic features to separate populations of blood cells in these individuals. When size of individuals (measured by mass) was correlated to the variation in cell complexity (measured by the standard deviation of side scatter, SS), we observed a positive correlation (r^2 = 0,58, p=0,038) between these two variables (Fig. 2A). Despite an observed increase in the variation of cell complexity in larger animals (Fig. 2A), similar populations of cells (gates 1-6) could be identified among all individuals (Fig. 2B-C).

We evaluated the relative abundance of CPSCs in the hemocoel of *Styela plicata* by the detection of fluorescently labelled ALDH+ hemocytes. The 6 populations of cells (gates 1-6) used in our analyses are shown in the scatterplot distributions of the aldehyde dehydrogenase

(ALDH+) cells and their corresponding control with ALDH inhibitor DEAB of one of representative individual (Fig. 2B). We applied a gating strategy based on apparent visual trends. Following a conservative approach, we selected six different gates for every recognizable population that was consistent among individuals (Fig. 2B). The gates reflected visual differences of blood cell populations (Fig. 2B, C; coordinates of the chosen gates polygons are provided in Table 3) and showed invariant aggregations repeatedly among the twelve analyzed individuals of different size. Five randomly selected cells for each gate are shown to highlight the variation of cell types we observed by imaging flow cytometry analyses (Fig. 2C). While cells on each gate varied notably in size, shape, and morphology (bright field, BF), the levels of fluorescence (ALDH+ activity) and cell complexity (side scatter, SS) assisted us in the assignment of the distinct cell populations (Fig. 2C). Gates 1, 2, and 3 showed distinct dispersion patterns and increasing degrees of complexity, as well as BAAA fluorescence (Fig. 2B-C). Gates 4, 5 and 6 indicated high ALDH activity and were located above relative intensity values of 3100 (Fig. 2B). When comparing the populations of blood cells (gates 1-6) between individuals, we consistently observed a significant increase in cell relative frequency of aldehyde dehydrogenase activity (ALDH+) in two populations of blood cells (gates 5 and 6, $p<0,01$), which also showed relatively high complexity (i.e. high SS) (Fig. 2B, D). We observed a significant increase of 7,36 ± 6,8 % ALDH+ cells within the enriched gates 5 and 6. In contrast, we observed a decrease in the relative frequency of aldehyde dehydrogenase activity (ALDH+) in two populations (gates 1 and 2, $p<0,05$) (Fig. 2B, D).

To test whether the two populations that showed a significant increase of Aldefluor enriched cells (gates 5 and 6) were part of a single population of cells, we performed multiple correlation analyses between individual size and gate values of all recorded cell measurements. Only one population of cells (gate 6) showed little or no variation in cell size among individuals of different size (Fig. 2E), suggestive of a homogenous population that may contain the CPSCs with hemoblast-like cell characteristics. In contrast, the other high ALDH+ population (gate 5) showed an increase in the standard deviation of blood cell area in bigger ascidians (Fig. 2E), suggestive of a more heterogeneous population of cell types within this gate.

Undifferentiated hemocytes are clustered in the subepithelial region of the S. plicata intestine

The general organization of the intestine tissue comprises three regions: (1) an epithelium constituted by a monolayer of columnar cells and a thin basal lamina. We nominated this

epithelium as mucosa (Fig. 3 A, C); (2) connective tissue and glands, in addition to compartments of cells (Fig. 3 A, B, E, F, G). We denominated this region as the submucosa; (3) a serosa layer, separated from the connective tissue above by a thin layer of muscles, and constituted by connective tissue and a simple cubic epithelium (Fig. 3 A, B, D). The serosa shares a common space with other organs through a series of epithelium-bound protrusions that face the coelom (Fig. 4 B). Immunofluorescence revealed a number of hemocytes in the subepithelial region of *S. plicata* intestine expressing different markers (Fig. 3): pHH3+ cells varied in their frequency, possibly depending on the intestine region. Clusters of putative hemoblasts at the submucosa showed proliferative activity in some of the cells proximal to glandular structures (pHH3+ cells varied from 5 to 40% of intestine cells; N=2, eight images analyzed; Fig. 4 H-I). In contrast, piwi+ cells consisted of young cells with morphological features of determination to a differentiated lineage in the subepithelial region of the intestine. Up to 4% of cells (N=2, 10 images analyzed; Fig. 4 J-L) were labeled in this region. We found no nonspecific labeling in the controls.

Ultrastructural observation revealed that, at the submucosal region, there are cells that appear to be from the hematopoietic lineage (Fig. 4 A, B, C). These cells have characteristics of undifferentiation: a heterochromatic nucleus that occupies almost all the cytoplasm, electron-lucent cytoplasm and few granules. Other cells appear to be more differentiated hemoblasts, mainly because the nuclear chromatin is more condensed and the nucleus is smaller in relation to its cytoplasm (Fig. 4 C). Occasionally, differentiation traits were observed in some hemoblasts, namely presence of granules, condensed chromatin and an irregular perimeter of the plasma membrane (Fig. 4 A, B, C). In Addition, we observed a granulocyte within the undifferentiated cell cluster, close to the hemoblasts (Fig. 4 D). A part of a gland in the submucosal layer can be seen in Figure 4 D. Below the glandular epithelium a hemoblast can be seen, immersed in fibrils present in the extracellular matrix that fills the organ. This hemoblast had heterochromatic nucleus, an apparent nucleolus, a cytoplasm filled with few granules and a relatively small nucleus / cytoplasm ratio. We also observe electron-dense granules in the apical portion of these epithelial cells.

Discussion

Characteristics of circulating cell populations in Styela plicata

The characterization of blood cell types in ascidians has historically lacked a uniform nomenclature in the literature due to methodology biases and subjectivity (Cima et al., 2001; de Leo, 1992; Wright, 1981). In this study, we have used key characteristics to determine categories that encompass all cells in the blood. Progenitor cells, in particular, are characterized in all classifications of hemocytes among ascidians. The characteristics of the hemoblast profile in *S. plicata* are: small size (≈4 μm), uniformity, a big nucleus, a nucleolus and a low cytoplasmic volume (Barros et al., 2009; Radford et al., 1998). These conserved morphological characteristics allowed us to identify this cell type after application of diverse techniques. Cell morphology observed on flow cytometry imaging shows a trend between gates on cell complexity, regarding shape and number of cytoplasmic elements. However, functional cell types known for this species (see Table 2) were not categorized through this assay.

We observed a high individual variation in the *S. plicata* hemocyte populations. Blood, as is related to quick response mechanisms of the body, is susceptible to various changes in cell type frequency. However, flow cytometry analysis allowed us to detect a positive trend in the relationship between the overall variation of cell surface complexity and size of the animal (measured through the operational variables side scatter and body weight, respectively). According to Yamaguchi (1975), *S. plicata* becomes sexually mature at about 40 mm in body length. It takes about 2 months in summer and nearly 5 months in winter for cultivated specimens to grow to this size (Yamaguchi, 1975). According to Yamaguchi's (1975) *S. plicata* growth curve, animals used in our flow cytometry assay could be between 1 and 5 months old.

These observations lead us to formulate three gates for cells that occupied a high range of ALDH activity. Among all gates analyzed, gates 5 and 6 showed a significant increase in cell proportion between Aldefluor treatments and controls. This is indicative of both gates covering a single population. However, correlation analysis brought a distinct difference between variation trends between gates. While gate 6 SS standard variation is not changed between sizes, gate 5 shows a significant correlation. Therefore, gate 6 is most likely enriched with a CSC population, assuming a self-renewing profile for this lineage. The fact that BAAA+ gates proportion increase was accompanied by a decrease in gates 1 and 2 suggests that cells shift their position between

the two regions. Lymphocyte-like cells, as they are most likely located in gates 1 and 2, as they fit a low granularity/low ALDH activity profile.

In *B. schlosseri*, the frequency of cells capable of differentiation and self-renewal increases tenfold in BAAA+ cells, in comparison to non-sorted cells. According to Laird et al. (2005), 10-20% of all *B. schlosseri* cells are BAAA+. Disregarding the vast difference between both models CSC potential, it is apparent that *S. plicata* may have lower concentration of circulating hemoblasts than *B. schlosseri*, more so assuming that cell total has a lesser frequency of stem cells than the blood.

The structure and function of circulating hemoblast populations in Styelidae possesses great complexity. The fact that potential niches of circulating hemoblasts could have already been present in a styelid ancestor, suggests that a degree of plasticity was inherent in this group at the time coloniality evolved. Flow cytometry imaging evidences that gate 6 ALDH+ cells mostly present hemoblast features (i.e. lack of granules, round morphology with prominent nucleus). Key modifications in stem cell characteristics may have allowed for coloniality to evolve as a result of novel selective interplays between lineages. Further insights into this evolutive transition will come from comparisons among species of interest in Styelidae.

The intestine as a possible hemoblast niche

The observed pattern of labeled cells at the intestine submucosa suggests a unique cell identity occurring at this site. A population of hemoblasts at this site is proliferating and confined to a physical matrix. In a number of developmental systems, CPSCs are controlled by a particular microenvironment, known as a "niche". Studies have revealed that the stem cell niche is composed by a group of cells in a specific localization in the tissue (Adams and Scadden, 2006). Additionally, the niche functions as a physical anchor for stem cells, apart from generating extrinsic factors that control their number and fate (Li and Xie, 2005).

Stem cell niches are characterized by a basal membrane that provides mechanical anchoring to the cells (Li and Xie, 2005). If progenitor cells in the intestine are acting as putative stem cells, the submucosa may be providing the basement membrane necessary for the niche maintenance. Cells that originate in this region are connected to blood circulation through epithelial protrusions of the serosa.

To test for the stem cell identity on candidate intestine hemoblasts, we used gene markers of stemness maintenance (piwi) and proliferation (pHH3) through IHC. Stem cells in non-model

organisms have been previously explored using this approach (Brown et al., 2009b; Kawamura and Sunanaga, 2010). For instance, piwi, a protein nuclear complex, is expressed in totipotent germline cells. This protein is considered a key regulator of gene expression (Sunanaga et al., 2010). Piwi belongs to the conserved PIWI/Argonaute superfamily of RNA interface effector proteins, which are essential for self-renewal and maintenance of germline and somatic stem cells in *B. schlosseri* (Kawamura and Sunanaga, 2010; Lin, 2007; Sunanaga et al., 2010). Furthermore, this protein complex has been described in sponges, planarians, ascidians, *Drosophila*, the zebra fish and mammals (Funayama, 2010; Kuramochi-Miyagawa, 2004; Qiao et al., 2002; Reddien et al., 2005; Tan et al., 2002; Vagin et al., 2006).

The ultrastructural analysis from the intestine of *S. plicata* revealed cells with the same characteristics already described for undifferentiated cells found in the hemolymph of this species (Barros et al., 2009). On the intestine submucosa, we observed a high frequency of cells with a high nucleus/cytoplasm ratio and few or no organelles. However, some cells contain granules in the cytoplasm. This is resembling of the arrangement of hematopoietic tissues in other invertebrates, such as the crayfish, in which the central cell is marginally bigger than the adjacent cells (Chaves da Silva et al., 2013). It has been hypothesized that the presence of undifferentiated cells in the mammal intestine is linked to its capacity for cell renewal (Simons and Clevers, 2011) and that intestine homeostasis is mediated by intestinal stem cells (Barker et al., 2007). Thus, our observations in *S. plicata* support the intestine as a part of the hematopoietic system, thus implying a high degree of functional plasticity in this organ.

In *B. schlosseri*, potential stem cell niches have been identified in the anterior ventral region of the endostyle (Voskoboynik et al., 2008) and in mesenchymal structures labeled as ventral cell islands (Rinkevich et al., 2013). Some authors also classify the aggregates of hemoblasts in the circulatory system as hematopoietic nodules or stem cell niches (Ermak, 1976; Kawamura and Sugino, 1999). Hemoblast presence has also been observed in the gonads of *B. schlosseri*, forming compact structures with high proliferation (Sunanaga et al., 2008). In our observations, even though hemocytes are widespread within body tissues (i.e. branchial basket, body wall and gonads; not shown), hemoblast aggregates are most apparent beneath the intestine epithelia. Furthermore, the clustered cells within each aggregate present less differentiation indicators, such as granules and vacuoles, whereas free cells surrounding the structures are partially or fully differentiated. Based on the synthesis of our results, we hypothesize that a putative stem cell population originates at the intestine and is present among circulating hemocytes in *S. plicata*. This is supported by morphological observations and antibody tests.

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TABLES

Table 1 - Cell morphotypes used to classify total hemocyte populations in *Styela plicata*

Cell morphotype	Diameter	Percentage	Possible types	Description
Small-round cells	2.8 – 6 µm	16.7 ± 9.3%	Lymphocyte-like cells, hemoblasts	Circular cells with a high nucleus/cytoplasm ratio. Granules scarce or absent..
Granular amoeboid cells	4 - 8 µm	9.9 ± 5.9%	Granulocytes and amoeboid cells in differentiation	Amoeboid cytoplasm with granular contents.
Large granulocytes	5 - 8.6 µm	46.2 ± 9.9%	Fully differentiated granulocytes, univacuolar refractile granulocytes	Rounded contour, numerous granular structures in cytoplasm, a large refractile vacuole may be present.
Vacuolated cells	5 – 20 µm	31.4 ± 8.8%	Simple univacuolar cells, compartment cells, morula cells	Cells with one or more prominent basophilic vacuoles in the cytoplasm.
Pigmented cell	8 - 13 µm	3.5 ± 3.9%	Pigment cell	Large cells, cytoplasm saturated with compartments filled with amber pigment.

Table 2 - Hemocyte types identified in *Styela plicata*

Cell type	Diameter	Identified content	Possible function	Reference
Granulocyte	4,2 – 5,2 µm	Heparin and Histamine	Similar to vertebrate basophiles	de Barros et al., 2007
Lymphocyte-like cell	3,1 – 4,8 µm	Nitric Oxide	Signaling in defence mechanisns	de Barros et al., 2009
Hemoblast	6,1 µm	none	Regeneration	Medina et al., 2014; Wright, 1981
Morula cell	8,8 – 16,1 µm	Phenoloxidase	Citotoxicity	(Cammarata et al., 1997)
Pigment cell	5,1 – 13,1 µm	Nitrogenatec compounds	Pigmentation	Wright, 1981

Table 3 - Gate polygon coordinates for *Styela plicata* hemocytes BAAA assay

Number	Intensity Ch 2	Intensity Ch 6	Number	Intensity Ch 2	Intensity Ch 6
Gate 1	-23,898	607,639	Gate 4	3086,707	6266,418
	-41,46	162,507		3121,691	131464,244
	-26,825	42,031		22918,511	239608,156
	25,86	-18,839		69700,32	105598,578
	121,56	-49,274		99031,196	55500,66
	339,387	31,886			
Gate 2	60,655	3080,355	Gate 5	3151,079	6592,449
	347,749	31,886		3151,079	3409,259
	1594,332	126,102		295800,401	3240,653
	224,286	17282,854		193582,719	20123,432
				99037,138	52755,863
Gate 3	225,362	17282,854	Gate 6	3215,249	3240,653
	1601,651	132,664		3215,249	269,881
	3121,691	256,531		289757,657	269,881
	3121,691	131464,244		289757,657	3240,653
	1002,719	79160,3			

FIGURES

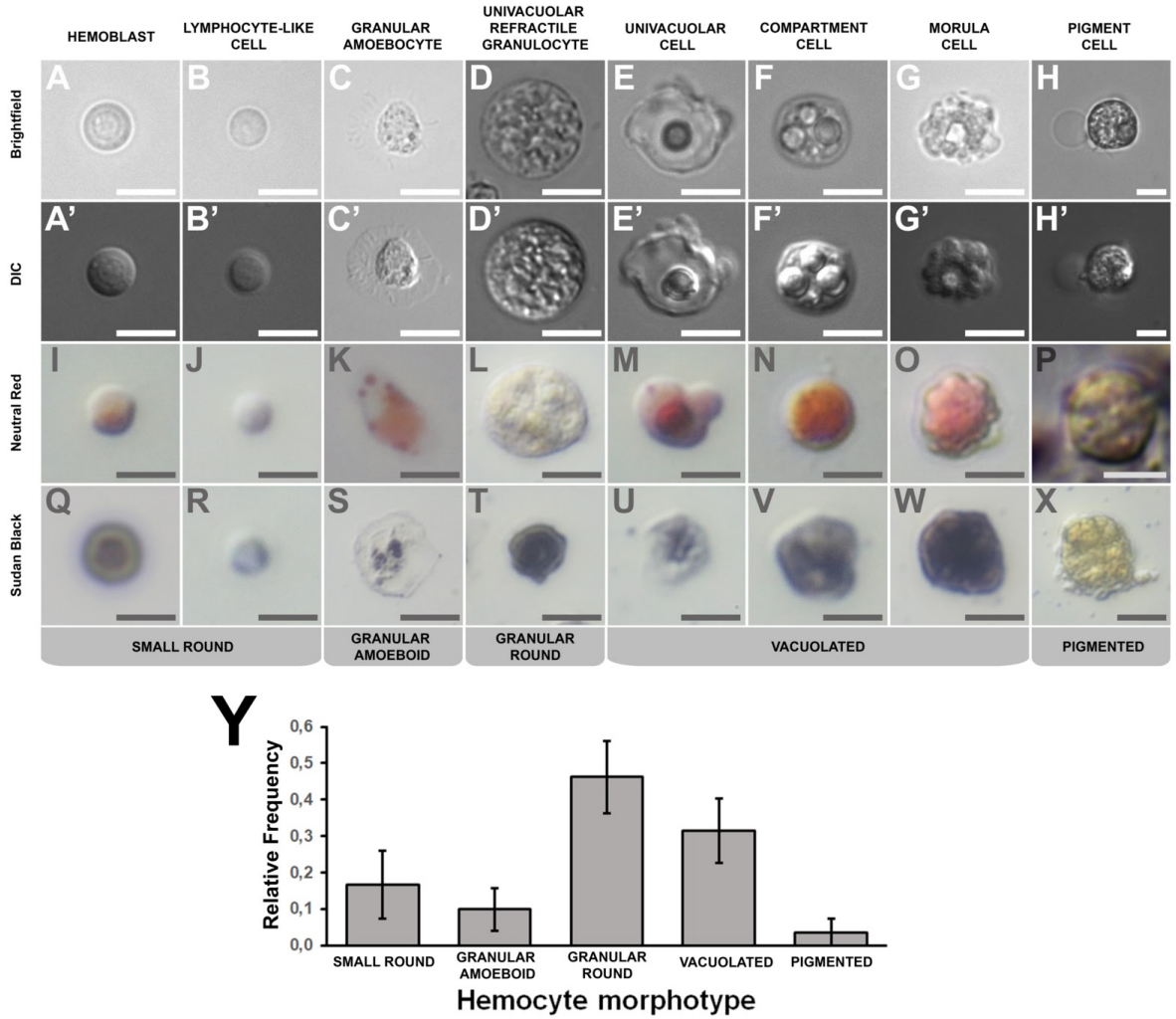

Figure 1 - *Styela plicata* hemocytes (A-X) and frequencies (Y). (A, A', I, Q) Hemoblast, one of the two types of *small round* cell with big nucleus (see text for explanation); (B, B' J, R) Lymphocyte-like cell, the other *small round* cell with big nucleus that is generally smaller than the hemoblast (A, A', I, Q); (C, C', K, S) Granular amoebocyte, includes *granular amoeboid* cells with characteristic scattered granules and an irregular membrane; (D, D', L, T) Univacuolar refractile granulocyte, large *granular round* cells; (E, E', M U) Univacuolar cell, *vacuolated* cell type with one conspicuous vacuole; (F, F', N, V) Compartment cell, includes *vacuolated* cells with 3-5 vacuoles; (G, G', O, W) Morula cell, *vacuolated* cells with numerous vacuoles; (H, H', P, X) Pigment Cell, includes all *pigmented* cell types; (Y) Relative frequencies of cell morphotypes show *granular round* cells as most abundant, whereas *pigmented* cells are least abundant; *small round* cells (hemoblast and lymphocyte-like cells) represent about a fifth of all cell types (n=4 individuals, approx. 300 images analyzed). Microscopy techniques used: (A-H) Bright field, (A'-H') DIC, (I-P) Neutral Red, (Q-X) Sudan Black. Hemocyte types (shown above) are based on de Leo (1992) and de Barros (2009, 2014), and morphotypes used to calculate frequencies in (Y) and in this study are shown below in grey tags. Scale bars = 5µm.

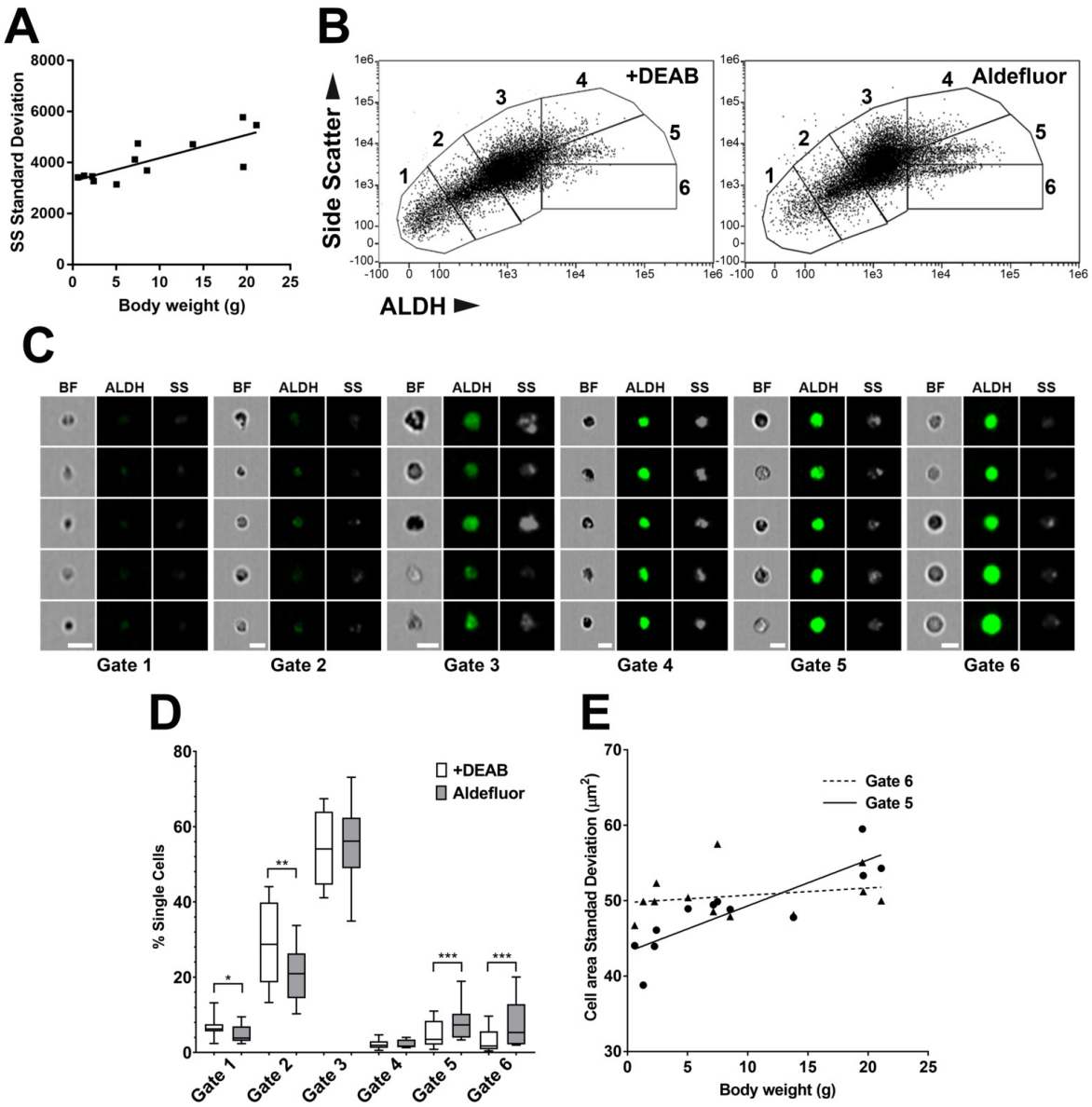

Figure 2 - Imaging flow cytometry analysis of hemocyte populations in *Styela plicata*

(A) Cell complexity of total hemocytes is shown (Side scatter Standard deviation) as a function of body weight (r^2 = 0,58, p=0,038; y = 172,3*x + 5078); (B) Gating strategy applied to SS/ALDH Scatterplots of one representative ascidian specimen (Gates 1-6); Gates 5 and 6 show an increase in cells in Aldefluor treated samples as compared to the ALDH inhibitor control (+DEAB); (C) Gallery of images from selected gates representing five cell morphologies; (D) Cell percentages corresponding to each gate on Aldefluor test samples vs controls, note the highly significant increase of cells in gates 5 and 6 (***, p<0,01) and the significant decrease in cells in gates 1 (*, p<0,05) and gate 2 (**, p<0,025), asterisks indicate significant differences using Wilcoxon matched-pairs signed rank tests; (E) Cell complexity of hemocytes in gates 5 and 6 is shown (Side scatter Standard deviation) as a function of body weight, gate 5 regression: r^2 = 0,73, p=0,0004, y=0,61x+43,21, gate 6 regression: r^2= 0,055, p=0,59, y=0,0962x+49,77. Note that cells in gate 5 do not change in cell complexity in ascidians of different size.

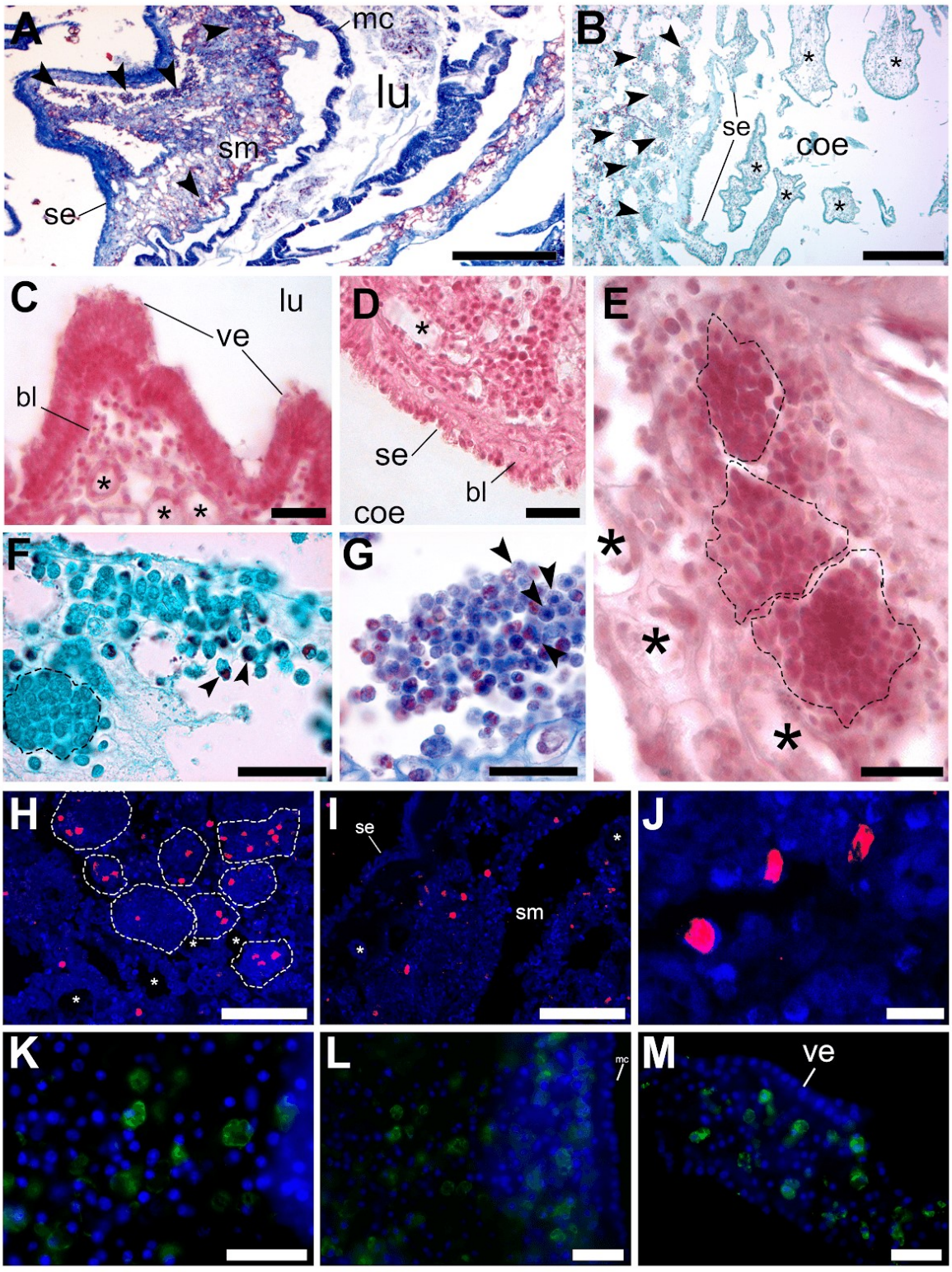
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Figure 3 - The cellular structure of the intestine of *Styela plicata* (A) Sagittal section of *Styela plicata* intestine showing its layers, clustered hemoblasts are highlighted (arrowheads) (B) The interface of the serosa with the body cavity (coelom). Hemoblast clusters are indicated with arrowheads. Serosa epithelium projections are highlighted. Asterisks mark projections of the serosa to the coelom. Note that the connective tissue contains hemoblast clusters. (C) Section detail of the mucosa, showing villi. Glandular structures can be seen beneath the epithelium (asterisks) (D) Section detail of the serosa. The asterisk marks a glandular structure. (E) Section detail of the submucosa. hemoblast clusters are delineated. Asterisks highlight glandular structures. (F) Detail of niches (delineated in black) located at the outer submucosa. Arrowheads point to free cells with conspicuous granules. (G) Niche within the submucosa. Arrowheads point to characteristic hemoblasts. (H-J) Phosphohistone (pHH3) immunofluorescence shows antibody reaction in the region of the intestinal epithelia in *Styela plicata*. All cell nuclei were stained with DAPI (blue) (H) Wide view of the submucosa region, showing hemoblast aggregations (delineated by dotted lines), glandular structures are highlighted with asterisks. Labeled cells are pseudo colored red. Dotted line delineates panel shown in (I) Wide view of the intestine subepithelial region below the serosa. Glandular structures are highlighted with asterisks. (J) Detail of labeled cells with pHH3 antibody in the submucosa. (K-M) Immunofluorescence showing antibody reaction with piwi in the subepithelial region of *Styela plicata* intestine. All cell nuclei were stained with the nucleus marker DAPI (blue) (K) Detail of the submucosa, labeled cells are pseudo colored green. (L) Detail of the subepithelial region. (M) Intestinal villi showing piwi+ cells at the subepithelial region. Staining techniques applied: Mallory-Cason trichrome (A, G), Hematoxylin and Eosin (C-E), Gomori trichrome (B, F), Immunocytochemistry (H-L). (se) serosa epithelium, (sm) submucosa, (mc) mucosa, (lu) lumen of the intestine, (bl) basal lamina (ve) villus extension, (coe) coelom, (m) mitochondria, (n) nucleolus. Scale bars = 5 mm in A; 2mm in B; 50 µm in C, D, E, F, G; 100 µm in H, I; 20 µm in H', K, L.

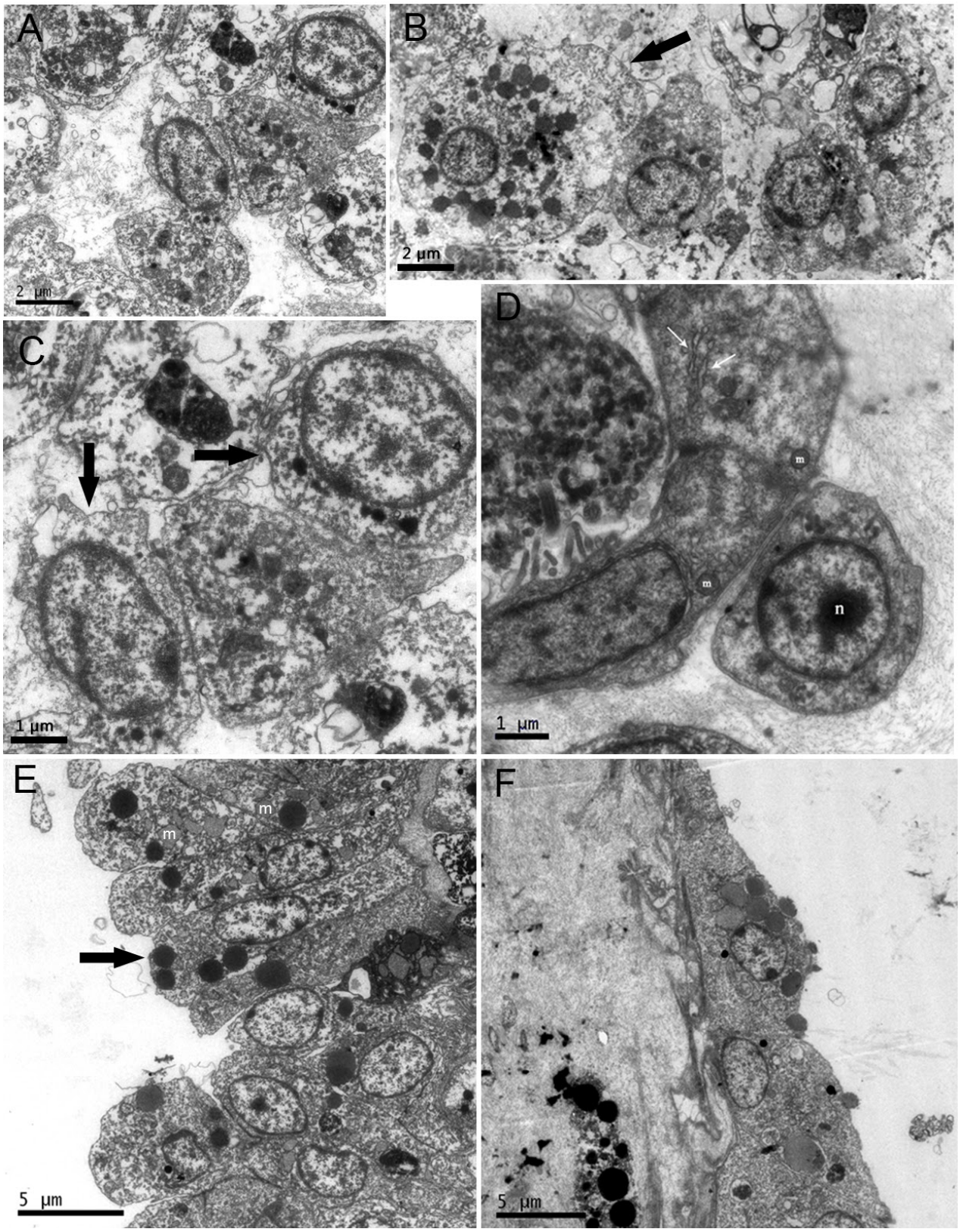

Figure 4 - Electron micrographs showing the *Styela plicata* intestine ultrastructure (A, B) Cell cluster in the submucosa showing hemoblasts in different maturation stages. (C) Higher magnification of (A). (D) Hemoblast associated with an intestinal gland. Arrowheads point to endoplasmic reticulum. (E) Mucosa layer cells. Electron-dense granules and mitochondria are conspicuous. (F) Serosa layer cells.

Final Considerations

This dissertation aims to characterize the hemocytes of solitary styelid ascidians and to discuss cell type diversity in an evolutionary context. In particular, I focus on circulatory stem cell (CSCs) as possible homologous cell types with hemoblast cells involved in budding of colonial styelids. There are previous studies that have tested the role of blood cells in the development of styelids (LAIRD; DE TOMASO; WEISSMAN, 2005; TIOZZO; BROWN; DE TOMASO, 2008; RINKEVICH et al., 2010; KAWAMURA et al., 2011; BRADEN et al., 2014; MEDINA et al., 2014; KASSMER; RODRIGUEZ; DE TOMASO, 2016) while other works have focused in the physiological function of the distinct blood cell types in ascidians (GOODBODY, 1975; WRIGHT et al., 1976; DE LEO, 1992; HIROSE; SHIRAE; SAITO, 2003; BALLARIN; KAWAMURA, 2009; BARROS et al., 2009). This study, therefore, intends to explore two novel aspects in the study of blood cells in the Styelidae: (i) systemic comparative studies of hemocyte populations across different species and (ii) a morphological, molecular, and functional analysis of CSCs in an evolutionary-developmental context to ultimately understand the origin of these cell types in the styelids.

In the first chapter of the dissertation I compared the blood composition and development of two species of solitary styelids: *Styela canopus* and *Styela plicata*. These species tend to adopt different strategies of settlement in the field: solitary, upright growth in *S. plicata* and aggregative settlement in *S. canopus*. I analyzed the development of both species up until the maturation of the juvenile, and the composition of their blood, based on simplified categories. In *Styela canopus*, I observed a transitory, extracorporeal vascular system after two weeks of metamorphosis. In addition, the circulating blood cells of this species have a lower abundance of vacuolated cells, and a higher abundance of small round cells. These differences could be related to immune processes related to the diverging tunic composition of these species.

It has been well supported that Styelidae includes two independent origins of coloniality, with all solitary species, including *Styela*, diverging early on in the Styelidae. However, the inclusion of additional colonial species to our current understanding of phylogenetic relationships could modify my current conclusions. For instance, only the *Polyandrocarpa* encompasses 29 colonial species (SHENKAR; SWALLA, 2011), from which only two were included in the phylogeny from Hiebert et al. (2018). One of these species, *P. zorritensis*, was found to belong to

a lineage with an independent origin of coloniality. In addition, an 18S-based phylogeny by Pérez-Portela et al. (2009) placed *S. canopus* as a sister species of *Polyandrocarpa zorritensis*. Inclusion of more colonial species in the phylogeny of Styelidae, particularly *Polyandrocarpa* spp., could bring better insights on the evolutionary relationship between aggregative settlement strategies and coloniality.

In the second chapter of the dissertation I ask whether the CSCs can be experimentally isolated in *Styela plicata*. I used imaging flow cytometry with an ALDH fluorescent substrate to establish a gating strategy that reflects putative hemocyte populations. From this assay, one out of the six gates encompassed ALDH+ hemocytes with morphologies similar to CSCs. Further in this chapter, I characterized cell types in the intestine submucosa to propose this tissue as a possible hematopoietic niche for this species in collaboration with I. Santos de Abreu (UFRJ). This region is populated with cell clusters that showed a characteristic CSC morphology. This hypothesis was supported by a significantly higher proportion of cells labelled with pHH3 and piwi antibodies, which are proliferation and stem cell markers respectively, as well as by ultrastructural analysis by electron microscopy. Further understanding on the identity of ascidian CSCs will be attained by studying expression profiles of isolated hemocyte populations. I recommend future studies to follow the flow cytometry guidelines presented here to carry out this experiment.

Hemocyte classification, particularly in tunicates, has been challenging due to the murky relationship between progenitors and terminally differentiated cells. The precise definition of cell types has generated some confusion that needs a better understanding of hematopoietic lineages and new approaches to characterize them. One possibility to approach this problem may come from characterizing complete transcriptomic expression patterns of individual cell types. As recently proposed, an unambiguous cell type identification could be developed by using a unique regulatory signature that includes cell type-specific core regulatory complexes (CoRC) (ARENDT, 2008). A CoRC comprises a set of transcription factors and co-factors that hypothetically enables the evolution of a cell-specific pattern of gene expression, which remains distinct from its evolutionary sister cell type (ARENDT et al., 2016). Identifying a CoRC in the transcriptomes of blood stem cells of styelids would provide a tool for defining functional cell types, as well as to infer evolutionary homology to the distinct blood cell types of different styelid species.

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Resumo

As ascídias da família Styelidae são diversas em modos de desenvolvimento, variando de espécies estritamente sexuais solitárias até colônias altamente integradas. As células-tronco circulatórias (CTCs) desempenham papéis fundamentais nos processos do desenvolvimento de ascídias styelídeas. Nas especies coloniais deste grupo, as CTCs permitem a brotação e são capazes de originar a linha germinativa em certas espécies. A função dessas células tem sido testada experimentalmente em modelos dentro de Styelidae. No entanto, a compreensão da colonialidade como uma novidade evolutiva requer reconstruir as características das possíveis CTCs ancestrais para Styelidae. Com o fim de abordar essa questão, este trabalho analisa a possível origem do desenvolvimento e a identidade de CSCs putativas entre populações de células sanguíneas de styelídeas solitárias. O primeiro capítulo desta dissertação teve como objetivo caracterizar e comparar as populações de hemócitos em dois espécies solitárias: *Styela plicata* e *Styela canopus*. Além disso, o desenvolvimento inicial, a metamorfose e a maturação do juvenil foram comparados em ambas as espécies. Após a metamorfose, *S. canopus* desenvolve brevemente uma rede de vasos extracorpóreos com numerosas ampolas terminais. Esses caracteres são geralmente associados a ascídias coloniais e não foram encontrados em *S. plicata*. Com relação às populações de hemócitos, morfotipos semelhantes estavam presentes em ambas as espécies. No entanto, o *S. canopus* apresenta menor frequência de células vacuoladas, o que pode ser devido a um nível reduzido de citotoxicidade na túnica em relação a *S. plicata*. Essas diferenças observadas entre *S. canopus* e *S. plicata* podem estar relacionadas a diferenças nos graus de gregariedade ou tamanho corporal entre as duas espécies. A fim de investigar possíveis abordagens para distinguir e isolar populações de CTCs em um modelo de styelídeo solitário, usei citometria de fluxo com adquisição de imagem. As CTCs putativas foram identificadas através da medição de parâmetros morfológicos e da atividade da aldeído desidrogenase (ALDH). A correlação entre estes parâmetros permitiu determinar 2 gates enriquecidos com tipos celuláres particulares. Uma diferença significativa foi encontrada na população ALDH+ dentro de um gate de células com baixa granularidade, sugerindo a presença de células-tronco circulatórias. Para examinar a biogênese das CTCs em *S. plicata*, foi realizada uma descrição de um nicho hematopoiético candidato nesta espécie. Um exame histológico exaustivo para células semelhantes a hemoblastos foi realizado e complementado com imunohistoquímica com marcadores de células-tronco (piwi) e proliferação (pHH3). Os perfis morfológicos e de

expressão do intestino sustentam a submucosa intestinal (SI) como nicho hematopoiético. Nesta região há agregações de células com morfolia indiferenciada, corroborada pela análise ultraestrutural. Além disso, a SI mantém alta proliferação celular e freqüência de células piwi+. As ascídias são consideradas modelos interessantes para investigar a reprodução assexuada e o desenvolvimento modular. Este estudo representa um avanço na compreensão dos processos, populações celulares e estruturas que podem estar relacionadas a facilitar o surgimento desta novidade evolutiva.

Abstract

Styelid ascidians are diverse in developmental modes, varying from strictly sexual solitary species to highly integrated colonies. Circulatory stem cells (CSCs) accomplish fundamental roles in developmental processes of styelid ascidians. In the colonial styelids, CSCs enable budding and are capable of giving origin to the germline in certain species. The function of these cells have been tested experimentally in models within Styelidae. However, the understanding of coloniality as an evolutionary novelty requires reconstructing the possible ancestral CSCs characteristics in Styelidae. To address this issue, this work analyzes the possible developmental origin and the identity of putative CSCs among blood cell populations. The first chapter of this dissertation aimed to characterize and compare the hemocyte populations in two solitary styelids: *Styela plicata* and *Styela canopus*. In addition, the early development, the metamorphosis and the early maturation were compared in both species. After metamorphosis, *S. canopus* briefly develops a network of extracorporeal vessels with numerous terminal ampullae. These characters are usually associated to colonial ascidians, and were not found in *S. plicata*. With respect to the hemocyte populations, similar morphotypes were present in both species. However, *S. canopus* shows a lower frequency of vacuolated cells, which may be due to a reduced level of cytotoxicity in the tunic relative to *S. plicata.* These differences observed between *S. canopus* and *S. plicata* may be related to differences in the degrees of gregariousness or body size among the two species. In order to investigate possible approaches to distinguish and isolate CSC populations in a solitary styelid model, I used imaging flow cytometry. Putative CSCs were identified through measurement of morphological parameters and aldehyde dehydrogenase (ALDH) activity. The correlation between these parameters allowed to determine 2 gates enriched with particular cell types. A significant difference was found on the ALDH+ population within a gate of cells with low granularity, suggesting the presence of cells among circulatory hemocytes. To scrutinize the biogenesis of CSCs in *S. plicata*, I present a description of a candidate hematopoietic niche in this species. An exhaustive histological survey for hemoblast-like cells was performed, and complemented with immunohistochemistry with stem cell (piwi) and proliferation (pHH3) markers. The morphological and expression profiles of the intestine support the intestinal submucosa (IS) as a hematopoietic niche. At this region there are aggregations of cells with and undifferentiated morphological profile, corroborated by ultrastructural analysis. Furthermore, the IS holds high cellular proliferation and frequency of piwi+ cells. Ascidians are considered interesting models to investigate asexual reproduction and

modular development. This study represents an advancement towards understanding the processes, cell populations and structures that may be related to facilitating the appearance of this evolutionary novelty.