Invertebrate Reproduction & Development, 2015
Vol. 59, No. S1, 33–38, http://dx.doi.org/10.1080/07924259.2014.944673

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Botryllus schlosseri, an emerging model for the study of aging, stem cells, and mechanisms of regeneration

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(*Received 15 March 2014; accepted 23 June 2014*)

The decline of tissue regenerative potential with the loss of stem cell function is a hallmark of mammalian aging. We study *Botryllus schlosseri,* a colonial chordate which exhibits robust stem cell-mediated regeneration capacities throughout life. Larvae, derived by sexual reproduction and chordate development, metamorphose to clonal founders that undergo weekly formation of new individuals by budding from stem cells. Individuals are transient structures which die through massive apoptosis, and successive buds mature to replicate an entire new body. As a result, their stem cells, which are the only self-renewing cells in a tissue, are the only cells which remain through the entire life of the genotype and retain the effects of time. During aging, a significant decrease in the colonies' regenerative potential is observed and both sexual and asexual reproductions will eventually halt. When a parent colony is experimentally separated into a number of clonal replicates, they frequently undergo senescence simultaneously, suggesting a heritable factor that determines lifespan in these colonies. The availability of the recently published *B. schlosseri* genome coupled with its unique life cycle features promotes the use of this model organism for the study of the evolution of aging, stem cells, and mechanisms of regeneration.

Keywords: stem cells; urochordate; aging; *Botryllus schlosseri*; regeneration; asexual development

Introduction

The aging process can be defined as a general loss in biological competence for both the individual cell and the organism as a whole. At the cellular level, it is expressed as decreasing replicative ability in proliferating cells and decreasing functional activity in post-mitotic cells (Geiger & Van Zant 2002; Van Zant & Liang 2003). Adult stem cells divide to produce new progeny that undergo programs of differentiation and maturation required to replace old or damaged tissues (Weissman 2000). The decline in tissue regeneration capacity has been attributed to a diminished responsiveness of tissue-specific stem and progenitor cells (Rossi et al. 2008; Conboy & Rando 2012; Signer & Morrison 2013). Adult stem cells are subjected to the effects of aging, which limit their ability to self-renew, differentiate, and maintain tissue homeostasis leading to loss of function (Conboy & Rando 2012; Signer & Morrison 2013). Short telomeres, oxidative stress, DNA damage, impaired metabolism, and deregulation of gene expression are all 'hallmarks' of aging that account for the impaired stem/progenitor cell function (Rossi et al. 2008; Beerman et al. 2014; Sousounis et al. 2014). Studying invertebrate models which are closely related to vertebrates support robust regeneration activities, and exhibit extensive stem cell-mediated regeneration capacities throughout adult life can provide fundamental insights into the process of aging and regeneration (Austad 2009). The potential of studying colonial organisms for aging studies was further emphasized by Skold and Obst (2011). The colonial ascidian *Botryllus schlosseri* belongs to the chordate subphylum Tunicata or Urochordata, a taxonomic group considered to be the closest living invertebrate relative of vertebrates (Figure 1(e), Delsuc et al. 2006; Voskoboynik, Neff, et al. 2013). It is an organism with several characteristics that render it a valuable model for the study of aging. This includes: (i) homeostasis, involving weekly, *de novo* robust tissue regeneration with adult stem cells mediating formation of all body organs including the heart, nervous system, respiration system, digestive system, endostyle, ovary and testis, throughout their life (ii) identified adult stem cells (Laird et al. 2005) and their niches (Voskoboynik et al. 2008; Rinkevich et al. 2013), (iii) colony life cycle including both sexual and asexual reproductions (Review in Manni & Burighel 2006; Manni et al. 2014), (iv) clonal replicates which demonstrate simultaneous senescence (Rinkevich et al. 1992; Lauzon et al. 2000), and can be altered under various conditions and treatments (Boyd et al. 1986; Voskoboynik et al. 2002, 2004), (v) a colonial life history which allows separation of one genotype (colony) into several clonal replicates and

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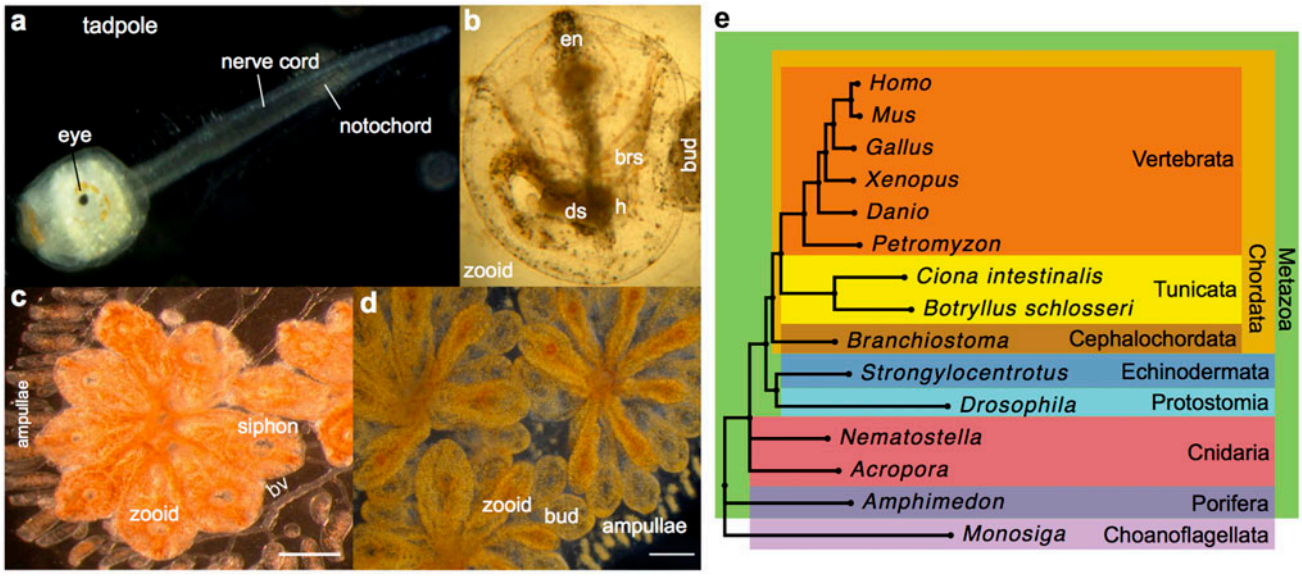

Figure 1. *B. schlosseri* anatomy, life cycle, and phylogeny. *B. schlosseri* reproduces both through sexual and asexual (budding) pathways, giving rise to virtually identical adult body plans. Upon settlement, the tadpole phase of the *B. schlosseri* lifecycle (a) will metamorphose into a founder individual (oozooid) (b), which through asexual budding, generates a colony. The colony includes three overlapping generations: an adult zooid, a primary bud, and a secondary bud, all of which are connected via a vascular network (bv) embedded within a gelatinous matrix (termed tunic). The common vasculature terminates in finger-like protrusions (ampullae; b–d). (c). Through budding, *B. schlosseri* generates its entire body, including digestive (ds) and respiratory (brs) systems, a simple tube-like heart (h), an endostyle (en) that harbors a stem cell niche, a primitive neural complex, and siphons used for feeding, waste, and releasing larvae (b–d). Each week, successive buds grow (d) and complete replication of all zooids in the colony, replacing the previous generation's zooids, which die through a massive apoptosis. (e) A phylogenomic tree produced from the analysis of 521 nuclear genes (40,798 aligned amino acids) from 15 species, including *B. schlosseri*. Scale bar-1 mm.
Source: DOI: 10.7554/eLife.00569.003.

a serial collection of whole body samples along their entire lifespan, offering a unique approach to characterize genetic changes during aging. Here, we review studies conducted over six decades that examined the *B. schlosseri* life cycle, with special emphasis on research that led to the discovery of the nature of the cells in colonial ascidians that mediate budding and highlight its potential for stem cell and aging research. The life histories of *B. schlosseri* and the availability of its genome (Voskoboynik, Neff, et al. 2013) further promote *B. schlosseri* as a model organism for the study of aging and the evolution of stem cell-mediated regeneration.

B. schlosseri *alternative reproduction pathways*

The life cycle of *B. schlosseri* includes both sexual and asexual reproduction pathways (Review in Manni & Burighel 2006; Manni et al. 2014). Despite differences in initiation, these two reproduction modes give rise to the same adult body plan (Figure 1). Sexual reproduction starts with fertilization and progresses through classic embryonic stages into a tadpole larva featuring chordate characteristics such as tail, notochord, neural tube, and striated musculature (Figure 1(a)). Upon hatching, the motile tadpole settles on a substrate and metamorphoses into an oozooid, with a sessile body plan, losing most of its morphological chordate characteristics (Figure 1(b)). The oozooid begins a cyclical budding process of asexual reproduction, forming a colony of genetically identical zooids and buds. The colony grows when more than one bud replaces the parent zooid. All zooids and buds are connected through a vasculature network and are embedded within a gelatinous tunic (Figure 1). A mature colony includes three overlapping generations: an adult zooid, a primary bud, and a secondary bud. Each zooid is an autonomous, filter-feeding individual comprising a heart, digestive tract, endostyle, branchial sac, neural complex, oral, and atrial siphons. Sexual reproduction commences upon the formation of ovaries and testis (Burighel & Cloney 1997; Figure 1). Asexual (palleal) budding begins with the formation of a small vesicle that breaks off from the parent epidermis and peribranchial epithelium and segregates into a blastula-like structure. As the cell proliferates, the vesicle undergoes a series of invaginations, differentiates, and is developed into an adult zooid (Manni et al. 2014).

The replacement of the zooids' generation occurs through a synchronized wave of massive apoptosis, in which the adult zooids are destroyed and taken over by their primary buds (Lauzon et al. 1993; Cima et al. 2010;

Figure 1(d)). Palleal budding is divided into several major developmental stages and is synchronized in continuous cycles throughout the colony (Berrill 1951; Sabbadin 1955), a cycle that continues throughout the entire life of the colony. Unlike most species, where the body is long lived and maintained by cellular replacements, *B. schlosseri* regenerates new colonial units on a weekly basis — its stem cells remain for life.

Under certain conditions, *B. schlosseri* can regenerate its entire body from the vasculature alone (vascular budding; Sabbadin et al. 1975; Voskoboynik et al. 2007). When all the developing buds and zooids are removed from a colony, regions of the vasculature become niches for budding. The entire body can be regenerated even if no pre-existing zooids are present. This suggests that pluripotent and/or multipotent stem and progenitor cells that initiate budding can migrate and that regions within the colony vasculature are suitable niches (Voskoboynik et al. 2007).

Stem cell-mediated chimerism and budding

In addition to their high regeneration activity, *B. schlosseri* stem cells can be mobilized and transplanted between genetically compatible individuals, inducing natural chimerism. Once transplanted, donor pluripotent stem cells proliferate, differentiate, home, and replace germline and or somatic host tissues (termed cell parasitism; Sabbadin & Zaniolo 1979; Pancer et al. 1995; Stoner & Weissman 1996; Stoner et al. 1999; Laird et al. 2005; Voskoboynik et al. 2008; Rinkevich et al. 2013). The ability of colonies to undergo a natural transplantation reaction and exchange stem cells is determined by a single polymorphic histocompatibility locus (Oka & Watanabe 1957; Sabbadin 1962; Scofield et al. 1982; Voskoboynik, Newman, et al. 2013). Colonies that share at least one allele will anastomose vessels upon contact (fusion), creating a chimera or natural parabiont. Pairs that do not share alleles develop an inflammatory immune response (rejection) between the colonies (Oka & Watanabe 1957; Sabbadin 1962; Scofield et al. 1982; Voskoboynik, Newman, et al. 2013).

The *Botryllus* histocompatibility gene (BHF) was isolated, encoding a 252-amino acid protein that is highly charged, partially unstructured, and has no recognizable domains or motifs (Voskoboynik, Newman, et al. 2013). Allelic variation of this polymorphic gene predicts fusion/rejection outcomes, where one amino acid difference can lead to rejection. Thus, molecular discrimination of sequence variation within this gene provides a barrier to chimerism and transfer of stem cells between rejecting colonies (Voskoboynik, Newman, et al. 2013). BHF recognition represents an ancestral recognition system regulating blood-based chimerism; future investigation of this gene will likely reveal new mechanisms of recognition.

The long-term ability of cells from one genotype to replace the germline or soma of the host led to the hypothesis that chimerism, cell parasitism, and budding are mediated by stem cells (Stoner & Weissman 1996; Stoner et al. 1999). When somatic cell parasitism in *B. schlosseri* induces the development of a foreign entity bud within the host colony, chimerism serves as a platform to test whether asexual budding is mediated by stem cells. In a set of serial engraftment assays, Laird et al. (2005) demonstrated that adult stem cells are responsible for a stable long-term chimerism and budding in *B. schlosseri*, by transplanting a single cell which expressed high enzymatic activity of aldehyde dehydrogenase. Using *in vivo* cell labeling, cell engraftment, and time lapse imaging, we (Voskoboynik et al. 2008) further demonstrated that the anterior ventral region of the subendostyle sinus (termed endostyle niche) harbors and exports somatic stem cells in *B. schlosseri* colonies. As few as 5–20 engrafted cells transplanted from the donor endostyle niche sufficed to generate somatic chimerism in compatible hosts. However, no germline chimerism was observed in this transplantation assay (Voskoboynik et al. 2008). Germline chimerism was detected following transplantation of cells from a cluster of cells flanking the endostyle, termed ventral cell islands (Rinkevich et al. 2013).

Induction of chimerism demonstrates the remarkable stemness capacity of the cells in these tissues (Voskoboynik et al. 2008; Rinkevich et al. 2013). In adult mammalian tissues, stem cells are stably maintained in their niche throughout most, if not all, of an organism's life. (Zhou et al. 2013). The niche achieves long-term maintenance of stem cells and controls their self-renewal by orchestrating multiple signaling inputs that prevent differentiation (Morrison & Spradling 2008). Although somatic tissues undergo weekly waves of apoptosis and phagocytosis, *B. schlosseri* colonies undergo regeneration and live for years. Repeated trafficking of stem cells into new niches prevents their destruction by degenerating tissues and enables self-preservation throughout life.

Programmed life span in B. schlosseri *colonies*

The rate of aging in *B. schlosseri* is plastic and amenable to change. Colonies grown in the field are characterized by short, sub-annual life spans (Grosberg 1988; Chadwick-Furman & Weissman 1995a, 1995b). For instance, life span in colonies raised in Monterey Bay varied significantly in response to seasonal variation (temperature, light, and nutrients), from about 3 months for spring-born colonies to 8 months for fall-born colonies (Chadwick-Furman & Weissman 1995a, 1995b). Laboratory-maintained colonies exhibit three broad groups of lifespan, either short (<0.5 year), medium (0.5–2 years), or long (2–8+ years; Sabbadin 1969; Boyd et al. 1986; Rinkevich et al. 1992;

Lauzon et al. 2000). However, when a specific parent colony (genet) is experimentally separated into a number of clonal replicates (subclones), subclones frequently undergo senescence simultaneously (Figure 2; Rinkevich et al. 1992; Lauzon et al. 2000). We have found that in independent genotypes, senescence could manifest itself in either random or synchronized fashion (Figure 2; Rinkevich et al. 1992; Lauzon et al. 2000). Simultaneous senescence suggests that a heritable factor determines lifespan in *B. schlosseri* colonies.

Lifespan and regeneration capacities in B. schlosseri

B. schlosseri colonies grow exponentially as juveniles, reaching a maximum size towards the last third period of its life. Toward the end of its life span, asexual reproduction begins to slow down and the number of individuals in the colony is reduced (Figure 2(c)). This suggests that an individual dies when it is no longer able to replace tissues. Bancroft (1903) reported that field colonies of *B. schlosseri* exhibited a series of regressive changes prior to death including decreased budding potential and shrinkage of individual zooids. Our studies on wild and laboratory-raised colonies confirmed his observations and further described the unique morphological changes that occur systemically during colonial senescence (Rinkevich et al. 1992; Chadwick-Furman & Weissman 1995a, 1995b; Lauzon et al. 2000). While nonrandom senescence occurs quickly, completed in 7 days from the onset of morphological changes, random senescence exhibits a gradual deterioration over a prolonged period of weeks and months (Lauzon et al. 2000).

Long-lived *B. schlosseri* colonies exhibit significantly higher regeneration capacities compared with short-lived colonies (Voskoboynik and Ishizuka unpublished data). Exposure to physiological stimuli such as the antioxidant Butylated Hydroxytoluene (BHT) affects both regeneration capacities and longevity (Voskoboynik et al. 2002, 2004). We demonstrated enhanced growth rates and dramatically increased the life span in *B. schlosseri* colonies in response to a single, short-term treatment with the antioxidant BHT (Voskoboynik et al. 2002). The BHT treatment also affected the structure of the endostyle and the composition of cells in its sinuses (Voskoboynik et al. 2004). These studies suggest that animals able to sustain regeneration activities throughout adult life, and maintain the ability to support robust regeneration activities, experience increased longevity.

As an organism ages, its phenotype undergoes modification in its functionality and morphology. Due to the complexity of each organism and the vast amount of aging-related phenomena that occur, it is very difficult to

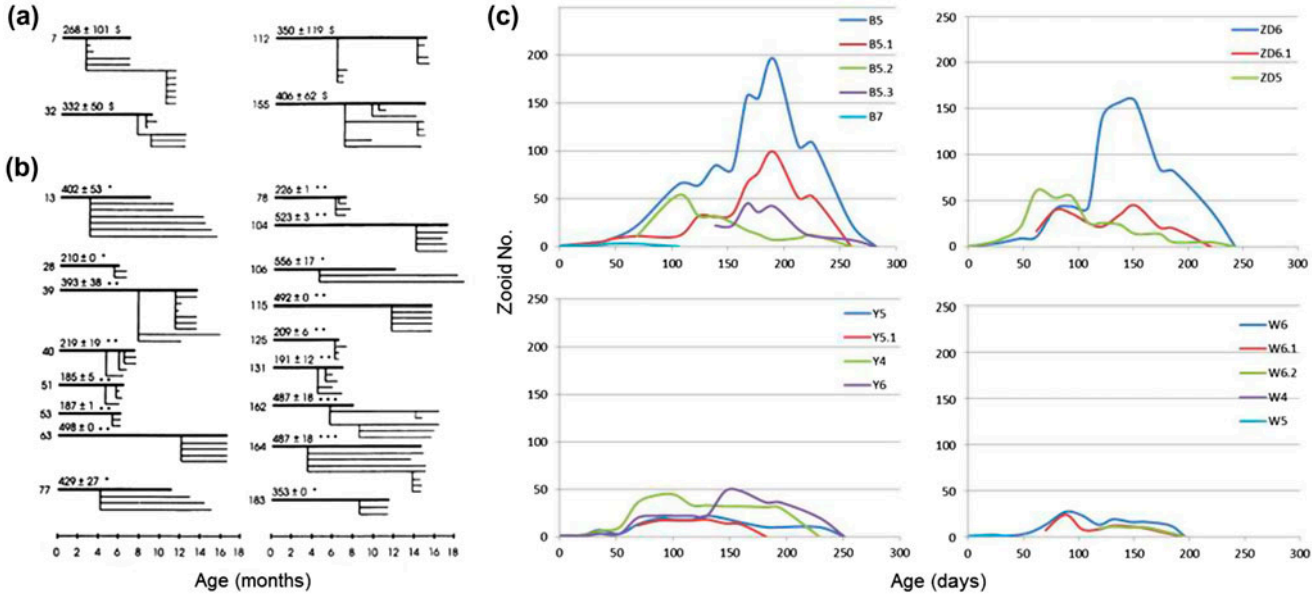

Figure 2. Longevity of ramets subcloned from four representative colonies of *B. schlosseri* undergoing random mortality (a) and the 17 genets expressing synchronized mortality (b). The colony numbers are marked on the left margins of the lines. The original part of each colony is marked by a thick horizontal line; a subclone subcloned from the original part, by a thin line. When more than one subclone was subcloned on a given time, their longevities are marked by parallel horizontal lines. The right-hand end of the horizontal line represents the age at death of each subclone derived from a specific genet. Numbers above each colony represent the average +−SD of all subclones belonging to a specific genet. Levels of significance between the absolute deviation in mortalities of subclones from each genet, compared with the sample mean of the 41 parents genets, are $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $, not significant (taken from Rinkevich et al. 1992). (c) Growth chart, as measured by zooid number of subclones from four different genotypes studied.

distinguish between phenotypes that cause aging and those which are the effect of it. The decline of tissue regenerative potential is a hallmark of mammalian aging. In colonial organisms, like *B. schlosseri*, individuals are transient structures; every week, some individuals die through massive apoptosis, while others are 'born' as buds of stem cells that replicate an entire new body. As a result, their stem cells, the only self-renewing cells in a tissue, remain throughout the entire life of the genotype and are the only cells that preserve the effects of time. Therefore, aging of the colony is, by definition, aging of the stem cells; most other cells are regenerated from these on a weekly basis. In this model organism, we can clearly define that stem cell aging is the root cause of senescence of the entire colony. Utilizing *B. schlosseri* as an invertebrate model organism, which is closely related to vertebrates and exhibits robust stem cell-mediated regeneration capacities throughout life, will provide an opportunity to study the correlation between robust regeneration and aging and to discover additional genes and key pathways that might be relevant to human aging.

Acknowledgments

We thank K.J. Palmeri and K.J. Ishizuka for thoughtful edits. The *Botryllus schlosseri* genome is publicly available at NCBI BioProject http://www.ncbi. nlm.nih.gov/bioproject/. A *Botryllus* genome browser is maintained and updated by our lab at http://botryllus.stanford.edu/botryllusgenome/browse/.

Funding

This study was supported by NIH [grant number 1R01AG037968], [grant number R01GM100315] awarded to I.L. Weissman, and A. Voskoboynik, and the Virginia and D.K. Ludwig Fund for Cancer Research awarded to I.L. Weissman.

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