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Review article

Sixty years of experimental studies on the blastogenesis of the colonial tunicate *Botryllus schlosseri*

Lucia Manni[a], Chiara Anselmi[a,*], Francesca Cima[a], Fabio Gasparini[a,*], Ayelet Voskoboynik[b], Margherita Martini[a], Anna Peronato[a], Paolo Burighel[a], Giovanna Zaniolo[a], Loriano Ballarin[a]

[a] Department of Biology, University of Padova, Italy
[b] Institute for Stem Cell Biology and Regenerative Medicine and Hopkins Marine Station, Stanford University, United States

ABSTRACT

In the second half of the eighteenth century, Schlosser and Ellis described the colonial ascidian *Botryllus schlosseri* garnering the interest of scientists around the world. In the 1950's scientists began to study *B. schlosseri* and soon recognized it as an important model organism for the study of developmental biology and comparative immunology. In this review, we summarize the history of *B. schlosseri* studies and experiments performed to characterize the colony life cycle and bud development. We describe experiments performed to analyze variations in bud productivity, zooid growth and bilateral asymmetry (*i.e.*, the *situs viscerum*), and discuss zooid and bud removal experiments that were used to study the cross-talk between consecutive blastogenetic generations and vascular budding. We also summarize experiments that demonstrated that the ability of two distinct colonies to fuse or reject is controlled by a single polymorphic gene locus (BHF) with multiple, codominantly expressed alleles. Finally, we describe how the ability to fuse and create chimeras was used to show that within a chimera somatic and germline stem cells compete to populate niches and regenerate tissue or germline organs. Starting from the results of these 60 years of study, we can now use new technological advances to expand the study of *B. schlosseri* traits and understand functional relationships between its genome and life history phenotypes.

1. The history of *Botryllus schlosseri*

The second half of the eighteenth century represented the golden age for both intellectual curiosity and studies on the marine fauna and flora. During that time European physicians, philosophers and naturalists began identifying different marine species. The colonial ascidian *Botryllus schlosseri* (Pallas, 1766), commonly known as the star ascidian or golden star tunicate, was one of these newly characterized species. *B. schlosseri* was initially classified as "plantanimal" or "zoophyte" based on its sedentary nature paired with animal features (*e.g.*, contractility). Rondelet (1555) was the first to describe *Botryllus* colonies and called them *uva marina* (Fig. 1A) while Pallas (1766) and Linnaeus (1767) named it *Alcyonium schlosseri*, Gärtner (1774), Bruguière (1792), and Renier (1793) adopted *Botryllus stellatus* and, finally, in 1816, Savigny called it *Botryllus schlosseri* (Brunetti et al., 2017).

The first microscopic description of *B. schlosseri* in scientific literature dates back to 1756, when a manuscript entitled: *An account of a curious, fleshy, coral-like substance*, by Schlosser and Ellis (1755–1756), was read at the Royal Society, in London. They named the species *Alcyonium carnosum asteriscis, radiis obtusis, ornatum* and described the development of the buds:*...all the interstices between the stars are fill'd with eggs of different sizes, each adhering by one end to a very fine capillary filament. The smallest eggs are globular, and as they advance in size, change to an oval figure; from thence they assume the shape of one of the radii of the star...* (Fig. 1B).

The species was also described by the German scientist Gärtner (1774) (Fig. 1C), and by the Italian scientist Spallanzani (1784) who, in his diary, described it as a new animal, unknown to him, and reported his observations on the replacement of the old generation zooids by a new generation: *August 20, 1784. This morning a new phenomenon occurred. The zoophyte collected yesterday, maintained in seawater, and observed today, did not have only six leaflets, but 12. In a day, then, 6 new leaflets appeared and these are very similar to the old ones. The zoophyte was circular and still is*. In his investigations in the North-Adriatic Sea, Spallanzani was supported by the naturalist Stefano Chiereghin (Chioggia, Italy), who rendered some drawings of marine animals and, among them, a detailed illustration of *B. schlosseri* (Gibin, 1997; Fig. 1D). The naturalists Olivi (1792) and

* Correspondence to: Department of Biology, University of Padova, Via Ugo Bassi 58/B, 35131 PD, Italy.
E-mail addresses: chiara.anselmi.3@phd.unipd.it (C. Anselmi), fabio.gasparini@unipd.it (F. Gasparini).

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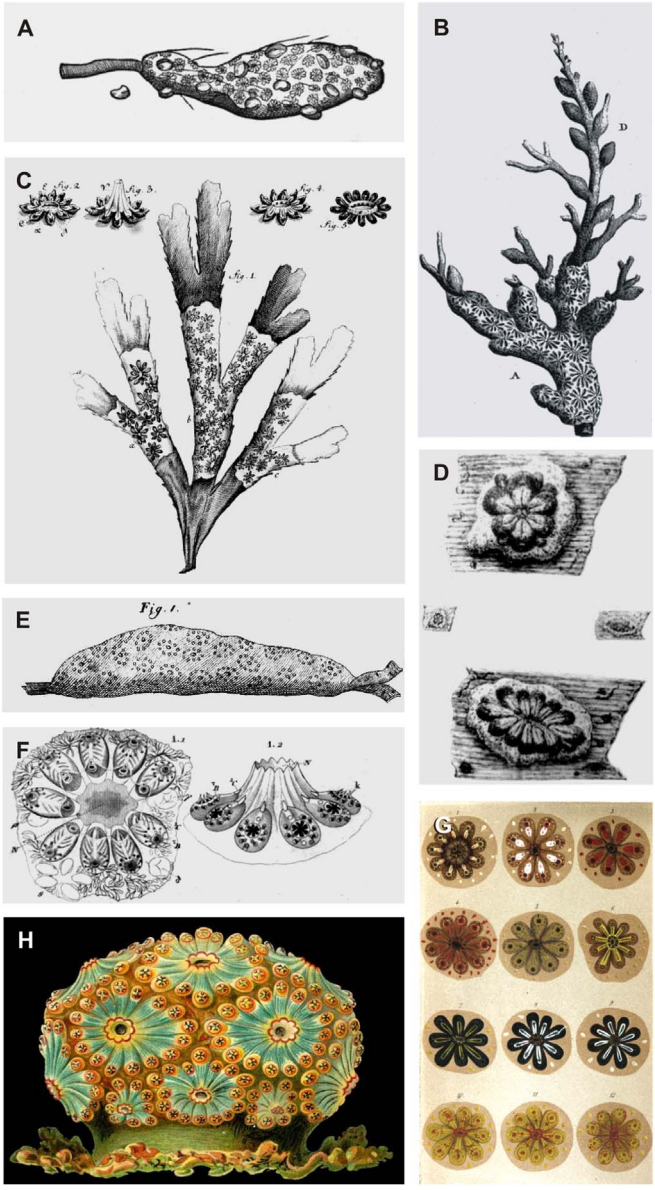

Fig. 1. Colonies of *Botryllus schlosseri*, as drawn by: Rondelet (1555) (**A**), Schlosser and Ellis (1755–1756) (**B**), Gärtner (1774) (**C**), Stefano Chiereghin (in Gibin, 1997) (**D**), Andrea Renier (in Gibin, 1997) (**E**), Savigny (1816) (**F**), Giard (1872) (**G**), and Haeckel (1899) (**H**).

Renier (1793), from Chioggia, also described *Botryllus*. According to Renier, buds are *eggs interspersed among the big corpuscles* (zooids), and the generation change as follows: *...the eggs of the Alcyonium, once developed, increase the number of the corpuscles and the total volume*. Renier was the first to describe the colonial vasculature: *...the crystalline substance* (the tunic) *contains internal small vessels that communicate with small vesicles* (the ampullae) *that contain various small opaque clustered globules* (the hemocytes)... and included a detailed illustration of the colony (Fig. 1E; Gibin, 2013).

In the nineteenth century, *B. schlosseri* was described by many authors, including Savigny (1816); Fig. 1F), Ganin (1870), Giard (1872), and Della Valle (1881). Giard (1872) studied colony pigmentation (Fig. 1G), and Haeckel (1899) described the colony anatomy (Fig. 1H). Metschnikow (1869), Hjort (1893) and Pizon (1893) described the process of bud development, later re-investigated by Berrill (1941a, 1941b, 1951), Watterson (1945), Sabbadin (1955), and Izzard (1973). In the first half of the last century, Bancroft (1903a) was the first to describe colony specificity: the ability of *B. schlosseri* to fuse or reject other colonies. Sabbadin (1955) succeeded in raising *B. schlosseri* colonies in the laboratory and began studying their life history. Classical genetic studies and fusion rejection assays, performed by Sabbadin (1962) and Scofield et al. (1982) on *B. schlosseri*, by Oka and Watanabe (1957a, 1960), Taneda and Watanabe (1982a, 1982b) and Taneda et al. (1985) on *Botryllus primigenus,* revealed that the ability to fuse in *Botryllus* is controlled by a single polymorphic gene locus with multiple, codominantly expressed alleles.

Burnet (1971) suggested studying *Botryllus* as a model for the evolution of self-recognition. He wrote: *although self recognition in ascidians is not analogous to the immunological processes of vertebrates, it presents a primitive type of' self and not self' recognition from which adaptive immunity may have evolved.*

Over the last 35 years electron microscopy (scanning, transmission microscopy, freeze-fracture techniques) (reviewed in Manni et al., 2007) has advanced the knowledge of *B. schlosseri* anatomy and development. The Weissman lab has focused on the genetic control of the fusion/rejection in *B. schlosseri*. Fusion or rejection occurs when two colonies touch, the blood vessels either fuse and create a chimera or reject forming inflammatory responses (points of rejection) (Sabbadin, 1962; Scofield et al., 1982). They determined that chimerism is a stem cell mediated phenomenon (Laird et al., 2005a) and that the biology of stem cell engraftment in *B. schlosseri* chimeras is regulated on four different levels: first, there is fusion or rejection (Scofield et al., 1982); second, if fusion occurs, the body of one partner is resorbed (Rinkevich et al., 1993; Corey et al., 2016); third, there is competition between somatic stem cells that circulate from one chimeric partner to another for asexual whole body development (Stoner and Weissman, 1996; Stoner et al., 1999; Laird et al., 2005a; Voskoboynik et al., 2008); and fourth, there is stem cell competition among germ line stem cells (Stoner et al., 1999; Rinkevich et al., 2013). Using defined homozygous and heterozygous *B. schlosseri* lines for distinct fusibility alleles that were developed by the late Saito in 1985–1987, the Weissman lab described hierarchies in allogeneic resorption (Rinkevich et al., 1993), sequenced the *B. schlosseri* genome and transcriptome (Voskoboynik et al., 2013a, 2013b), and discovered *BHF*, the gene that controls fusion and rejection outcomes (Voskoboynik et al., 2013b). Fusion between colonies requires at least one shared *BHF* allele while rejection occurs when no *BHF* alleles are shared (Voskoboynik et al., 2013b). Genetically distinct strains have somatic stem cells that, in a chimera, vary in their vulnerability to be resorbed, undergo competitions to “win” or “lose” differentiated tissue (akin to regeneration), and to win or lose germline niches (Rinkevich et al., 1993, 2013; Stoner and Weissman, 1996; Stoner et al., 1999; Laird et al., 2005a; Voskoboynik et al., 2008).

Sixty years of research on *B. schlosseri* asexual reproduction, stem cell biology, and stem cell competition within chimeras has directed studies in mammals that revealed stem cell competition during mammalian development, aging and cancer (Weissman, 2015).

2. The star ascidian: the colony and its blastogenetic cycle

A *B. schlosseri* colony begins its life as a tadpole-like larva (the product of sexual reproduction) that metamorphoses into an oozooid (the first zooid of the colony) (Fig. 2). The larva has two buds, one on the right side of its body wall (formed of the epidermis, the peribranchial epithelium, and the mesenchymal derivatives between the two epithelia), the other on the left (Sabbadin, 1958). Following metamorphosis, the bud on the oozooid’ right side develops into the first blastozooid (the zooid derived from asexual reproduction). Through asexual reproduction, new blastozooids are formed and arrange themselves in star-shaped systems, with a common cloacal siphon in the center. The number of individuals in a colony increases when more than one bud replaces the blastozooids of the old generation. The colony expands as a system divides into two or more systems after the development of approximately 12 or more blastozooids. All the systems

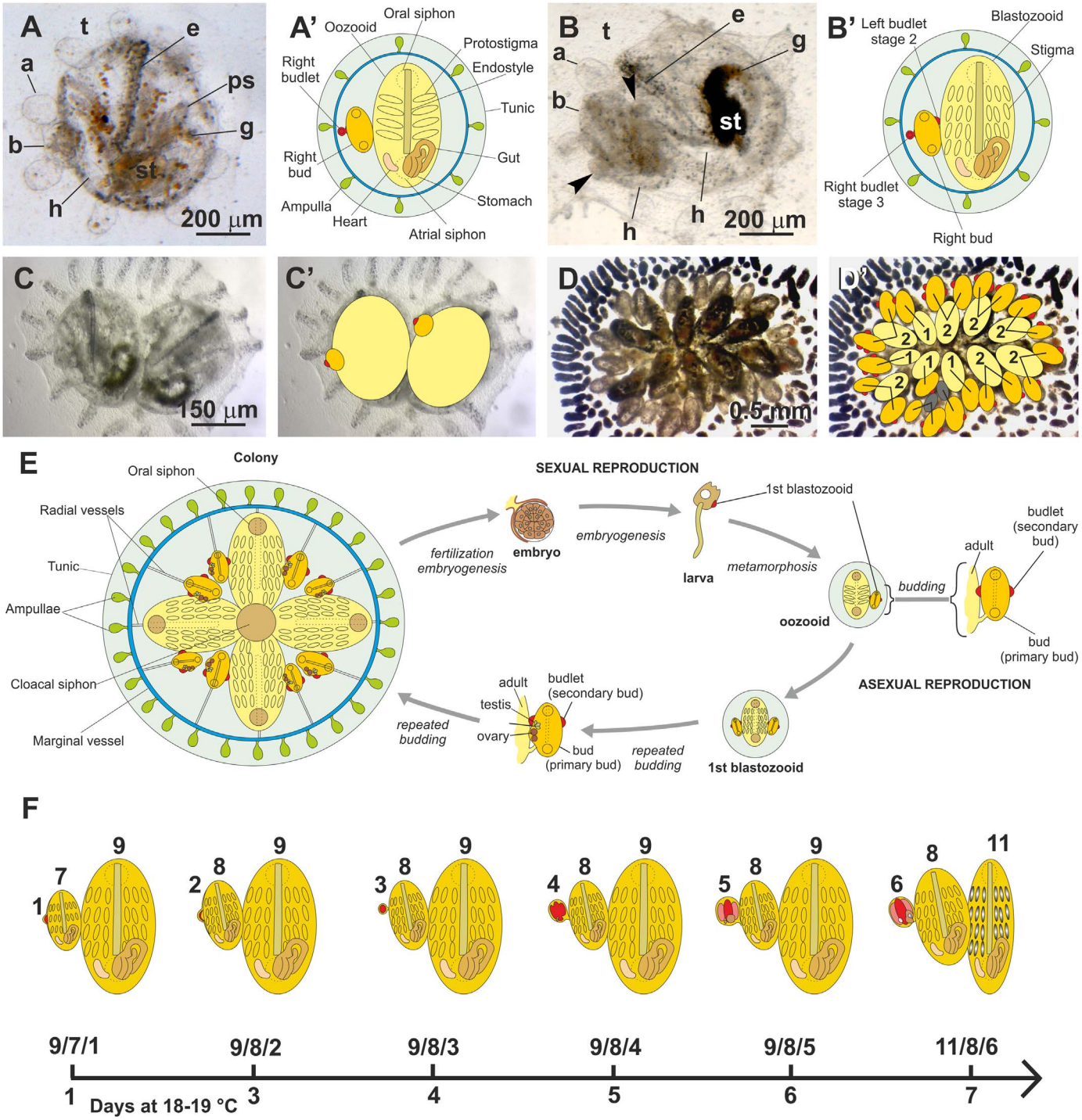

Fig. 2. Ventral view of an oozooid (**A**), an adult blastozooid of the first blastogenetic generation (**B**), a young (**C**) and old (**D**) colony. **A′–D′**: schematic drawings of **A–D;** zooids, buds and budlets are marked with different colors; in **C′–D′**, drawings are superimposed to images. Only the right bud (**b**) is present in **A** and **B**; the single budlets is poorly recognizable in **A**; budlets are present on both sides in **B** (**arrowheads**). Note that the young colony in **C** possesses fewer buds (only the right ones) than the older colony in **D**, where most of the zooids (marked with number 2) bear two buds and only a few (marked by number 1) bear only one bud. Black lines link each bud to its parent. Some buds bear three budlets. The two grey zooids are adult individuals in early TO; their buds are still developing and survive their parent. **a**: ampulla; **e**: endostyle; **g**: gut; **ps**: protostigma; **st**: stomach; **t**: tunic. **E**. Life cycle of *B. schlosseri* (modified by Gasparini et al., 2014). **F**. Blastogenetic cycle of a colony. Each adult blastozooid filters for about one week at 18-19 °C before being resorbed at the TO. Colonial phases are indicated by a combination of three numbers separated by slashes; developmental stages of adults, buds and budlets are indicated above the zooids (modified from Manni et al., 2014).

of a colony are embedded in a common, gelatinous tunic and are connected to each other by vessels containing hemolymph.

Eleven, morphologically-distinguishable blastogenetic developmental stages from bud to adult zooids were introduced by Berrill (1941a) and modified by Sabbadin (1955) and Izzard (1973) (Fig. 2F). This is the reference staging method used today, and has been described in an anatomical and developmental ontology that has been recognized by the tunicate community (Manni et al., 2014). Some authors adopt the four phase (A-D) method suggested by Watanabe (1953) for the Japanese species *B. primigenus,* based on the rapid (four day) blastogenetic cycle of colonies reared in the warm water temperature of the Japanese Sea. This method cannot document zooid develop-

mental changes and irregularities in the synchronization of the different blastogenetic generations coexisting in colonies.

Three blastogenetic generations are usually observed: the adult filtering zooids and their (primary) buds and budlets (secondary buds). Colonial developmental phases are defined by the blastogenetic developmental stages of the three generations and expressed with a formula of three numbers, separated by slashes, each number referring to the blastogenetic developmental stage of adult zooids, primary buds and budlets, respectively (*e.g.*, 9/8/4).

Cyclical generation replacement, called takeover (TO), occurs every week (at 18-19 °C). During this phase, the entire parental generation of zooids in a colony synchronously ceases filtering and is resorbed while the asexually-derived generation of buds mature and replaced it. Primary buds become the new zooids, budlets become the new primary buds and a new budlet generation grows. The TO phase involves massive programmed cell death (PCD) of zooid organs *via* apoptosis followed by programmed removal of cell corpses by circulating phagocytes within approximately 24–36 h (18-19 °C) (Lauzon et al., 1992; Manni et al., 2007; Franchi et al., 2016). The blastogenetic cycle, *i.e.* the interval between the siphons opening of two following adult zooid generations, lasts 7 days (at 18-19 °C). The lifespan of each individual in a colony, from its appearance as a bud primordium to the end of its filtering activity at TO, lasts about 3 weeks at 18-19 °C (Manni et al., 2007). When colonies at different blastogenetic developmental phases fuse to create chimeras, the development of one partner will speed up in order to assure a rapid equalization of the phase differences (Watanabe, 1953, 1962).

In the following sections, we will review some of the aspects of blastogenesis. In particular, we will focus on experimental studies of bud removal and the consequent effects on zooid growth and lifespan, duration of the blastogenetic cycle, blastogenetic capabilities, left-right asymmetry and regenerative capabilities. These topics will be discussed in the context of the developmental and evolutionary biology of ascidians.

3. Leaflets and flowers: morphological observations of palleal budding

Palleal budding is the phenomenon that astonished Spallanzani: how can leaflets (buds) in the zoophyte (zooid) double, while remaining similar to the old zoophyte? However, if the eminent researcher had followed the zoophyte's growth today, he would have discovered an oval zooid, being replaced by two buds that grew from its body wall. The primitive tools available to Spallanzani did not allow him to observe the appearance of budlets and follow their development.

The new budlet arises as a disc-shaped thickening (blastogenetic developmental stage 1) of the peribranchial epithelium of a bud at the blastogenetic developmental stage 7. The bud primordium, containing candidate pluripotent stem cells, arches perpendicularly to the bud wall that forms a hemisphere (stage 2) and then skews towards the anterior end of the parental bud. At this stage, the budlet anterior-posterior and dorsal-ventral axes are already established (Sabbadin et al., 1975; Manni et al., 2007). Body axis seems to be related to the vascularization of the budlet, as the entrance of the affluent vessel (coming from the bud) marks the posterior end of the budlet (Izzard, 1973; Sabbadin et al., 1975). A detailed description of budlet organogenesis can be found in Manni et al. (2007).

4. Genes associated with palleal budding

Current knowledge regarding genes and pathways associated with blastogenesis is limited. The majority of the available molecular data is descriptive and aims to verify gene expression patterns in buds *vs* adult zooids. For a minute number of genes, gene silencing experiments were performed, but the exact mechanism for their role in blastogenesis is still far from being understood. Here, we summarize the list of genes that were identified so far.

Laird et al. (2005b) identified Athena, a gene, unique to tunicates that is highly transcribed when a new budlet appears during the TO phase, when compared to other blastogenetic developmental phases. Genetic knockdown of *Athena* results in blastogenesis defects ranging from the delay of budlet development and growth impairment to altered organogenesis and developmental failure of the left buds (Laird et al., 2005b).

Another gene highly transcribed at TO is mortalin, a highly conserved chaperone member of the hsp70 family, involved in various functions ranging from stress responses to control of cell proliferation and inhibition of apoptosis (Londono et al., 2012). Its transcript is present in normal zooids and absent in zooids undergoing resorption. Allosteric inhibition of the protein leads to severe alteration of zooid morphology, in particular in the development of: digestive system, endostyle and gonads (Ben-Hamo et al., 2018). When compared to adult tissues, bud tissues express higher levels of the mortalin proteins (Ben-Hamo et al., 2018), PL10 and cadherin (Rosner et al., 2006, 2007).

Tissues of budlets and buds stained with antibodies raised against β-catenin, indicate a role of the Wnt pathway in blastogenesis, also supported by the interference of Wnt agonists and antagonists with normal bud development (Rosner et al., 2014; Di Maio et al., 2015).

The involvement of the TGFβ pathway is suggested by the immunopositivity of bud tissues to anti-TGFβ and the alteration in bud development induced by TGFβ agonists (Rosner et al., 2014).

Rosner et al. (2014) also found that the antibody for p-Mek1/2, an enzyme of the MAPK/ERK signal transduction pathway, stains bud tissues, whereas colony exposure to p-Mek1/2 inhibitors resulted in malformed buds (Rosner et al., 2014).

An orthologue of *ptx* is also transcribed in bud tissues: it is located in the gut epithelium, the oral siphon rudiment, and the developing neural complex (Tiozzo et al., 2005). The knock-down of this gene results in budding impairment and colony death (Tiozzo and De Tomaso, 2009).

The development of musculature during budding has been studied by *in situ* hybridization of muscular actin and troponin-T (Degasperi et al., 2009).

Furthermore, during blastogenesis, orthologues of vertebrate *Six1/2*, *Six3/6*, *Eya* and *FoxI*, associated with placode formation, are transcribed in the cells lining the forming branchial slits and siphons in the buds (Gasparini et al., 2013).

Internal tissues of the developing buds of the congeneric colonial species *B. primigenus* express nanos, a protein typically expressed by stem cells, repressing apoptosis and maintaining cell pluripotency (Sunanaga et al., 2008).

A new set of differentially transcribed genes in the epithelia of the bud primordia of *B. schlosseri* has been recently identified. It includes genes for transcription factors related to stemness and development, telomere maintenance, tumor suppression, and signal transduction pathways (Ricci et al., 2016a, 2016b).

Corey et al. (2016) studied the cellular and molecular framework underlying loss of tolerance to one partner within a natural *B. schlosseri* chimera. In this experiment, one chimeric partner is eliminated in a process of allogeneic resorption. A few days before the resorption of one chimeric partner, the development of the buds in the resorbing partner is halted (termed developmental arrest; Corey et al., 2016). Although this study was focused on the resorption event in a chimera, the comprehensive sequencing analysis described revealed upregulated expression of genes and pathways in normally developing budlets *vs* budlets with halted development (Corey et al., 2016). These upregulated genes and pathways are associated with embryogenesis and development. The top ranked genes differentially expressed in the budlets (secondary buds) of the resorption winner include: the transcription regulator *hist1h3b*, the anti-apoptotic gene *g2e3*, genes associated with embryonic development like *tbx1*, *six1* and *hmx1*, heart and stem cell proliferation *osr1*, and *neurog3*

which is involved in neurogenesis and endocrine cell development (Corey et al., 2016).

Many of the genes known to be associated with development, regeneration and stem cells are expressed in the buds. Using next generation sequencing technologies to map in detail the genetic program of blastogenesis might shed light on the robust regeneration capacities of colonial tunicates.

5. Stem cells and palleal budding

Adult stem cells are multipotent, self-renewing progenitor cells uniquely capable of both reproducing themselves and differentiating into a diverse range of specialized cell types. Studies in vertebrates revealed that tissue specific stem cells persist throughout adult life and are essential for the repair and regeneration of specific organs such as skin, brain and blood (review in Weissman, 2015). Identification and isolation of tissue specific stem cells is challenging and demands the design of experiments that can demonstrate self-renewing ability and multidifferentiative potential.

In recent years, the interest in marine invertebrate stem cells has rapidly grown due to the potential of better understanding fundamental biological processes (*e.g.*, senescence, regeneration, cell reprogramming) (Ballarin et al., 2018).

In *B. schlosseri* chimeras, the buds and gametes of one or both chimeric partners become a blend of both genotypes or, in some cases, are completely replaced by the cells of one partner (Sabbadin and Zaniolo, 1979; Pancer et al., 1995; Stoner and Weissman, 1996; Stoner et al., 1999). This chimerism persists even when the blood vessels connecting the fused colonies are disconnected, and it follows genetically heritable hierarchies for germline “winners” and “losers” (Sabbadin and Zaniolo, 1979; Stoner et al., 1999). The reproducibility and longevity of this phenomenon led to the hypothesis that chimerism is mediated by blood borne stem cells (Sabbadin and Zaniolo, 1979; Rinkevich and Weissman, 1987; Pancer et al., 1995; Stoner and Weissman, 1996; Stoner et al., 1999).

By transplanting a single cell which had expressed a high enzymatic activity of aldehyde dehydrogenase and a set of serial engraftment assays, Laird et al. (2005a) revealed that adult stem cells are responsible for a stable long-term chimerism in *B. schlosseri*. Criteria for a candidate stem cell were both the induction of long-term, stable, multilineage chimerism and demonstrated self-renewal potential (Laird et al., 2005a).

In vivo cell labeling, cell engraftment, and time lapse imaging showed that the anterior ventral region of the subendostylar sinus (termed endostyle niche) in *B. schlosseri* zooids harbors and exports somatic stem cells, and that the cell islands which are located along the endostyle harbor germline stem cells (Voskoboynik et al., 2008; Rinkevich et al., 2013). The subendostylar sinus is a hemolymphatic sinus ventral to the endostyle, the long glandular groove in the ventral side of the branchial sac (Burighel and Brunetti, 1971). The endostyle has an iodine-concentrating activity and is considered a homolog of the vertebrate thyroid gland (Burighel and Cloney, 1997; Ogasawara et al., 1999). In *B. schlosseri*, *in-situ* hybridization and immunostaining of the endostyle in zooids and buds reveal unique expression patterns of site-specific factors that are linked to developmental regulation and stem cell activity including β-catenin, Piwi, Oct4, STAT, Raldh, RAR, pSmad2 and more supporting the key role of the endostyle as a niche for stem cells involved in bud development (Voskoboynik et al., 2008; Rinkevich et al., 2013).

The prospective isolation of germline and somatic stem cells in *B. schlosseri* (Laird et al., 2005a) and the identification of stem cell niches in this organism suggest that palleal budding in colonial ascidians is mediated by tissue specific stem cells that migrate through the colony vasculature and seed developing buds. However, although studies done so far suggested that budding in *B. schlosseri* are stem cell mediated phenomena (Laird et al., 2005a; Voskoboynik et al., 2008; Rosental et al., 2018), we can not exclude the possibility of dedifferentiation. In the ascidian *Polyandrocarpa misakiensis,* budding has been suggested to depend on the transdifferentiation of the peribranchial epithelium, where cells acquire new differentiation markers (Kawamura and Fujiwara, 1994, 1995). Comprehensive lineage tracing of individual cells that use transgenic lines will be needed to prove transdifferentiation. Future studies that will investigate the cellular and molecular mechanisms underlying palleal budding in tunicates will advance our knowledge on stem cell mediated regeneration processes.

6. Interactions among coexisting generations: experiments of bud removal

To better understand the asexual development of a colony and the relationships between the zooids, buds and budlets, oozooids were settled on slides and their development was followed for 7 blastogenetic generations (49 days) (Sabbadin, 1955, 1958; Gasparini et al., 2015). Colonies were observed every day and the number and position of buds, zooid size and blastogenetic developmental phases were recorded (Fig. 3). Fourteen colonies were used as a control, and the others were used for experiments of bud removal (budectomy). These experiments, reviewed below, provided basic knowledge regarding homeostatic and regenerative capacities of budlets, asymmetric blastogenetic potential, colony growth rate, length of the blastogenetic cycle, and the interactions among coexisting generations.

6.1. Blastogenetic capabilities

The number of budlets produced by buds on zooids of the first blastogenetic generation is lower than that produced by buds of the following generations (generations 1–7; Fig. 4A, Suppl. Table 1). Under normal conditions, both sides of the bud can form bud primordia and each bud can originate up to four budlet primordia. Buds possess an asymmetrical blastogenetic potential, *i.e.*, they produce more budlets on the right side of the body than on the left (Fig. 4B, Suppl. Tables 2, 3). In addition, right anterior budlets are slightly advanced in development with respect to the left and posterior budlets (Sabbadin, 1958). On both sides, posterior budlets are preferentially resorbed with respect to the anterior ones (Fig. 4B, C; Suppl. Table 3) (Sabbadin, 1958; Watkins, 1958). Therefore, the right anterior budlets are more likely to develop into adult zooids. In order to study the asymmetric blastogenetic potential of buds, *i.e.* the different ability of the two sides to produce budlets, budectomy experiments were performed on young colonies (Fig. 3A–C). The aim of the experiment was to verify if budlet removal on a side resulted in a blastogenetic potential variation on the opposite side, *i.e.*, if there is a competition between the two budding sites. Indeed, when all the budlets except one are removed, the blastogenetic potential of the colony is altered. Specifically, the remaining budlet, once bud, forms more budlets than usual, *i.e.* up to three budlet primordia on the right side and up to two on the left (Fig. 5A). In addition, budectomy enhances budlet ability to complete their development: the number of budlets (right posterior, left anterior and posterior) going through developmental arrest is significantly reduced in surgically altered colonies in comparison to the controls (Suppl. Table 5). Conversely, the number of posterior bud primordia, and the number of left anterior budlets that resume normal development are significantly higher as compared to control. In addition, in experimental and control colonies, budlet productivity is lower in younger generations than in older ones (Fig. 5A, Suppl. Table 6).

In conclusion, these results show that under normal conditions the right side produces a higher budlet number (up to 3) than the left. Following removal of the buds on the right side, the left side of the buds and its posterior locations can support normal development. They also demonstrate that budectomy increases the blastogenetic capabilities of colonies.

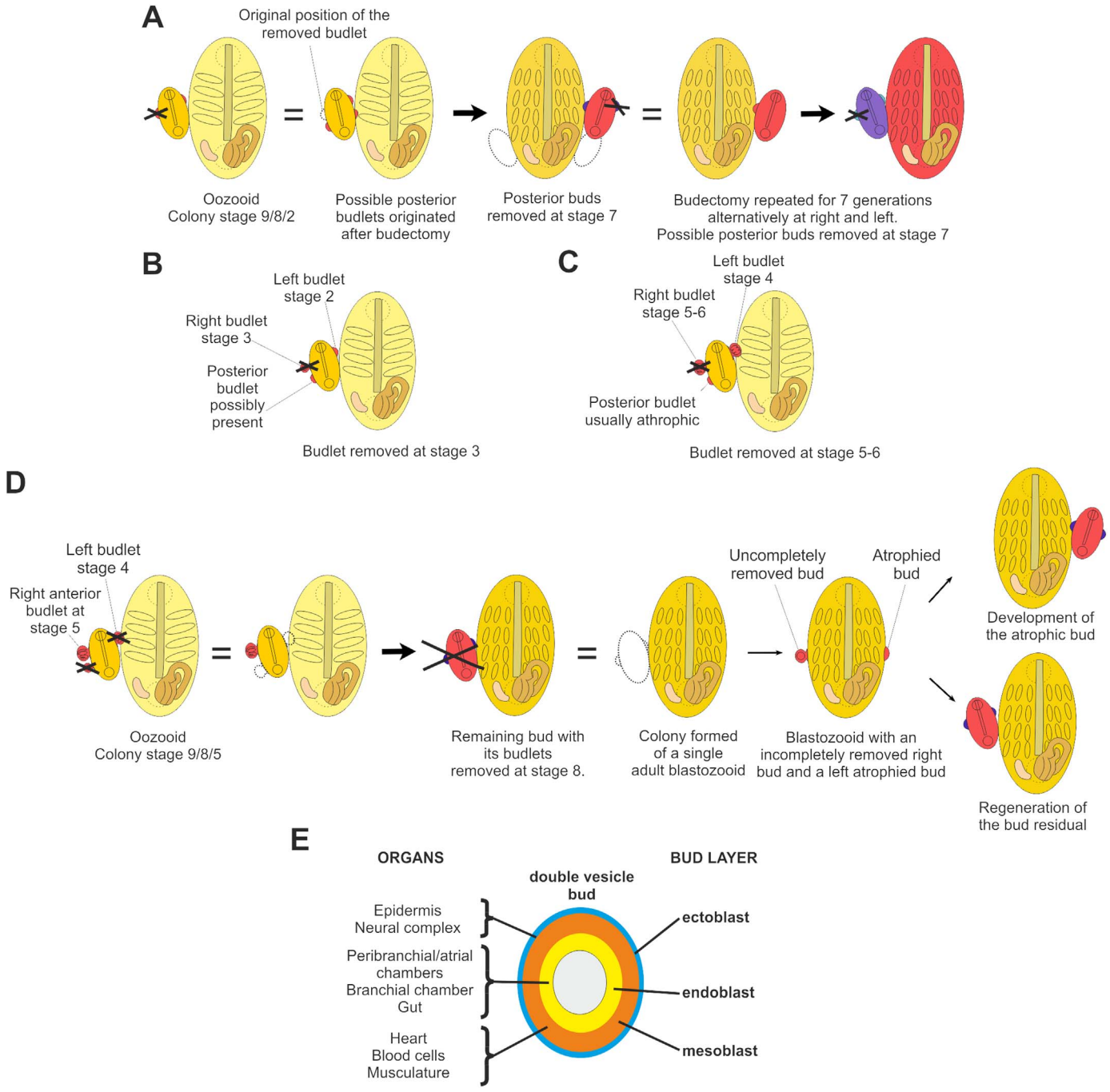

Fig. 3. Experimental plan of bud removal experiments performed by Sabbadin (1958). **A–C**. only one bud was left in buds; removal carried out at colonial phase 9/8/2 (**A**), 9/8/3 (**B**), and 9/8/5–6 (**C**). The same individual (as budlet, bud, or zooid) is marked by the same color over generations; the original position of removed zooids is indicated by dotted line; removals are indicated by crossed lines. **Arrow**: change of generation; = colony after removal. **D**. Removal of buds at stage 8 leads to colonies with adult zooid and its atrophied bud or bud remnants. The final effect is the development of the atrophied bud or the regeneration of the bud remain. **E**. Scheme of the double vesicle stage budlet showing organ derivation from bud layers (modified from Manni and Burighel, 2006).

6.2. *Homeostatic and regenerative capability of budlets*

Under normal conditions a high percentage (around 50%) of budlets undergo degeneration during development (Fig. 4C, D; Suppl. Table 3, 4). Most of these budlets are the posterior ones and those on the left side. They can persist as “atrophied” budlets (*i.e.*, budlets unable to complete their blastogenetic development) on the bud/adult body wall.

A specific budectomy experiment was performed to verify the ability of atrophied budlets to resume development. As the budlets of the first blastozooid reached the blastogenetic developmental stage 5–6, all the budlets but the right anterior one were removed; once the remaining budlet developed into bud, it was itself removed, creating a colony of a single adult zooid (Fig. 3D). This two-step removal experiment was thought to guarantee colony survival, since the contemporary removal of all the budlets frequently caused colony death. In the single-zooid colonies, remnants of an incompletely removed budlets (or remnant of the anterior right bud) could regenerate new budlets that expedite their development and reach the double vesicle stage (stage 3) (Fig. 3D). Moreover, atrophied budlets survived on the adult zooid body wall could resume development. The experiment revealed high regeneration capacities in budlets and demonstrated that the atrophied ones can

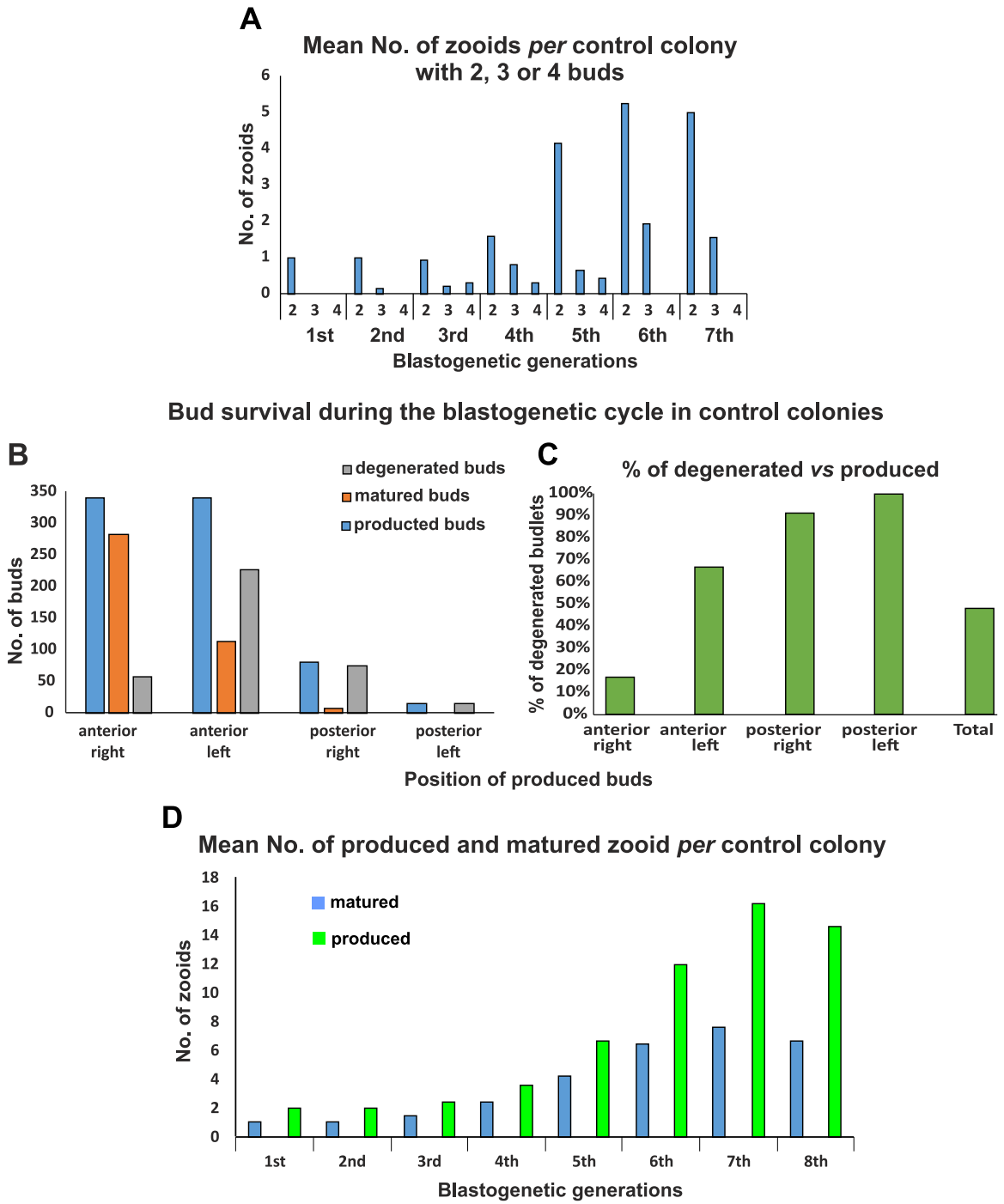

Fig. 4. A: Mean number of adult zooids per colony with 2, 3 or 4 buds in the first seven blastogenetic generations (data in Suppl. Table 1). **B**: number of budlets produced (light blue bars) that matured to buds (orange bars) or underwent atrophied degeneration (grey) in the anterior right, posterior right, anterior left and posterior left sides of the buds of control colonies (data in Suppl. Table 3). **C**: percentage of degenerated budlets in the same locations (data in Suppl. Table 3). **D**: mean number of budlets produced *per* blastogenetic generation in a control colony (green bars) and mean number of them reaching the adult stage (light blue bars) (data in Suppl. Table 4).

develop into normal buds. In a few cases, even two budlets formed from an atrophied single budlet, suggesting that the atrophy state is not due to budlet inability to develop, but it is the result of competition between blastogenetic generations.

During budlet regeneration or atrophied budlet development, the achievement of the double vesicle stage represented the first sign of normal blastogenetic development recovery. The double vesicle stage is considered "a triploblastic vesicle of the gastrula type" (Brien, 1968), based on its organogenetic capacities: the outer vesicle is formed by the epidermis and will give rise to the zooid epidermis, whereas the inner vesicle and the mesenchyme will form all the internal tissues of the zooid (Fig. 3E) (Manni and Burighel, 2006; Manni et al., 2007; Ricci et al., 2016a).

It is important to note that the parental bud in colonies at advanced developmental phases do not develop new budlets (budlet-ectomy performed on or after stage 7). This suggests that the blastogenetic capabilities are inhibited in buds after the developmental stage 7, a stage where the budlets developed from a disc on the wall of the bud. These experiments demonstrated that the blastogenetic competence is restricted in time and space. The factors that determine and restrict it are unknown.

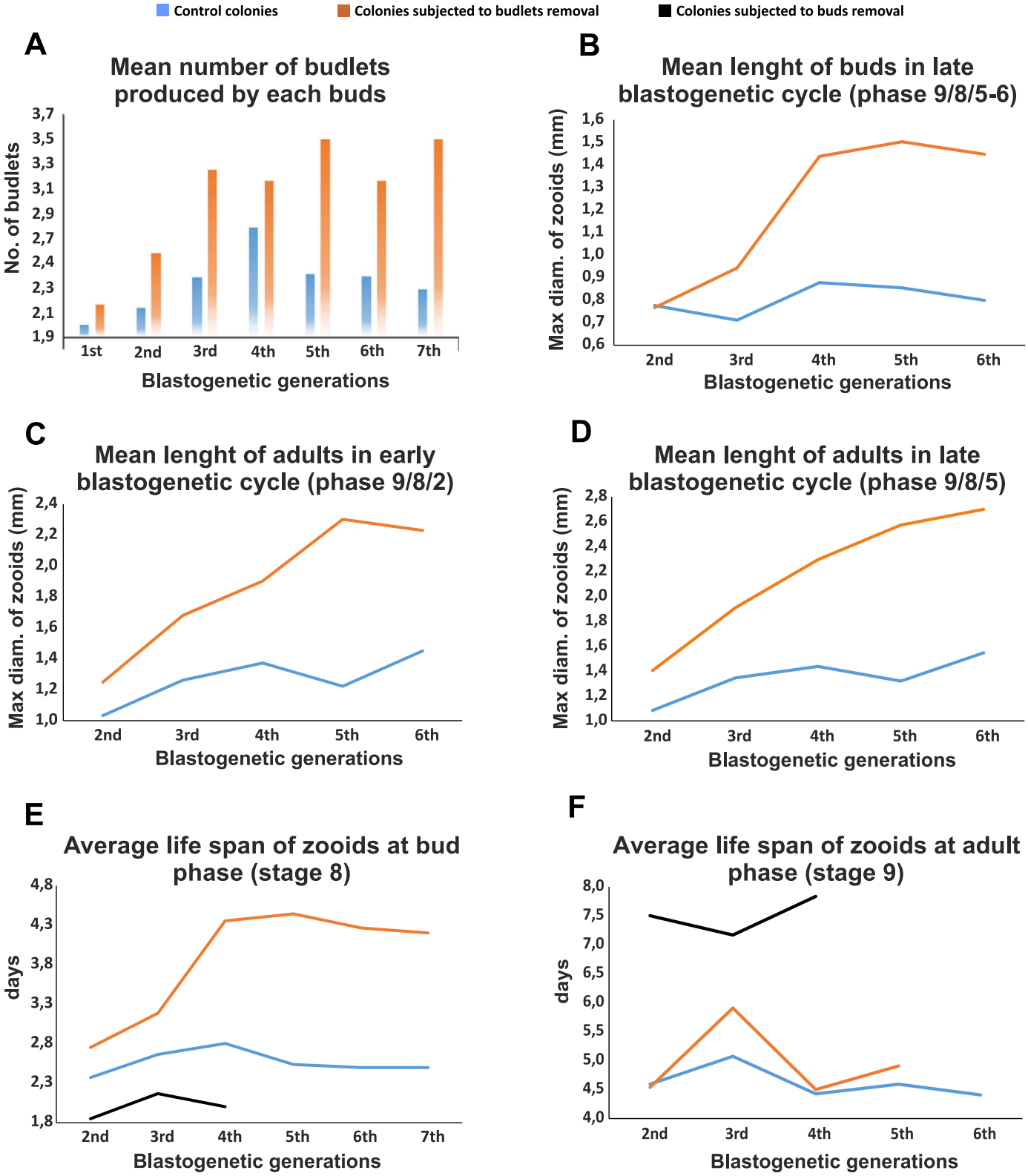

Fig. 5. A. mean number of budlets produced by control buds (light blue bars) and by single budlets, after the removal experiments, once become buds (orange bars), in the first seven blastogenetic generations. **B–D**: mean length of buds at blastogenetic developmental stage 8 (**B**) and of adults at developmental phase 9/8/2 (**C**) and 9/8/5 (**D**) in control colonies (light blue lines) and in colonies subjected to budlet removal (orange lines) (data in Suppl. Tables 7, 8). **E–F**: mean duration of blastogenetic developmental stage 8 (**E**) and 9 (**F**) in control colonies (light blue) and in colonies subjected to removal of budlets (orange lines) and of buds at stage 8 (black lines) (data in Suppl. Table 11).

6.3. Zooid growth during blastogenesis

The size of the zooid is dependent on its bud size: it changes over generations, with a general trend to increase (Berrill, 1941a, 1941b; Sabbadin, 1958).

Budectomy experiments were used to analyze zooid growth and to investigate if it is influenced by competition between budlets developing on the same bud, and by competition among generations coexisting in the colony (Sabbadin, 1958; Lauzon et al., 2002).

The budectomy experiment showed that when all buds but one were removed from the zooids, the size of the following generation zooids was larger than the first ones (Fig. 5B–D; Suppl. Tables 7, 8). Possible explanations are: i) in experimental colonies, the single maturing bud per generation does not need to compete for nutrients with other buds, and it solely benefits from parental degeneration at TO; ii) once adult, this zooid supports the development of only one bud; iii) bud and adult generation time (*i.e.*, stages 8 and 9), in experimental individuals, last longer than in controls (see below), so that they can grow for a longer period of time.

Bud and/or adult zooid removal was also used to study the involvement of buds/zooids and the vascular system on regulating programmed cell death (PCD) and cell clearance during the TO phase (Lauzon et al., 2007). This study revealed: a bud-independent signal that activates PCD in old zooids and a bud-dependent, survival signal that acts in short-range fashion *via* the colonial vasculature and requires mature buds (Lauzon et al., 2007). The importance of the availability of an adequate quantity of nutrients for bud development is suggested by the requirement of appropriate phagocytosis for the onset of a new blastogenetic cycle, as indicated by the severe impairment of bud development when phagocyte activity is inhibited (Voskoboynik et al., 2004). Therefore, the same kinds of turn-over processes occurring in long-lived animals *(i.e.*, continual death and disposal of aged cells counterbalanced by regeneration from stem and progenitor cells) ensure the growth of the colony by cycles of death and regeneration of its constituent zooids (Lauzon et al., 2002).

6.4. Synchronization among generations and duration of the blastogenetic cycle

The blastogenetic cycle is characterized by synchronized development of all buds and budlets in the colony. Zooids degeneration during the TO phase is also synchronized. Budectomy can perturb the synchronization among generations and influence the duration of the blastogenetic cycle providing information on the relationships between adult zooids and their buds (Sabbadin, 1958; Lauzon et al., 2007). When colonies grow in unfavorable environmental conditions, the number of budlets and buds decreases, and adult zooids tend to be resorbed before they are fully developed. When this happens, primary buds mature to functional zooids earlier. Stress conditions also perturb synchronization in 34% of colonies observed (Sabbadin, 1958). Under extreme environmental conditions, colonies can survive with only two generations of individuals (zooids and primary buds), with reduced blastogenetic and growing capabilities. This occurs when the older generation is resorbed, and buds enter the adult stage before their budlets are capable of budding. (Suppl. Tables 9–11). Conversely, when budlets and buds are removed (Fig. 3D), adult zooids remain active for a longer time compared to the controls (Fig. 5F; Suppl. Table 11).

Furthermore, the incomplete removal of advanced right buds can induce the regeneration of new budlets from bud remnants when adults have already entered TO. The removal can also trigger the development of atrophied budlets on the opposite side (Fig. 3D). These buds are smaller than those developed from normal budlets, and the time they take to develop is significantly shorter (Fig. 5E, Suppl. Table 11), since they are forced to open their siphons and begin filtration as soon as possible for colony survival, with limited or no energy provisions for their growth from the parental zooids (Sabbadin, 1958).

The above observations indicate a close relationship between buds and adults: the growth of the former is dependent on the resorption of the latter at TO. Once buds are removed, zooids need more time to bud new generations that will replace them; conversely, as reported, precocious resorption of the adult zooids determines a faster development of buds to become adults.

More than three coexisting generations were observed in several zooids, where all but one of the buds were removed (see colony group C in Suppl. Table 9). In these experiments, the extension of the adult state beyond the normal period of blastogenesis might explain this phenomena. Considering that a longer adult lifespan causes a longer bud lifespan (Fig. 5E–F; Suppl. Table 11), a new generation of primary buds appears before the generation change (Sabbadin, 1958). This new primary bud generation can give rise to budlets (fourth generation) that, once at the blastogenetic developmental stage 7, are able to bud a fifth blastogenetic generation of budlets before the adult zooids complete their resorption at TO. This anomalous phase can be indicated by the formula 11/9/8/7/1. Similar phenomena has been observed when a chemical treatment (BHT) prevented the resorption of the colony zooids in B. schlosseri (Voskoboynik et al., 2004) and also in *Symplegma reptans* (Sugino and Nakauchi, 1987), an ascidian belonging to the same family – Styelidae – as *Botryllus*.

7. Left-right axes determination in blastogenesis: the *situs inversus viscerum*

In ascidians, zooids have a marked bilateral asymmetry: the digestive tract is located on the left of the branchial basket, laterally to its posterior tract, and the heart is located ventrally, at the bottom of the branchial chamber, on the right side of the midline marked by the endostyle. Occasionally, the bilateral asymmetry of viscera appears reversed: the digestive tract extends to the right of the branchial chamber, and the heart is placed on the left of the midline. This condition, known as *situs inversus viscerum* (*SIV*) (Figs. 6 and 7), is rare (< 0.1%) in nature: only a single oozooid, out of 1500 in *B. schlosseri* (Sabbadin, 1956) and few reversed blastozooids out of many thousands in *Metandrocarpa taylori* (Watanabe and Newberry, 1976) were found. These zooids are able to multiply regularly by budding and produce normal gametes.

As previously reported (Fig. 4A; Suppl. Table 1), in small colonies, *e.g.* those formed by the oozooid and its bud and budlets, or by zooids belonging to the first blastogenetic generations, usually only the right budlet can develop. In such small colonies (at the developmental phase 9/8/2), the removal of the single bud at the blastogenetic developmental stage 8 can result in: i) the resumption of development of the left budlet (most frequent event); ii) the recovery of development of a posterior bud, if present; iii) the regeneration of a new bud from an incompletely removed bud; iv) the death of the colony left without any bud (Fig. 4; Sabbadin, 1956, 1958). In most of the cases where colonies survive, buds having resumed their development maintain the parental *situs viscerum* (Table 1) (Fig. 6A). This is the rule in the case of regenerated buds after incomplete removal. However, in 12% of the cases of development from an atrophied bud, especially when the adult zooids undergo a precocious TO before the formation of viscera in the budlet (stage 4), *SIV* appears (Table 2) (Figs. 6B, 7). The occurrence of *SIV* in incompletely removed buds that have regenerated, was reported in *S. reptans* (Sugino and Nakauchi, 1987) and can also be experimentally induced in *P. misakiensis* (Kawamura and Watanabe, 1982; Oda and Watanabe, 1981, 1982). The probability to obtain buds with *SIV* decreases in older blastogenetic generations. The highest frequency (17.64%) is observed in oozooids; it decreases to 13.38% in the 1st blastozooid and to 7.54% in colonies formed by the second blastozooids and their buds. However, when buds at developmental stage 8 are destroyed (tearing of the body wall and viscera removal) without affecting their budlets, and circulation between the adult zooid and

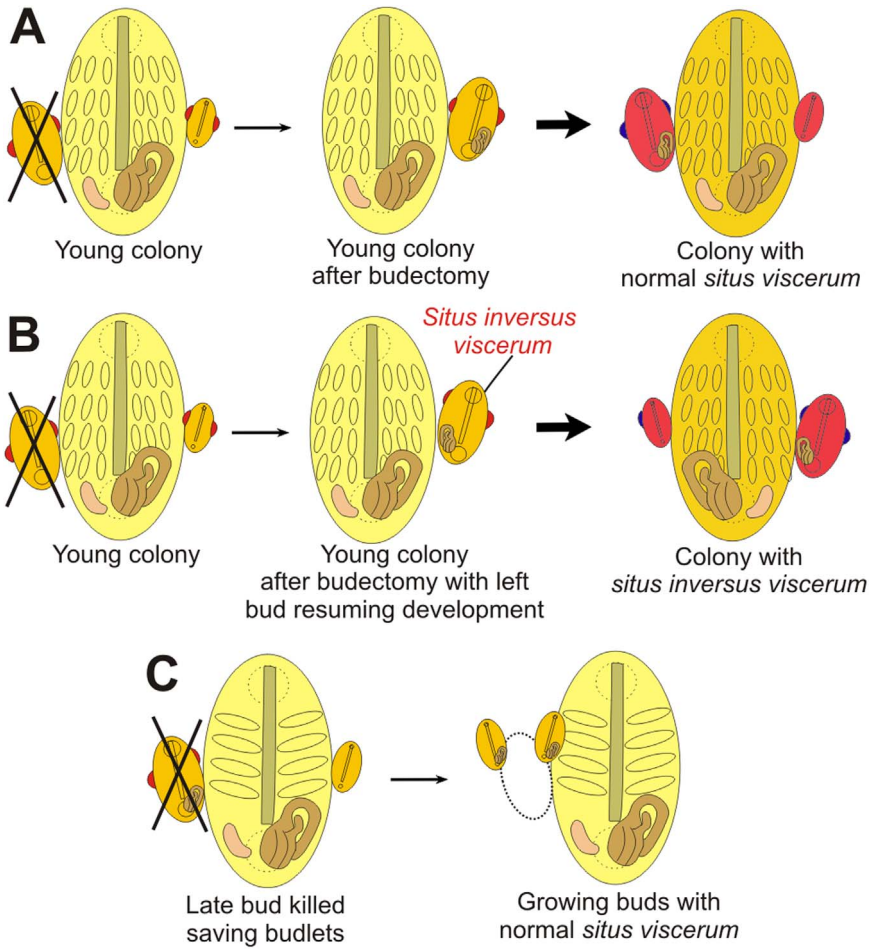

Fig. 6. Experiments of bud removal causing *SIV* appearance (Sabbadin, 1960). Color code and symbols as in Fig. 4. Ventral view. **A**: a high percentage of zooids derived from atrophied budlets resuming development after the removal of the right anterior bud having reached the blastogenetic developmental stage 8 maintain a normal *situs viscerum*, evidenced by the gut on the left side and the heart on the right side of the body. **B**: if buds removal occurs when colonies are approaching TO, *SIV* can develop (note that gut and heart have an opposite position with respect to endostyle, as compared with colonies with normal *situs viscerum*). **C.** In the case buds are killed without affecting their budlets, the parental asymmetry is maintained and *SIV* never appears.

the bud remains intact (Fig. 6C), the parental asymmetry is maintained and *SIV* never appears (Sabbadin, 1960). Repeated experiments indicate that, once present, *SIV* always originates in the buds, and is preserved in the subsequent blastogenetic generations (up to 20) as zooids with *SIV*. In this way, large colonies of hundreds of individuals can be obtained with *SIV* in all zooids (Sabbadin, 1956, 1960). However, the inverted asymmetry cannot be transmitted sexually as colonies with *SIV* undergoing sexual reproduction always originate colonies with normal *situs viscerum*. When genetically compatible colonies with normal asymmetry are left to fuse with colonies with *SIV*, zooids with opposite asymmetry coexist in the same chimeric colony and their buds maintain the asymmetry of their parent zooids (Fig. 8) even when adult *SIV* zooids are ablated to enhance the influence of normal zooids. This suggests that *SIV* is an epigenetic phenomenon, not controlled by factors diffusing from the common circulation, and indicates that the determination of bilateral asymmetry is very precocious, present in the budlets as early as developmental stage 3. Probably, the parental control occurred directly through the peribranchial epithelium, from which budlets derived and upon which they depend for their polarity (Sabbadin, 1966).

In normally asymmetric zooids of *B. schlosseri*, the blastogenetic potential is higher on the right side, whereas the ability to mature gonads is higher on the left side (Gasparini et al., 2015). Once a zooid develops an inverted bilateral asymmetry, its blastogenetic and gonadogenetic potentials are also reversed (Sabbadin, 1956, 1960; Gasparini et al., 2015). In colonies with normal asymmetry, 73% of buds emerge on the right side and 27% on the left one, whereas inverted colonies produce 25% of right buds and 75% of left buds (Sabbadin, 1960). In gonadogenesis, gonads are usually observed on the left side in 68% of the observed control zooids, whereas, in *SIV* colonies, the right side produced more eggs in 76% of the zooids (Sabbadin, 1960).

8. An alternative asexual reproduction: the vascular budding

Vascular budding (termed whole-body regeneration when induced experimentally; Rinkevich et al., 1995; Voskoboynik et al., 2007) was first described in botryllid ascidians more than two hundred years ago (Savigny, 1816) and observed again by Giard (1872). However, it was denied by Metschnikow (1869), who was convinced that buds originated only from the body walls (palleal budding). Bancroft (1903b) and Herdman (1925) again described the process. In species of the genus *Botrylloides*, vascular budding is generally associated with the process of aestivation or hibernation, during which colonies resorb their zooids to overcome the adverse periods. Zooids appear again, formed by tunic vessels, when environmental conditions turn mild (Bancroft, 1903b; Oka and Watanabe, 1959; Burighel et al., 1976; Rinkevich et al., 1995, 2007a, 2007b; Brown et al., 2009; Atsumi and Saito, 2011). Conversely, in *B. primigenus*, vascular budding is a physiological process that occurs continuously near the leading edge of the colony, contributing to the growth of the colony (Oka and Watanabe, 1957b). A physiological vascular budding has also been reported in the stolido-

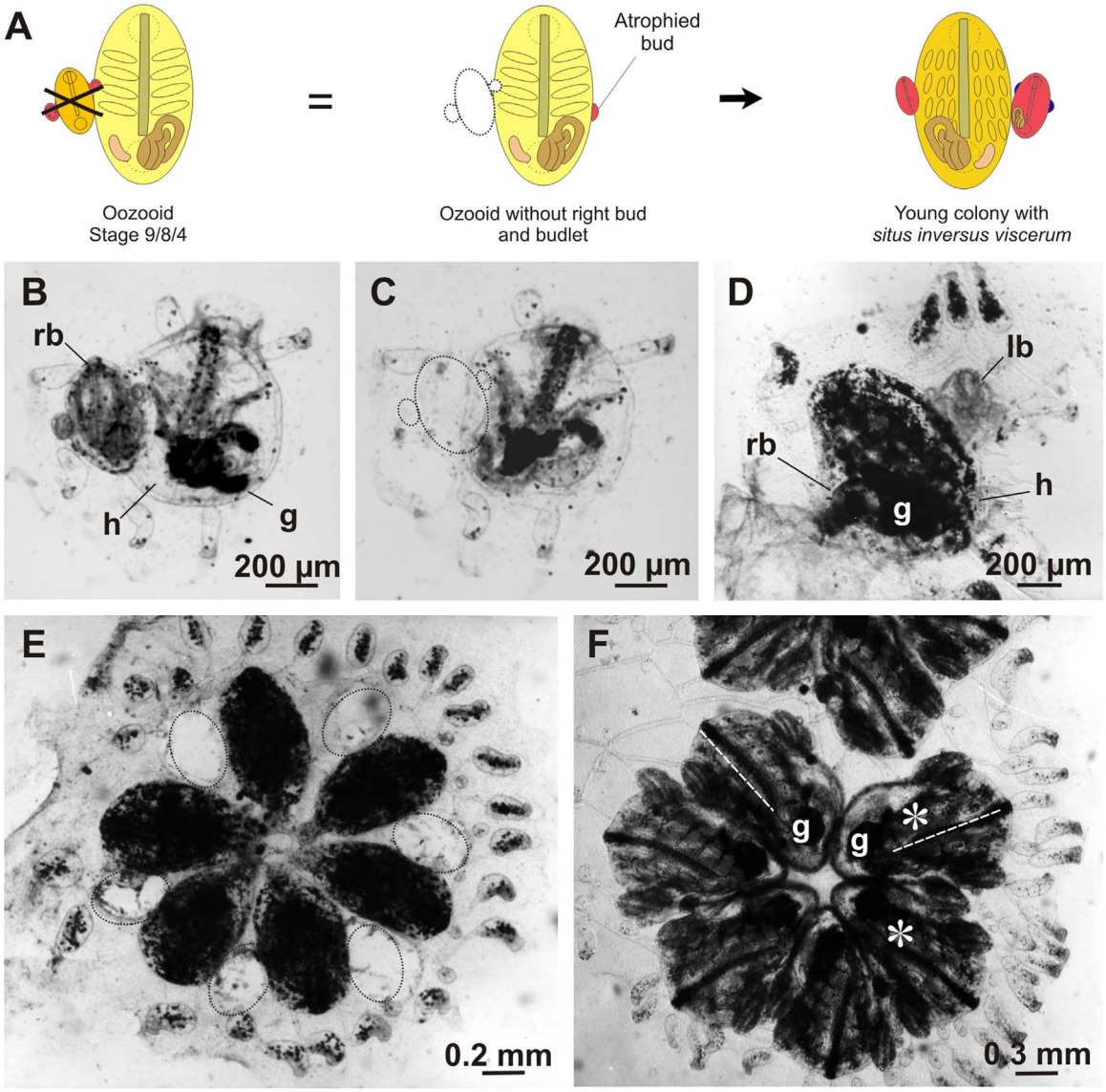

Fig. 7. A: *SIV* induction in the first blastozooid by removal of the oozooids right bud. **B**: control oozooids. **C**: oozooids deprived of its bud. **D**: blastozooid with *SIV* deriving from the recovery of the atrophied left budlets. **E**: colony with removed buds. The recovery of its atrophied budlets resulted in a colony (**F**) with two *SIV* zooids (**asterisks**). **g**: gut; **h**: heart; **lb**: left budlet; **rb**: right budlet; the endostyle is marked with a dotted line.

branch styelid *Symplegma brakenhielmi* (Gutierrez and Brown, 2017) and the phebobranch *Perophora viridis* (Freeman, 1964).

In *B. schlosseri*, vascular budding is usually repressed and occurs only after the removal of all the zooids and the buds from the colonial matrix (*i.e.*, the tunic and the common vasculature) in colonies approaching or undergoing TO (Fig. 9A–D; Milkman, 1967; Sabbadin et al., 1975; Voskoboynik et al., 2007; Kürn et al., 2011; Ricci et al., 2016a) and implies a sequence of morphological abnormal developmental steps (Voskoboynik et al., 2007). Vascular buds maintain the asymmetry of the parental colony (Table 3), suggesting that the colonial matrix has a role in the transmission of the bilateral asymmetry to newly-formed vascular buds (Sabbadin et al., 1975).

Table 1
Percentages of buds with normal or reversed bilateral asymmetry of viscera (*situs viscerum*) obtained in experiments of late bud extirpation from young colonies and comparison with bilateral asymmetry of the parental zooid.

Asymmetry of parental zooid	**Asymmetry of buds**					
	Normal			Reversed		
	Buds with normal growth	Restored buds	Buds with late development	Buds with normal growth	Restored buds	Buds with late development
Normal **Percentage**	21 **77**	4	21	5 **23**	–	9
Reversed **Percentage**	1 **5**	–	1	26 **95**	4	8

Table 2
Percentages of buds with normal or reversed bilateral asymmetry of viscera (*SIV*) in relation to the functional conditions of the parental zooid and its asymmetry at the moment of bud differentiation.

Asymmetry and functional condition of parental zooid	Asymmetry of buds	
	Normal	Reversed
Normal, filter-feeding	100	–
Reversed, filter-feeding	–	100
Normal, at TO	75	25
Reversed, at TO	–	100

Unlike palleal buds, vascular buds are formed by the epidermis lining the vasculature and circulating cells (hemoblasts), featuring stem cell properties (Oka and Watanabe, 1957b; Rinkevich et al., 1995; Voskoboynik et al., 2007), able to originate both the soma and germ line (Sunanaga et al., 2006; Voskoboynik et al., 2007). A bud primordium appears as an aggregate of hemocytes, adhering to the vessel epithelium, that show the morphology of stem cells, such as small size and high nucleus-cytoplasm ratio (Freeman, 1964; Oka and Watanabe, 1957b; Voskoboynik et al., 2007; Rinkevich et al., 2007b, 2008). In botryllid ascidians, these cell aggregates appear at the bases of the ampullae (the blind endings of the tunic vessels) and, later, organize to form a double vesicle stage, critical for bud organogenesis and normal bud development (Oka and Watanabe, 1957b; Rinkevich et al., 1995). A heart-like organ is the first organ to develop in the vascular buds, supporting efficient blood flow to the regenerating area and essential to zooid development (Voskoboynik et al., 2007). Vascular budding is part of the normal life cycle of *Botrylloides leachii* (Rinkevich et al., 1995, 2007a). In this species, the process occurs in five stages (Zondag et al., 2016; Blanchoud et al., 2017). In the first stage, lasting 15 h, wound healing takes place, then, a restructuring of the vessel architecture and of the ampullae occurs leading to the formation of small regeneration niches (stage 2), followed by the contraction of the tissues (stage 3) and the homing of stem cells in the regeneration niches (stage 4). Finally, the competition among the various stem cell aggregates (stage 5) leads to the maturation of a single bud per experimental fragment (Rinkevich et al., 2007a, 2007b, 2008; Zondag et al., 2016; Blanchoud et al., 2017).

9. Genes associated with vascular budding

Recently, the expression pattern of a number of genes related to vascular budding have been studied in various botryllid species except *B. schlosseri*.

Vascular buds do not express *piwi* in *B. primigenus* (Sunanaga et al., 2010). However, a role of hemocytes lining the vessel epithelium, able to proliferate and expressing *piwi*, has been postulated in the formation of the bud primordia in *Botrylloides violaceus* (Brown et al., 2009) and *B. leachii* (Rinkevich et al., 2010). Analogous to palleal budding of colonial ascidians (Kawamura et al., 1993, 2013), vascular budding in *Botrylloides* requires the presence of retinoid acid (RA): RA inhibitors block the process, whereas RA agonists accelerate bud formation and increase the number of buds per experimental fragment (Rinkevich et al., 2007a). In addition, serine proteases are also required, as serine protease inhibitors alter the development of vascular buds in *Botrylloides* (Rinkevich et al., 2007b). This is probably in relation with the role of the enzyme in remodeling the extracellular matrix (Rinkevich et al., 2007b). This hypothesis agrees with the observation that the transcription of a trypsin-like serine protease increases upon RA treatment in the budding ascidian *P. misakiensis* (Ohashi et al., 1999). In botryllid ascidians, the transcripts for aldehyde dehydrogenase, an enzyme involved in RA synthesis, and a serine protease similar to the mammalian urokinase-type plasminogen activator, are mainly located in stem cell populations (Laird et al., 2005a) but are also present in other cells including circulating phagocytes (Rinkevich et al., 2007a, 2007b). The latter are deeply involved in innate immune responses of botryllid ascidians (Franchi and Ballarin, 2017). This suggests a key role of these cells in vascular budding, in addition to their ascertained role in palleal budding (Voskoboynik et al., 2004) and implies the involvement of innate immune responses in morphogenetic events of colonial ascidians. According to this,

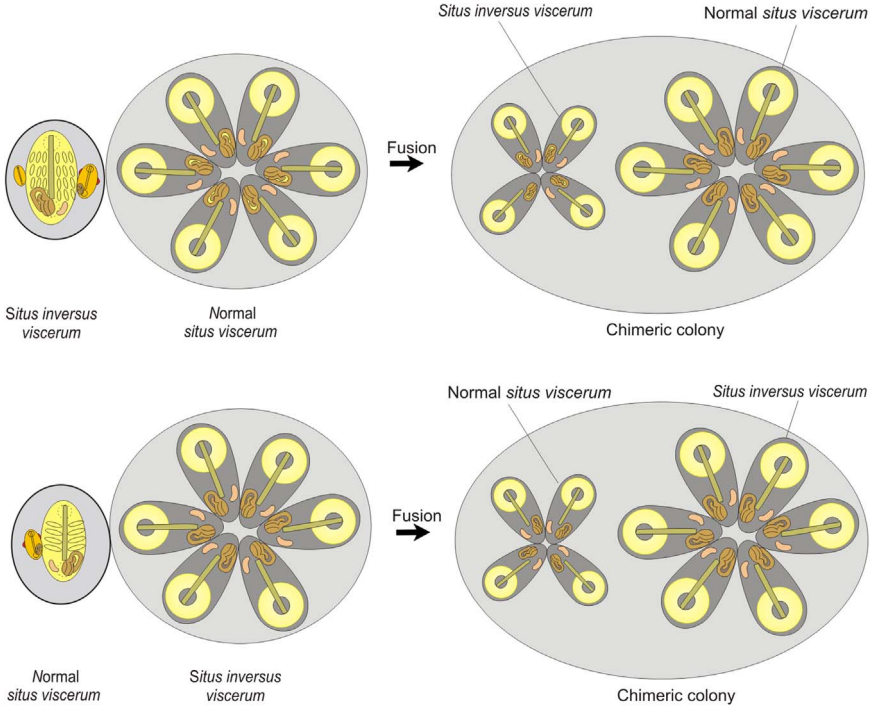

Fig. 8. Effect of colony size on bilateral asymmetry in parabiosis experiments. Young colony with *SIV* fused with a larger, genetically compatible colony with normal *situs viscerum* (top) and young colony with normal *situs viscerum* fused with a larger, genetically compatible colony with *SIV*. In both cases, the chimeric colony maintains the original bilateral asymmetry of the zooids over generations.

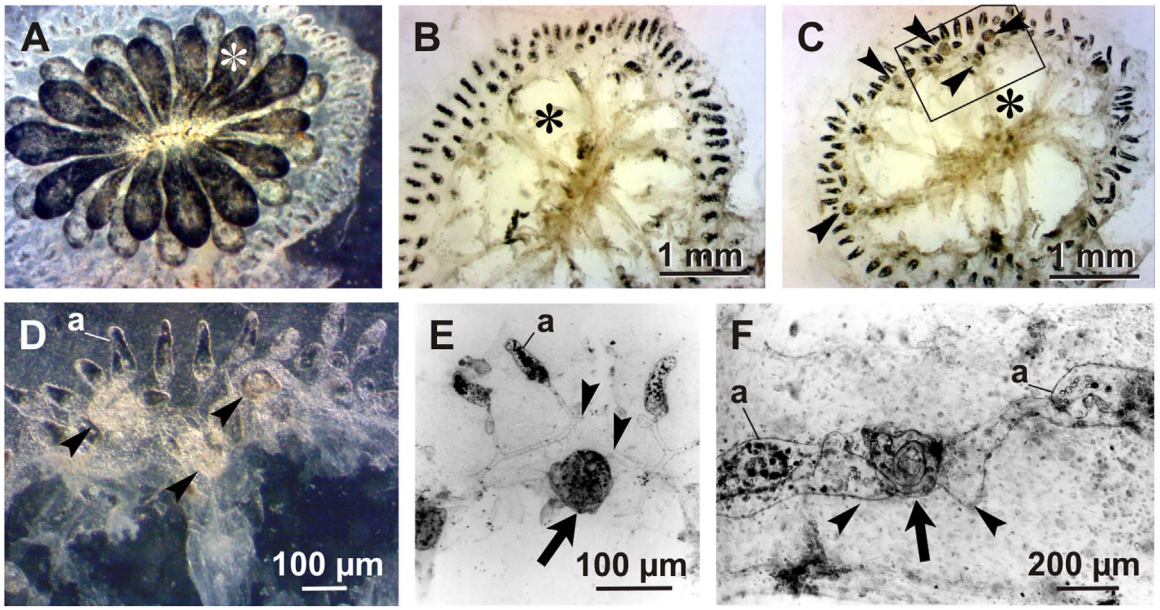

Fig. 9. A–D: vascular budding. **A**: colony before the removal of zooids and buds. **B**: remaining colonial matrix. **C–D**: vascular buds (**arrowheads**) in the colonial matrix. The asterisks mark the location of a reference zooid in the colonial matrix. The squared area in **C** is enlarged in **D. E**: isolated palleal bud (**arrow**). Note the truncated edges of the marginal vessel (**arrowheads**) **F**: isolated palleal bud (**arrow**) contacted by two ampullae (**arrowheads**). **a**: ampulla.

Table 3
Percentages of palleal (isolated in place or transplanted) and vascular buds with normal or reversed bilateral asymmetry of viscera (*situs viscerum*) in relation to the bilateral asymmetry of the parental colony.

Buds	Asymmetry of the parental colony	Asymmetry of buds	
		Normal	Reversed
Palleal, isolated	Normal	100	–
	Reversed	–	100
Palleal, transplanted	Donor and recipient reversed	–	100
	Donor normal, recipient reversed	100	–
Vascular	Normal	97	3
	Reversed	–	100

B. leachii vascular budding is associated with the differential transcription of various immune genes codifying for membrane and soluble proteins acting both as receptors or adhesion proteins (*e.g.*, lectins, integrins) and effectors (*e.g.*, complement factors, serine proteases) (Rinkevich et al., 2007b). An increased transcription of genes involved in cell signaling and codifying for transcription factors has also been revealed by ESTs derived from early stages of vascular buds (Rinkevich et al., 2008).

10. Strategies for survival in isolated or transplanted buds and blastogenetic regeneration

When all the adult zooids and budlets in a colony are removed, the remaining buds (at blastogenetic developmental stage 8) can reach adulthood even if all the left buds and about 40% of the right buds undergo resorption (Zaniolo et al., 1976). Once they become adults, only the right buds are able to develop to full functionality.

When buds are isolated from the common vasculature, vascularization is required for their survival as development does not progress without the connection to the tunic vessels (Zaniolo et al., 1976) (Fig. 9E–F). New branches sprout from the colonial marginal vessel attracted by the isolated buds: they join the buds always on the right side (or on the left side in case of buds with *SIV)*. In normal blastogenesis, the entrance of the affluent vessel marks the posterior end of the bud in both vascular and transplanted buds (Sabbadin et al., 1975; Zaniolo et al., 1976). Sometimes, isolated buds can form their own vascular system and are progressively resorbed without reaching adulthood, allowing the development of their right bud, which can form a functional adult. In this case, when a new adult is formed, its buds are at the blastogenetic developmental stage 4 or 5, so that, for a short period of time, the budlet generation is lacking and two, instead of three, generations are present (Zaniolo et al., 1976).

Palleal buds removed from the parental zooid at developmental stages 1–3, and transplanted in the colonial matrix of a genetically compatible colony, can grow if vascularized and maintain their bilateral asymmetry (Sabbadin et al., 1975). Once isolated, they can also be cultured *in vitro*, where they can survive up to 5 months (Rabinowitz and Rinkevich, 2003, 2004). If cultured in an artificial medium at later developmental stages (4–5), they form epithelial monolayers expressing cadherin, PL10, piwi and mortalin (Rabinowitz and Rinkevich, 2011; Ben-Hamo et al., 2018). However, cells stop dividing after 24–72 h, without producing any permanent cell line (Rabinowitz et al., 2009; Rabinowitz and Rinkevich, 2011). Collectively, the study of the development and senescence of these transient cell cultures from dissected buds evidences that, under *in vitro* conditions, the normal growth processes are replaced by different developmental pathways, but also that internal clocks programming cyclical death, are replaced by new biological mechanisms with different timetables (Rabinowitz and Rinkevich, 2004).

In the Japanese species *P. misakiensis* (Kawamura and Fujiwara, 1995) and *B. primigenus*, (Kawamura et al., 2006), the establishment of stable cell lines from bud tissues was reported. The results are still debated as a contamination by traustochytrids protists is suspected (Rinkevich, 1999).

The term **"**blastogenetic regeneration**"**, introduced by Sugino and Nakauchi (1987), indicates the regeneration of a colony from fragments of buds through the emission of buds from the bud remnants that, after healing of the cut surfaces, are progressively resorbed. The process was initially described in *B. schlosseri* (Majone, 1977), in young colonies (at the developmental phase 9/8/4), where both adults (stage 9) and budlets (stage 4) were removed as well as the posterior part of the buds (stage 8). The anterior fragments, containing the oral siphon, the neural complex, part of the branchial basket and of the

endostyle remained connected to the tunic circulation *via* the radial vessel. In these fragments, the internal tissues lose their morphology and are progressively resorbed, and new vascular connections with the colonial marginal vessel replace the original radial vessel. Five to six days after the operation, new budlets sprout from the bud remnant: only one of them reaching adulthood. Up to 17% of the zooids obtained by blastogenetic regeneration, have inverted asymmetry (Majone, 1977). A similar regeneration process was described in *S. reptans* (Sugino and Nakauchi, 1987), together with a normal regeneration of bud fragments through a morphallactic process, also reported in *P. misakiensis* (Oda and Watanabe, 1982; Sugino and Nakauchi, 1987).

11. Future perspectives

Colonial ascidians are representative of tunicates, the sister group of vertebrates. They are the only chordates capable of asexual reproduction and vascular budding. Colonial ascidians are ideal models for the study of tissue regeneration and development, due to their diverse reproductive strategies, relatively short lifespan, simple morphological and genomic organization, and easy experimental use. The studies described above detail the varied strategies *B. schlosseri* colonies undertake to survive and propagate. These strategies include: variations in bud developmental potential, zooid growth potential, and duration of a generation cycle, and under certain conditions the number of coexisting generations and the number of coexisting genotypes (formation of chimeras). Old and young generations coexisting in the colony, create a continuous balance between the energy provided by the adult zooids sustaining the colony and the energy required to support bud development. Today, new molecular and gene manipulation tools can be used to study these biological phenotypes. The anatomical and developmental ontology of *B. schlosseri* asexual development is now available, allowing the use of a controlled and shared vocabulary among different laboratories (Manni et al., 2014). The hypothesis of the close evolutionary relationship between tunicates and vertebrates (Delsuc et al., 2018; Kocot et al., 2018; Giribet, 2018) is also supported by analysis of hundreds of nuclear genes from 15 species, including the colonial tunicate *B. schlosseri* (Voskoboynik et al., 2013a) and outcomes of single gene/structure/pathway studies on *B. schlosseri* asexual reproduction (Degasperi et al., 2009; Gasparini et al., 2013, 2016). The genomes of *B. schlosseri* and *B. leachii* have been published (Voskoboynik et al., 2013a; Blanchoud et al., 2018), and transcriptomes covering different reproductive/regenerative traits are available (Voskoboynik et al., 2013a, 2013b; Campagna et al., 2016; Corey et al., 2016; Kowarsky et al., 2017; Rosental et al., 2018; Ricci et al., 2016b). Methods to label, sort and transplant specific cell populations, and assayed cell differentiation capacities have been developed (Laird et al., 2005a; Voskoboynik et al., 2008; Lauzon et al., 2013; Rinkevich et al., 2013; Corey et al., 2016; Rosental et al., 2018). In addition, powerful imaging systems allow the tracing of labeled cells *in vivo via* the transparent body of young colonies (Voskoboynik et al., 2008; Rinkevich et al., 2013; Rodriguez et al., 2017; Rosental et al., 2018), and methods to silence specific genes (Laird et al., 2005b; Rosner et al., 2006, 2007, 2013; Tiozzo and De Tomaso, 2009; Voskoboynik et al., 2013b; Ricci et al., 2016a) are available. Moreover, powerful statistical approaches take advantage of coloniality, in which each individual zooid is essentially a biological replicate and differences among individuals derive only from external perturbations (*e.g.*, experimental variations) (Gasparini et al., 2014; Manni et al., 2018).

Altogether, the knowledge gained through 60 years of experimental studies of *B. schlosseri,* and the methods and databases developed, render *B. schlosseri* an excellent model for the study of stem cell mediated regenerative processes, development, chimerism and senescence.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.09.009.

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