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Whole-Body Regeneration and Developmental Competition in Two Botryllid Ascidians

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular, Cellular, and Developmental Biology

by

Shane Mahdi Nourizadeh

Committee in charge:

Professor Anthony de Tomaso, Chair

Professor Carolina Arias

Professor Dennis Clegg

Professor William Smith

June 2021

The dissertation of Shane Mahdi Nourizadeh is approved.

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Carolina Arias

_____

Dennis Clegg

_____

William Smith

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Anthony de Tomaso, Committee Chair

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Curriculum Vitae – June 2021

Shane Nourizadeh

Education:

2014 - B.S., Cell and Developmental Biology (GPA 3.49) – University of California, Santa Barbara, CA

Research:

2014 Fall – 2021 Spring

Graduate student researcher in the lab of Professor Anthony De Tomaso: Researching the mechanism of whole-body regeneration in the animals *Botryllus schlosseri* and *Botrylloides diegensis*. This model organism maintains the capacity to rebuild its entire body plan when segments of the blood vessels are all that remain after surgical removal of the main bodies (vasculature runs outside this animal). Understanding the genes and pathways involved in triggering stem cells to confer this regenerative property will have important biomedical implications and can enhance our overall understanding of stem cell activity.

2012 Summer

INSET (Internships in Nanosystems Science, Engineering, and Technology) intern: Decreasing the size of a cancer therapy drug and adsorbing antibodies onto its surface to increase the bioavailability and selectivity to targeted cells.
Title of project: Engineering Pure Drug Nanoparticles for Cancer Therapy.

Publications:

- Nourizadeh, S., Kassmer, S.H., Rodriguez, D., De Tomaso, A.W., Paper in Progress. Whole-body regeneration and developmental competition in two botryllid ascidians.
- Kassmer, S.H., Nourizadeh, S., De Tomaso, A.W., 2018. Cellular and Molecular Mechanisms of Regeneration in colonial and solitary Ascidians. Dev. Biol. DOI.doi.org/10.1016/j.ydbio.2018.11.021
- Rodriguez, D., Nourizadeh S., De Tomaso, A.W., 2018 The biology of the extracorporeal vasculature of Botryllus schlosseri. Dev. Bio. DOI:10.1016/j.ydbio.2018.10.013

Mentorship:

2018 Spring - 2020 Spring

Undergraduates:
Shiloh Kluding (2018-2020) – *RNA interference protocol design and testing.*
Serhat Suzer (2019-2020) – *Whole-body regeneration immunofluorescence.*

Outreach and Leadership:

2017 Fall - 2019 Spring
Graduate Student Mentor Program: Attended seminars on diversity and held quarterly meetings with three incoming doctoral students to aid in their transition into graduate school. Provided mentorship to students regarding their coursework, project design, committee formation, and project consideration.

2011 Spring - 2019 Spring
Annual Friday Night Science chemistry show organizer and participant: Helped the local community college strike enthusiasm and interest in STEM fields by bringing chemistry to life through the design and display of chemistry demonstrations in front of live audiences of 200+ people.

2011 Spring - 2012 Spring
SACNAS (Society for the Advancement of Chicanos and Native Americans in Science) secretary position: Our group planned coat drives, handed out Thanksgiving turkeys to single parents, and raised money using a raffle to send members of our group to the national SACNAS science conference in San Jose.

Awards and Scholarships:

2020 – Broida-Hirschfelder fellowship
2019 – Doreen J. Putrah Cancer Research Foundation conference travel fellowship
2017 – Shing Chang candidacy advancement fellowship
2017 – Doreen J. Putrah Cancer Research Foundation conference travel fellowship
2016 – Yzurdiaga UCSB departmental fellowship
2015 – Ralph M. Parsons fellowship
2014 – Dean's Honor Roll
2012 – Elevator speech contest winner
2012 – Sonrisa scholarship from Allan Hancock College
2011 – SACNAS community college of the year award

Posters:

- Nourizadeh, S.; Kassmer, S., Rodriguez, D.; DeTomaso, AW: Poster presented at 10th annual Tunicate conference, Villefranche-sur-mer France, July 4th-11th, 2019
- Nourizadeh, S.; Kassmer, S.; DeTomaso, AW: Poster presented at 9th annual Tunicate conference, Manhattan NY, September 21th-27st, 2017
- Nourizadeh, S.; Barua, S.; Mitragotri, S: Poster presented at 39th SACNAS national conference, Seattle WA, October 29th-31st, 2012

ABSTRACT

Whole-Body Regeneration and Developmental Competition in Two Botryllid Ascidians

by

Shane Mahdi Nourizadeh

Examples of metazoan regeneration can be seen within all animal phyla; however, the ability to restore lost tissues upon injury varies greatly. Some species can only partially replace tissues and organs, while others can repeatedly regenerate entire limbs, severed spinal cords, and portions of their brain. There are also extraordinary cases of whole-body regeneration (WBR) where animals can produce entire bodies from small fragments of leftover tissue after injury. A few species of botryllids can even utilize circulating blood borne stem cells for WBR. These animals are in the subphylum tunicata, and evolutionarily classified alongside vertebrata in the phylum Chordata.

Botryllids are marine invertebrates that grow by repeated rounds of asexual reproduction to form a colony of clonally derived individuals called zooids. Under normal conditions, zooids are regenerated *de novo* on a weekly basis, and a colony increases in size by expanding the number of individuals. Two distinct processes that differ by the source of new bodies are responsible for zooid regeneration in the botryllids. In the first, called

blastogenesis, new zooids arise from a multipotent epithelium of a pre-existing zooid. In the second, termed vascular budding (VB) or WBR, mobile cells in the vasculature are the source of the new zooid. In some botryllid species, blastogenesis and VB occur concurrently. In others, blastogenesis is exclusively used for expansion while WBR only occurs following injury or exit from dormancy.

We studied WBR in two related species: *Botryllus schlosseri* and *Botrylloides diegensis.* Both have an extracorporeal vasculature that develops outside the bodies to connect all individuals within a colony. We isolated blood vessels to induce WBR by removing all zooids, primary buds, and secondary buds. This surgery was performed under a dissecting microscope with scalpels and extra-fine tip forceps to prevent damage to the vasculature. Regeneration was then analyzed using time-lapse microscopy with the animal being maintained in a temperature-controlled seawater recirculation basin. We compared and characterized early stages of WBR utilizing these days-long videos. Our data suggest that unlike other botryllid species, new zooid growth following injury in *B. schlosseri* is not due to WBR, but instead through ectopic development of tissues leftover from the blastogenic process.

Despite these differences, we did find a common theme in the two species: developmental competition results in only a single zooid reaching maturity. We utilized this phenomenon to control the number of buds and their spatial orientation in *B. schlosseri* and found that competition is reversible and mediated by circulating factors and/or cells. This represents a new model to study resource allocation and competition within an individual.

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I. Introduction

A. Importance of regeneration research in medicine

The process of regenerating human tissue through use of cultured stem cells and activation of endogenous stem cells is an emerging field known as regenerative medicine. To successfully employ tissue regeneration in humans, we first must understand how these processes unfold in a wide range of model organisms. Currently, regeneration biology seeks answers to why many invertebrates and some vertebrates are more capable of regenerating lost tissues when compared to mammals. Advancement in this field requires elucidating *in vivo* signaling mechanisms used for niche formation, stem cell engraftment, and environment specific pluripotent cell differentiation.

While regenerative medicine holds vast potential in treating a myriad of conditions, many of the molecular mechanisms needed to employ therapies most effectively have yet to be investigated. Current data show that stem cells injected in a suspension have a low probability of homing to their target, engrafting, and differentiating[1]. The more effective treatments utilize stem cells adhered to an extracellular matrix mimicking biomaterial[2], however; signals needed for intercalation into existing tissues should be further investigated to improve cell survival[3–5].

Knowing the mechanisms employed by model systems will remove current misunderstandings in regenerative medicine and improve efficacy. Determining the temporal and spatial aspects of gene expression will lead to increased cell survival and

enhanced binding/intercalating with suitable bio-substrates. Future studies will open opportunities for understanding how cells respond to tissue damage, and novel strategies will utilize genetically compatible cells derived directly from patients to eradicate autoimmune pathologies caused by syngeneic cells.

B. Animal regeneration

Current regeneration strategies among model organisms involve two fundamental mechanisms: morphallaxis and epimorphosis. Morphallaxis is the rearrangement of pre-existing tissue to replace lost tissues, which occurs in *Hydra* where head regeneration can continue even when cell proliferation is blocked. A population of cells in the gastric column undergo differentiation to replace the missing head based on positional cues along the body axes[6]. Epimorphosis is when injury triggers the proliferation of adult stem cells, and this occurs in planarians, mice, and zebrafish. They can regenerate missing body parts through the formation and proliferation of a blastema[7]. Some species are even capable of employing both mechanisms to regenerate lost tissue depending on injury location[8].

The roles for known developmental signaling pathways (e.g. Wnt, BMP, FGF) in regeneration are being uncovered, and even though many organisms share common developmental pathways, not all maintain commensurate ability to replace lost tissues. Animals such as *C. elegans* and *D. melanogaster*, in which these signaling pathways have been studied in detail, are unable to regenerate missing body parts, therefore, regeneration is defined by the microenvironment and cellular context.

C. Tunicates

Tunicates are invertebrates that live exclusively in marine environments. They are classified in the phylum Chordata and are primitive relatives to the vertebrates[9]. Tunicates are comprised of three classes, Appendicularia, Thaliacea, and Ascidiacea, but only the latter two produce a tunic. All are, however, capable of biosynthesizing cellulose microfibrils via cellulose synthase, and are the only metazoans with this ability[10,11].

Out of 2815 species of ascidians, 61.5% are colonial[12]. Solitary ascidians like *Ciona intestinalis, Styela clava, and Halocynthia roretzi* have zooids that live separately within individual tunics, whereas zooids of colonial species like *Botryllus schlosseri, Botrylloides diegensis, and Symplegma brakenhielmi* share a common tunic and are connected via an extracorporeal vasculature.

To further the knowledgebase on regeneration, our lab studies *Botryllus schlosseri*, a diploid Tunicate with a 725 million base-pair genome on sixteen chromosomes. Recent genome sequencing has shown this species to have homologs for over 600 human genes related to a multitude of processes including cancer and immunity[13]. They can regenerate entire bodies from minimal tissue fragments. Therefore, characterizing this activity will allow for comparative gene analyses with other regeneration processes in the animal kingdom, as well as with human wound healing signaling pathways. In *Botryllus schlosseri,* researchers have applied *in situ* hybridization techniques with stem cell markers to pinpoint sources of tissue responsible for regeneration. However, the source of these cells is still not well understood.

D. Botryllus schlosseri animal overview

The invertebrate chordate species, *Botryllus schlosseri*, can be found near almost all continents in open shore habitats permanently adhered to docks, rocks, mussel shells, and other surfaces[14]. These animals have two modes of reproduction, and during the sexual lifecycle[15], embryogenesis gives rise to a free-swimming larval tadpole that eventually settles onto a suitable substrate and metamorphoses into a sessile invertebrate adult body, called a zooid. The adult then gives rise to more zooids through a four-stage (A-D) asexual budding process called the blastogenic cycle[16], and even though each zooid is a complete and independent filter feeding individual with a complex body plan including siphons, a heart, GI tract, nervous system, and gonads, they tend to tightly associate with two to fourteen other zooids to form star-shaped clusters known as systems (Fig. 1). All systems are enclosed within a protective and translucent cellulose-based extracellular matrix called a tunic, and when multiple systems reside under a single tunic, they are collectively referred to as a colony. All zooids in a colony are connected via a common extracorporeal vasculature that runs throughout the tunic and serves as a conduit for their complex hematopoietic system. The network of external blood vessels is formed from a single layer of ectodermally derived tissues that allow components of the blood to be shared between all animals within a colony[17,18]. Vessels extend to the tunic periphery and terminate into close-ended oval shaped ampullae that are involved in colony allorecognition[19], adherence to substrate surfaces, and asexual reproduction in some species. This vascular setup is dynamic and in conjunction with other tissues is largely involved in the ability for *Botryllus* to regenerate even after severe injury to all zooids in a colony.

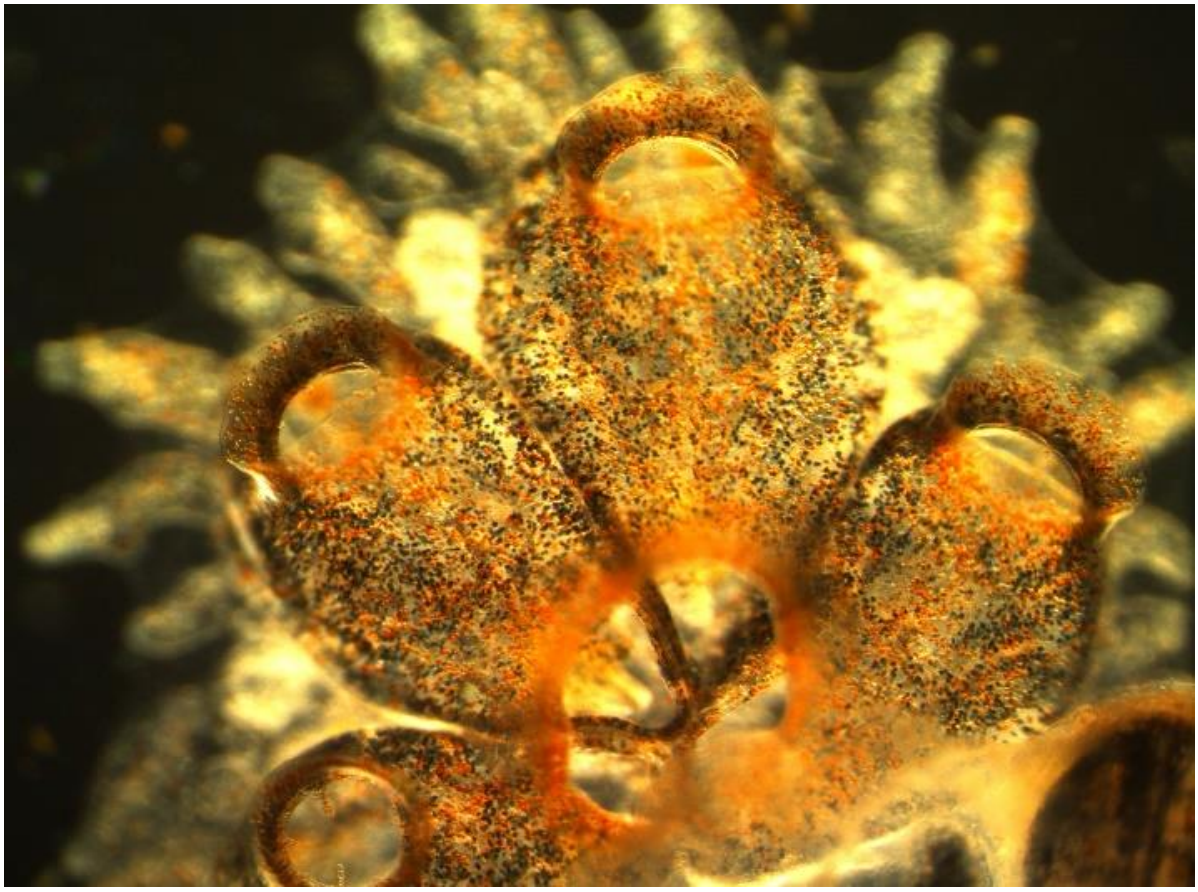

Figure 1: Dorsal view showing four zooids of *Botryllus schlosseri*.

No other chordate possesses the ability to regenerate an entire body from minimal tissue and studying this mechanism in a mostly translucent vasculature will allow for an understanding of spatial and temporal aspects of receptors, ligands, morphogens, and growth factors responsible for orchestrating tissue reorganization and regeneration.

E. Botryllus schlosseri regeneration

The major mechanisms underlying whole-body regeneration in *Botryllus schlosseri* are not well understood because the tissues implicated in initiating this process have yet to

be conclusively identified. Early researchers looked to other related species to gain clues, and histological studies done in *Botrylloides leachii* show that an aggregation of hemocytes correlates with regeneration[20]. However, they were unable to elucidate the origin of cells leading to early developing structures. Subsequent studies looked more at *Botryllus schlosseri* vasculature and detected *piwi* expression in non-migratory cells that appeared to be lying flat against the inner epithelium upon injury[21]. As regeneration proceeded, the *piwi* expressing cells were found in circulation and eventually clustered. Therefore, it was concluded that cells in contact with the epithelium become migratory upon injury and aggregate to provide early cell contributions for WBR. Knockdown of *piwi* with siRNA resulted in failure to regenerate, indicating its role during the initial stages. The original tissue type of these *piwi* expressing cells could not be confirmed. Therefore, it is still unclear which cells give rise to regenerating zooids, and whether the initial stages involve proliferation of stem cells (epimorphosis), or rearrangement of existing tissues (morphallaxis). Studies have also examined asexual stage dependency on vascular budding, and which suggest that this process is confined to stage D of the blastogenic cycle, and stages A through C are incapable of employing this mechanism of recovering from major tissue loss[22]. They also show that the blood flow and number of blood cells in circulation are invariable throughout the four stages of blastogenesis, suggesting that increased hemocyte concentration does not correlate with potential to bud from blood vessels.

Because whole-body regeneration occurs from blood vessels, this dissertation begins by reviewing the blood vasculature of *Botryllus schlosseri*. This is followed by a review of solitary and colonial ascidian regeneration to give a broad overview of

mechanisms and methodologies employed in closely related animal studies. It finishes with a closer look at whole-body regeneration in two colonial botryllids: *Botryllus schlosseri* and *Botrylloides diegensis*.

II. The biology of the extracorporeal vasculature in *Botryllus schlosseri*

Delany Rodriguez∗, Shane Nourizadeh, Anthony W. De Tomaso

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A. Introduction

Tunicates are marine invertebrate chordates considered the closest living non-vertebrate to vertebrates[9,23]. *Botryllus schlosseri* belong to the polyphyletic sub-phylum Tunicata (tunicates), class Ascidiacea (ascidians), and family Styelidae; a family where both solitary and colonial organisms have been identified[24–28]. Ascidians can reproduce both sexually and asexually (Fig. 2.1B)[15,18,25,27]. *B. schlosseri* embryonic development results in a tadpole larva with typical chordate characteristics, but then undergoes metamorphosis to give rise to a sessile invertebrate known as an oozooid. This initial oozooid then expands the colony through a lifelong asexual budding process termed blastogenesis, in which genetically identical zooids are added (Fig. 2.1A). Each adult individual has a fully functional heart, gastrointestinal tract, nervous system, and germline.

The *B. schlosseri* vascular system is a combination of two structures. First, within each zooid exists an open circulatory system consisting of: a tubular heart, lacunae, and tissues bathed in blood[29,30]. Second, a large ramified transparent extracorporeal vasculature forms a network of monolayered vessels that extend out to the periphery of the colony and terminate into specialized structures known as ampullae (Fig. 2.1C and D).

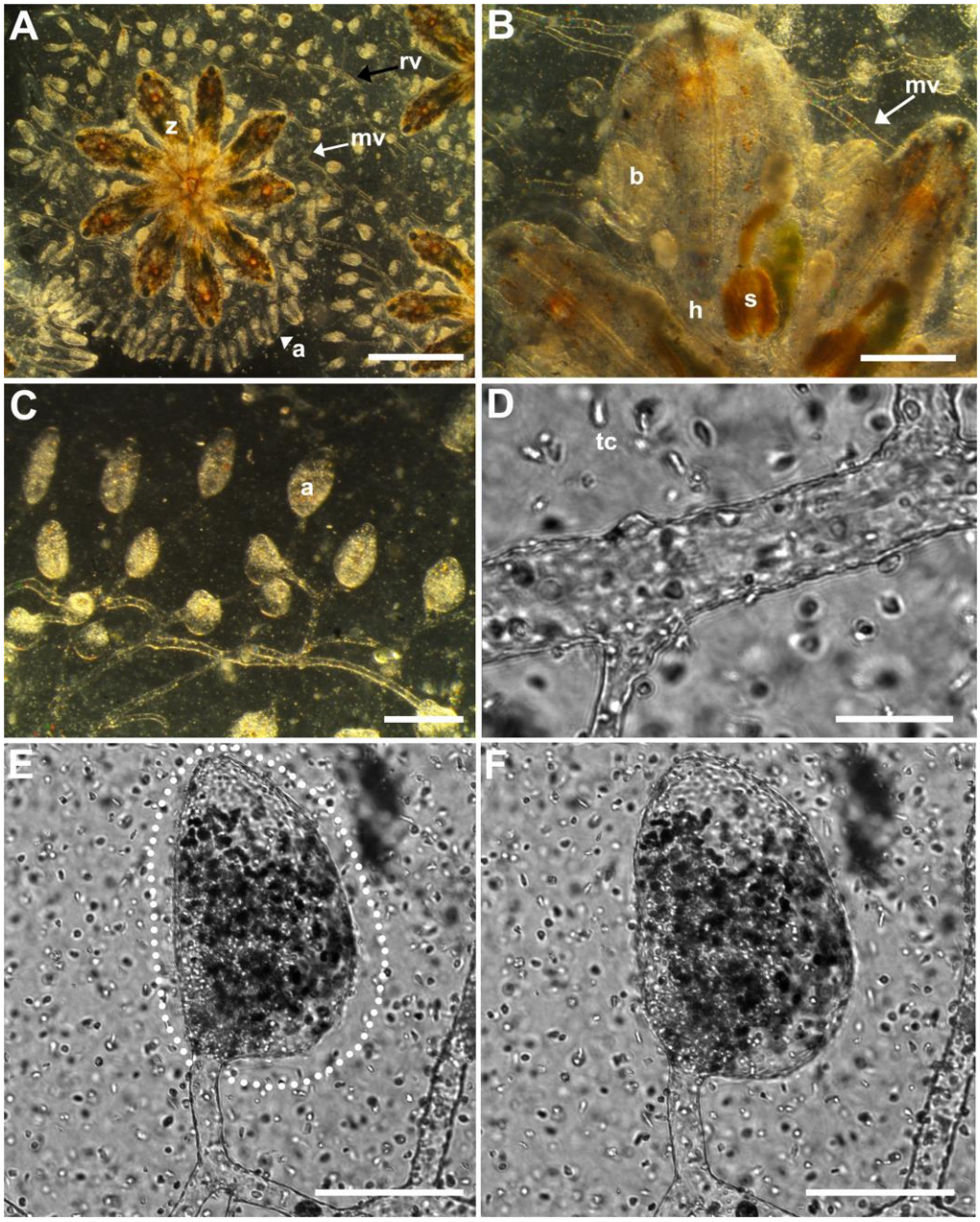

Figure 2.1. The extracorporeal vasculature of *Botryllus schlosseri*. A) Light micrograph of a *Botryllus schlosseri* colony; the zooids (z) arrange themselves into star-shaped structures called systems. A colony can consist of multiple systems, all interconnected by a large common extracorporeal vasculature. One main blood vessel (mv) directly connects all individuals. From the mv the vasculature extends out (radial vessel (rv)) to the periphery of the colony and terminates into pouch-like structures called ampullae (a). **B)** Close up light micrograph of a zooid. Although the lacunae and sinuses in the body are not easily visualized, organs such as the heart (h) and stomach (s) are easy to identify as well as the asexually reproducing bud (b). **C)** A close view of the periphery of the colony showing ampullae. **D)** Zoom-in of a blood vessel embedded in the tunic and showing tunic cells (tc). **E and F)** Zoom-in of a contracting and relaxing ampullae, respectively (dotted line on E provides comparison). Scale Bars: A) 2 mm, B) 1 mm C) 500 µm D) 100 µm E and F) 50 µm.

Ampullae are protrusions that adhere to a substrate, and are also involved in other biological processes as discussed later (Fig. 2.1E and F). Some are elongated and positioned at the periphery of the colony, while others are found in the extracorporeal vasculature where they are spherical. The intricate network of anastomized blood vessels, including ampullae and zooids are fully embedded in an extracellular matrix (ECM) known as the tunic. This tunic has a composition that includes four main macromolecules; collagens, proteoglycans, glycoproteins, and cellulose[11,27,31]. Many cells are also found within the tunic; some that are directly in contact with the blood vessels and others that are highly mobile (Fig. 2.1D)[32]. In *B. schlosseri*, the tunic is thin and transparent, which allows for easy observation and manipulation of the vasculature[27,33].

The extracorporeal vasculature, also described as the colonial circulatory system[34], can be divided into four components: 1) marginal/main vessel, 2) radial/peripheral vessels, 3) ampullae, and 4) an intricate network of anastomosed blood vessels that spread around the vascular bed and connect components 1–3. The main vessel is a continuation of the zooid's open circulatory system, and each zooid has at least two epidermal connections to the extracorporeal vasculature: one directly to the main vessel, and a second one to the peduncular vessel[29]. The radial vessel connects to each zooid, including primary buds but excluding secondary buds, to the surrounding vasculature. It is formed by the growth and fusion of two converging close-ended extrusions, one from the zooid epidermis and the other from a nearby vessel (Fig. 2.1A). Peripheral ampullae are sac-like structures delimited by a monolayer epithelium that is columnar, but only at the tip of the leading edge ampullae. Ampullae are involved in adhesion and contractile, and collectively provide

continuous blood flow (Fig. 2.1E and F; discussed below). All of the components of the extracorporeal vasculature are highly regenerative and characterized by their ability to expand with angiogenic-like properties[27,30,35–37].

B. Similarities and differences with vertebrate vasculature

Vertebrate vasculature

Blood vessels are tubular structures that transport blood throughout the body, and are formed by two mechanisms: vasculogenesis and angiogenesis[38–40]. During vasculogenesis, precursor cells are differentiated into endothelial cells that give rise *de novo* to a primitive vascular network formation[39]. In contrast, angiogenesis is defined as the formation of new blood vessels from preexisting ones[41]. Vertebrate tubule formation initiates from a simple epithelial pocket that sprouts branches to extend into a web-like network of interconnected tubes[42,43]. Defects in tube formation often lead to major disorders, one example being ductal plate malformations[44]. Tubular structures are diverse in size, shape, and origin, yet they have a common characteristic; they are a wrapped epithelium with the cells apical surface lining the lumen. Vertebrate blood vessels are lined on the inside with mesodermally derived endothelial cells[45–47]. They are externally layered by a basement membrane (BM) that is further surrounded by smooth muscle. The vessel morphology is characterized by having the apical side of the cell surfaces lining the lumen, and BM and smooth muscle cells located abluminal. Endothelial cells are essential for vertebrate angiogenesis because they express vascular endothelial growth factor (VEGF)

and angiopoietin receptors that control this process. Angiogenesis first takes place during embryogenesis, and continues to occur throughout life to provide regeneration (e.g. injury), renewal conditions (e.g. menstruation), and unwanted tumor vascularization[38,48–51]. Vertebrate angiogenesis first requires the degradation of BM followed by: extension of exploring filopodia, proliferation, endothelial cell migration towards angiogenic stimuli, secretion of new BM, recruitment of perivascular cells, and finally lumen formation. All of these biological processes are regulated by VEGF[52–54]. VEGF stimulus causes a local degradation of the BM, is followed by cell migration, and leads to the formation of blind tubes[39,55]. The most common mechanism of vertebrate angiogenesis is sprouting, which usually occurs in early processes such as retinal vasculature formation during eye development[56,57]. The other type of angiogenesis is known as intussusceptive, or splitting angiogenesis, and is poorly understood. In general, it refers to the formation of new blood vessels by splitting existing ones into two[58].

Botryllus **vasculature**

It has been hypothesized that endothelial cells lining the blood vessels of vertebrates evolved from invertebrate hemocytes sporadically binding to the BM of invertebrate blood vessels[59]. In *B. schlosseri,* the blood vessels are a simple squamous epithelium whose apical side faces the tunic, and basal side along with BM faces the lumen (Fig. 2.2). These blood vessel cells are also myoepithelial and can contract. The tissue

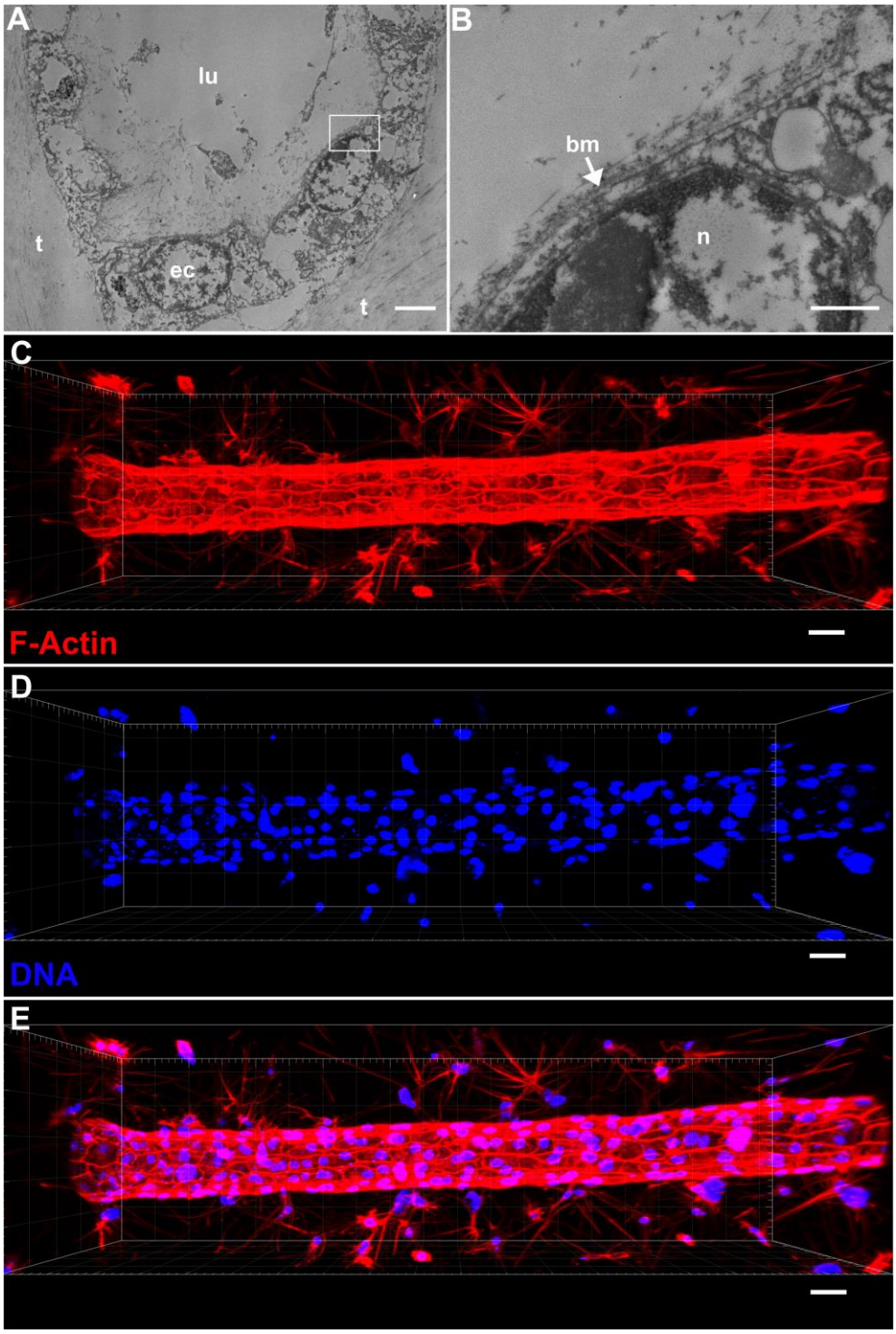

Figure 2.2. Architecture of a *Botryllus* blood vessel. A) Transmitted Electron Micrograph of frontal plane of a blood vessel with the lumen (lu) in the center surrounded by a monolayer of endothelial cells (ec), and the tunic (t) directly outside. **B)** A zoom-in from A (white frame) depicting an endothelial cell with the basement membrane (bm) facing the lumen right across from the nucleus (n). **C-E)** Projection of a confocal z-stack of a blood vessel stained with Phalloidin (C) and DAPI (D) and the merged imaged of both (E). Scale bars A) 200 nm B) 500 nm C, D, E) 50 µm.

architecture with the BM directly facing the lumen and blood, allows circulating cells to directly attach to the BM. Evolutionarily it can be postulated that amoebocytes, or a similar cell type, are the cells that endothelial cells derived from.

Botryllus vascular cells are characterized by having apicolateral tight junctions[34], and utilizing angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF) and receptors (VEGFR-1, VEGFR-2, and EGFR). Gasparini et al. showed that antibodies against vertebrate angiogenic growth factors and receptors can cross-react and detect *Botryllus* protein homologs[34]. These antibodies showed positive staining in regenerating regions of the vessel epithelium and were also detected at the active apex of peripheral ampullae. Interestingly, the transcript for VEGFR was detected, using fluorescent *in situ* hybridization (FISH), in blood vessels and ampullae, but not in the epithelium lining the lacunae and sinuses of the zooids (Fig. 2.3)[35,37]. In contrast to the antibody staining, FISH revealed a positive signal for all vascular cells including ampullae and potentially some tunic cells. The abundance discrepancy between mRNA transcripts and proteins has been observed before and it is understood that these two do not always correlate or have the same spatial localization[60]. *Botryllus* vascular cells, however, do seem to have high levels of BsVEGFR mRNA, perhaps indicating that these cells can rapidly respond to VEGF stimulus for tissue repair and remodeling.

Botryllus vascular cells express vascular progenitor cells markers such as CD133 and VEGFR-2, and a marker for differentiated vasculature, VE-Cadherin[35]. To study the role of BsVEGFR during homeostasis, siRNA was administered for three weeks. During the first two

weeks, the budding and physiology of the colony appeared normal. At the beginning of the third week the typical star-shaped zooid organization became disorganized, and the extracorporeal vasculature became leaky. Blood cells started to spill into the tunic while the ampullae became deflated, and the overall tunic appearance changed from transparent to opaque[37]. In conclusion, BsVEGFR plays a key role regulating homeostasis in the extracorporeal vasculature.

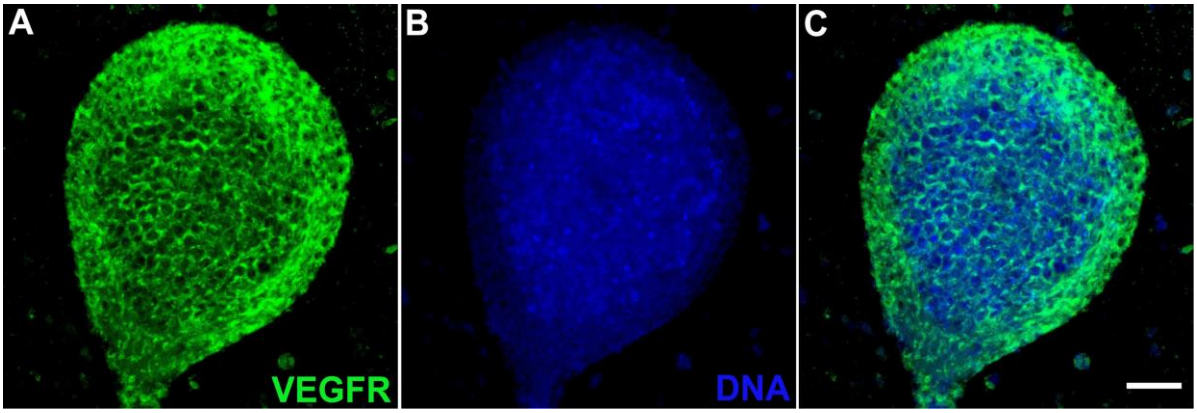

Figure 2.3. Fluorescent in-situ hybridization of BsVEGFR. A) Expression of BsVEGFR on ampullae, riboprobe shown in green. **B)** Same ampullae counter stained with DAPI shown in blue. **C)** Merge image from A and B. Scale bar 25 µm.

It has been shown that certain types of vertebrate tumors have blood spaces that are not lined by an epithelium. These tumors can trigger *de novo* vessel formation through a poorly understood mechanism known as vasculogenic mimicry[61]. These vessels are capable of distributing plasma and blood, thereby mimicking the function of normal vessels[62]. Tumors displaying vasculogenic mimicry have been shown to upregulate genes involved in angiogenesis and vasculogenesis[63]. It has been suggested that novel and

potentially useful anticancer therapies can be found by studying the molecular mechanisms of invertebrate vascular tube formation given its similarities between vasculogenic mimicry in tumor growth[36,61].

The two major differences between invertebrate and vertebrate blood vessels are the presence of the vertebrate endothelial cell layer, and that *Botryllus* extracorporeal vasculature growth does not requires degradation of a BM. The similarities between angiogenesis, however, are found in the signaling pathways controlled by VEGF and VEGFR. Because of these differences and similarities it has been suggested to use the terms "endothelial angiogenesis" when referring to vertebrates and "myoepithelial angiogenesis" when referring to invertebrates[64,65].

C. Angiogenesis (natural expansion of the colony)

Angiogenesis has been defined in vertebrates as the process where new blood vessels are formed from preexisting ones. This includes the process of remodeling and expansion of blood vessels in both physiological and pathological conditions such as recovering from trauma/surgery, and the vascularization of tumors, respectively[66,67]. Angiogenesis starts *in utero* and continues through the adult life of an individual[68]. For example, vessel regression is normally observed during luteolysis, and the involution of the mammary gland[69–71]. Defects in angiogenesis can lead to diseases such as age-related wet macular degeneration, diabetic retinopathy, and cancer[72].

In *Botryllus*, the extracorporeal vasculature propagates by extending the vessel network through the tunic in a mechanism similar to vertebrate angiogenic sprouting[29,30,34,36,37]. As new individuals are added, the extracorporeal vasculature adapts and grows to meet the demands of a larger colony. The natural expansion of the extracorporeal vasculature starts with a thickening of the vessel wall which will either form a blind tube to fuse with other vessels, or differentiate into an ampullae[30,34,35]. During the expansion process, blood vessels are formed to connect newly developing buds[34]. Ampullae development starts behind the outer ring of existing ampullae, and then elongates outward to the same extent. The apex of each ampullae uses filopodia to extend into the tunic. Cells of the extending ampullae are characterized by their basal nucleus, and by having membrane bound secretory vesicles discharge their contents directly into the tunic. It has been observed by transmitted electron microscopy (TEM) that cells on sprouting apexes undergo epithelial-to-mesenchymal transition during ampullae formation, stop proliferation and migration, and retain their capability to produce tunic[34].

D. Vascular regression

Angiogenesis is often thought of as the growth of blood vessels; however, it includes and is balanced by vascular regression in order to prune excess vessels formed during growth[73]. It has been shown that milk-producing breast mammary glands undergo vascular regression after weaning[74], so to ensure blood supply for constantly changing tissues, a homeostatic combination of blood vessel growth and regression is necessary[75].

Induced vascular regression is a major target for new anti-angiogenic related drugs and therapies to treat vascularized tumors[76,77].

At the end of each budding cycle in *Botryllus*, zooids undergo a massive apoptotic event and their corpses are engulfed by blood borne phagocytes[37,78–81]. This event is crucial to allow the new zooids to occupy the vacant space of the previous generation[82–86]. During this process, termed "take over", there is a massive rearrangement of the extracorporeal vasculature. It is characterized by regression of all blood vessels as the zooid corpses are pulled to the center of each system, followed by expansion to allow more space for new zooids and buds[85]. This can be considered as a natural regression followed by a natural expansion of the extracorporeal vasculature.

Recently, our group has shown by FISH that lysyl oxidase 1 (LOX1) homolog in *Botryllus* is expressed by vascular cells[18]. LOX1 is involved in the cross-linking of collagen and elastin, is secreted by blood vessels[87], and is crucial for ECM stability and remodeling. Through pharmacological inhibition of LOX activity using BAPN, a specific small molecule inhibitor, we were able to manipulate the stiffness of the BM, which caused vascular regression of the entire extracorporeal vasculature within 16h (Fig. 2.4A and B). This coordinated blood vessel regression maintained blood flow without bleeding and displayed a disrupted collagen arrangement. A 10-fold increase in apoptotic cells was observed in regressing vessels; however, only a subset of vascular cells become apoptotic rather than the entire vascular tissue. This type of apoptosis, termed anoikis, where programmed cell death is induced by an improper or lack of attachment to the ECM[88].

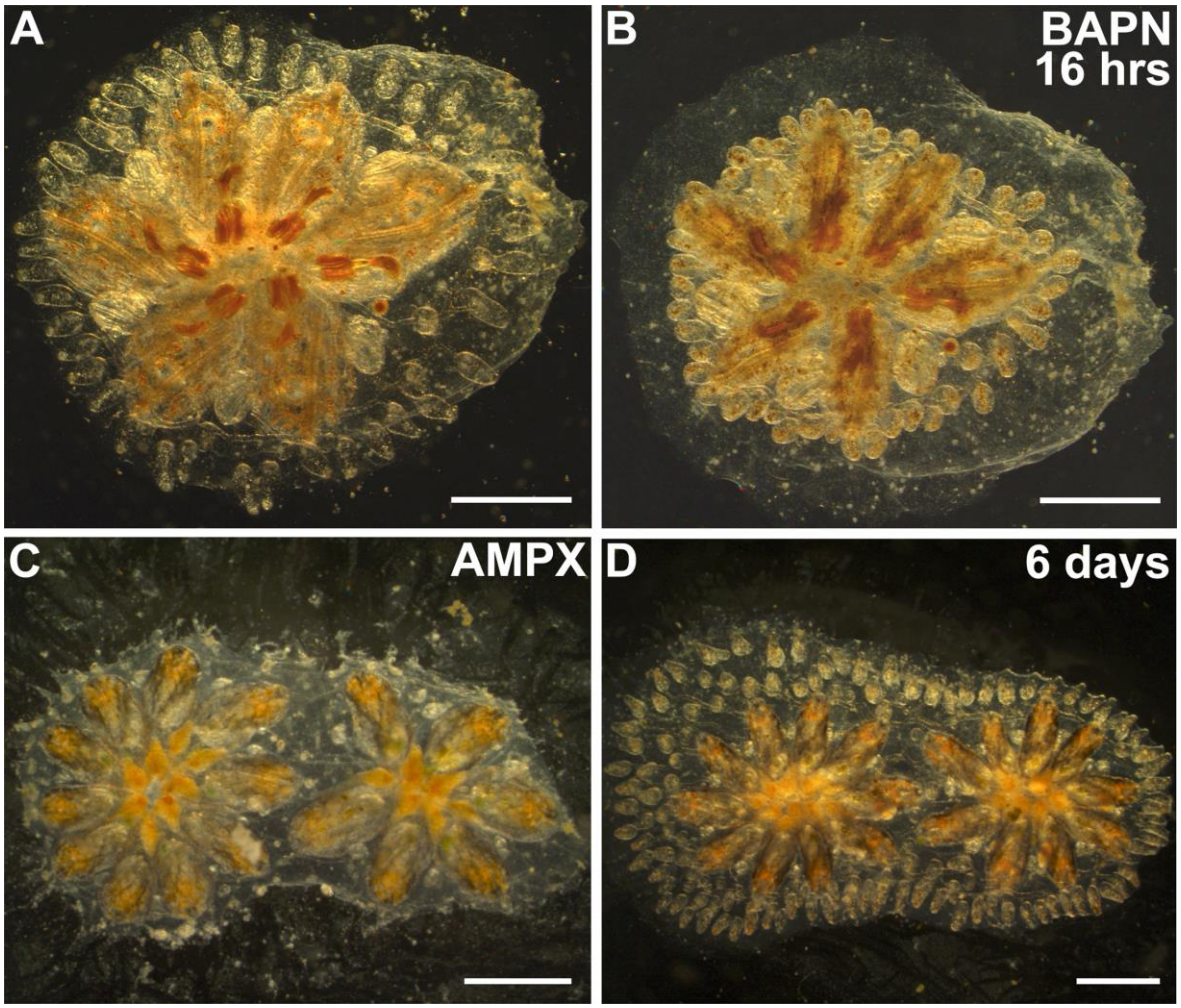

Figure 2.4. Vascular regression and regeneration. A) Bright field image of a young colony before induced regression. **B)** Same colony as after induced vascular regression by incubation of BAPN for 16 h. **C)** Bright field image of a colony right after ampullectomy (all the extracorporeal vasculature and ampullae have been removed except for the vessels interconnecting zooids). **D)** Same colony as C 6 days after ampullectomy; all newly regenerated extracorporeal vasculature has extended out to the same area as before the surgery. Scale bars A-D 2 mm.

Apoptotic cells are extruded directly into the lumen while being ingested by circulating phagocytes; blood-borne phagocytes are key in removing cells that are extruded basolaterally across the BM during induced vascular regression. Further analysis of the integrin pathway by inhibition of Focal Adhesion Kinase (FAK) showed similar vascular

regression patterns to LOX 1 inhibitor, and BAPN regressing colonies showed low levels of FAK phosphorylation when compared to controls. Our results suggest that disruption of collagen crosslinking induces cells to undergo anoikis due to their lack of integrin-mediated binding to the disrupted ECM.

E. Vascular regeneration upon injury

Botryllus extracorporeal vasculature can completely regenerate vessels that have been injured through damage or surgical removal (Fig. 2.4 C and D). To study vascular regeneration, the vasculature and peripheral ampullae are surgically removed to leave behind only the main vessel. This type of surgery is known as an ampullectomy[30,35,37]. It has been shown that *Botryllus* blood vessel angiogenesis can be induced by injecting human pro-angiogenic growth factors, VEGF and EGF, into colonies following ampullectomies[36]. Upon surgical removal of ampullae and radial vessels, blood leakage occurs for five to ten seconds until blood cells clot at sites of injury to stop the bleeding[30,36,37]. Blood flow then returns to normal about one-hour post-surgery. In the case that the extracorporeal vasculature is partially removed (*i.e.* only half) it has been observed that the remaining ampullae reposition toward the site of surgery. The apexes of these ampullae become active and in some cases penetrate the regenerating area to aid in the regrowth process[30]. Newly formed ampullae can be observed around 24h after surgical ablation[30,35–37]. The post-surgery vessel stumps become actively involved in vessel regeneration, and new vessels will also form from branching points or start as sprouts that rapidly bifurcate from

the main vessel. Some cells detach from the active apex of sprouts and migrate into the tunic. Detaching cells change their tight junctions from apicolateral to basal position while their neighbors maintain apicolateral. The apical surface of a detaching cell extends out filopodia into the tunic, and as this cell protrudes, it detaches and leaves the lumen intact. As the detaching cells move out, it maintains basal tight-junctions with neighbors, and neighbors eventually converge and restore apicolateral tight junctions providing continuous integrity to the BM[30].

The regeneration of the extracorporeal vasculature has been classified into five stages: 1) blood clotting followed by small bulb formation, 2) bulb formation growth and becoming round in shape, 3) the structure becomes oval, 4) the main vessels have completed regeneration and are actively extending newly formed ampullae, and 5) the ampullae gain back their average size. In adult colonies it takes 3–7 days to fully regenerate the vessel network to the same extent as before surgery[30,34,35,37]. In contrast, an oozooid can regenerate all ampullae in about 24h[37]; however, it is important to note that in the case of an oozooid, the ampullae are proximal to the zooid and do not have expanded vasculature. Therefore, young colonies containing 2–5 zooids with expanded extracorporeal vasculature may take longer, approximately 3–7 days, to fully regenerate.

siRNA-mediated knockdown of VEGFR prevented regeneration and formation of new blood vessels and ampullae after ampullectomy[37]. The zooids did not present abnormalities; they continued budding, and blood flow was present. In contrast, in the BsVEGFR knockdown without ampullectomy, the zooids lost their characteristic star-shaped association and presented a random position in the colony[37]. The

VEGFR siRNA results were phenocopied using a pharmacological inhibitor of VEGF receptors (PTK787), which was able to inhibit both vessel and ampullae regeneration for 120h post-ampullectomy. The authors concluded that the extracorporeal vasculature serves as an important cue to modulate the spatial organization and distribution of the zooids in a colony. Similarly, mouse pancreas organogenesis requires growth factors to dictate apicobasal polarization to maintain proper tissue organization and architecture [89,90]. During pancreas organogenesis, individual cells acquire apicobasal polarity with the apical side facing the lumen, and the basal side attached to the ECM. These polarized cells form rosettes that fuse into a luminal plexus, which in-turn forms a tubular monolayer epithelium attached to a basement membrane[90]. It has been shown that EGFR is capable of modulating both morphogenesis and cell differentiation in a context specific manner, thereby modulating apical polarity through pancreatic organogenesis[89].

Braden et al. developed a novel vascular lineage tracing method that was also used as a marker for isolating vascular cells[35]. Lineage tracing was obtained taking advantage of proteins, in this case Bovine Serum Albumin, bound to a pH stable Alexa Fluor that can maintain fluorescence inside the low-pH lysosomal environment (Fig. 2.5). Cells that engulf these pH stable protein-bound fluorophores undergo proliferation and equally partition lysosomes between daughter cells which maintain the label. The molecular mechanism underlying specific uptake of this conjugate into *Botryllus* vascular cells is not well understood, yet *Botryllus* vascular cells intake and retain it, which serves as a cell lineage tracing tool. Using this label, it is possible to study the role of vascular epithelial cells in vascular regeneration, proliferation, mobility, and differentiation into ampullae. Braden et

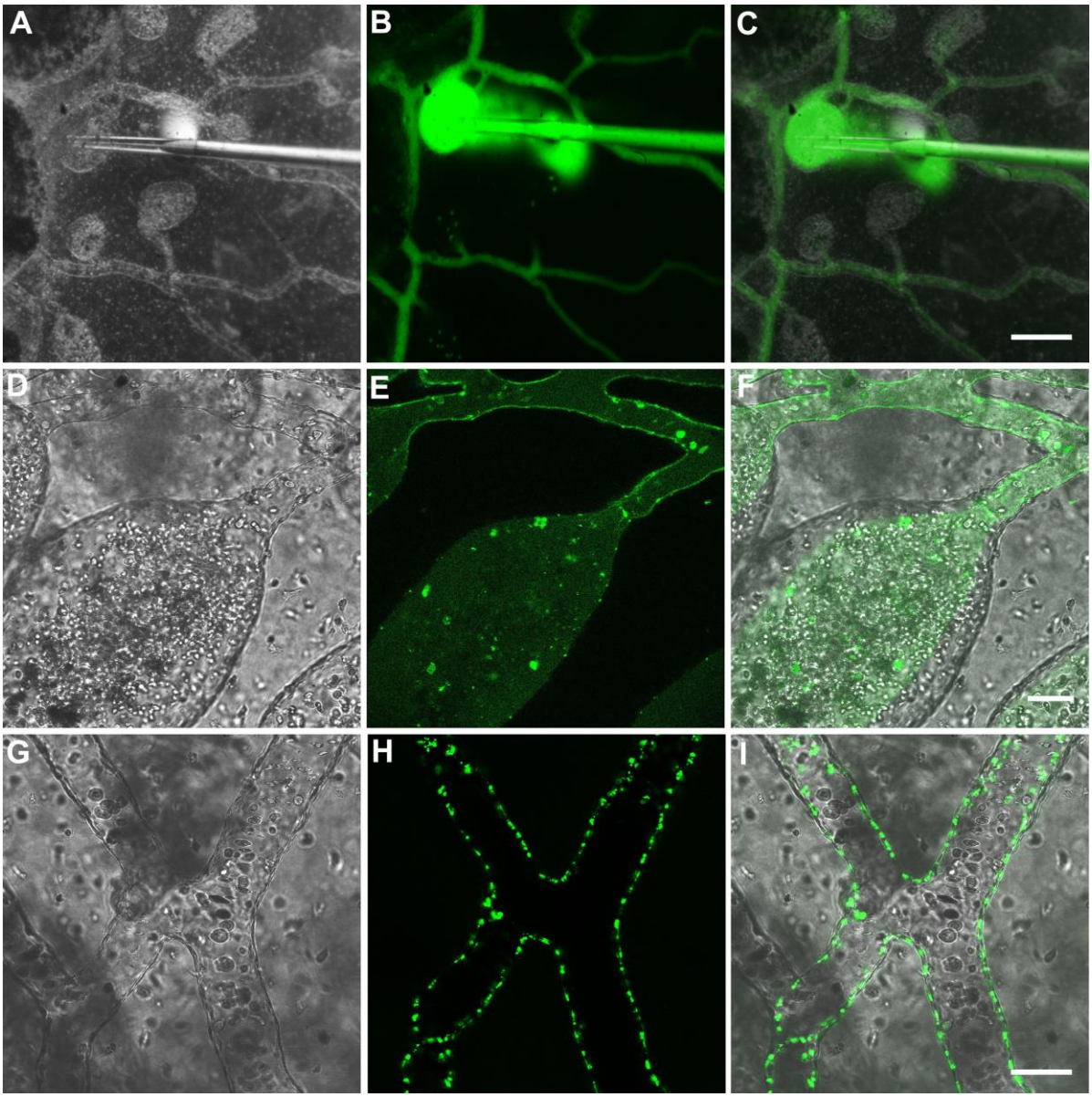

Figure 2.5. Microinjections of BSA-488 into the extracorporeal vasculature. A) Bright field image of a microinjection into an ampulla showing the glass needle injecting directly into the blood stream. **B)** Fluorescent micrograph showing diffusion of BSA-488 and distribution throughout the extracorporeal vasculature. **C)** Overlay of A and B. **D)** Bright field image of a microinjected ampulla 10 min after injection. **E)** Fluorescent micrograph showing the BSA-488 distribution through the extracorporeal vasculature; note that the vascular cells have not up taken the BSA-488. Some auto florescent circulating pigment cells are detected in the blood stream. **F)** Overlay of D and E. **G)** Bright field image of blood vessels following 24 h of microinjection. **H)** Fluorescent micrograph showing the BSA-488 uptake by the cells of the extracorporeal vasculature, note that the absence of circulating BSA-488. Some auto florescent circulating pigment cells are detected in the blood stream. Scale bars A-C 250 µm D-I 50 µm.

al. also showed that regenerative resident cells within the vascular tissue have the potential to form newly regenerated blood vessels and differentiated ampullae, and that *Botryllus* blood vessel regeneration occurs through a combination of proliferation and differentiation[35]. Only a few studies have addressed the molecular mechanism underlying regeneration of blood vessels in *Botryllus*.

In mice, mutation of Vascular Endothelial (VE) Cadherin is lethal due to vascular defects during embryogenesis[91,92]. These studies concluded that VE-Cadherin plays a key role in the survival of endothelial cells involved in embryo vascularization. CD133, also known as Prominin-1, is a stem cell/progenitor marker expressed at the apical side of several tissues and may play a role in regeneration of tubular epithelial after injury[93–96]. It has been shown that injury to ampullae causes a local up-regulation of Bs-Cadherin in damaged tissue similar to how endothelial VE-Cadherin responds to injury of blood vessels[97,98]. CD133 is expressed by regenerating ampullae, which is in accordance with its importance in regulating branching morphogenesis[35,99]. It has been proposed that CD133 may have a role in ampullae differentiation[35].

F. Self and non-self recognition

Several reviews have thoroughly covered this process[100–104]. Ampullae are implicated in adherence of the colony to a substrate, and are involved in the allorecognition process between two genetically distinct colonies[104]. When the ampullae of two compatible colonies come in contact, they can initiate anastomosis and form blood

chimeras. In contrast, when colonies are genetically incompatible, the tunic partially fuses and the ampullae leak cells to form a rejection site[101,104–106]. The allorecognition process is dictated by a single locus known as the fusion/histocompatibility (FuHC) locus. Individuals with at least one identical allele are fusion compatible, and those with none are incompatible and trigger rejection[105,107,108]. It has been shown that Fester, Uncle Fester, HSP40L, and BHF play a key role during fusion and rejection, and can be used to predict rejection outcomes[105,109–113]. Allorecognition proteins are differentially expressed on ampullae compared to blood vessels[109–111]. *Botryllus* allorecognition system is highly polymorphic. It has been suggested that its function is to limit vascular anastomosis because blood-based chimeras result in a stem cell competition for the germline[114–117]. Ampullae can thus be considered the gatekeepers of invasive parasitic germline stem cells.

Ampullae are distinct tissues when compared to blood vessels. The arrangement of epithelial cells on the leading edge are columnar instead of the simple squamous epithelium that make the blood vessels. Ampullar epithelial cells also express allorecognition genes that are not otherwise expressed in any other tissue[104,108]. The fusion event is rapid, can happen overnight, and involves remodeling of the tips between fusing ampullae. It has been observed that blood-borne phagocytic cells are present in the fusing ampullae, indicating that they may play a major role during this process[118]. In the hours following fusion, newly fused ampullae will change their morphology to a blood vessel that is indistinguishable from any other[19,35,118]. Vascular and ampullar epithelial cells have different cellular identity, yet they have the plasticity to differentiate into the other cell

type, therefore the fusion process is an ideal scenario to study the molecular mechanisms underlying this differentiation process.

G. Anastomosis and parabiosis

Parabiosis comes from Greek words: para meaning alongside, and bios meaning life. It can be defined as the union between two organisms that share a common vasculature and therefore blood supply. Parabiosis in mammals is usually accomplished through surgery, although it can occur naturally during abnormal development, resulting in conjoined individuals[119]. Experimental parabiosis in mice has led to major breakthroughs in transplantation, cancer, and rejuvenation of aged organs[120]. Natural occurring parabiosis in *Botryllus* is not age restricted, meaning that it can be achieved between colonies of different ages.

Once fusion of blood vessels between two distinct genotypes occur, they become blood sharing chimeras, and two types of cell competition occur: somatic and germline[114–116,121]. It has been shown that *Botryllus* colonies have long-lived germline stem cells that recurrently migrate to the newly established niches inside developing buds[27,122]. A mobile germline is critical in maintaining sexual reproduction in a colony that constantly replaces adult zooids on a weekly basis. Clusters of follicle and germline progenitors become mobile and migrate to niches in both primary and secondary buds during a specific phase of the budding cycle[123]. The migration of germline progenitors to niches is directed by a sphingosine-1-phosphate gradient in the secondary buds[124]. Because of the mobile

nature of germline progenitors, fusion between two colonies results in a phenomenon known as germline parasitism, where gametes and progeny of one genotype can be completely replaced by the other[117]. The parasitism is persistent through the budding cycles and even after the colonies are disconnected[125]. It has been shown that germline stem cells can be prospectively isolated and transplanted directly into the bloodstream of a fusible host colony to recapitulate germline parasitism[114,115]. This is also true for somatic pigment cell parasitism, although we do understand that cell lineages for somatic and germline are separate.

Parabiosis experiments have been performed between young and old mice to study the effect of young blood in different tissues of the old fusion partner. In these experiments, cardiac hypertrophy in old mice was dramatically reduced and accompanied by decreased cardiomyocyte size after four weeks of parabiosis[126,127]. GDF11 was identified as a circulating factor in young mice responsible for rescuing cardiac hypertrophy in old mice. Research has shown that aging causes a decline in blood flow leading to a reduction in neural stem cells. Using heterochronic parabiosis caused aged cerebral vasculature to undergo a remodeling, which led to greater blood flow and proliferation of neural stem cells[128]. Young and old vessel fusion may reveal how these differently aged blood vessels and individuals influence each other, and *Botryllus* natural parabiosis is a great model system to study this unique situation.

H. Conclusions and future directions

The extracorporeal vasculature of *Botryllus schlosseri* plays a key role in the life of a colony because it: delivers nutrients to all individuals, regulates the orientation and organization of zooids, dictates the directionality of the expansion and growth of the colony, decides if a fusion or rejection event will happen, serves as a reservoir for germline stem cells, and plays a role in the maturation of developing oocytes. The health of the vasculature is so vital to the survival of a colony that it rapidly regenerates upon injury. In extreme cases when all zooids are removed, it is even able to maintain blood flow and give rise to new zooids[129].

Many aspects related to the remarkable plasticity of the vasculature remain poorly understood: what signals induce vascular epithelial cells to proliferate, or to undergo apoptosis? How do the cells interact with the ECM and how do changes in the ECM affect the behavior of the epithelial cells? What signals regulate differentiation into the columnar epithelium of ampullae, and back into blood vessel epithelium?

Because of the accessibility of the BM facing directly to the lumen, *Botryllus* vessels are an ideal model to study mechanotransduction and how epithelial cells respond to chemical or physical stimuli. It has recently been shown that by using small molecule inhibitors it is possible to "soften" the BM, and it would be ideal to study "tightening" the BM using similar approaches.

Long-term effects of parabiosis between age-mismatch colonies and the potential consequences in the physiology of the blood vessels of both parabionts have not been

studied in *Botryllus* blood chimeras. We are currently investigating the role of the extracorporeal vasculature and its effects on the senescence of the entire colony.

To better understand the evolution of branching morphogenesis and blood vessel formation it would be ideal to compare different species of colonial ascidians and their ability to respond to either induced regression or expansion of their extracorporeal vasculatures. Earlier comparisons have been done, but more modern molecular tools will allow us to understand the specific mechanism behind angiogenesis of invertebrate blood vessels[130]. In future studies, analysis of transcriptomics and proteomics of purified populations of vascular cells will identify key genes, proteins, and signaling pathways involved in all different biological aspects of the extracorporeal vasculature.

III. Cellular and molecular mechanisms of regeneration in ascidians

Susannah H. Kassmer∗, Shane Nourizadeh, Anthony W. De Tomaso

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A. Introduction

The ability of some animals to replace missing body parts has fascinated biologists for hundreds of years and is widely distributed among the different metazoan phyla[131]. Many invertebrate organisms, including examples of the hydrozoans, anthozoans, echinoderms, annelids, holothuroids, platyhelminthes, and tunicates, have robust regeneration capacity, yet only very few of these have been studied in detail. In contrast, most vertebrates have a very limited capacity to regenerate, except for some amphibians and teleosts that can replace appendages, tail, spinal cord, and heart.

Regeneration in many cases is dependent on the formation of a blastema; a mass of proliferating cells that give rise to newly differentiated cells to replace missing tissues. In vertebrates, epimorphic regeneration occurs by de-differentiation of lineage-restricted cells that subsequently acquire the ability to proliferate and give rise to differentiated progeny[132]. In many invertebrates, regeneration is dependent upon proliferation of pluripotent or multipotent stem cells that are present in the adult animal and capable of giving rise to a blastema.

Invertebrate chordates such as cephalochordates and tunicates have extensive regeneration capacities[133,134]. In colonial ascidians such as *Botrylloides leachii*, the entire

body can regenerate from small fragments of extracorporeal vasculature, and these animals are the only chordates that are capable of whole-body regeneration. Solitary ascidians such as *Ciona intestinalis* can regenerate distal structures upon injury, such as the cerebral ganglion and the oral siphon, so long as proximal structures are intact[135,136].

Regenerative processes involve the activation of many conserved developmental signaling pathways such as Wnt, Notch, TGF-beta, and retinoic acid. Although these pathways are shared among all metazoan organisms, not all these organisms are capable of regeneration. The question is how and in what specific cellular context are these pathways activated and regulated in regenerating species. During embryonic development, many transient pluri- and multipotent cell types are generated, but the persistence of these into adulthood differs between species, as does the ability of the more differentiated cell types to reassess these developmental pathways[137].

It has been suggested that regeneration may be an ancestral chordate trait that has been lost in some lineages during vertebrate evolution[138], and recent studies suggest that highly conserved developmental signaling pathways, such as Notch, play important roles during regeneration in ascidians[7,139].

Since tunicates are the closest living invertebrate relative to the vertebrates, the study of regeneration mechanisms in these animals will deepen our understanding of the evolution of regeneration across animal species and may to help advance biomedical approaches in the future.

Phylogenies based on both morphological and molecular data suggest that the Tunicates are monophyletic. Coloniality is found in different families of ascidians and appears to have evolved independently[25]. Many species of ascidians have remarkable regeneration capacities; however, the cellular and molecular basis of regeneration has been studied in any detail only in colonial ascidians of the family Botryllidae, and in solitary ascidians of the Cionidae, and therefore this review is focused mainly on those species where those data are available.

B. Whole-body regeneration in colonial ascidians

Colonial ascidians of the family Botryllidae, such as various species of *Botrylloides* and *Botryllus*, are characterized by integrated colonies in which all zooids develop within a common tunic and share a vascular system with a constant blood flow (Fig. 3.1). Asexual reproduction in these species occurs by palleal bud formation, which ensues synchronously and is regulated by colony-wide developmental processes. During palleal budding, a new individual grows by formation of buds from a specialized epithelium in the adult. Some species in the family of the Botryllidae have the striking ability to regenerate entire new bodies from small pieces of extracorporeal vasculature. This process, known as whole-body regeneration (WBR), has been described mainly in different species of *Botrylloides*, such as *Botrylloides violaceus* and *Botrylloides leachii*, and appears to follow identical steps in these species. A staging system has only been established for *Botrylloides leachii*[139], but based on

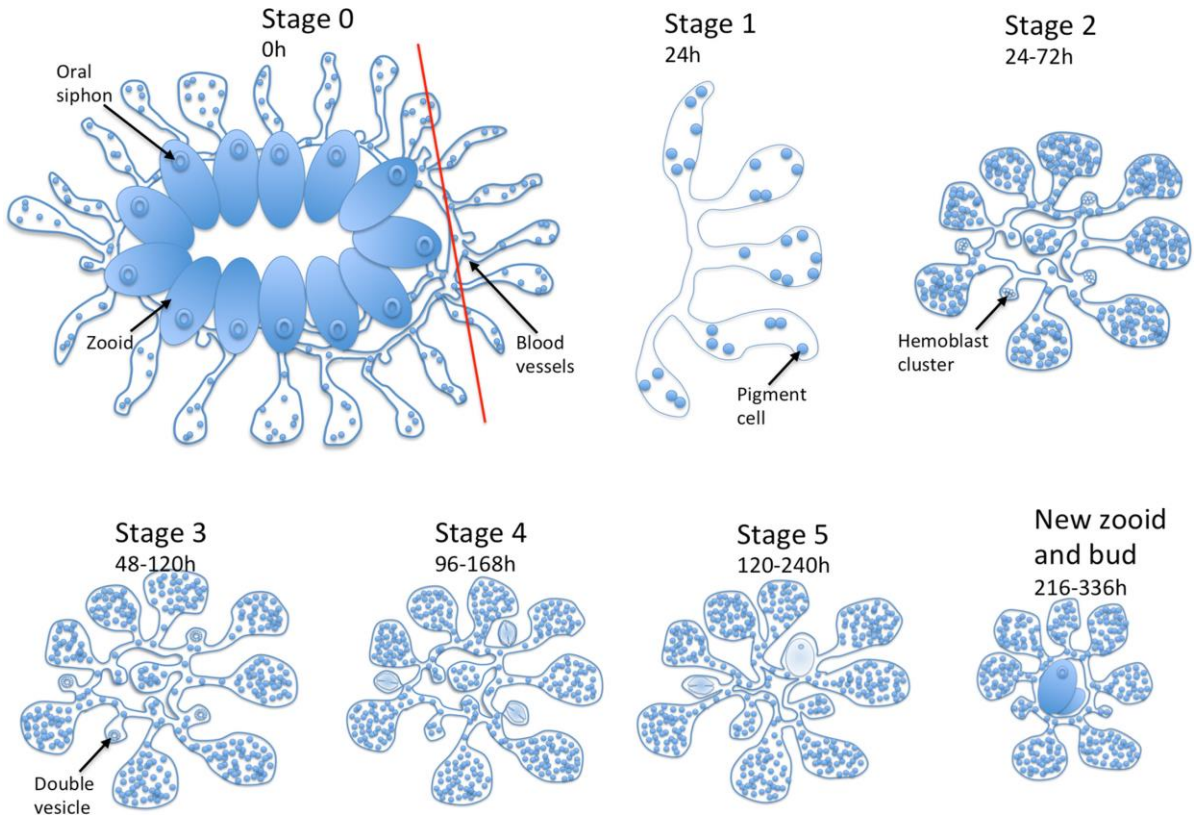


Figure 3.1. Whole-body regeneration from pieces of vasculature in *Botrylloides*. Genetically identical zooids share a common tunic and extracorporeal vasculature. When a piece of vasculature is cut away from the colony (stage 0), blood flow resumes within 24 hours (stage 1). Blood vessels undergo remodeling and become condensed and highly pigmented (Stage 2). Hemoblasts aggregate in niches formed by pockets of vascular epithelium. At stage 3, a double vesicle develops inside the regeneration niche. This tissue grows and undergoes invaginations (stage 4), followed by organ formation. At stage 5, the growing body has a heartbeat and opens the oral siphon. Once fully mature, the new body will begin palleal budding and give rise to a new colony.

the available literature, WBR follows the same basic pattern and stages in all species that are capable of it. Whether all Botryllidae have the capacity for whole-body regeneration is currently under investigation. (Of note, no other part of the colony can regenerate a whole-body, although developing palleal buds can continue development into fully functional zooid when separated from the adult.) During WBR, a tiny fragment of blood

vessel that is separated from the rest of the colony is enough to give rise to a complete and functional new body within 7–14 days. The timing of WBR in *Botrylloides* is highly variable and depends on the species, the health of the colony at the time of injury, water quality and temperature, and possibly other factors. In Fig. 3.1, we provide a schematic of the morphological stages of regeneration with approximate timing.

After a piece of blood vessel is cut from the colony, blood flow via ampullar contractions[140] resumes within 24h. In the next few days, blood vessels undergo extensive remodeling, become rounded, condensed, and highly pigmented (Fig. 3.2). The terminal ampullae shrink, new vessel connections are formed, and the vascular system contracts into a dense network. Within this dense vascular tissue, an opaque mass of cells becomes apparent. In *Botrylloides violaceus*, Oka and Watanabe[138] observed the gathering of 15–20 small, undifferentiated cells (hemoblasts) under the epidermis of a blood vessel, followed by cell divisions that resulted in formation of a hollow blastula-like structure about 3 days following isolation of the blood vessel. The epidermis then continues to wrap itself around this vesicle, resembling the double vesicle stage commonly seen in palleal (asexual) budding (Fig. 3.1). Histological sections[141] show the development of an epithelial sphere surrounded by mesenchymal cells around 9 days post zooid removal. After the vesicle stage, a fold develops from the internal side of this sphere to become the endoderm, and regeneration begins to resemble the development of an asexual palleal bud. One report describes formation of a single-layered sphere of cells within the lumen of a blood vessel as early as two days after isolation from a colony[142], and although multiple buds can initially

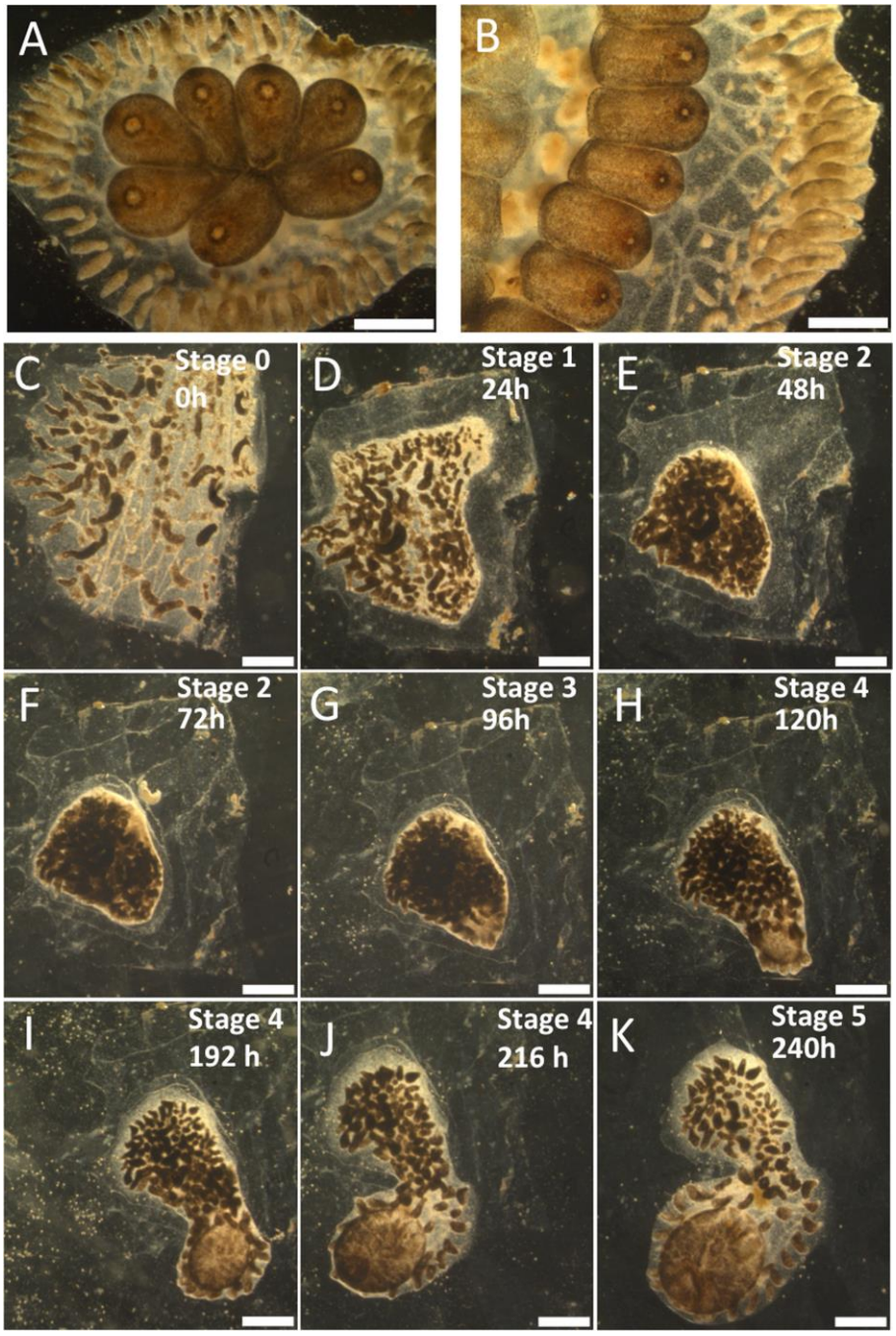


Figure 3.2. Brightfield morphology of whole-body regeneration in *Botrylloides leachii*. A: Brightfield image of whole *Botrylloides leachii* colony. **B)** Brightfield image of the extracorporeal vasculature of *Botrylloides leachii*. Scale bars in A and B: 1mm. **C-K)** Brightfield images of regeneration stages as outlined in Figure 1. C: Stage 0, blood vessel after surgical separation from colony. **D)** Stage 1. Within 24h, blood flow resumes, and vessels begin to remodel. **E and F)** Stage2. Blood vessels remodel, become condensed and highly pigmented. **G)** Stage 3. A non-pigmented area (regeneration niche) becomes visible, containing a double vesicle. **H-J)** Stage 4. The regenerating tissue within the double vesicle grows and germ layers begin to differentiate, followed by organ formation. **K)** Stage 5. The regenerated body has developed a heartbeat and opens the oral siphon.

form, only one will continue to develop into a zooid[141]. Heart activity starts before the development of other organs is complete, and the fully mature zooid will begin filter feeding after ten days of regeneration. This new zooid then begins asexual reproduction to replace the entire colony[138]. In the closely related colonial ascidian *Botryllus schlosseri*, WBR requires a larger section of intact vasculature, and some reports suggest that it only occurs when surgical removal of bodies is performed during a specific stage of asexual reproduction, known as takeover, when zooids die and are replaced by palleal buds[143,144].

Bodies form naturally from within blood vessels in some Botryllidae as part of their normal life cycle. This process is known as 'vascular budding' and is highly reminiscent of the steps observed in WBR of *Botrylloides*. In *Botryllus primigenus*, small, undifferentiated hemoblasts aggregate in the blood vasculature and give rise to a solid mass that reorganizes into a hollow vesicle, then subsequently undergoes morphogenesis to develop a body[145]. Vascular buds form in a similar fashion in a closely related species *Botryllus tuberatus*[146]. In the closely related colonial ascidian *Symplegma brakenhielmi* (family Styelidae), zooids are embedded in a common tunic and surrounded by a communal vascular system in a similar fashion as in *Botrylloides*, but colonies appear less organized[147]. Formation of palleal and vascular buds occurs simultaneously and asynchronously.

During *Symplegma* vascular budding, evagination of the vessel epithelium is observed, and small, round cells accumulate. The vesicle then expands to form a sphere, and an inner layer of cuboidal epithelium forms an inner vesicle before continuing organogenesis[147]. These observations suggest that whole-body regeneration and vascular budding are highly similar processes that may share a common origin and common

mechanisms. The only difference may be the signals that initiate the process: In the case of WBR, separation of the vessels from the colony is required to trigger formation of bodies from vessels, whereas in species with vascular budding, injury is not required, and the signal is always "on". Oka and Watanabe[138] suggested that vascular budding is a primitive and ancestral type of budding that has been suppressed in some colonial ascidians in favor of palleal budding. Separation of blood vessels from the rest of the colony may artificially remove this suppression and trigger vascular budding.

Evidence for this idea comes from recent study showing that even in Botrylloides leachii, vascular budding can occur without injury, and is induced by stressors such as decreased temperatures and unfavorable conditions[148]. Under such circumstances, the colony can enter a hibernation stage (termed: Torpor), where all bodies are resorbed and only a condensed aggregate of blood vessels remains. This dormant state can last for months, however; once conditions improve, a new body will regenerate from within the blood vessels and give rise to a new colony. Morphologically, this development of bodies from hibernating vessels is very similar to whole-body regeneration induced by injury. In the wild, a colony may recover from various factors such as unfavorable environmental conditions or injury from a predator in this way.

In addition, budding behavior may be a mode of adaptation to environmental conditions, as it can differ between populations of the same species isolated from different regions, as seen in *Symplegma brakenhielmi*. Colonies from Panama only undergo vascular budding, whereas colonies from Brazil undergo both palleal and vascular budding[147]. This suggests either environmental influence on vascular budding, or intrinsic genetic diversity

between local populations, thus caution must be taken when comparing results from samples of the same species collected in different parts of the world. Maintaining genetically inbred strains that are shared between laboratories would be ideal, however; this is complicated by the low fitness of, for example, inbred *Botryllus schlosseri* colonies. Other colonial ascidians are very difficult to propagate in the laboratory and need to be collected from the wild, therefore more effort is needed to overcome these limitations in the future.

C. Cellular origin of whole-body regeneration

To date, the cell types that give rise to the tissues that are formed during whole-body regeneration or vascular budding have not been clearly identified. It remains to be determined if newly formed somatic tissues arise from a population of stem cells, whether these stem cells are pluripotent or have lineage restricted potential, and whether dedifferentiation plays a role. Morphological observations suggest that vascular buds in *Botrylloides*, *Botryllus primigenus*, *Botryllus tubaratus*, and *Symplegma* originate from an undifferentiated population of blood borne cells. In colonial ascidians, small, undifferentiated cells in the blood or coelomic fluid have been termed "hemoblasts"[145,149]. These cells appear undifferentiated, have a transparent cytoplasm and no nucleolus, and have been implicated in contributing to somatic tissues during asexual budding, whole-body regeneration, and vascular budding[149]. An increase in the population of hemoblasts is observed 15h post injury during whole-body regeneration in *Botrylloides leachii*[140].

During whole-body regeneration in *Botrylloides violaceus*, Piwi expression is seen in circulating hemoblasts temporally near the early vesicle stage and occasionally within the epithelium of a vesicle[141]. This suggests that Piwi-positive hemoblasts integrate into the vesicle. During stages of organogenesis, many Piwi-positive cells are found adjacent to tissue layers, with occasional presence within a layer[141]. siRNA mediated knockdown of Piwi expression inhibits whole-body regeneration[150]. These data suggest that hemoblasts contribute to somatic tissues during whole-body regeneration.

In colonial ascidians, mobile germline stem cells expressing vasa are present in the blood and give rise to gonads during asexual reproduction[114,124,151,152]. Piwi, like vasa, is also expressed in blood borne germline progenitors[149,152]. Piwi and vasa belong to a group of genes that are normally associated with the germline. Interestingly, in some invertebrates these same genes can be expressed in multipotent cells that are not fully germline restricted, and thus have somatic potential[153]. In some animals, an irreversible separation of germline and soma occurs early in embryonic development, but many adult flatworms, cnidarians, and sponges contain multipotent or totipotent stem cells that give rise to various adult cell types, including the germ line[153]. The function of these cells depends on piwi, vasa, tudor, and pumilio - genes that were first identified in germline stem cells. Therefore, in ascidian species that can undergo whole-body regeneration, the vasa/piwi-positive population of hemoblasts may contain both germline- and somatic progenitors, and there may be a primitive population of cells not restricted to either germline or soma. In single cell transplants between genetically distinct *B. schlosseri* colonies, germline and somatic engraftment never came from the same cell, suggesting that separate lineages of

somatic and germline stem cells exist, at least in this species[114]. More markers need to be identified to discriminate between potential somatic stem cells, germline stem cells, and pluripotent stem cells that may exists within the "hemoblast" population amongst different Botryllid species.

In colonial ascidians that undergo vascular budding as part of their normal life cycle, blood borne stem cells may naturally have a wide differentiation potential. When vascular budding is suppressed under normal conditions, like in *Botrylloides*, these stem cells may be dormant before activating upon stress or injury. Future studies will reveal what those suppressing or activating signals are. It is also possible that the differentiation potential of cells within the vasculature may be induced to change upon injury. When no bodies and buds are left, de-differentiation of mobile or non-mobile cells may give rise to progenitors that lead to the formation of new bodies. De-differentiation is the basis for limb regeneration in vertebrates and is a very effective means of producing new tissue from pre-existing cells[132]. Whether de-differentiation is involved in either whole-body regeneration or vascular budding is currently unknown.

It is evident that the vasculature plays an important part in whole-body regeneration and vascular budding, but its exact role has not been fully characterized. Upon separation from the rest of the colony, the remaining blood vessels undergo extensive remodeling and re-shaping, eventually forming pockets or niches where hemoblasts aggregate (Fig. 3.1). How and why this remodeling occurs and how it leads to the formation of niches has not been investigated, though it has been suggested that the epithelium that lines the blood vessels can develop into the ectoderm of vascular buds[145]

by giving rise to the outer wall of the double vesicle. A double walled vesicle is also formed during palleal budding, with the outer wall derived from the epidermis of the parent zooid, and the inner wall, which gives rise to the organs, from the peribranchial (atrial) epithelium of the parent zooid. The gut primordium originates from the atrial epithelium. Hemoblasts migrate into the space between the two layers of the double vesicle – their contribution to palleal bud formation is not well understood, but genetic tracing in *Botryllus schlosseri* confirmed that at least the intestine of a palleal bud does not originate from a mobile progenitor[154]. Oka and Watanabe[145] describe vascular budding in *B. primigenus* as mesoblastic, wherein mesenchymal cells circulating in the bloodstream form the inner wall of the vesicle. If this is true, then the very first steps of palleal budding and vascular budding occur by very different mechanisms and from different cellular sources, however; both lead to the formation of a double vesicle. After that, bud development in both cases is strikingly similar[144].

Some evidence for a contribution of mobile hemoblasts to somatic tissues comes from other colonial ascidians. *Perophora viridis* undergoes stolonial budding. Colonies irradiated with X-rays do not form stolonial buds, but bud formation resumes upon injection of coelomic hemoblasts[155]. In *Symplegma reptans*, detailed electron micrographs strongly suggest that hemoblasts develop into body muscle cells around the developing siphon during palleal budding. Myofilaments appear in the cytoplasm of hemoblasts that aggregate around the epidermis of the oral siphon[156].

In the future, lineage-tracing experiments will hopefully provide insight into the differentiation potential of different hemoblast populations. Efforts are currently underway

to generate transgenic colonial ascidians which will enable more precise cell tracking, gene manipulation, and help provide a more detailed analysis of the cellular sources underlying whole-body regeneration and vascular budding. Current resources for colonial ascidians include: Published genomes for *Botrylloides leachii* and *Botryllus schlosseri*[143,157] mRNA seq and transcriptome database[15]. Fluorescent in situ hybridization[158], qPCR, small molecule inhibitors[124]. siRNA–mediated gene knockdown has been published by several labs using different approaches, and so far, there appears to be no consensus on the proper technique for this approach[149,150,159]. The success of this technique appears to be highly dependent on the gene and context. Microinjection of embryos is currently being attempted, and if successful will hopefully generate CRISPR mutants and transgenic lines soon.

D. Pathways involved in whole-body regeneration

A large-scale gene expression study identified a few pathways that are upregulated during whole-body regeneration in *Botrylloides leachii*[160]. During the first 24h after injury, some highly conserved pathways that have previously been shown to be involved in development and regeneration in other chordates, such as Wnt, Notch, TGFbeta and Hedgehog, are upregulated. TGFbeta is required during axolotl limb and xenopus tail regeneration, and the Wnt pathway is involved in regenerative processes in many species, like zebrafish, planarians, and axolotl[132]. During the early stages of whole-body regeneration, cell-cell and cell-matrix-adhesion as well as metabolic pathways and

apoptotic pathways are upregulated. During later stages, translational processes as well as cellular component biogenesis are upregulated, such as 58 genes in the ribosomal pathway – consistent with increased synthesis of proteins. Wnt, Notch and TGFbeta are down regulated in later stages of regeneration[139]. Their expression may only be transiently required during the early stages of regeneration. The Wnt pathway is known to play important roles in morphogenesis during asexual reproduction[161], but its function during the early stages of whole-body regeneration has not yet been characterized.

Retinoic acid signaling controls cellular differentiation during embryonic development and controls the formation of the blastema during zebrafish fin regeneration[162]. During whole-body regeneration in *Botrylloides leachii*, retinoic acid receptor is expressed in aggregates of hemocytes, and retinoic acid synthesis inhibitors block regeneration[163].

Transcription factors associated with germ layer specification during embryogenesis, such as FoxA1, GATAa, GATAb, Otx, Gsc and Tbx2/3 are expressed in developing vascular buds in a similar fashion as during asexual reproduction[144]. These results suggest that whole-body regeneration in colonial ascidians is controlled by a similar set of pathways as regenerative processes in other animals. The exact functions of these pathways during these complex processes leading to the formation of an entire new body remain to be investigated.

E. Regeneration from multipotent epithelia in colonial ascidians

Colonial ascidians contain multipotent epithelial tissues such as the epicardium, septum, and atrial epithelium[164]. These tissues normally give rise to buds during asexual reproduction but can also replace missing body parts in the adult animal upon injury. One example is *Polyandrocarpa misakensis*. When cut in half, the anterior part of the adult zooid does not contain the posterior components such as the esophagus, stomach, and intestine; however, it regenerates these organs within a week, and they are formed from the atrial epithelium near the cut surface[164,165]. Within the first 48h, the atrial epithelium invaginates into the mesenchyme space and gives rise to the gut primordium. The invaginating epithelial cells change their appearance to cuboidal, while the other parts of the atrial epithelium remain squamous. The stomach and intestine differentiated 3–4 days after the amputation, and inhibitors of retinoic acid (RA) synthesis suppressed regeneration and gut formation. Addition of 13-cis RA rescues regeneration, further suggesting that RA is required for the regeneration of the gut[165].

A recent study investigated the mechanisms of de-differentiation of the atrial epithelium during regeneration and found a correlation between de-differentiation and autophagy[166]. Ultrastructural observations show that the numbers of autophagosomes, lysosomes, and secondary lysosomes increase remarkably, concomitant with mitochondrial degradation and exosome discharge. Autophagy had been previously shown to play roles in regeneration blastema of planarians[167] and zebrafish[168].

In Clavelinidae, when the thorax containing the pharynx, brain, and siphons is surgically removed from the abdomen, a new thorax is regenerated from the multipotent

epicardium[164]. It will be interesting to investigate whether all tunicate species that contain multipotent epithelia are able to regenerate adult body parts.

F. Regeneration of distal body parts in Ciona

In solitary ascidians such as *Ciona intestinalis*, regeneration capacities are more restricted than in colonial species. These animals only reproduce sexually, and are capable of partial body regeneration, in which only some parts of the body can regenerate the missing parts of an animal. Specifically, distal body parts can be replaced from proximal parts, but only if a portion of the branchial sac (Fig. 3.3) is present, and distal parts are unable to regenerate the proximal parts. Even after the most basal bisection, only the basal parts that contain the branchial sac will regenerate the distal parts, and middle portions of trisected animals containing the branchial sac will regenerate only the distal parts[135].

Ciona is one of the largest of all ascidians and regenerative experiments are comparatively easy to perform. Along its proximal (base) to distal axis it contains the visceral organs (heart, gonad, stomach, and intestines), the pharynx containing a large branchial sac, the neural complex, and the oral and atrial siphons (Fig. 3.3). The oral siphon is composed of muscular tissue, nerves, vasculature, epidermis and eight oral pigment organs (OPO) – sensory receptors rimming the siphon opening. The oral siphon can regenerate within a month after removal.

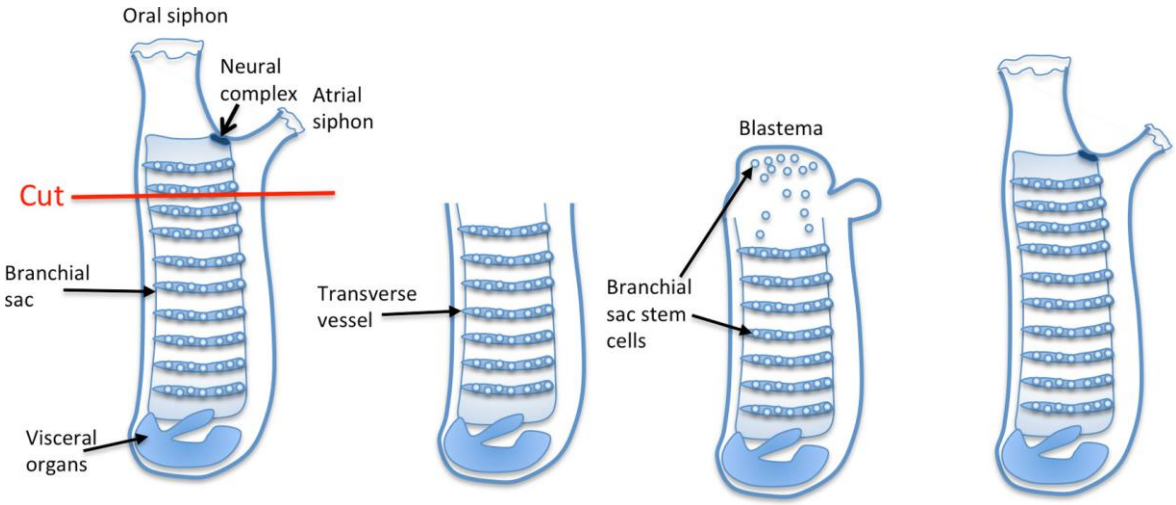


Figure 3.3. Siphon regeneration in *Ciona*. Surgical bisection removes distal structures such as the oral siphon and the neural complex. The proximal parts contain the branchial sac and visceral organs. Cells within the transverse vessels of the branchial sac begin proliferating. A blastema forms at the cut site, containing potential stem cells that have migrated from the branchial sac. The blastema differentiates into a new oral siphon and neural complex.

During regeneration of the oral siphon, a blastema of proliferating cells is formed about 4 days after injury. Within 10 days, the OPO and circular muscle fibers are regenerated, before the new oral siphon grows to full length. Interestingly, a large wound in one of the siphons causes the creation of a new siphon tube in the wounded area, and the same phenomenon was discovered in another solitary ascidian, *Styela clava*[135].

All the distal tissues of the ectopic siphon were formed in the correct proportions. *Ciona* lives for about 1 year and grows continuously until death, but regeneration capacity declines with age, and regrowth of the oral siphon becomes progressively slower during aging[169].

G. Role of stem cells in oral siphon regeneration

Elegant experiments by W. Jeffrey show that cells from the branchial sac are involved in distal regeneration of the oral siphon. As mentioned above, the middle portions of trisected animals, which contain the branchial sac, can also regenerate the distal parts[136]. As soon as 3h after injury, dividing cells within the transverse vessel of the branchial sac incorporates the nucleoside analog Edu (5-ethynyl-2-deoxyuridine), and many labeled cells can be seen in the lymph nodes. These same cells also label with alkaline phosphatase (AP) and an anti-piwi antibody, suggesting that they are undifferentiated, and possibly stem cells. High AP activity is a traditional marker of pluripotency in mouse and human stem cells[170]. At this early stage of regeneration, none of the cells in the regenerating oral siphon incorporate Edu, however; after a 24h labeling period with Edu, and a 10-day chase, Edu positive cells appear in the regeneration blastema at the oral siphon. This suggests that branchial sac stem cells migrate to the oral siphon regeneration blastema. In transplantation experiments, Edu-labeled branchial sacs were removed, stained with neutral red and transplanted into the branchial sac of a host animal. After bisection, Edu positive cells were detected in the regenerating oral siphon of the host. In older animals that have reduced regeneration capacity, AP and piwi staining is weaker, suggesting declining numbers of branchial sac stem cells. These data strongly suggest that cells within the branchial sac that are recruited into the cell cycle upon injury are stem cells that can give rise to regenerating structures within the oral siphon. Longer cell tracking experiments will be needed to assess incorporation of their progeny into differentiated tissues of the regenerated oral siphon, such as the cerebral ganglion, and single cell

transplantation would clarify if they are multipotent at the single cell level. To assess the source of the cells replacing the OPO, the remaining siphon stump that was left after OS amputation at the distal tip was cultured as an explant. New OPO were regenerated in the distal region of the explants, indicating that local cells give rise to the new OPO[171].

H. Pathways regulating oral siphon regeneration

Gene expression analysis of regenerating oral siphons by microarray showed that members of the notch-signaling pathway are upregulated during regeneration. These include the ligands delta and jagged, and the notch receptor. Expression of delta1, notch and hes-b were detected in regenerating tissue, including the blastema[7]. Notch was also expressed in the lymph nodes along the transverse vessel of the branchial sac, where potential stem cells are located. Chemical inhibition of notch signaling blocked siphon regeneration and prevented proliferation of branchial sac stem cells. Notch signaling is involved in amphibian tail regeneration[172] and is required for blastema cell proliferation during teleost fin regeneration[173,174].

Another study used RNA-seq to identify 472 mRNAs that are differentially expressed during oral siphon regeneration. Among them are apoptosis related genes, proliferation related genes, TGF-beta pathway activators and transcription factors[175]. 23 micro-RNAs were also differentially expressed. Categories of biological processes upregulated in the early stages of regeneration included wound healing, stress response, activation of morphogenetic processes and signaling. At day 3, Wnt and hedgehog signaling

related genes are transiently upregulated. TGF-beta activators were upregulated postamputation, and treatment with an inhibitor of TGF-beta signal transduction completely blocked regeneration. These results emphasize the idea that regeneration in ascidians is controlled by conserved pathways that are also involved in regeneration in animals from other phyla.

I. Central nervous system regeneration in Ciona

In addition to being able to regenerate oral siphons, *Ciona* is capable of central nervous system regeneration. The nervous system of tunicates consists of the cerebral ganglion, nerves within the body wall, the visceral nerve, the dorsal strand plexus, and sensory structures. The cerebral ganglion contains most of the neurons of the central nervous system (CNS), and nerves extend from the ganglion to the siphons, body wall and caudal viscera. The neural complex (NC), which contains the central ganglion (brain), the neural gland, and the ciliated duct and funnel, can regenerate after removal of the distal part of the body.

After removal of the neural complex, including part of the pharynx and the body wall, the neural complex regenerates completely within about a month[176]. The cellular origin of the regenerated neural complex is poorly understood and likely involves more than one cell type. GnRN positive neuroblasts from the dorsal strand are initially detected near the regenerating cerebral ganglion after NC ablation, and subsequently develop axonal processes and become incorporated into the posterior portion of the regenerating

ganglion. Dahlberg et al. reported that new structures originated from the residual neural structures on all sides of the ablation area[176]. At the tip of each nerve, incorporation of the proliferation marker Edu indicated the formation of a blastema which gives rise to new nerve cells. The neural complex regulates the contractile behaviors of the muscular body wall and siphons, including the so-called "cross-siphon" reflex[177,178]. These behaviors are gradually restored after ablation as connections are re-formed between nerve tracts of the CNS and the regenerating ganglion[176].

J. Regeneration in other solitary ascidians

Polycarpa mytiligera can regenerate its gut and branchial sac. Following gentle squeezing of the tunic these animals can eviscerate a large portion of their gut through the oral siphon[179]. The disemboweled part of the digestive tract is usually composed of the gut loop, including the stomach, and a large endocarp (a projection of the body wall into the atrial cavity) attached to the gut. A torn branchial sac, an esophagus and rectum are left behind in the animal. Twelve days after evisceration, animals had regenerated a completely new gut with fecal pellets and a mucus thread in the branchial sac, implying active filter-feeding[179].

K. Conclusions

Undifferentiated cells that are likely multipotent or pluripotent stem cells seem to be involved in both WBR in colonial ascidians as well as in distal regeneration in solitary

ascidians. In *Botrylloides leachii*, undifferentiated hemoblasts that express piwi appear to be involved in regeneration, and in *Ciona*, piwi-positive stem cells from the branchial sac are required for siphon regeneration. These cells are mobile, appear to be maintained as a pool of undifferentiated cells and proliferate in response to injury. In *Ciona*, it has been demonstrated that branchial sac stem cells give rise to regenerating tissues. In *Botrylloides*, this question remains to be investigated. Likewise, it remains to be formally tested whether these undifferentiated cells are self-renewing stem cells or whether they potentially arise from de-differentiation. Likewise, it will be important to test whether these putative stem cells lineage restricted, multipotent, or pluripotent, and whether they overlap with the population of mobile germline stem cells. Germline stem cells in colonial ascidians express piwi, and therefore, it is possible to speculate that a population of totipotent stem cells with germline and somatic potential could exist in these animals.

WBR and distal regeneration share similar set of signaling pathways and molecular mechanisms that are activated upon injury. These include Notch, Wnt, TGFbeta, and hedgehog. All these pathways have conserved roles in regeneration across phyla[180], where they are required for the proliferation of blastema cells or are involved in regulating differentiation. Some striking cross-species similarities in the functions of these pathways during regeneration are beginning to be uncovered. In zebrafish, Notch regulates blastema cell proliferation in zebrafish[174] and plays a similar role in *Ciona* where inhibition of Notch reduces proliferation of branchial sac stem cells[7]. The roles of these pathways have not yet been experimentally tested during whole-body regeneration, but it is likely that the roles of

some of these molecular pathways are shared between different modes of regeneration in tunicates.

While regeneration in some tunicates appears to be linked to the presence of undifferentiated cell types, de-differentiation seems to play a role in other cases. In *Polyandrocarpa misakensis*, the atrial epithelium that had been classified as multipotent shows clear ultrastructural signs of de-differentiation prior to giving rise to new organs[164,165]. Once appropriate tools become available, such as transgenics, future studies will aim assess whether multipotent epithelial tissues found in colonial ascidians, such as the epicardium, septum, and atrial epithelium, are truly multipotent, and whether they undergo de-differentiation during asexual reproduction and regeneration.

Some theories suggest that regenerative capacity is linked to the number of resources invested into the adult body, but because of the high cost of this process, it is selected against if investment in reproduction increases fitness of the species[137]. In colonial ascidians, bodies are transient structures that are required for sexual reproduction and feeding, whereas the vasculature is permanent. The bodies carry the gonads that are replaced every few days by asexual reproduction. The ability for whole-body regeneration from the vasculature may have been selected for because it is vital for these organisms to be able to replace lost bodies to continue sexual reproduction.

Within the chordates, the range of regenerative capacities is very broad and ranges from wound healing associated with scarring (humans, mice) to the capacity of fully regenerating limbs and spinal cords without scars (amphibians) to whole central nervous system regeneration (*Ciona*), and, finally, to the extreme case of WBR (*Botrylloides*).

Because of their phylogenetic position as invertebrate chordates, the study of regeneration in tunicates is vital to understanding the evolution of this wide range of regenerative capacities within the chordates. Tunicates seem to be the only chordates that have undifferentiated, multipotent stem cells that are involved in regeneration. In all other cases of regeneration in chordates (axolotl limbs and spinal cord, zebrafish fins and hearts), the new tissues arise from terminally differentiated cells that re-acquire proliferation capacities and de-differentiate within their pre-defined lineage. Future studies will investigate the cellular and molecular mechanisms underlying the regenerative processes in our close relatives, the tunicates, and will be crucial for the advancement of human regenerative medicine.

IV. Whole-body regeneration and developmental competition in two botryllid ascidians

Shane Nourizadeh*, Susannah H. Kassmer, Delany Rodriguez, Anthony W. De Tomaso

A. Introduction

Ascidians (subphylum tunicata) are marine chordates and the closest living invertebrate relatives to vertebrates[181]. Sexual reproduction leads to a pelagic chordate tadpole larva that swims to find a suitable substrate[182], then settles and undergoes metamorphosis to a sessile adult individual. The resulting invertebrate body plan is called an oozooid; and has little resemblance to the larval form[183–185]. The oozooid is a filter feeder with a complex body plan that includes incurrent and excurrent siphons, central and peripheral nervous system, pharynx for respiration and feeding, stomach, intestines, gonads, circulatory system, tube-like heart, and glands for endocrine signaling[186–189]. After metamorphosis, there are two divergent life histories among ascidians. Most species, such as *Ciona robusta*, grow by increasing in size and become sexually mature during their one-year lifespan. However, many species are colonial[190] and propagate asexually. This process, called budding, generates multiple independent individuals (called zooids) with identical body plans to that of the oozooid[138,145,147,191,192]. Budding occurs throughout the life of a colony and can lead to thousands of clonal zooids, all derived from a single larva[193]. In the botryllids, all adult and developing zooids (buds) are connected by a common vasculature that ramifies throughout the colony. Outside of the bodies, vessels radiate distally and terminate into close-ended oval shaped structures called ampullae that encircle the colony

periphery (Fig. 4.1A, B). All bodies and blood vessels are embedded within a cellulose based tunic, a defining feature of this phylum. Among the colonial species there are multiple asexual budding pathways that have arisen independently, and the diversity and phylogeny of these processes have been extensively reviewed[25,194].

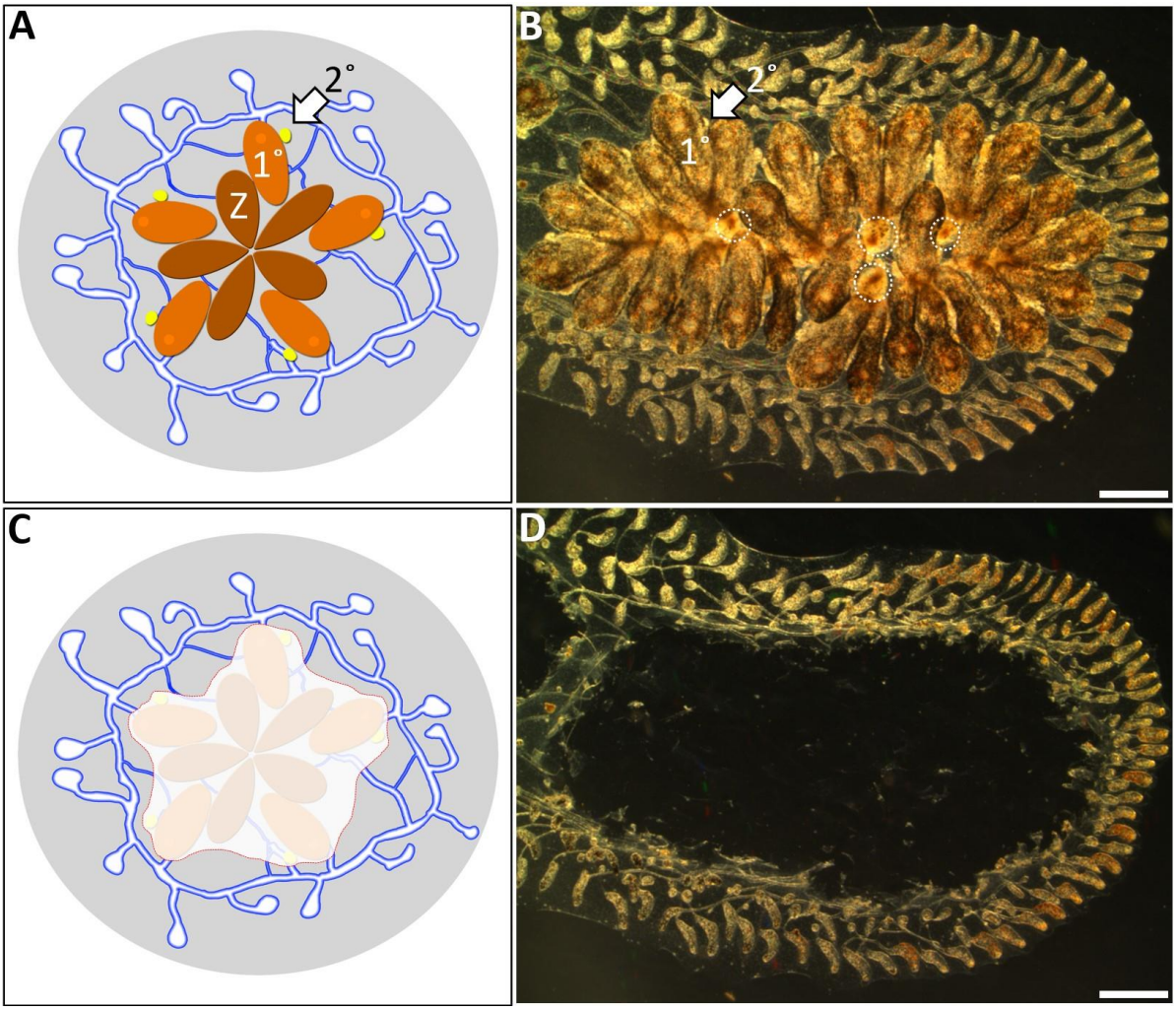


Figure 4.1: Surgery performed on *Botryllus schlosseri* to induce whole-body regeneration. A) Illustration showing a system of *B. schlosseri*. Depicted are zooids in brown (Z), primary buds in orange (1˚), and secondary buds in yellow (2˚). **B)** Pre-surgery darkfield image of a colony being grown on a glass slide. Zooids are almost entirely resorbed (shown in white dashed circles). **C)** Illustration shows excision of zooids and primary/secondary buds, but vascular tissue remains intact. **D)** Post-surgery image of same colony in panel B two minutes following the removal of all zooids and developing bud tissues. Scale bars = 1.0mm.

Individual genotypes of colonial ascidians such as *Botryllus schlosseri* and *Botrylloides diegensis* can consist of thousands of zooids and survive for years; however, the zooids themselves are short-lived (Supplemental Fig. 4.1A, labeled Z1-3). In these species, new zooids regenerate weekly through a process called blastogenesis (Supplemental Vid. 4.1). Blastogenesis is organized into three concurrent generations that are spatially arranged and developing synchronously (Supplemental Fig. 4.1B). Zooids, the oldest generation, are the only animals in a colony with an open oral and atrial siphon for filter feeding and sexual reproduction. Primary buds are the second oldest generation and are found proximal to the zooids. They are in the last stages of development (Supplemental Fig. 4.2C-F, white arrow). Secondary buds are the youngest generation and located proximal to the primary bud (Supplemental Fig. 4.1D-F, black arrow). Secondary buds originate from a region of the primary bud called the peribranchial epithelium, and buds originally derived from this tissue are called palleal buds. At the end of each blastogenetic cycle, all adult zooids die and are resorbed through a coordinated process of apoptosis and phagocytosis known as takeover[85,195]. Primary buds migrate to the newly vacated area to open their siphons and begin feeding; this designates them as adult zooids. Secondary buds become primary buds and continue development, and new secondary buds initiate development (Supplemental Video 4.1). In *B. schlosseri*, zooids are arranged into star-shaped groups known as systems. Zooids occupy the center, and primary buds and secondary buds are distal, respectively. In *B. diegensis*, the budding process is the same, but the zooids are arranged linearly.

In colonial species of the family Styelidae, in which *B. schlosseri* and *B. diegensis* are classified, another asexual budding mode exists, called vascular budding. Here, the source of the new bud is a population of circulatory cells that aggregate within the blood vessels to form into a blastula like structure and develop into a new bud in situ. This process is morphologically equivalent to palleal budding. Both palleal and vascular budding are utilized differently among *botryllid* species. For example, *Botryllus primigenus, Botryllus tuberatus, Botryllus delicatus, Botrylloides gascoi,* and their phylogenetic outgroup *Symplegma brakenhielmi,* can simultaneously form palleal and vascular buds[145,147,196,197]. Others, like *Botrylloides violaceus, B. diegensis and B. schlosseri,* undergo colony expansion exclusively through palleal budding[147].

Vascular budding is also observed in two other situations in the botryllids: response to injury and exit from seasonal dormancy. When vascular budding is induced by injury, it is also referred to as whole body regeneration (WBR), and only occurs following a surgical stimulus that involves isolating portions of the extracorporeal blood vasculature by excising all zooids and buds[139,141,142,147,150,163,198–200]. This stimulus triggers vascular rearrangement and initiates blood cell aggregation; the first step of vascular budding. The zooid that develops then switches back to palleal budding to eventually regenerate the entire colony. Vascular budding is also utilized to exit seasonal dormancy. Environmental perturbations trigger dormancy, which cause zooids and developing palleal buds to resorb and blood vessels to coalesce until conditions improve[201]. Dormancy can last for months, and during that time the colony resembles the early stages of WBR: the vessels have remodeled into an opaque bed and multiple presumptive vascular buds at the earliest stages of

development: aggregates and blastula-like structures; are present[148]. When environmental conditions return to normal, these buds complete development, begin feeding, and initiate palleal budding to regenerate the colony.

We have been coupling transplantation and prospective isolation studies to identify the cells which initiate WBR[202], and one of our aims was to compare this process between *B. diegensis* and *B. schlosseri.* One interesting observation is that the requirements for WBR in the two species are disparate. In *B. diegensis*, WBR is simple and robust: isolating a small 2mm^2 area of peripheral vasculature at any adult life stage will trigger vascular bud development, and a zooid will develop to maturity in around ten days with an efficiency >90%. In contrast, previous publications in *B. schlosseri* from multiple labs around the world have found that WBR requires a few strict conditions. These include using an entire vascular network from a large colony; a size that is ten times larger than that required for *B. diegensis*. It is also reported that surgical removal of the zooids and buds must occur during a specific 36h window during takeover (Supplemental Video 4.1). If these requirements are not met, WBR is not successful[143,144,192,203].

Previous studies have clearly shown that WBR in *Botrylloides* occurs from the vasculature[139,142,163], and that the source for development is a population of circulating cells[202]. None of the previous studies in *B. schlosseri* show any evidence of the early stages of vascular budding, while the aggregation of circulatory cells and early morphogenesis within the vasculature has been clearly shown in multiple species of *Botrylloides*.

The initial goal of this study was to rigorously analyze the early stages of WBR in *B. schlosseri* to determine the source of the new bud and try to understand why the

requirements for successful regeneration were much more stringent. Through our analysis we found that the zooid which develops after surgical isolation of blood vessels in *B. schlosseri* is not due to WBR, but instead relies upon ectopic development of secondary or incompletely resorbed buds derived from the blastogenic process. While analyzing the timing and activity of ectopic development in *B. schlosseri* we also found that if multiple buds survived surgery, that they would compete for survival with only one winning and completing development. An analogous competition between developing vascular buds has also been shown to occur during WBR in *B. leachii*[163,204]. We followed up on these observations and discovered that a reversible mechanism of competition exists in *B. schlosseri* and is mediated through the vascular network.

B. Materials and methods

Animals:

Colonies of *Botryllus schlosseri* and *Botrylloides diegensis* were collected from Santa Barbara Marina in California, USA. Hatches were collected on glass slides and raised in a mariculture room with constant flowing seawater and temperature ranging from 19°-21°C. They are fed algae every hour, and glass slides are cleaned of parasites every two weeks using Kim-wipes and soft synthetic bristle paint brushes (size number 2).

Surgeries

Botryllus schlosseri: Removal of tissue was performed using the following tools: fine forceps (Dumostar/55), micro-surgery scissors (FST/15400-12), and razor blades (Personna/0.009RD). Surgeries were done under a stereomicroscope (Zeiss Stemis 2000) at magnifications between 40x and 50x. Animals were cleaned using a round-tip paint brush a day prior to surgery to reduce negative effects from parasitism, and subsequently left to develop in stagnant 0.5µm filtered seawater. When a feeding siphon opened, animals were transferred to mariculture tanks.

Botrylloides diegensis: Separation of zooids from blood vasculature was accomplished using a razor blade (Personna/0.009RD). Animals were transferred to a new slide, and vasculature regenerated while adhered to original slide in 0.5µm filtered seawater at temps ranging from 19°-21°C.

Removal of all zooids and developing buds from Botryllus schlosseri*:* Zooids, primary buds, and secondary buds were cut out using micro-surgery scissors. All bodies were then removed at once using fine forceps. A paintbrush was then used to remove unwanted debris left after surgery.

Removal of all zooids to leave an anterior primary bud fragment with secondary bud in *Botryllus schlosseri*: Zooids, and all but one anterior primary bud and secondary bud were

cut out using micro-surgery scissors. Bodies were then removed at once using fine forceps. A paintbrush was used to remove unwanted debris left after surgery.

Removal of all zooids to leave only a developing secondary bud in *Botryllus schlosseri*: Tissue was excised using a new razor blade and fine forceps. A cut was made to gape the excurrent siphon region, and resorbing zooids were teased out of the surgical hole using forceps. The primary buds were then opened down the middle from the anterior most point, and the two sections were carefully pulled out using forceps while being careful not to pull or damage the secondary bud.

C. Results

Disparities in injury response between closely related species

Previous studies on whole body regeneration (WBR) in *Botryllus schlosseri* concluded that there were three requirements for zooid development from isolated vasculature: 1) experimental colonies must be large, having nine or more zooids[143]; 2) the marginal vessel (central blood vessel that connects all zooids and ampullae) must be left intact following ablation of the zooids and buds[192] (Supplemental Fig. 4.2); and, 3) surgery required ablation of the zooids and buds when the zooids are resorbing during the takeover process[143,144]. To make sense of these qualifications, we attempted to replicate previous experiments in *B. schlosseri* by carefully removing zooids and developing buds from large colonies to isolate blood vasculature and induce WBR (Fig. 4.1). We made

detailed observations by carrying out longitudinal studies and recording time-lapse videos starting immediately following surgery. While collecting data for both species, *B. schlosseri* and *B. diegensis* (Fig. 4.2), we never observed a zooid develop from an isolated blood vessel in *B. schlosseri* (Fig. 4.2C); however, zooid development in *B. diegensis* was common. In both species, the vascular network initially reacted to colony damage by rapidly clotting up severed vessels to prevent blood loss. Next, the vasculature actively remodeled within the tunic matrix, with major differences observed between the two species. After three days of reorganization, the tissues in *B. diegensis* coalesced into a compact mass (Fig. 4.2E).

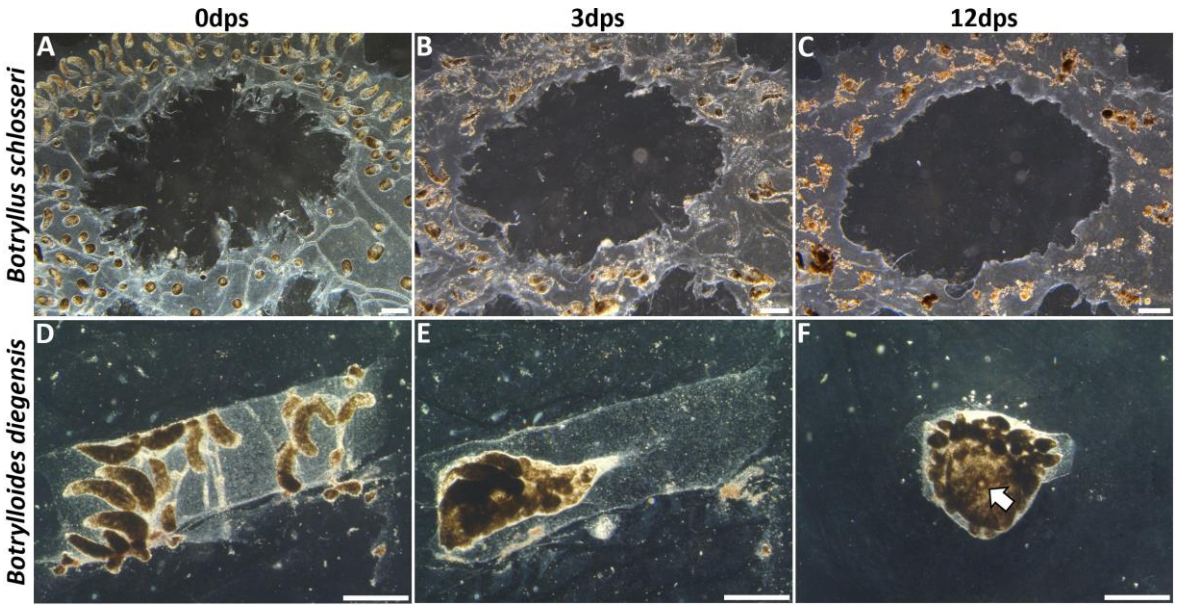


Figure 4.2: Comparison of whole-body regeneration (WBR) surgeries between *Botryllus schlosseri* and *Botrylloides diegensis*. A) Darkfield image of *B. schlosseri* after surgery. Ten zooids were excised, and blood circulation remained strong throughout vasculature. **B)** After three days, blood continued to flow, and vasculature had rearranged within the tunic. **C)** After 12 days, blood flow had stopped, and the vasculature showed increased pigmentation. No sign of WBR, and vessel migration had ceased. **D)** Darkfield image of *Botrylloides diegensis* after surgery. Minimal vasculature and approximately 20 ampullae were left after removal of zooids and buds. **E)** Three days post-surgery the vasculature had condensed. **F)** At day 12 post-surgery an open oral siphon (arrow) and atrial siphon have developed. Ampullae have begun to spread outward and sexual budding resumes. dps = days post-surgery. Scale bars represent 0.5mm.

In contrast, *B. schlosseri* vessels went through a characteristic global regression, followed by vessel re-extension that at times retraced the pathway taken for regression (Fig. 4.2, Supplemental Vid. 4.2). These retraction and re-extension processes are consistent among genotypes and take approximately 24h.

Unremoved secondary buds migrate to vasculature and continue development

After twelve time-lapse experiments with *B. schlosseri* we observed a WBR event following zooid ablation (Supplemental Vid. 4.3). However, through retrospective analyses of high-resolution images, we noticed small developing bud tissues had been inadvertently left behind following surgery. The observed tissues migrated away from their original location through the tunic, and restored contact with the peripheral blood vessel. Once fused with the circulatory system these tissues increased in size and continued to develop as if seemingly derived from the blood vasculature.

To follow up on these results, we performed over 150 surgeries to ensure removal of all zooids and developing bodies from large, stage D colonies of *B. schlosseri* (Supplemental Fig. 4.3A-C). Experiments included five distinct genotypes from the Santa Barbara harbor on the Pacific coast of California (Supplemental Table 4.1). We only scored animals that restored colony-wide circulation and showed robust blood flow throughout the observation window (n=128); therefore, in over 85% of our experiments we analyzed vascular rearrangement and blood circulation for up to twelve days following surgeries. None of these experiments provided evidence that WBR could be induced through injury. Instead, we observed characteristic vascular remodeling (described above), followed by

eventual constriction of vessels, cessation of blood flow, and necrosis of remaining tissues (Supplemental Fig. 4.3D-F). If a bud appeared, we could trace its origins to outside of the vasculature. These findings led us to believe that *B. schlosseri's* inability to regenerate from vasculature is not genotypically constrained. Even when we performed surgeries on very large colonies (n = 8) that were >5x the reported minimal size requirement, there was no indication of regeneration (Supplemental Fig. 4.5). In contrast, *B. diegensis* robustly and repeatedly underwent WBR from minimal vascular tissue under the same mariculture conditions (Fig. 4.2D-F).

In summary, when zooids developed after surgery in *B. schlosseri*, we could always retrospectively identify a previously undetected transparent tissue that was outside of the vasculature. Following surgery, tissues rapidly migrated and re-attached to the vasculature to provide the source of the new zooid. They initially appeared near the peripheral vasculature furthest away from the zooids and were most likely secondary buds that we missed during surgical ablation. At this point in the blastogenic cycle, secondary buds are small (250x100µM), and lack pigmentation. It would be easy to miss ablating them, particularly since the peripheral vasculature cannot be damaged for WBR to occur, thus one would avoid cutting close to the vessel (Fig. 4.2A). We found the most critical time point of these observations were the initial hours after surgery, during which we observed tissues migrating from their original position to fuse with vasculature (Fig. 4.3A-C). This phenotypically appeared as though a zooid developed directly from the remaining vasculature (Fig. 4.3D-J), but we could always predict where the zooid would arise following surgery when detailed images were scrutinized for migrating tissues.

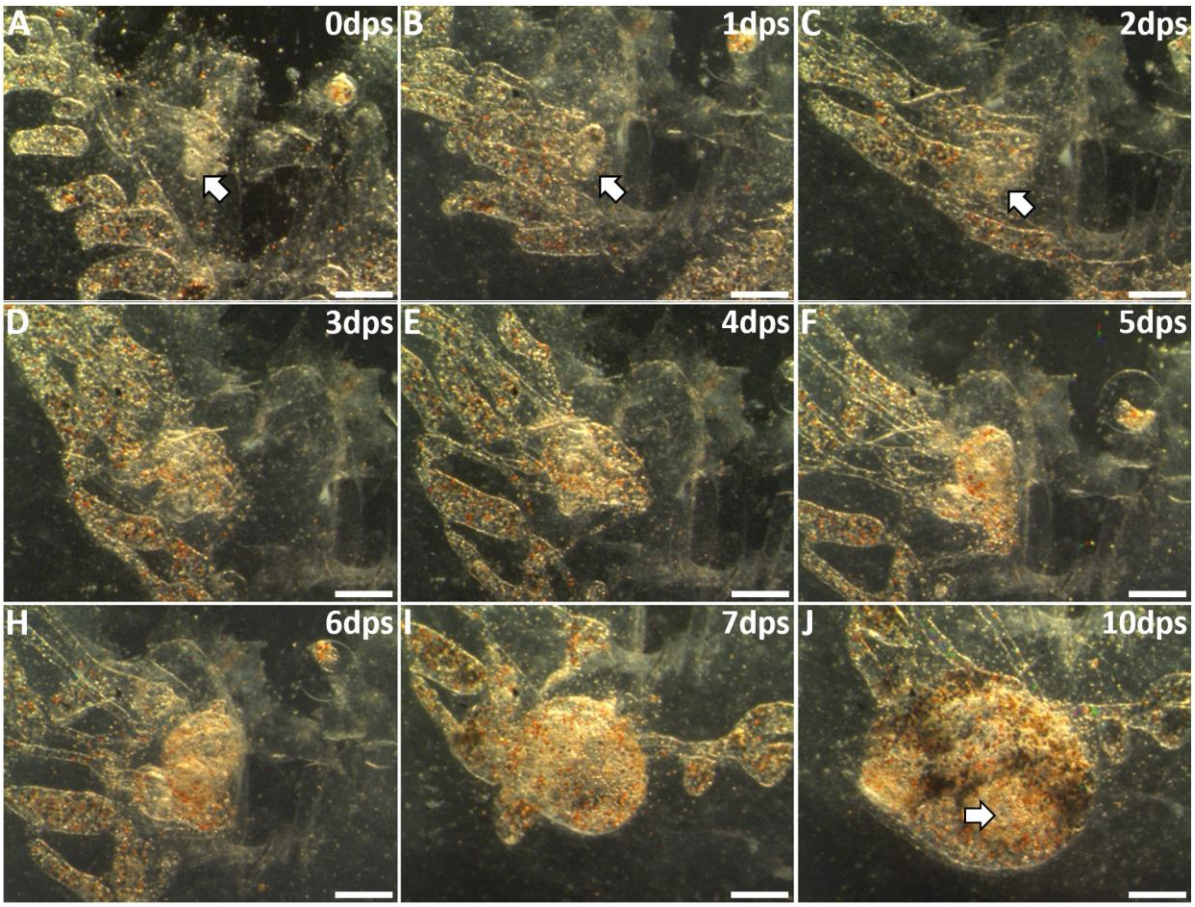


Figure 4.3: Secondary bud migration and development following removal of zooids and other developing buds. A) Post-surgery darkfield image showing vasculature, tunic, and a single remaining stage-D secondary bud (white arrow). **B)** After 24 hours, the secondary bud (white arrow) had been vascularized and is indistinguishable from the vasculature. **C and D)** At 2- and 3-days post-surgery, the secondary bud continued development and remains difficult to discern from the vasculature. **E-I)** The secondary bud shows pigmentation and is now evident under the microscope. The heartbeat developed during this 5–7-day time frame which allowed for easy detection. **J)** Ten days post-surgery the secondary bud opened a siphon and was actively feeding. dps = days post-surgery. Scale bars = 250µm.

Injury and characterizing development of remaining tissues

Developing buds are often near the marginal vessel, situated partially underneath the zooid, thus it is possible to leave fragments of primary buds with secondary buds after surgery. We followed up on previous observations by removing all zooids at stage D and

purposefully leaving combinations of primary and secondary bud tissues to characterize the response. We initially carried out two experiments: leaving only intact secondary buds or leaving fragments of the primary bud coupled to the secondary bud. In both cases, the remaining tissue migrated from its original location, re-attached to the peripheral vasculature, then completed development into a zooid, as we had seen previously. When part of the primary bud was left, it was resorbed by the developing bud, and the zooid developed in 89% of the cases. When only a secondary bud was left, this decreased to 48% (Table 4.1; Supplemental Fig. 4.5). In many cases when only a secondary bud was left, we observed that the resulting zooid had an abnormal phenotype. This including being shifted

Table 4.1: The potential of various body tissues (whole or partial) to develop a post-surgery zooid.

Tissues Tested with Vasculature	Develop a heartbeat (%)	Open siphon (%)	N
None	0	0	128
Partial primary Bud	0	0	20
Secondary bud + Partial primary bud	90	89	73
Secondary bud	78	48	27
Fragmented secondary bud	25	6	16
Secondary bud with reduced vasculature (≤3.5mm^2)	10	0	10
Secondary bud with reduced vasculature (6mm^2)	25	25	4

Seven surgery permutations were performed to determine how much tissue was required for a zooid to develop after injury. Vascular tissue and tunic alone were insufficient to recover from loss of all zooids and associated buds. When only the anterior region of a primary bud was left to develop, it became resorbed into the existing tissue, but no zooid formation occurred. When a secondary bud was associated with that same portion of the primary bud, a zooid developed. Furthermore, secondary buds alone can complete development 48% of the time, but if damaged, survivability drops to only 6%. Secondary buds did not survive with less than 20 ampullae after surgery; however, leaving more than 20 ampullae was sufficient to support full secondary bud development and asexual budding.

sideways in the tunic such that the siphons pointed to the left or right, rather than dorsally (Supplemental Fig. 4.6). However, this this does not impinge on the next generation of palleal buds. One interesting observation regarded differences in the timing of development following these two surgeries. Normal palleal budding in lab-reared colonies at stage D have secondary buds that are six days old, form a pumping heart three days later (day 9), and open the siphon five days after the heart (day 14). When we observed development following surgeries in which the secondary bud remained attached to the primary bud anterior region (Fig. 4.4A-B), it required on average three days for heart formation and six days to open a siphon (Fig. 4.4C). Thus, secondary buds developed normally when remaining primary bud tissues were present. When we performed surgeries to leave only the secondary bud (Fig. 4.4D-E), it required six days for hearts to pump, and twelve days for siphon opening (Fig. 4.4F), an approximate two-fold delay vs unmanipulated palleal budding. This is the exact same range for WBR to occur in previous studies[143,144,192,203].

If WBR in *B. schlosseri* is due to tissues leftover by accident during ablation, they would not be left in a controlled fashion, as in the previous experiments. We next characterized the level of damage that could occur to a secondary bud and still result in zooid development. We removed all but one intact secondary bud from a large colony at stage D, then injured that bud and observed the results. Our experimental injury applied pressure to the tunic above the bud without tearing into the tunic, until gross morphology was perturbed (Supplemental Fig. 4.7B). The reason for this approach was that secondary buds did not survive direct surgical cuts. Interestingly, 25% of damaged secondary buds

developed a pumping heart (Supplemental Fig. 4.7C), but only one out of sixteen cases did we observe the damaged bud develop into a mature zooid with open siphons. While this injury model is not replicating what may have happened in previous studies, it does suggest that a relatively undamaged secondary bud is required to generate a zooid. These experiments also provide strong evidence that WBR does not occur in *B. schlosseri*: a single secondary bud was purposefully left and damaged prior to revascularization, but no WBR event was observed under these controlled conditions. Importantly, in the case where development did occur, it did so from the damaged bud.

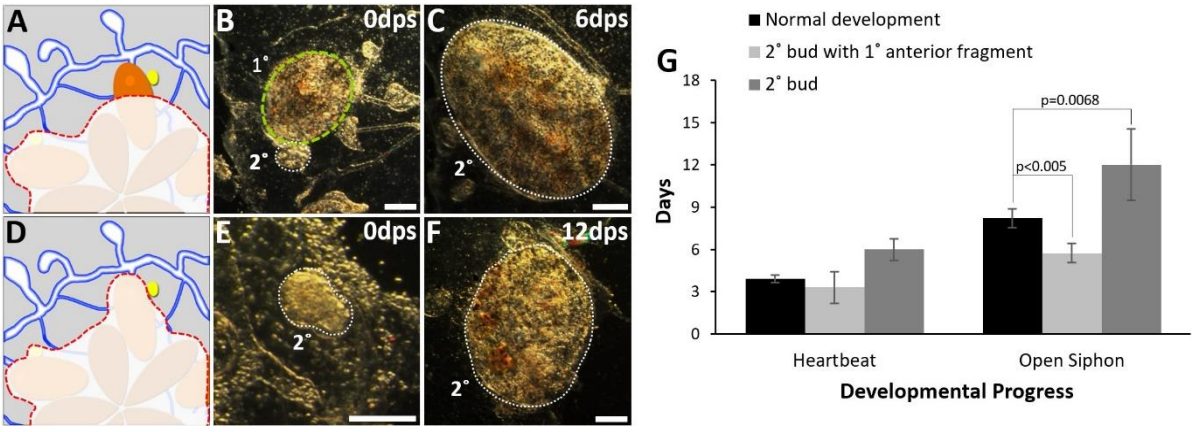


Figure 4.4: Post-surgery timing of secondary bud development. A) Illustration showing surgery to isolate secondary bud and anterior region of associated primary bud. **B and C)** Post-surgery darkfield images of those bud tissues at day 0 and 6, respectively. **D)** Illustration showing surgery performed to isolate a secondary bud alone. **E and F)** Post-surgery darkfield images of secondary bud tissue at day 0 and 12, respectively. The siphon opened at day 13. **G)** Quantitative results comparing the timing of development to heartbeat and siphon opening during normal budding, secondary/primary bud, and secondary bud alone. dps = days post-surgery. Scale bars = 0.25mm.

Isolated secondary bud survival has a vascular tissue size requirement

Previous studies reported that a continuous marginal blood vessel and approximately 10x more vascular area was required for WBR in *B. schlosseri* versus *B. diegensis.* Additionally, the ablation must take place during takeover, when adult zooids are dying and being phagocytosed in stage D (Supplemental Video 4.1). Taken together, this suggested that *B. schlosseri* requires more energy via catabolism of the remaining tissue versus *B. diegensis*, where only a small portion of the vasculature is required to support WBR (Fig. 4.2).

To address this potential difference in energetic demand, we determined the minimal size of remaining vasculature that was required to allow successful post-surgery zooid development of a single secondary bud (Table 4.1). Secondary buds left with ≤3mm^2 of total tissue area did not survive (Fig. 4.5A-F), whereas isolated secondary buds (Fig. 4.5G) with tissue area ≥6mm^2 (Fig. 4.5H) developed and continued asexual budding (Fig. 4.5I, Supplemental Vid. 4.4). Surprisingly, the time to complete development was equivalent whether we used an entire vascular network (Fig. 4.4), or only a 6mm^2 section (Fig. 4.5). In other words, an area of vasculature larger than the minimum size did not expedite the developmental process. These data show that there is a vascular tissue size requirement for secondary bud survival, but it is <10% of the size required for successful WBR as described previously[143].

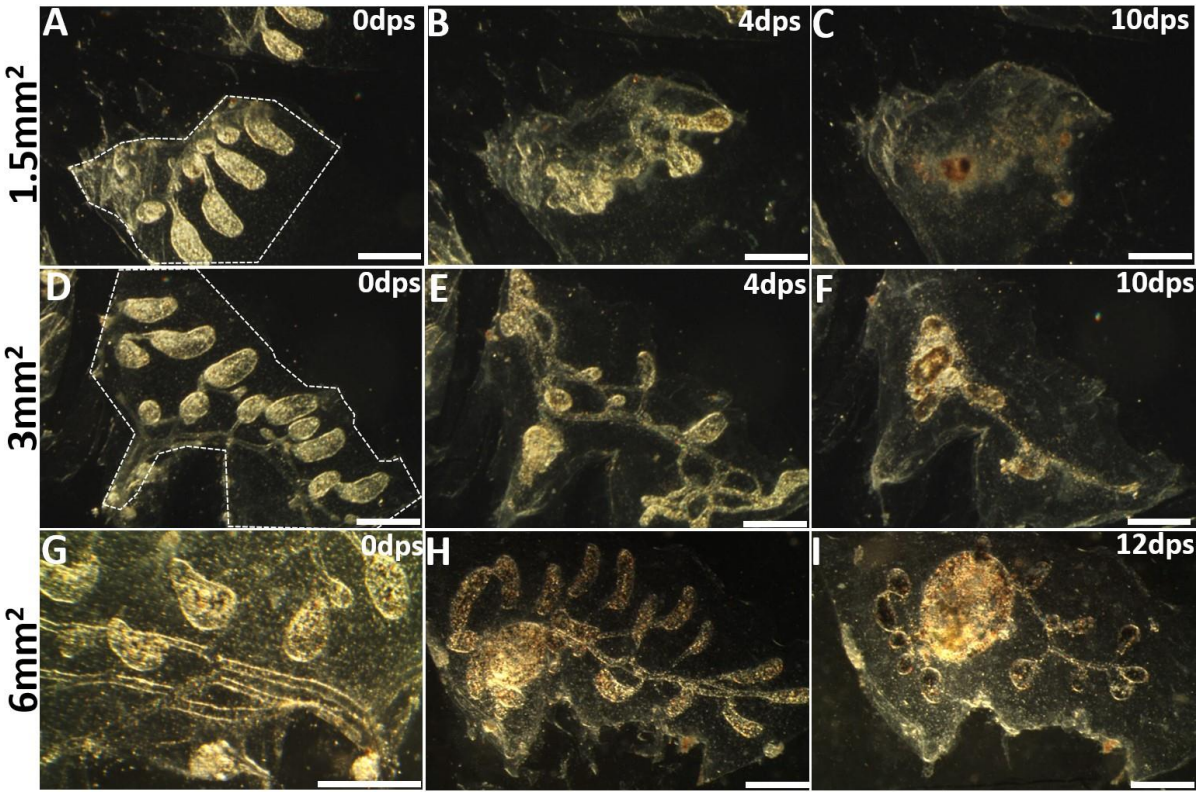


Figure 4.5: Complete post-surgery secondary bud development has a vasculature size requirement. A-C) Secondary buds that were left to develop with a tunic area of <2mm^2 did not survive and loss of all activity was seen by day 10. **D-F)** Vasculature and tunic with an approximate area of 3.4mm^2 with a single secondary bud did not survive but showed longer activity than experiments having less tissue resources. **G-I)** Full development of a secondary bud was observed with a tunic area of 6mm^2 or more. dps = days post-surgery. Scale bars = 0.5mm.

Secondary buds compete for sole survivor

Previous studies on WBR in *B. diegensis* have revealed that while multiple vascular buds are initiated following surgery, only a single zooid completes development. This observation was consistent over a large range of vascular tissue. A single zooid can develop from a 3mm^2 fragment (Fig. 4.2D), so larger fragments around 40mm^2 (Supplemental Fig. 4.8) could theoretically support the development of multiple zooids, but that is not observed. This suggests that buds compete for resources during WBR, and previous

studies suggested competition occurs at the blastula-like stage[201,205]. If previous results documenting WBR in *B. schlosseri* were actually due to ectopic development of remaining secondary buds, we wondered why these experiments also resulted in the development of only a single zooid[143,144,192,203]. We next asked if competition also exists between developing secondary buds in *B. schlosseri*.

To assess the presence of interbud competition, we analyzed the outcome of leaving multiple secondary buds after surgically removing all zooids and primary buds. When two isolated secondary buds in *B. schlosseri* were left after surgery, the result was a single surviving zooid. We next examined the outcome when three secondary buds were left (Fig. 4.6). Seven days after surgery, hearts pumped in all three buds, but their sizes varied (Fig. 4.6C-F). By day thirteen, only one persisted and opened its siphons to become an active filter-feeding zooid (Fig. 4.6G). The other two developing secondary buds, which were always sharing blood with the winner, died, and resorbed into the vasculature (Fig. 4.6H), similar to takeover. Because these competing buds were derived from a single system, we next checked if secondary buds originating from separate systems within the same colony could compete at longer distances (Fig. 4.7A). The surgery performed left behind two developing buds spaced 1cm apart (Fig. 4.7B). They both developed pumping hearts by day six (Fig. 4.7C) but on day thirteen only a single zooid developed while the other was resorbed (Fig. 4.7D). Whatever is mediating the interactions between the buds can operate at these distances.

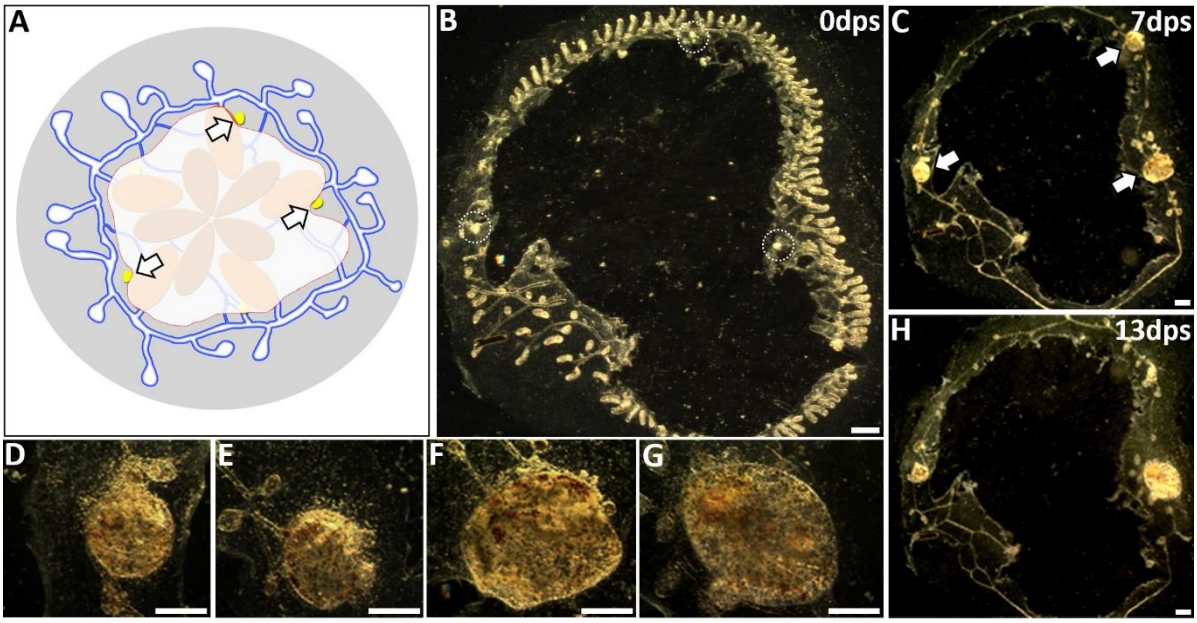


Figure 4.6: Three post-surgery secondary buds lead to a single zooid through competition in *Botryllus schlosseri*. A) Illustration showing surgery performed to remove zooids, primary buds, and all but three secondary buds (white arrows) **B)** Darkfield image of post-surgery. Secondary buds are demarcated (white circles). **C)** Seven days post-surgery. All three secondary buds (white arrows) have pumping hearts. Higher magnification of each bud is shown in panels D through F. **D)** Bud from left side of panel C. **E)** Bud from top of panel C. **F)** Bud from right side of panel C. **G)** The single zooid that fully developed is shown magnified in panel G. **H)** Zoom out of colony at day 13. Two other buds were in the process of resorption. dps = days post-surgery. Scale bars = 0.5mm.

Competition provides winner secondary bud with more resources

A zooid that develops from a single secondary bud (Fig. 4.8A-B) is significantly smaller than a control zooid derived from palleal budding (Fig.4.8J). However, leaving two secondary buds (Fig. 4.8F-G) gave rise to a single zooid (Fig. 4.8H-I) of similar size to the control (Fig. 4.8J). Interestingly, when multiple secondary buds are left, they grow synchronously until the heart begins beating (Fig. 4.8J), and differences in the growth rate are not apparent until after this point in development. This remains true even if three or more buds are left. However, when multiple secondary buds remain, the winner completes

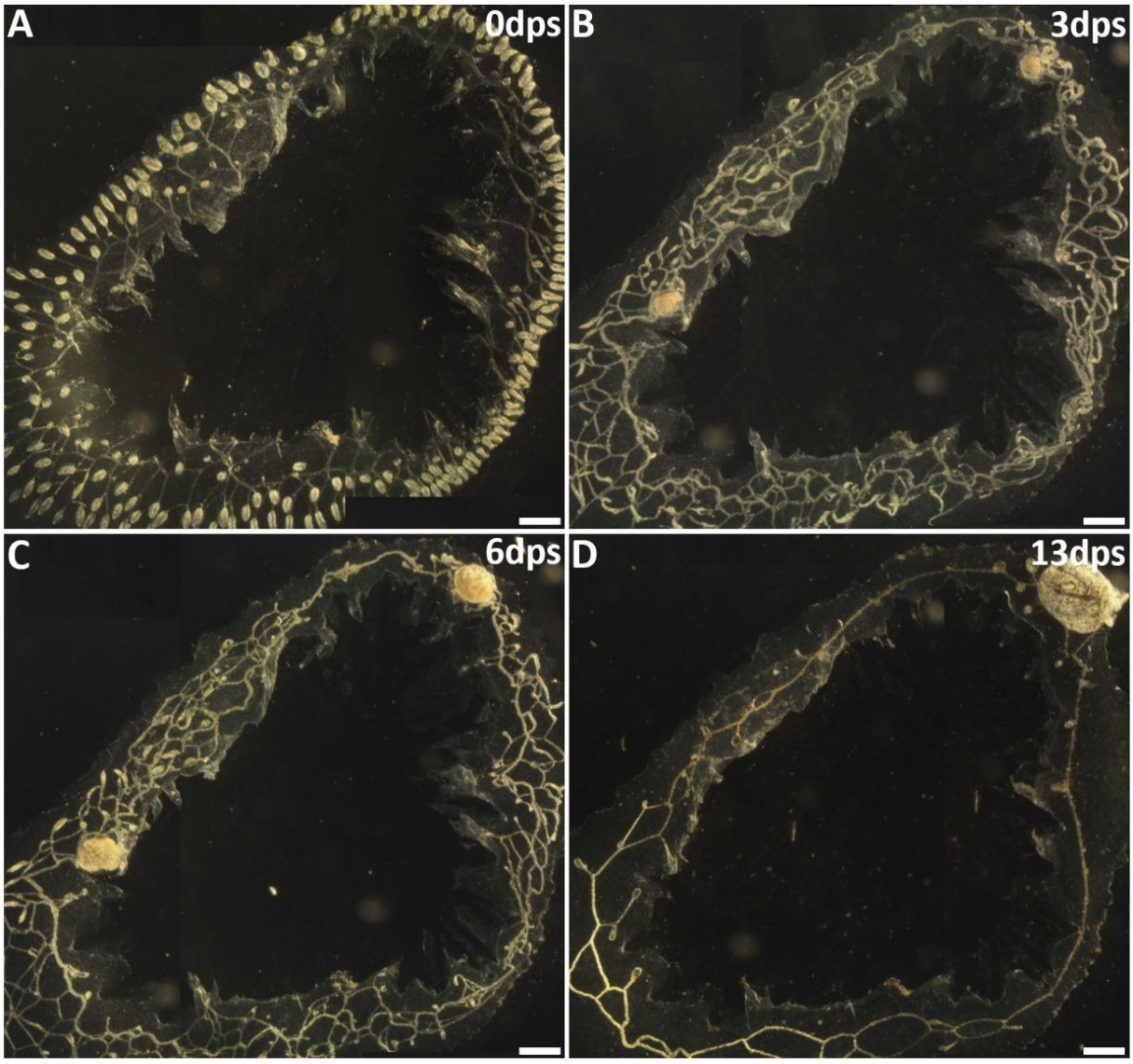


Figure 4.7: Secondary buds from separate systems can compete at a distance. A) Zooids, primary buds, and all but two secondary buds were removed from a three-system colony. **B)** Both secondary buds had migrated to fuse with the vasculature and commensurately increased in size. **C)** The secondary bud (left side) had reached its maximum size before being developmentally suppressed by the winner secondary bud (right side). **D)** A single secondary bud persisted, and the loser secondary bud had completely resorbed. dps = days post-surgery. Scale bars = 1mm.

development on day eight, four days faster than when a single secondary bud is left after surgery. This demonstrates that competition is causing the death and resorption of the

loser buds to reallocate those resources to the winner. Competition is not visually apparent until after the hearts have completed development.

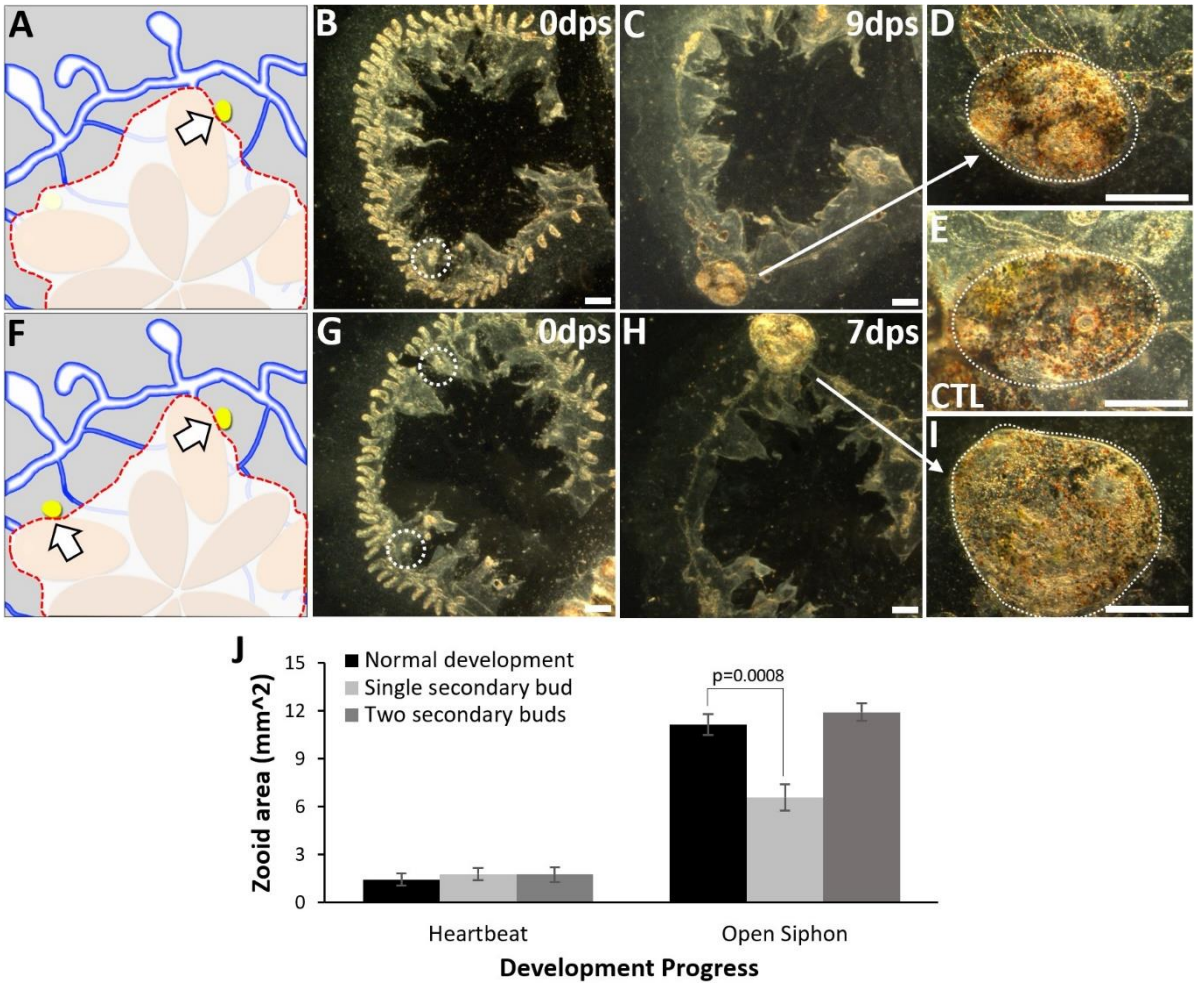


Figure 4.8: Loser bud resorption increased size of winner bud. A) Illustration showing surgery performed to isolate secondary bud (white arrow). **B and C)** Post-surgery darkfield images of secondary bud (white circle) at day 0 and 9, respectively. **D)** Higher magnification of zooid in panel C at time of siphon opening. **E)** Size of a control zooid upon siphon opening. **F)** Illustration showing surgery performed to isolate two secondary buds (white arrows). **G and H)** Post-surgery darkfield images showing secondary buds (white circles) at day 0 and 7, respectively. **I)** Higher magnification of panel C zooid at time of siphon development. J**)** Quantitative analysis comparing areas of isolated secondary bud(s) vs normal/blastogenic bud. Heartbeat and siphon opening used as developmental landmarks. **K)** Comparing the heart's beats-per-minute (BPM) between normally developing secondary buds and post-surgery secondary buds on first day of heart activity. dps = days post-surgery. Scale bars represent 0.5mm.

Growth inhibition is due to circulatory factors and is reversible

To narrow down the tissues possibly mediating competition, we did the same experiments leaving two secondary buds. This time we severed the blood vessels 48h later when the two buds were nearly equal in size but without a functional heart. The shared tunic was left intact so that we only disrupted the vascular connections (Supplemental Fig. 4.9B). In this case, secondary buds sharing tunic but not blood both developed to zooids (Supplemental Fig. 4.9D). These findings show that factors in the blood are responsible for the signals driving competition between secondary buds.

To narrow down the timing and mechanisms of competition, circulation was severed following visual changes in growth rates between the two buds. Colonies with two secondary buds (Fig. 4.9B) were left to develop following completion of heart development until one bud (presumed to be the winner) was growing steadily, and the other (presumed to be the loser) was not increasing in size. By day eight we observed that the smaller secondary bud started shrinking, indicating it was beginning to die (Fig. 4.9C, right side). At that observational timepoint, the vasculature was severed. Within four days, the loser secondary bud had substantially increased in size, opened its siphons, and started to bud (Fig. 4.9D, right side).

We repeated this experiment, but this time allowed the smaller bud to decrease in size to a point where we could observe accumulation of pigmented cells in the body. This is characteristic of the later stages of apoptosis and phagocyte resorption, and at that point severed the vascular connection between them (Fig. 4.10). During the next few days, the loser bud increased in size, decreased in pigmentation, and eventually opened its siphons.

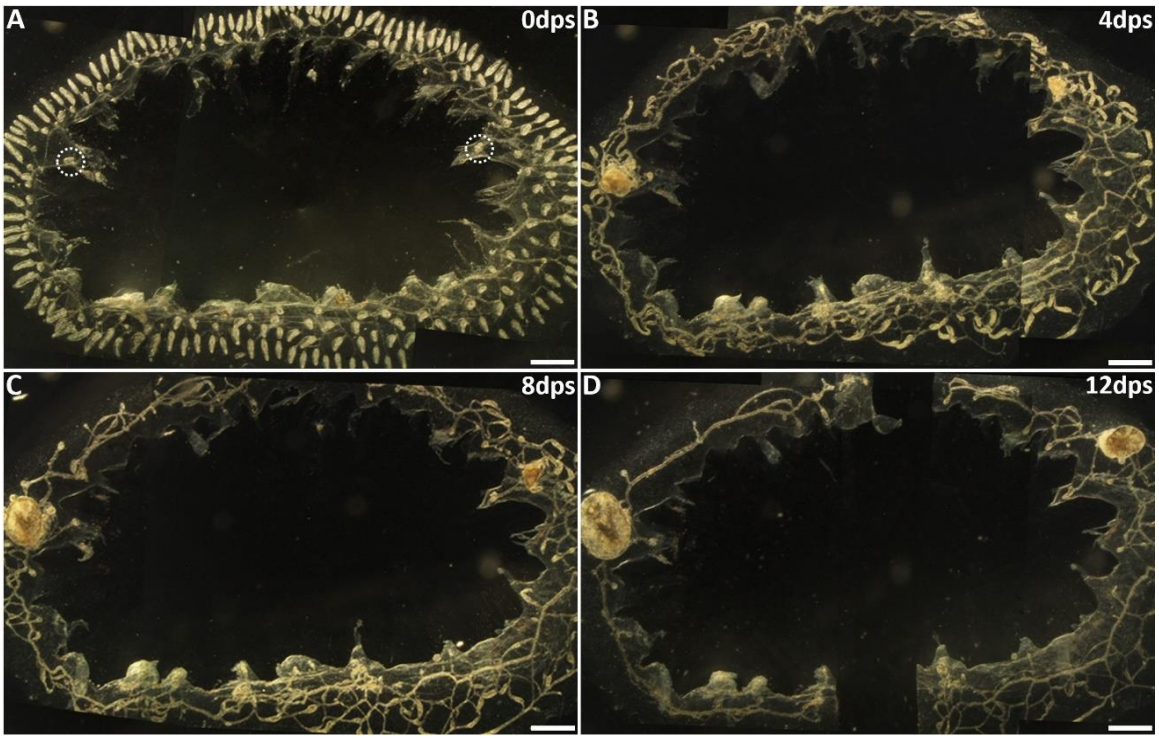


Figure 4.9: Secondary bud resorption is reversed after blood-borne crosstalk severed. A) Post-surgery darkfield image of vasculature with two secondary buds (white circles) connected through the blood vasculature. **B)** At four days post-surgery, both secondary buds had fused to the common vasculature and continued development. **C)** On day eight, the left secondary bud was identified as the purported winner because the right secondary bud had ceased growth. After the image was taken, a section of blood vessels between secondary buds was removed. **D)** By day 12 the loser bud had opened a siphon and began asexually budding. dps = days post-surgery. Scale bars = 1.0mm.

These data indicate that although both buds have the potential to develop, signaling from a winner secondary bud creates a continuously repressive environment for the loser. Importantly, this also shows that a partially resorbed primary bud can reverse its fate and complete development.

In summary, both WBR in *B. diegensis* and bud competition in *B. schlosseri* clearly show that a botryllid colony can shift metabolic resources within an individual to promote

survival. It shows that this process includes repression of one of the buds mediated via the shared circulation. Secondary buds can detect and respond to vascular injury by migrating and reconnecting to the vasculature. They also sense the presence of competition between multiple buds. These observations together suggest that this is an injury response. The colony responds to injury by shifting metabolic resources to increase the chances of survival and ensuring that a single bud reaches maturity. Afterward, the colony can feed and resume normal palleal budding.

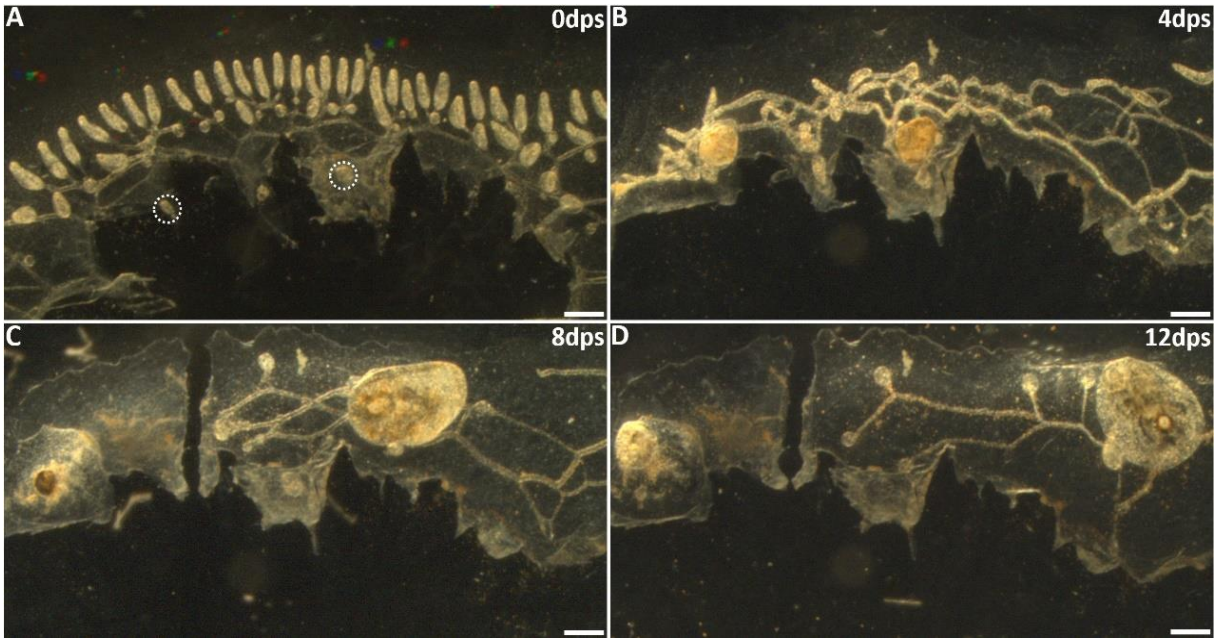


Figure 4.10: Loser bud resorption reversed at late stage of separation from winner bud. A) Post-surgery darkfield image of vasculature and two secondary buds (white circles). **B)** Both secondary buds developed to a proportional size by day four. **C)** Blood vessels between zooids were severed 24 hours prior to imaging. Loser secondary bud showed heavy pigmentation and was greatly reduced in size. **D)** Five days after separation, the loser secondary bud had increased in size and opened a siphon. dps = days post-surgery. Scale bars = 0.5mm.

D. Discussion

We initiated these experiments to understand why the size, circulation, and timing requirements for successful WBR could be so different between *B. schlosseri* and *B. diegensis*. We had initiated experiments designed to identify the cells responsible for WBR in *B. schlosseri* using a rescue assay subsequently used successfully in *B. diegensis* [202], but our results were inconsistent. We backtracked and carried out control experiments, following published protocols[143,144,192,199] but could not repeat previous results. While we did see what appeared to be WBR several times, retrospective analyses of time lapse videos revealed that the source was always a piece of tissue inadvertently left following the ablation surgery which we observed migrating and reconnecting with the vasculature and developing into a functional zooid (Figures and Videos). We followed this up with controlled experiments in which portions of buds were left behind, and they replicated every result that has been previously published for WBR on *B. schlosseri*; from the time to development (6-12 days) to the presence of a phenotypically abnormal zooid in the first generation[143,144,192,199] (Supplemental Table 4.2).

The simplest explanation of these results is that there are genetic or environmental differences between our local *B. schlosseri* population and those used in other studies. *B. schlosseri* is an introduced species to California and there certainly could have been a genetic bottleneck in the founding population or large environmental differences that resulted in the loss of WBR. In addition, as described above, there is plasticity to the use of palleal and vascular budding among the botryllid species. There is also a report of budding plasticity within one species from different populations: *Symplegma brakenhielmi* from

Panama only expand through vascular budding, whereas this same species in Brazil also uses palleal budding[147]. Conversely, there are no clear longitudinal studies or histological evidence of the earliest stages of WBR; blood cell aggregation and blastula formation within the vasculature, that are clearly shown for other species. We also could not find evidence of these stages using pluripotency markers or EdU lineage tracing (not shown), which worked robustly in *B. diegensis*[202]. Moreover, if WBR is a survival strategy that has evolved for colony survival following devastating injury, the requirements for successful zooid development- a large colony, continued circulation, and will only regenerate a functional zooid during a 36h window each week- seem unlikely to be strongly selected for. Yet, it is those requirements that are consistent among different populations that have been studied[143,144,192,199].

Given these observations, it is intriguing that in *B. schlosseri* a secondary bud clearly responds to surgical separation from the circulation by migrating away from its original location and reattaching to the colonial vasculature leading to further development. These observations have been made previously in experiments where only the zooids were ablated[192], and interestingly, the ability to migrate completely correlates with the divergent responses of the vasculature following ablation of the zooids between the two species. In *B. diegensis*, the vasculature remodels and forms into a large mat with very little visible cell movement, within which multiple vascular buds are initiated. In contrast, the vasculature in *B. schlosseri* undergoes a very typical regression then expansion, maintaining the anatomy and robust blood flow via pumping of the ampullae. It is difficult to believe this is a coincidence and suggests that ectopic development of leftover blastogenic tissue is the

response to injury, as it can occur with only a small amount of vasculature. We have culminated our findings into a model that illustrates how *B. schlosseri* is able to survive a devastating injury that exterminates all feeding and nearly all developing bodies in the colony (Fig. 4.11).

WBR also occurs when colonies are exiting dormancy. Previous studies have utilized *B. diegensis* and *B. leachi*, where the entry and exit of dormancy due to seasonal fluctuation in temperature has been thoroughly documented[145,204,206]. In both species, the entry into dormancy involves resorption of all zooids and buds followed by vascular remodeling visually equivalent to that stimulated by surgery induced WBR. Histological sections of the resulting vascular mats during dormancy[201] (*i.e*, weeks to months following the resorption event) reveal the presence of cell aggregates and blastula like structures; the first two steps in WBR that appear to be the dormant state of vascular buds. However, there is one highly significant difference following this early stage of development: multiple zooids will arise when a dormant vascular mat becomes re-activated, while only a single zooid will survive following surgically induced WBR[142]. This has several implications for the present study. First, the entry into dormancy involves resorption of a large amount of biomass, and it may be that the increased concentration of recycled metabolites allows multiple zooids to develop. This suggests that competition seen here and in other WBR studies[163] may be due to liming energetic resources to support development of the zooid. In addition, competition may also occur during exit from dormancy, as there are significantly more dormant buds than those that complete development[145,204]. However, in both *B. schlosseri* (this study) and *B. leachii*[163], the minimum amount of biomass to support

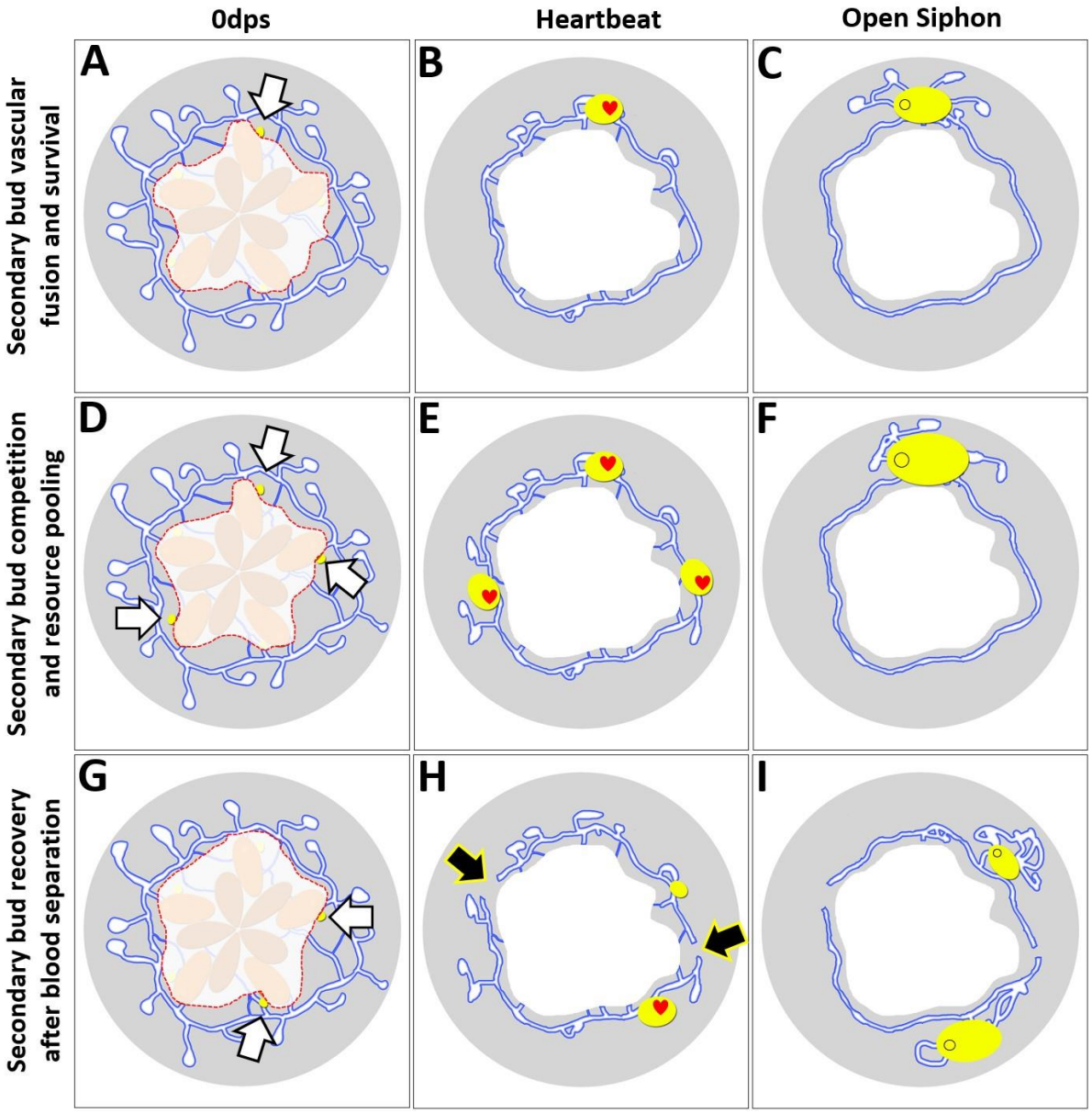


Figure 4.11: Scenarios of post-surgery secondary bud development in *Botryllus schlosseri.* A) Illustration showing surgical isolation of a single secondary bud. **B)** By day six, the vasculature is seen coalescing around the secondary bud, and a beating heart is observed. **C)** The siphon opens on average by day 12 and most of the vasculature collapses except for the area surrounding the newly developed zooid. **D)** Illustration showing surgery performed to isolate three secondary buds. **E)** All secondary buds form heartbeats even if they share the same blood vasculature. **F)** Only one zooid opens a siphon. Other secondary buds are resorbed into the blood vasculature and provide extra growth to the remaining zooid. **G)** Illustration showing surgery performed to isolate two secondary buds. **H)** Secondary buds are separated from blood communication (black arrows) **I)** Two zooids are present because blood-borne competition was inhibited, and prospective loser bud was capable of full development.

zooid development has been defined, yet competition occurs even when the amount of biomass is well above that minimum (Fig. 4.6) (Supplemental Fig. 4.8). In other words, only one zooid completes development even though there is enough vasculature to theoretically support development of multiple individuals. Another observation here was that leaving part of a primary bud with the secondary bud, or two secondary buds, resulted in a larger zooid (Fig. 4.8). In contrast, leaving excess vasculature had no effect on the speed or size of development (Fig. 4.4). Presumably, biomass is recycled via phagocytic cells in the blood through either engulfment of dying buds or cell extrusion during remodeling of the vasculature[18]. Why there would be differences in the recycled tissue, and how these levels are monitored are outstanding questions.

The competition we have observed here is another example of what appears to be colony wide regulation of asexual development that has been described in multiple studies. One of the most interesting aspects of palleal budding in the botryllids is that it is synchronized throughout the colony, which is mediated by factors in the circulation. Moreover, in species where palleal and vascular budding occur simultaneously, the vascular buds eventually synchronize with the palleal buds[145]. The role of the circulation in controlling growth also extends to WBR in *Botrylloides*: the vasculature must be completely isolated, as the presence of a single zooid or bud blocks both vascular remodeling and cell aggregation, suggesting that palleal buds and zooids secrete factors that inhibit WBR.

An analogous process occurs in *B. schlosseri*, where there is an asymmetry to the zooid body plan that is also seen during palleal budding of its daughter buds. When looking at the center of a system from the ventral side, the heart is on the left of the zooid. Every

week each zooid can give rise to 1-4 new zooids, as up to two daughter buds can initiate development on each side of the adult. In lab-reared colonies, there is usually a single primary bud visible on each side of the zooid, but the primary bud developing on the right (heart) side of the zooid is often significantly larger than that on the left. Moreover, in some cases the smaller bud dies and is resorbed with the zooid during takeover, suggesting that if it does not reach a certain size, it does not progress to the next generation. Interestingly, if the faster growing bud is surgically ablated a few days before takeover, the smaller one will rapidly increase its growth rate and survive. Thus, the smaller bud responds to the absence of the larger bud, increases its growth rate and instead of dying, replaces its parental zooid. It is not clear if the two buds are competing for resources, or if the smaller bud is redundant and the zooid is shifting resources when the larger one is ablated. What is fascinating is that this is a proximal event that is autonomous to each zooid and only its own daughter buds, thus whatever mediates this process is either very short lived, or not in the circulation.

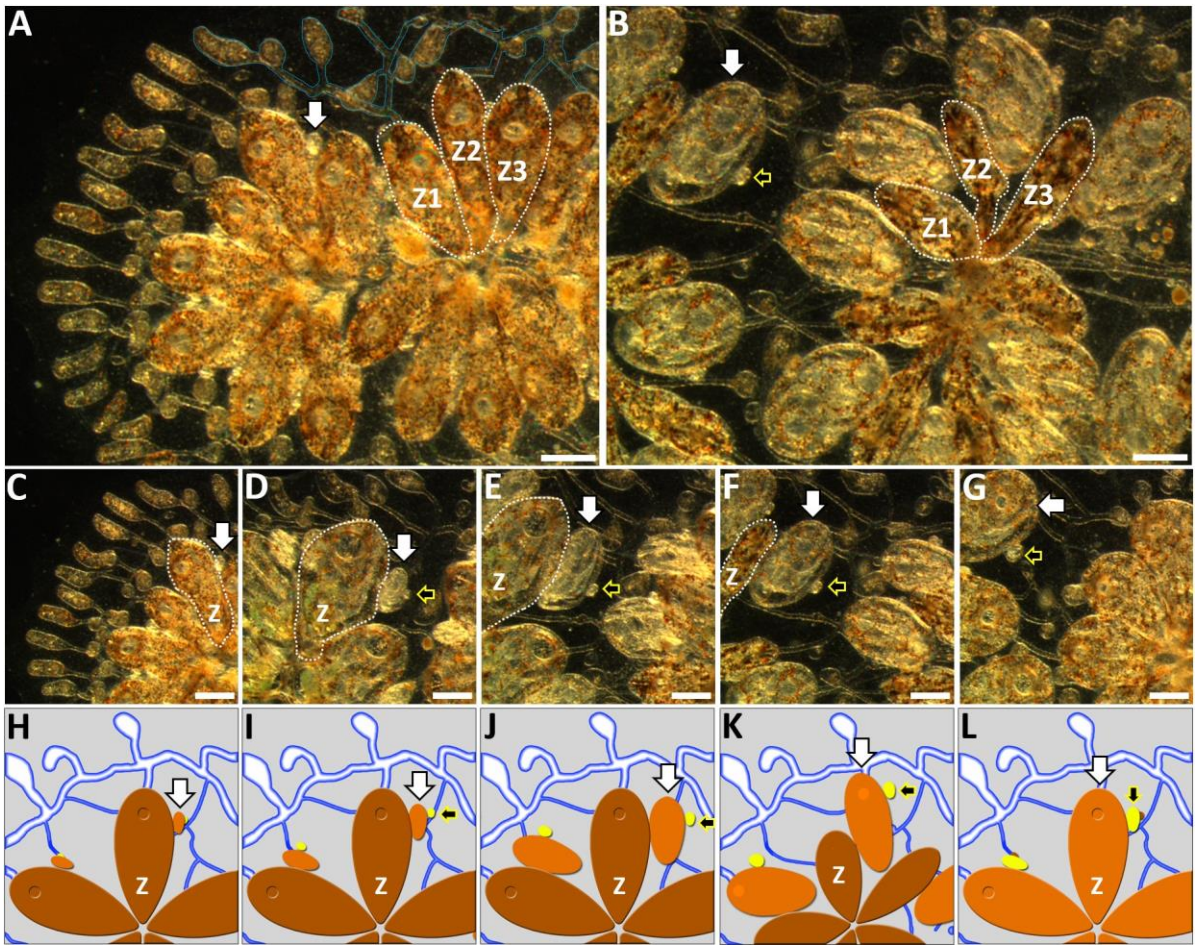
An equivalent monitoring of growth and proximal signaling also occurs between zooids and buds. It has been shown that the zooid has a seven-day zooid lifespan that is completely independent to the presence of the bud, as takeover occurs even if all the primary and secondary buds are surgically removed from a colony[207]. As a result of this programmed zooid life span, if no primary buds complete development, the colony dies[207]. In an elegant set of experiments, it was shown that individual zooids monitor early developmental stages of their daughter primary buds, and if any are ablated or damaged, that zooid will undergo takeover up to 48h early. In contrast, neighboring zooids with

unmanipulated buds have a normal seven-day lifespan. This has been termed crosstalk[207], and together suggests that each zooid monitors growth of its own buds, and will either shift resources between them or die early in order to recycle nutrients to the next asexual generation to buttress colony survival. Similarly, the interaction between remaining secondary buds described here and during WBR in *Botrylloides*[145,205], which in this case occur following a surgical stimulus and are clearly mediated via the circulation (Figs. 4.8-4.10), suggest that competition has evolved to ensure that a healthy zooid develops. How this occurs is not clear, but the ability to experimentally control the number and spatial orientation of secondary buds represents a new model to study the cellular and molecular mechanisms underlying these colonial monitoring and competitive processes.

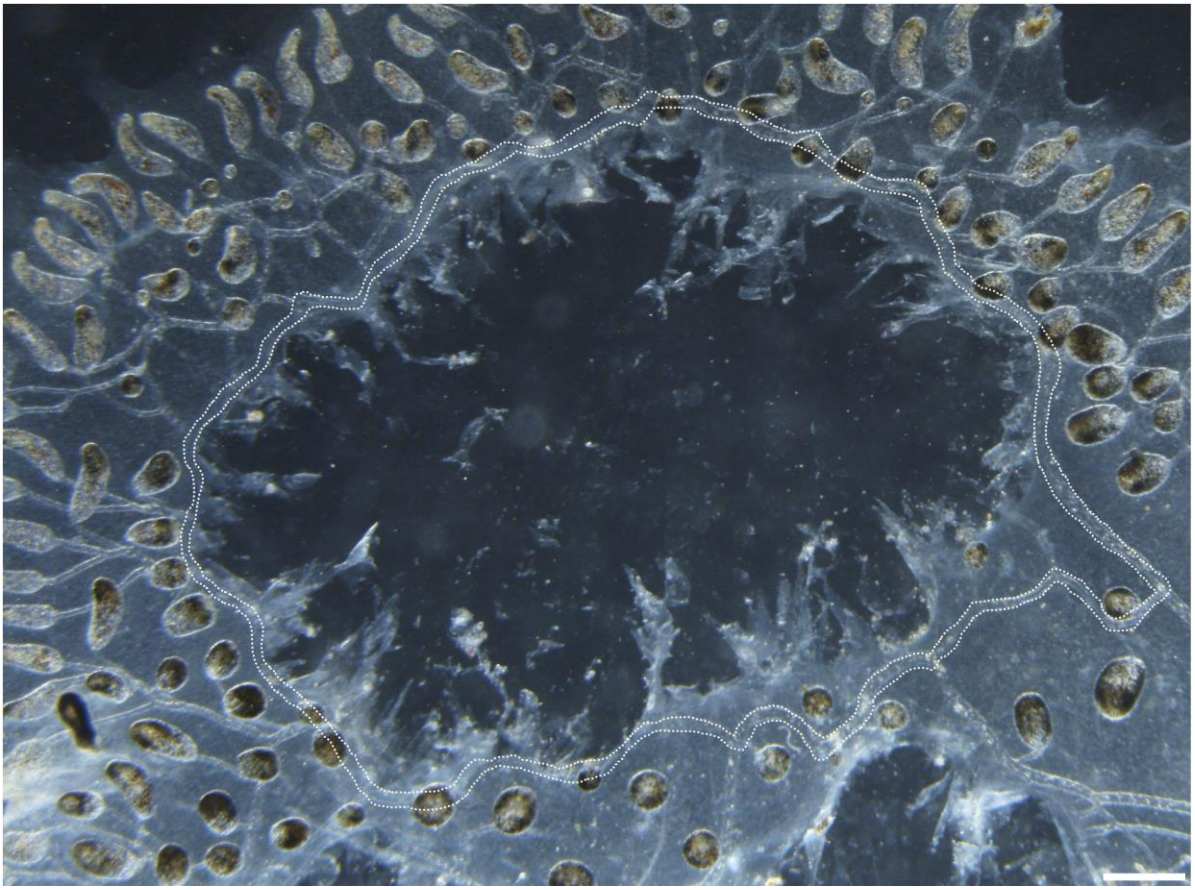
Finally, whether our results are species specific, or due to differences in ecotypes, it should be noted that this is not the first time that there has been a debate regarding the presence of vascular budding in a *Botryllus* species. The observation that palleal buds can migrate from their site of development and re-attach to the colonial vasculature appear elsewhere. In a paper in 1896 on the budding process, Ritter comments that:

"Metchnikoffs (1869) denial that buds are produced by the vessels in Botryllus, Giard (1872) raises the objection that if this were true it would be impossible to explain "la production d'etoiles multiples et distantes dans le cormus d'un Botryllien." The remoteness of the young buds from any older zooids in Goodsiria (now Metandrocarpa dura) has likewise frequently proved a stumbling-block to me in seeing how they could in such cases have been produced in the usual way, i.e. from the wall of the peribranchial sac. But I have given much attention to the point, and am quite convinced that in reality this is their only source. Herdman (1886) expresses the opinion that the ampullae of the vessels will be found to give origin to the buds, but such is apparently not the case here anymore than it is in Botryllus."

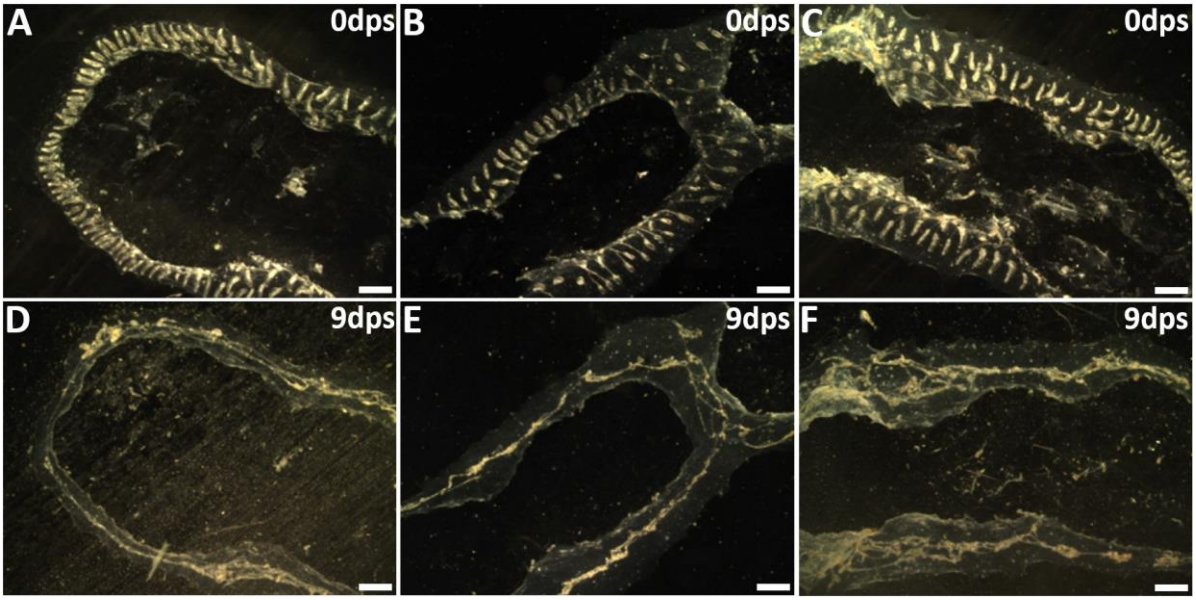
E. Supplemental Figures



Supplemental Figure 4.1: Asexual budding cycle in *Botryllus schlosseri*. A) Darkfield image of 15-zooid colony. The blue dashed lines demarcate an extracorporeal vasculature that allows for shared blood flow amongst the colony. Zooids (white dashed lines) and a developing primary bud (white arrow) grow concurrently. **B)** Colony in panel A after six days. Zooids undergo takeover and are replaced by the primary buds (white arrow). Also shown is a third generation, the secondary bud (black arrow), growing directly from the primary bud epithelium. Panels C through G show intermediate stages. **C)** During stage A1, the zooid's siphon opens, the primary bud is visible (white arrow), and the secondary bud is nascent. **D)** At stage B1, the secondary bud (black arrow) has formed a double vesicle. **E)** Stage C is where organogenesis is occurring in the secondary bud (black arrow). **F)** Stage D is takeover, where zooids are resorbed and replaced by the subsequent generation. **G)** After 7 days, what was initially the primary bud, is now a filter feeding zooid (white arrow) with an open siphon. The secondary bud has developed into the primary bud (black arrow), and the process repeats. **H-L)** Illustrations following blastogenesis in C-G, respectively. Scale bars = 0.5mm.



Supplemental Figure 4.2: Marginal blood vessel demarcation in *Botryllus schlosseri*. This blood vessel interconnects all zooids and developing bodies within in a system of zooids. The marginal vessel is adjacent to the surgical perimeter; therefore, removing all zooids, primary buds, and secondary buds without damaging the marginal vessel is the primary challenge in performing this assay properly. Scale bar = 0.5mm.

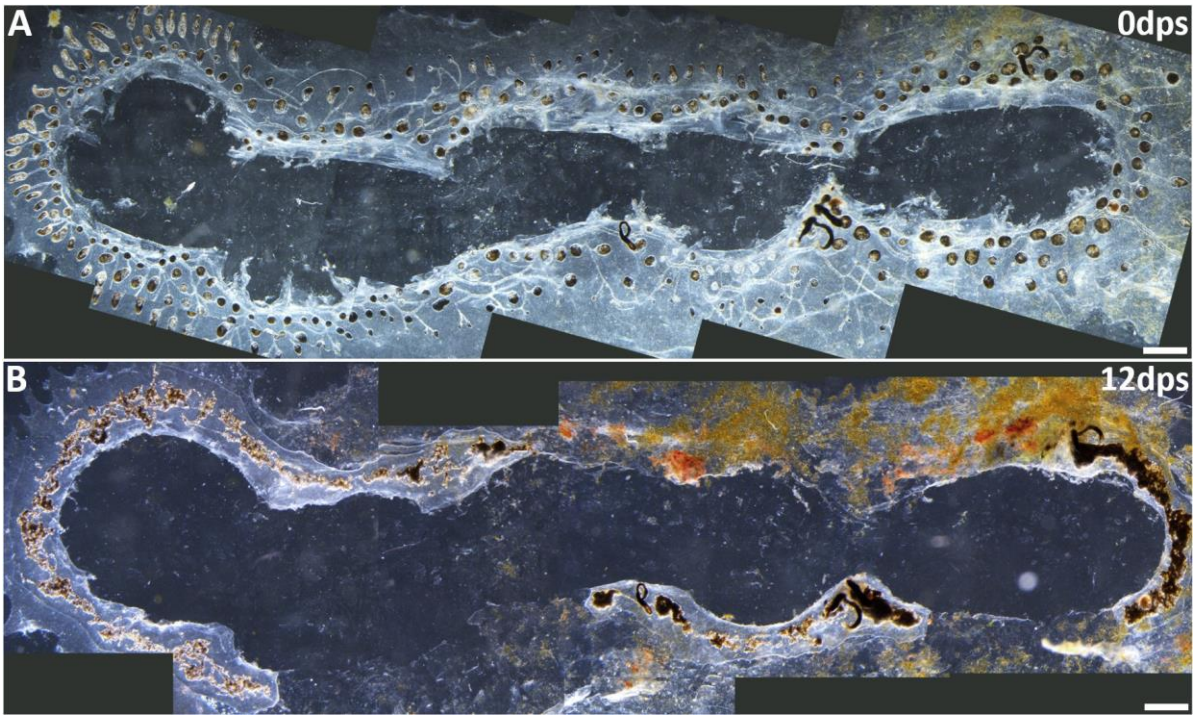


Supplemental Figure 4.3: Experiments to induce whole-body regeneration in *Botryllus schlosseri*. A-C) Darkfield images of post-surgery colonies of *B. schlosseri* at day zero. Zooids and all developing buds were removed. **D-F)** Same systems shown in panels A-C, respectively, at day nine post-surgery. Zero colonies regenerated a zooid (n=126). dps = days post-surgery. Scale bars = 0.5mm.

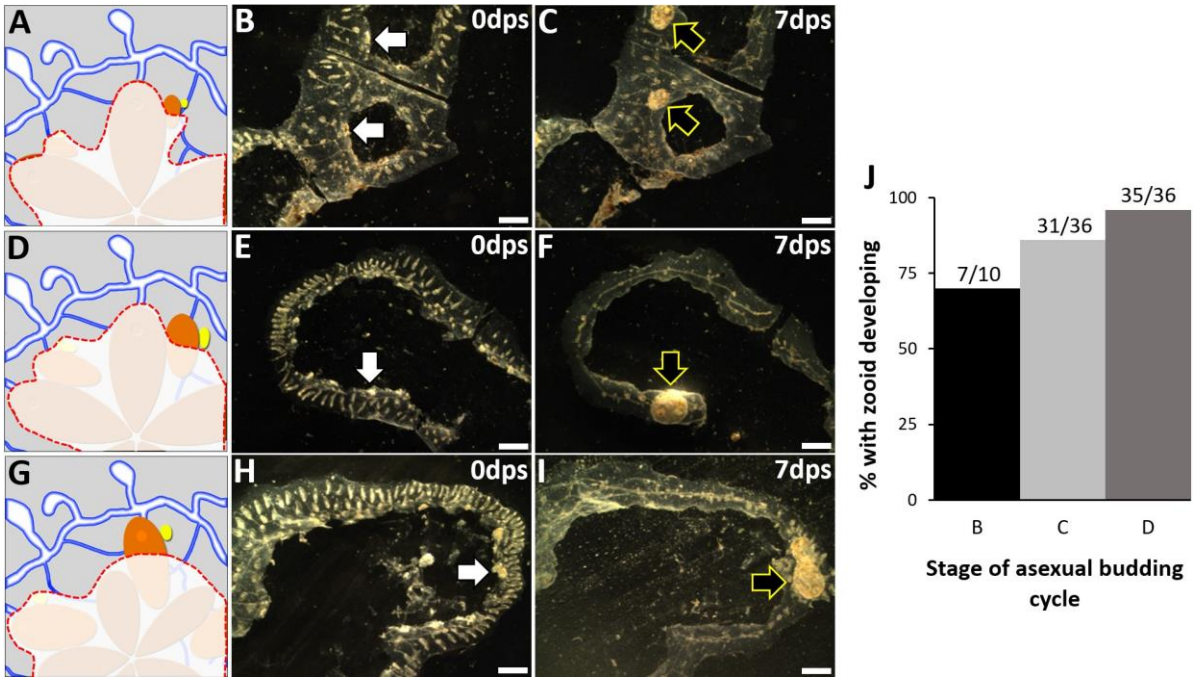
Supplemental Table 4.1: Whole-body regeneration potential between different genotypes.

Genotype	N	Zooid development without secondary bud (%)	Zooid development with secondary bud (%)
1	15	0	50
2	5	0	42
3	5	0	44
4	89	0	60
5	12	0	51

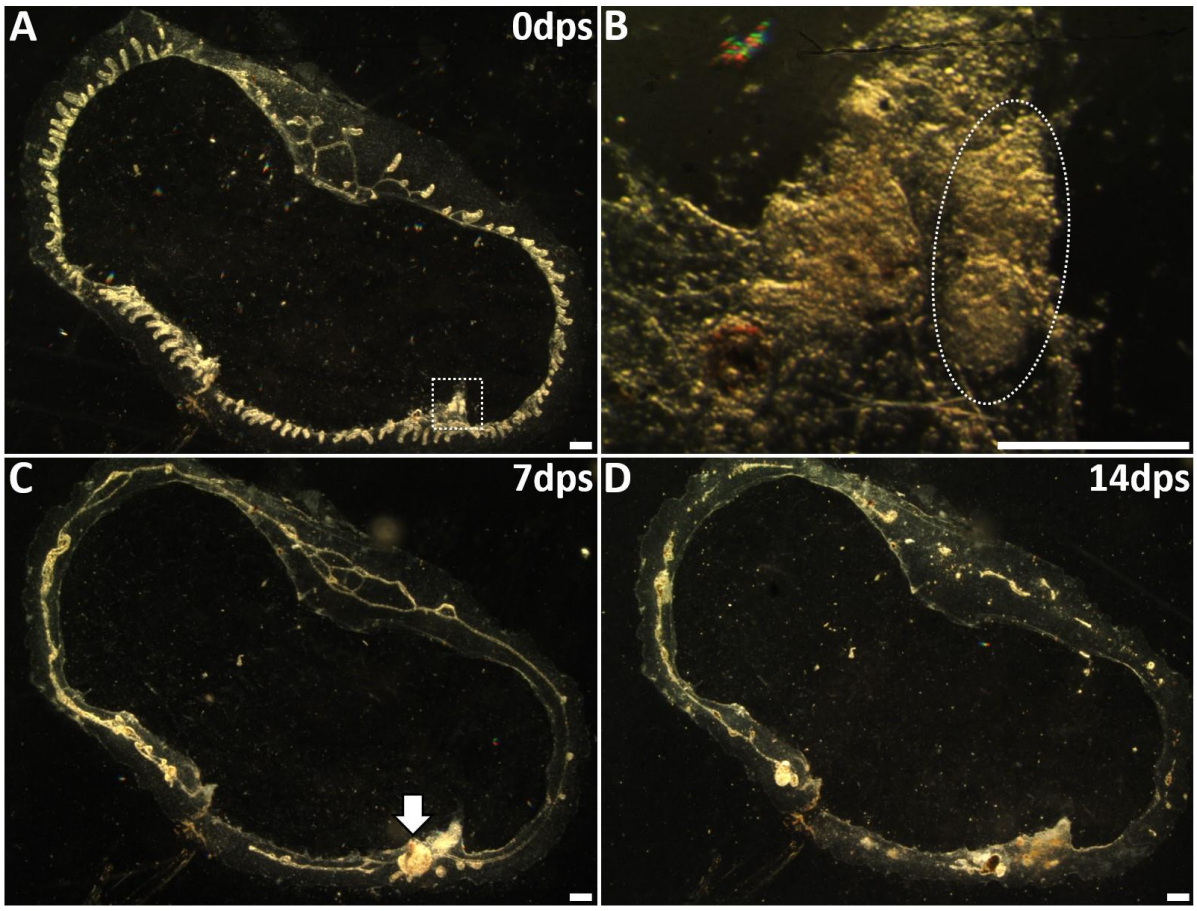
To assess whole-body regeneration (WBR) capability across genotypes, multiple individual colonies strains were examined. We performed from 5 to 89 surgeries on each genotype, but no vasculature gave indication for a WBR event. Animals were collected at the Santa Barbara Marina in California.



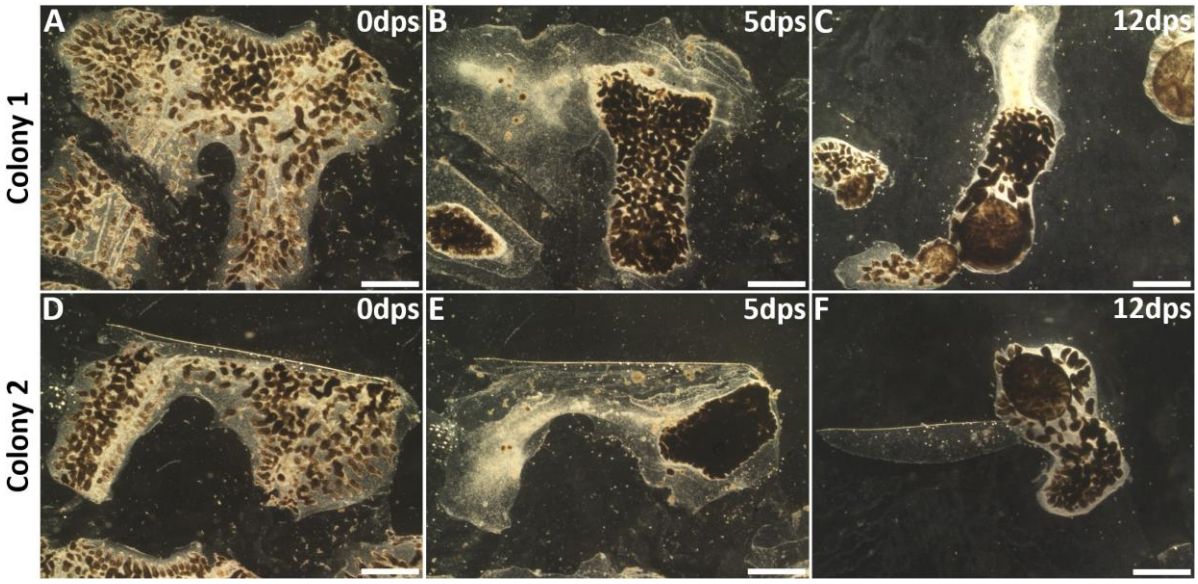
Supplemental Figure 4.4: Large colony surgery to increase chances of inducing whole-body regeneration. A) Post-surgery darkfield image of a five-system colony. There were approximately 250 ampullae all connected by a ring of vasculature with vigorous blood flow. Animal was maintained in filtered seawater and no evidence of a developing bud was detected. By day 12 the blood flow had ceased, hyper-pigmentation was present, and tissue movement had halted. dps = days post-surgery. Scale bar = 0.5mm.



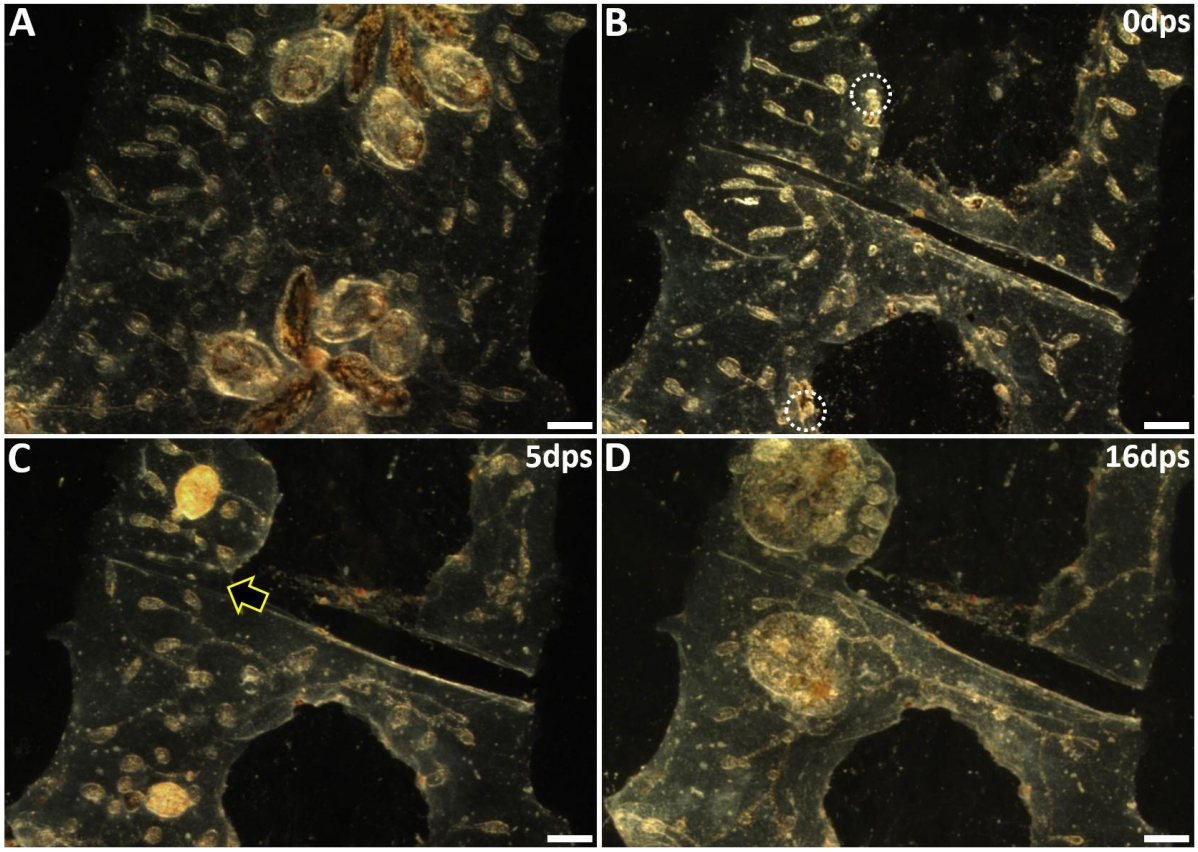
Supplemental Figure 4.5: Post-surgery secondary bud development with anterior primary bud fragments. **A)** Illustration showing surgery performed at stage B1 to isolate anterior half of the primary bud with secondary bud. **B and C)** Darkfield images of post-surgery B1 colony at day zero and seven, respectively. **D)** Illustration showing surgery performed at stage C1 to isolate the anterior half of the primary bud with secondary bud. **E and F)** Darkfield images of post-surgery C1 colony at day zero and seven, respectively. **G)** Illustration showing surgery performed at stage D (takeover) to isolate the anterior half of the primary bud with secondary bud. **H and I)** Darkfield images of post-surgery D1 colony at day zero and seven, respectively. **J)** Quantitative analysis of post-surgery zooid development with bud tissues left behind at stages B-D of the asexual life cycle. Zooids developed in 70%, 86%, and 97% of surgeries when performed in stages B, C, and D, respectively. dps = days post-surgery. Scale bars = 1mm.



Supplemental Figure 4.6: Assessing the potential for whole-body regeneration from damaged secondary bud. A) Post-surgery darkfield image of colony of *B. schlosseri* with a single secondary bud left behind that has been fragmented through the application of external pressure via forceps. **B)** Magnified view of fragmented secondary bud from panel A. **C)** Secondary bud developed a heartbeat at day seven. **D)** Secondary bud resorbs by day 14, blood flow has ceased, and the vasculature has collapsed. dps = days post-surgery. Scale bars = 0.5mm.



Supplemental Figure 4.7: WBR from large vascular beds in *Botrylloides diegensis.* A-C) A single zooid regenerating from a relatively large patch of vasculature. **D-F)** Solitary zooid regeneration from two patches of ampullae connected by a single blood vessel. dps = days post-surgery. Scale bars = 2mm



Supplemental Figure 4.9: Post-surgery secondary buds develop independently while sharing tunic but not blood in *Botryllus schlosseri*. A) Darkfield image of two systems within the same colony. **B**) One secondary bud was isolated from each system (white circles). **C)** By day five both buds had developed a beating heart. These buds were connected via the tunic; however, the blood vasculature had been severed (black arrow). **D)** Both buds developed into filter-feeding adults. dps = days post-surgery. Scale bars = 0.5mm.

Supplemental Table 4.2: Summary of how secondary bud isolation events explain WBR observations.

WBR observation	**Observation explained by isolated secondary bud experiments**
Stage dependency	Proximity to vasculature
Vascular requirement	Proximity to vasculature
Inconsistent timing	Whether partial primary bud included
Abnormal first generation	Physical perturbations
Appearance from vasculature	Migration within 24 hours to fuse with vasculature
Large colony size	Increased opportunity for tissues to evade surgery

The characteristics and requirements for WBR in *Botryllus schlosseri* match six observations when only a single secondary bud is isolated with vascular tissues after removal of all zooids and other buds.

V. Concluding remarks and future directions

A. Whole-body regeneration in Botryllus schlosseri

Through our work we have resolved a long-standing question regarding the tissues required for colony regeneration in the marine invertebrate chordate, *Botryllus schlosseri*. This ascidian has been suggested to undergo whole body regeneration (WBR) after an injury that causes death to all adults (zooids) and developing bodies (primary and secondary buds) in a colony. The surgical assay used to induce regeneration in this species requires carefully dissecting out all three simultaneously existing generations within a colony, which leaves behind a bed of extracorporeal vasculature that is protected by a translucent protective tunic covering. Previous reports indicate that a zooid will develop directly from within the isolated vasculature in *B. schlosseri*[143], and that piwi+ blood cells associated within the inner vessel walls detach and contribute to stem cell based WBR[150]. We sought to identify early niche formation through time-lapse brightfield microscopy to stage this process since it had not been previously undertaken.

Our findings contradict the current literature on *B. schlosseri* and show that the vasculature, tunic, and cells that reside within these tissues are incapable of WBR. We show that secondary buds accidentally left after surgery survive and eventually restore the entire colony by migrating to the vasculature and continuing development. Since these buds already contain the cells necessary for development, it appears that what was thought to be WBR, is ectopic development of a bud derived from the natural homeostatic asexual budding process of this animal. The reason these buds eluded researchers in the

past is because after surgery they become indistinguishable from the vasculature within 24h, after which they seemingly develop from within the vasculature.

We were able to show that secondary bud survival after surgery follows the trends discussed in previous WBR reports regarding developmental timeline and competition for survival when multiple buds are left. This study followed up on recent articles published in *Developmental Biology* regarding this topic[144,150] and presents an important insight into the burgeoning field of WBR studies in colonial ascidians. Knowledge of which animals possess this ability offers a deeper evolutionary understanding for when WBR emerged.

B. Developmental competition in Botryllus schlosseri

Our focus on analyzing the vasculature after removing all zooids and most buds led to the discovery that isolated secondary buds compete for survival. During homeostatic blastogenesis, zooids and buds can be resorbed at the will of the colony at any time during the life cycle. The decision to resorb a body is likely caused by shifts in environmental factors and metabolic needs but how each animal regulates this seemingly independent phenomenon within a colony is not well understood. Because there are three generations concurrently growing in a mature colony, it is challenging to elucidate the genetic factors and tissues involved in regulating synchrony and developmental regulation due to low signal to noise ratio caused by other metabolic processes.

Our assay on isolated secondary buds opens the opportunity to study these interactions using a reductionist approach. When two secondary buds are left behind, sometimes they both survive, and other times they compete for survival. We now know

that the communication between them travels through the bloodstream because severing the shared vascular connection blocks competitive effects on one-another. What is intriguing is that the outcompeted bud can reverse course when inhibiting signals from the winner are prevented. One simple explanation is that injured secondary buds go out of sync and trigger programmed competition when assessing each other's developmental progress within a shared vasculature. After surgery, the secondary buds must migrate to the vasculature for reconnection, which may cause timing discrepancies between multiple buds. We observe that secondary bud development does not resume until blood connection is restored, so it may be that a further-along developmental stage is what dictates a winner in this context. Our studies show that buds are not overtly competing until after heart development, so the tissue or cells that drive this process may not immediately take effect when two nascent buds begin sharing blood after surgical isolation. Another explanation is that evolutionarily, pooling resources into a single bud gives a higher survival rate and stronger budding capacity. This seems paradoxical because our studies show a small vascular requirement for survival. Two buds developing to filter feeding zooids would theoretically be advantageous to colony survival because of the increased nutritional uptake through algae consumption. The mechanisms that determine winner outcomes are future experiments.

C. Implications of this dissertation

This study has raised questions as to which other species truly can undergo WBR. Secondary bud migration, whether homeostatic or injury induced, appears to have caused

confusion in the field for centuries because of the small size of buds and their ability to migrate and merge with the vasculature at locations distant from their parent bud. Studies regarding WBR done in *Symplegma brakenhielmi* and *Botrylloides violaceus* may need to be revisited for these reasons. We have ended a centuries long argument about this process in *B. schlosseri* that was reiterated in a review[208]. Manni states that…

"Vascular budding (termed whole-body regeneration when induced experimentally) was first described in botryllid ascidians more than two hundred years ago [by Savigny in 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)."

Our research on *Botryllus schlosseri* regarding the lack of WBR shifted our attention to a sister genus, *Botrylloides*. While regeneration in *Botrylloides leachii* has been studied since 1995[142], our lab was able to bring *Botrylloides diegensis* into the spotlight because we showed that FACS isolated integrin-alpha-6+ cells are necessary for WBR[202].

D. Future directions

Botryllus schlosseri now allows for studies in developmental competition. Future experiments should investigate how long it requires for each secondary bud to migrate to vasculature prior to competition, then determine if the winner is simply chosen by developmental progress. We did not follow up on colonies to determine budding capacity in subsequent generations; therefore, this should also be assessed to learn if pooling resources via competition increases longevity and blastogenic potential. Competition clearly leads to a larger resulting zooid; therefore, this gives following generations more building blocks and likely augments long-term survivability for colony expansion.

B. diegensis is of interest now because of the ability to isolate stem cells and inject them to rescue WBR. Current technologies allow subsequent experiments in the field to assess various regeneration related activators, repressors, histone modifications, epigenetic markers, miRNA, and transcription factors. How these cells respond to injury, home to the regeneration niche, and organize through cell-to-cell communication will aid in our understanding of pluripotent activity and healing responses. When transgenics and CRISPR biotechnologies become proven techniques in *Botrylloides*, this model organism will become vital to advancing our knowledge for stem-cell based regenerative medicine.

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