Review Article

Gonad Development and Hermaphroditism in the Ascidian Botryllus schlosseri†

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

Molecular Cellular and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA 93106, USA

*Corresponding author: Delany Rodriguez
Email: drodriguez@lifesci.ucsb.edu
Telephone: 805-893-7280

Running Head: Gonad Development in Botryllus schlosseri

Keywords: Gonad resorption, gonad regeneration, fertility, colonial ascidian.

The authors would like to acknowledge the Grant Funding NIH RO1-AG037699.
DR was supported by a postdoctoral fellowship from the California Institute of Regenerative Medicine.

Quote: “The unique features of gonad formation in lab-reared Botryllus schlosseri [include] its ability to reversibly transition between different states of fertility, ranging from a complete absence of gonads to a male state to a hermaphrodite state.”

Additional Supporting Information may be found in the online version of this article.

Received 17 December 2015; Revised 18 April 2016; Accepted 15 May 2016
Molecular Reproduction & Development
This article is protected by copyright. All rights reserved
DOI 10.1002/mrd.22661

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/mrd.22661]
Abstract

The colonial ascidian *Botryllus schlosseri* is an ideal model organism for studying gonad development and hermaphroditism. *B. schlosseri* has been reared in laboratories for over half a century, and its unique biology allows investigators to probe the processes of germ cell migration and gonad formation, resorption, and regeneration. Following metamorphosis, colonies of *B. schlosseri* show a synchronized and sequential fertility program that, under standard laboratory conditions, begins with a juvenile stage with no visible gonads and subsequently develops testes at 9 weeks followed later by the development of oocytes – thus resulting in hermaphroditic individuals. The timing of oocyte production varies according to the season, and adult *B. schlosseri* colonies can cycle among infertile and both male and hermaphrodite fertile states in response to changing environmental conditions. Thus, these acidians are amenable to studying the molecular mechanisms controlling fertility, and recent genomic and transcriptomic databases are providing insight to the key genes involved. Here, we review the techniques and approaches developed to study germ cell migration and gonad formation in *B. schlosseri*, and include novel videos showing processes related to oocyte ovulation and sperm discharge. In the future, this valuable invertebrate model system may help understand the mechanisms of gonad development and regeneration in a chordate. This article is protected by copyright. All rights reserved.
Introduction

Ascidians are sessile, filter-feeding animals that belong to the Chordate subphylum Tunicata, which is composed of about 3000 species that are divided among four classes: Ascidiacea, Thaliacea, Appendicularia, and Sorberacea. The most important characteristic of ascidians that places them into the chordate phylum is their tadpole-like larvae, which contain all the main chordate features – bilateral symmetry, a notochord, a hollow dorsal nerve cord, pharyngeal slits, an endostyle (glandular structure), a ventral gut, and a post-anal muscular tail. Following metamorphosis into the adult, however, these features are lost.

Tunicates are the closest living relatives of vertebrates (Delsuc et al. 2006a; Delsuc et al. 2006b), and can be found around the world in both benthic and pelagic zones. Their entire bodies are embedded in a ‘tunic’, a tissue composed of proteins, carbohydrates, and cellulose (termed tunicin). Depending on the species, this tunic can be thin or thick, translucent or opaque, gelatinous or stiff. Most adult tunicates are soft-bodied, filter-feeding marine organisms that have a sedentary lifestyle. Tunicates can be further classified into two groups: solitary (e.g. Ciona intestinalis, Ciona savignyi, Halocynthia roretzi, Molgula manhattensis, Styela clava) or colonial (e.g. Botryllus schlosseri, Botryllus primigenus, Botrylloides violaceus, Didemnum vexillum).

*B. schlosseri* is cosmopolitan, grows in shallow, calm waters, and is easy to collect from marinas nearly worldwide. Colonies are easy to maintain and cross in mariculture (Fig. 1) (Sabbadin 1958; Manni et al. 2007; Gasparini et al. 2015) as they adapt to growth on glass slides and can survive on a feed of unicellular green algae (Fig. 1C, D, and E).

*B. schlosseri* has been used as a model since the 1950s to study a variety of biological processes, among them sexual reproduction. In-depth and detailed reviews about asexual reproduction in the wild and in the lab (Manni et al. 2007) and other biological processes –
such as allore cognition, asexual reproduction, and germ cell parasitism – can be found elsewhere (Rinkevich and Weissman 1992; Kawamura and Sugino 1999; Magor et al. 1999; Rinkevich 2002; De Tomaso 2006; McKitrick and De Tomaso 2010; Nydam and De Tomaso 2011; Rosengarten and Nicotra 2011; Gasparini et al. 2015; Taketa and De Tomaso 2015).

This review focuses on the unique features of gonad formation in lab-reared *B. schlosseri* (Fig. 1C - F), particularly on its ability to reversibly transition between different states of fertility, ranging from a complete absence of gonads to a male state to a hermaphrodite state. Furthermore, the feasibility of genotypically tracking these different states in time allows for the longitudinal characterization of the molecular mechanisms underlying fertility in a single individual.

**Structure of the colony and body plan**

Ascidian larvae are dispersed into the marine environment. When then settle, these tadpoles metamorphose into the adult body plan, which is sessile and shows a clear anterior-posterior and left-right asymmetry (Fig. 2). The metamorphosed adult (‘oozooid’) has an incurrent oral and an e xcurrent atrial siphon, pharynx, stomach, heart, intestine, central and peripheral nervous system, a branchial basket (a complex structure functioning in food collection and respiration), an endocrine gland, and a hematopoietic system with at least 8 different blood cell types (Sabbadin 1955; Ballarin and Cima 2005; Manni et al. 2007) (Fig. 2). All the individuals in the colony, known as ‘zooids’, are interconnected by an extra-corporeal circulatory system that ramifies throughout the tunic, surrounding the periphery of the colony and extending to terminal specialized finger-like extensions known as ampullae.

Each zooid is an independent body with the same body plan as the metamorphosed oozooi d, and does not depend on the rest of the colony – despite the shared vasculature. Zooids arrange themselves into groups of two up to 20 individuals, and assemble into star-
shaped structures known as ‘systems’ (Figs. 1 and 2A). Systems that are surgically separated from the colony and transferred to a new substrate will continue to grow. Thus, it is possible to analyze the same genotype under different conditions, or at different time points as the colony ages. A single ‘genotype’ is a colony of genetically identical, but independent, individuals linked by a common vasculature.

Blastogenesis (Asexual Reproduction)

Colonial tunicates are the only chordates with the ability to reproduce both sexually and asexually. *B. schlosseri* undergoes a weekly asexual reproductive cycle, called ‘blastogenesis’, beginning at the oozooid stage until the end of its life (6 months to >2 years in the lab). This asexual reproduction is accomplished by orderly and synchronized ‘budding’ (growing a new body from the side) of every individual within a colony (Figs. 2 and 3) (Berrill 1941; Berrill 1941b). The bud grows into a new body, which then replaces the original body as it dies and is resorbed, a process known as ‘takeover’.

Temperature influences blastogenesis and can drastically affect the lifespan of the zooid, shortening it from three weeks at 10°C to thirteen days at 26°C (Gasparini et al. 2015). In laboratory colonies reared at 19°C, the development of a bud into a new zooid takes 14 days, during which it transitions through two intermediate stages (or ‘generations’) known as secondary and primary bud; all three generations can co-exist at any given time. Zooids are connected to primary buds, which in turn are connected to secondary buds.

Budding can occur on both sides of the zooid (left and right), although the right side is often dominant whereas the left buds are slightly delayed when compared to the right buds. The development of left buds sometimes arrests, causing them to degenerate into a mass of cells (Gasparini et al. 2015). A single zooid can give rise to 1-4 buds, thus ensuring that the original zooid will be replaced within the colony at the end of its lifecycle. The left-right
asymmetry of the budding cycle is maintained over the lifespan of the colony, with buds on the right side of the zooid being larger and developing slightly faster than those on the left side. The colony grows when more than one bud is formed by a zooid, thus adding more individuals and therefore expanding in size (up to hundreds of individuals). The zooid will die after a lifespan of seven days, even if primary bud development is delayed (Lauzon et al. 2007), resulting in a takeover in which a slightly immature new body takes the place of the dying zooid.

Several methods have been proposed to define the stages of blastogenesis; we will use the most modern one proposed by Lauzon et al. (2002), which consists of seven stages – A1, A2, B1, B2, C1, C2, and D (Fig. 3) – based on visual morphological features (for comparison, the staging method by Sabbadin (1955) can be found in Supplemental Table 1, which correlates the staging between the two methods). Blastogenesis starts at stage A1, when a disc-like thickening of the primary bud’s peribranchial epithelia protrudes out and continues to extend, initiating a new secondary bud. At stage A2, the secondary bud twists towards the anterior hemisphere of the adult zooid. Stage B1 is characterized by the (secondary) bud forming an arch that will close off, forming a double-layered vesicle by stage B2. Organogenesis starts at stage C1, when the outer layer of the vesicle gives rise to the epidermis and the inner vesicle forms all the inner organs (branchial and peribranchial chambers, gut, and nervous system). Organogenesis is completed at stage C2, although the secondary bud continues to grow. During stage D, or takeover, the fully developed adult (former primary bud) will replace the adult zooid while the former secondary bud becomes the new primary bud. The zooid closes both siphons (oral and excurrent) before being replaced. Apoptosis and phagocytosis by blood-derived macrophages completely reabsorb the dying zooid as the new zooid is moved into the now-vacant position of the former zooid.
As colonies age or when they experience adverse environmental conditions, only the dominant bud (right) will complete development, and thus the colony undergoes no net growth – indeed, some may even regress if some adult zooids fail to produce or complete development of any of their buds – after each blastogenetic cycle. Death of the entire colony occurs when both heartbeat and budding halts, and the last generation of zooids dies with nothing to replace them (Munday et al. 2015).

From Embryogenesis to Metamorphosis

*B. schlosseri* utilizes internal fertilization, and the embryo completes development at about 5 to 6 days post-fertilization (Fig. 4). Since the embryo develops within the zooid, larva development and hatching have to complete within one blastogenic cycle (about 7 days). Embryo development is thus very closely linked to blastogenesis, and eggs are ready to be fertilized once the oral siphons on the new zooid open (stage A1).

The first cell division of the zygote (Fig. 4A) starts around 3 hours post-fertilization. The embryo keeps dividing until it reaches the blastula (stage A2 of the mother zooid) (Fig. 4B), and then gastrula one day later (stage B1) (Fig. 4C). The tail-bud stage is reached by stage B2 of blastogenesis, and the stage known as ‘tail wrap-around’ develops (followed by the stage known as ‘mid-tail’ embryo) (Fig. 4D) over the next couple of days (stages C1-C2) (Fig. 4D-H). Larva hatching occurs at late-C2-to-early-D stages, prior to or coinciding with the onset of takeover and right before the zooid permanently shuts down its siphons (Fig. 5) (Sabbadin 1955; Manni et al. 1999; Manni et al. 2007). The larvae hatch through the oral siphon of the mother (Fig. 5), and are capable of swimming freely for a short period of time (up to 24-36 hours) (Berrill 1950; Berrill 1975; Hiscock 2007).

The tadpole larva is 1.5 mm in length, and its head is equipped with a compound organ capable of sensing both light and gravity (known as photolith) and three adhesive papillae for...
attachment (Brunetti and Burighel 1969; Boyd et al. 1990; Manni et al. 1999; Sorrentino et al. 2000). The larva also contains eight ampullae in the mouth region that are involved in substrate adhesion. These sensory functions allow the free-swimming larva to identify and land on a suitable substrate for metamorphosis – i.e. mussels, pillings, Styrofoam floats in harbors and marinas, eelgrass, tunic of solitary ascidians such as Styela, Ciona, etc.) (Fig. 1A and B) (Brunetti and Burighel 1969), which takes place within 36 to 48 hours post-attachment and results in the formation of a fully functional oozooid (Fig. 4H-L). Larval metamorphosis begins with tail reabsorption (Fig. 4H-I), progresses to an intermediate metamorphosis stage known as ‘bell-shape’ (Fig. 4J), and is complete when an oozooid is formed (Fig. 4K-L). The ampullae extend out radially in a rapid manner and adhere to the substrate.

The larvae also contain the bud primordium that initiates the first round of blastogenesis, giving rise to the blastozooid, which will be replaced with the oozooid after the first takeover (Sabbadin 1955b). Both blastozooid and oozooids are considered juveniles, and are characterized by the absence of gonads (described in detail below).

**Early germ line specification during embryogenesis in B. schlosseri**

The expression of the vasa family of DEAD box helicases is generally restricted to germ cells in all metazoans, so vasa has been used as a marker to identify the germ-cell lineage in many species (Mochizuki et al. 2001; Extavour and Akam 2003). In solitary ascidians such as *Ciona intestinalis*, germ-line progenitors are maternally specified, and germ cells enter into the gonad rudiments during metamorphosis (Yamamoto and Okada 1999; Fujimura and Takamura 2000; Takamura et al. 2002; Shirae-Kurabayashi et al. 2006). In *B. schlosseri*, the earliest vasa expression can be found at the cortex of fertilized eggs and at the vegetal pole of the embryo during the first cell division (Brown et al. 2009). The spatio-temporal localization of vasa mRNA is equivalent to that found in solitary ascidians (Shirae-
Kurabayashi et al. 2006; Brown and Swalla 2007): A population of scattered cells in the head of the tadpole larvae following segregation into a pair of posterior blastomeres (Brown et al. 2009). In adult colonies of many botryllid species, vasa is expressed in circulating cells of the extracorporeal vasculature (Sunanaga et al. 2006; Brown and Swalla 2007; Sunanaga et al. 2008; Rosner et al. 2009).

**Germ Cells and Blastogenesis**

*B. schlosseri* contains long-lived germ-line stem cells that migrate to their niche inside developing buds. This mobility ensures that the germ line is maintained in a situation wherein colony turnover is high, and adult zooids are replaced on a weekly basis – i.e. a new niche is being formed each week during blastogenesis, so germ-line precursors must home to these newly established niches before their current zooid dies.

Langenbacher and De Tomaso (2016) identified a novel homolog of the TGF-β family member, named *tgf*-β, that is expressed by follicle cell progenitors, differentiated follicle cells, and support cells enclosing the maturing gametes. Almost all vasa-positive germ cells in *B. schlosseri* juveniles associate with *tgf*-β-positive follicle progenitors, together forming clusters consisting of as many as 10 cells with a 1:1 or 2:1 ratio of follicle progenitors-to-germ cells. These clusters reside in niches in both the primary and the secondary bud, as well as in the extracorporeal vasculature.

Clusters appear to be restricted to the primary bud niche at blastogenetic stages A1, A2, and B1, and mobilize between stages B2 and C1, when clusters are detected in the secondary bud niche – indicating a rapid 24-hour migration from old to new niches. An incremental increase in the number of small clusters of >10 cells at stages B1 and B2 suggests that a cluster may begin to fragment as it becomes migratory, which could aid their...
transition between niches. Kassmer et al. (2015) recently showed that the migration of germ-line progenitors is directed by sphingosine-1-phosphate (S1P). These migratory germ-cell precursors express known germ cell markers (vasa, piwi, and pumilio), and their in vitro migration towards S1P is dependent on integrin alpha-6 activity – a characteristic that can also be used to prospectively isolate this cell population. S1P signaling was further shown to be necessary for germ-line progenitor cells to home to secondary buds in vivo. Secondary buds generate S1P via sphingosine kinase, whereas the rest of the somatic tissues express lipid phosphate phosphatase, which helps maintain a S1P gradient that directs the homing of germ-line progenitor cells.

Consistent with the mobile behavior of their progenitors, immature, previtellogenic oocytes can also migrate in the blood stream for several consecutive blastogenetic cycles until they mature. The physiological basis for this phenomenon is unknown (Izzard 1968; Mukai 1977; Sabbadin and Zaniolo 1979; Manni et al. 2007; Rodriguez et al. 2014; Gasparini et al. 2015).

**Various Stages of Fertility: Juvenile, Male, Hermaphrodite, and Infertile Adult.**

Juvenile colonies take about 8-10 weeks post-metamorphosis to develop their first gonads. Gonads always develop sequentially: Testes form first, resulting in a male colony. Oocyte development always occurs after mature testes are present in the colony, resulting in colonies that are hermaphrodites. Following the initial acquisition of fertility, colonies can randomly switch between a male or hermaphrodite state. Fertility can also be completely lost, resulting in infertile adult colonies. The signals that drive *B. schlosseri* colonies to achieve different stages of fertility are not well understood.

Gonad development varies from zooid to zooid, and is intimately synchronized with the blastogenetic cycle. In large, expanding colonies, newly added zooids may take a couple of
blastogenetic cycles before developing gonads, thus some zooids develop gonads before others. Even though gonad development is bilateral, the gonadogenetic potential of a primary bud is greater on the left side, therefore the zooid tends to have more mature gonads on its left side (Sabbadin 1955b; Manni et al. 2007; Rosner et al. 2013; Gasparini et al. 2015). Secondary buds that grow on the right side, however, have higher overall gonadogenetic potential than the left-derived secondary buds when they eventually become primary buds. The gonadal rudiment is formed de novo from clusters of germ cells migrating from the bloodstream; the niches that they seed become the gonads when the germ cells differentiate (Manni et al. 1994; Rosner et al. 2013; Kassmer et al. 2015).

*Juvenile stage (no gonads)*

Neither oozooids nor the first generation of zooids develops gonads (Fig. 6A, A’, B, and B’). The timing of sexual maturation thereafter varies between genotype; on average, the testis will form in some zooids around 9 weeks post-metamorphosis. Through the first few weeks post-metamorphosis, however, undifferentiated gonad blastemas may be visible in secondary buds, and these blastemas eventually form testes that may be abortive or not fully functional (Sabbadin 1960; Sabbadin and Zaniolo 1979; Rinkevich et al. 1998).

Expression of genes required for germ cell specification (*vasa, piwi, and nanos*), can be detected by PCR and in situ hybridization in primitive germ cells that are present in both juveniles and adults (Brown et al. 2009; Sunanaga et al. 2010; Kawamura and Sunanaga 2011; Rinkevich et al. 2013; Rosner et al. 2013; Langenbacher et al. 2015). Germ-line progenitors also circulate between primary and secondary buds, even in juvenile colonies (Langenbacher and De Tomaso 2016), indicating the ubiquitous and continuous presence of germ-line precursors.
Male stage

Colonies of the same age do not always simultaneously develop gonads (Sabbadin 1955b; Manni et al. 2007; Gasparini et al. 2015). Testes form in the mesenchymal space between the epidermis and the peribranchial epithelium, on either the right or left side of the zooid (Burighel 2000). During the male stage, which may last up to nine blastogenetic generations (Gasparini et al. 2015), each zooid will develop two testes on the gonadal niches next to the bud sites (Fig. 6C, C’, D, and D’).

The testes are multi-lobed (Figs. 6C, C’, D, and D’, 7, and 8A), and they discharge sperm continuously through the oral siphon at stages B1 and B2 (Video S1) – which is after ovulation at stage A1, thus preventing self-fertilization. Spermatogenesis is initiated at stage A1 in the mature testes of the zooid, and sperm maturation continues until stage B2 (Manni et al. 2007).

The majority of genes up-regulated at stages A1 and A2 in fertile adults are, by homology, involved in spermatogenesis and sperm formation. Indeed, Gene Ontology analysis of fertile animals revealed an enrichment of genes related to male meiosis, spermatogenesis, cilium, meiosis, spermatid development, filopodium assembly, and flagellar motility in stages A1, A2, B1, and B2 of fertile animals (Rodriguez et al. 2014). The gene expression study also identified novel gene markers for both testes (tetraspanin-8, testis-specific serine/threonine protein kinase-1, and vitellogenin-1) and developing testes (otoancorin) that exhibit specific spatiotemporal expression profiles (Fig. 8), thus providing new markers to study the formation, maintenance, and regeneration of testes.

Female stage (Hermaphrodite)

As with testes, ovaries form in the mesenchymal space between the epidermis and the peribranchial epithelium (Burighel 2000). The ovary is composed of one to four functional...
oocytes, each with their corresponding envelope (Izzard 1968; Sabbadin and Zaniolo 1979; Manni et al. 1994). Three types of cells surround the developing oocyte: test cells, outer follicle cells, and inner follicle cells. The chorion (or vitelline coat) separates the test cells from the follicle cells. Each oocyte can be considered an independent ovary, consisting of the gamete, the surrounding cells, and an oviduct.

Oocyte development starts with the accumulation of undifferentiated progenitor cells on either side of the inner closed vesicle at stage B2. Oocyte precursors are first seen in secondary buds and continue their maturation process within maturing in primary buds; however, loss of premature oocytes may occur over a couple of cycles (several weeks after the testes form). Oocyte maturation on the primary bud is classified into 5 stages, based on diameter. Stage-1 oocyte precursors are characterized as having a high nuclear to cytoplasm ratio and have a diameter of 10 µm. At stage 2, the oocytes increase in diameter up to 50 µm. Stage-3 oocytes become larger, up to 80 µm in diameter. Vitellogenesis is initiated during stage 4, when surrounding cells secrete nutrients into the oocyte and the oocyte diameter increases to 120 µm. At stage 5, vitellogenesis is almost complete, yolk granules increase in size, and the oocyte has reached 220 µm in diameter. Mature B. schlosseri eggs are, on average, 300 µm in diameter. A few asexual generations are required for oocytes to complete their maturation process (Mukai and Watanabe 1976; Sabbadin and Zaniolo 1979).

One egg usually ripens in the primary bud, and is ovulated when the primary bud transitions from juvenile stage D to adult stage A1 (Figs. 3 and 9). An ovulated egg is transferred from the side of the primary bud into the branchial basket, where it sheds most of the outer follicle cells. At this point, siphons of the new zooid open at stage A1, thus allowing sperm to reach the egg for fertilization (Fig. 9) (Milkman 1967; Manni et al. 2007; Gasparini et al. 2015). Between one and four mature oocytes are ovulated for fertilization (Manni et al.
When fertilization occurs, the inner follicle cells, the peribranchial epithelium, and the oviductal epithelium form the placental cup (Zaniolo et al. 1987).

The left side of the zooid produces larger testes with more lobes, a greater number of eggs (usually up to two eggs in laboratory conditions), and more embryos, whereas the right side testes is smaller testes, and only one egg is produced (colonies rarely developed two eggs on their right side at their peak of fertility under lab conditions) (Sabbadin 1955; Sabbadin and Zaniolo 1979; Manni et al. 2007; Gasparini et al. 2015). When a primary bud with eggs on both sides is transitioning to become a zooid during takeover, the left side ovulates first (Fig. 9 and Video S2).

**Gonad resorption (infertile adults)**

In the wild, sexual reproduction is arrested in response to adverse changes in environmental conditions. Resorption of gonads can last for several blastogenetic cycles (Fig. 6G, G’, I, and I’) (Sabbadin 1955b; Manni et al. 2007; Rodriguez et al. 2014; Gasparini et al. 2015). Even in laboratory mariculture systems with natural seawater flow, where the temperature of the water is maintained at 19°C and food is constant, colonies can arrest sexual reproduction. First, egg production completely stops and no egg precursors can be found in the colonies, returning them to a male-only state. Under more extreme conditions (in the wild or in a mariculture system with temperatures at or below 16°C and/or in starvation conditions) the colonies will completely reabsorb their testes (Sabbadin 1958; Manni et al. 2007), resulting in an infertile state. During this period, budding is reduced to one bud per zooid, and the overall growth of the colony is arrested. Once environmental conditions improve, however, colonies can regenerate both testes and eggs. The mechanisms
underlying such phenotypic plasticity, the role that the environment plays in modulating fertility, the processes governing germ cell precursor homing to the niche in the developing buds, and how differentiation into eggs or testes is regulated remain poorly understood.

**Key Genes Related to Hermaphrodite Fertility**

Recent comparative transcriptome analysis of infertile versus fertile (hermaphrodite) colonies at each stage of the blastogenetic cycle (stages A1 through D) revealed a number of genes that regulate fertility. Differential expression analysis identified as few as 7 (stage C1) and to as many as 647 (stage B1) differentially expressed genes at each stage of blastogenesis (data are available at www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62112) (Rodriguez et al. 2014).

Gene Ontology analysis of fertile animals revealed an enrichment of genes related to female gamete generation at stages A1 and A2. Genes related to cell adhesion, female gamete generation, and apolipoprotein binding were up-regulated at stages B1 and B2. Stages C1, C2, and D, however, were enriched for genes related to oocyte maturation, oocyte growth, lipoprotein metabolic process, regulation of cholesterol transport, positive regulation of estrogen receptor, extracellular matrix disassembly, response to corticosteroids stimulus, and estrogen receptor. Novel gene markers were also identified for maturing oocytes in the primary bud – such as *vitellogenin-1*, *low-density lipoprotein*, *zona pellucida-1*, and *tolloid-protein like1* – as well as markers for earlier stages of oogenesis (stage 2 oocytes) – *estradiol-17β dehydrogenase-8*, *p-selectin*, *testis-specific serine/threonine protein kinase-1*, and *tretraspanin-8*.

Markers for primitive follicle cells, such as *tgfβ-1* and *vitellogenin-1*, will help uncover the origin and function of these cells, which can also be found in circulation as well as
surrounding both maturing oocytes and testes of the primary bud (Rodriguez et al. 2014; Langenbacher et al. 2015). Research in the near future will undoubtedly focus on how these genes contribute to gametogenesis and maintenance and regeneration of the gonads, and should help address many questions regarding *B. schlosseri* fertility, including: What is the origin of both embryonic and post-embryonic follicle cells? How is asexual and sexual reproduction balanced? What favors the gonadogenetic potential of one side compare to the other? What are the molecular mechanisms that control gonad resorption? And what are the mechanisms regulating the ability to cycle in and out of fertility? Answering these questions will help us understand the evolution of gonad development and hermaphroditism.

**Directions for the field in the near future**

Bioinformatic resources – such as comparative transcriptomics (Rodriguez et al. 2014), the genome sequence (Voskoboynik et al. 2013), and the ontology of anatomy and development (Manni et al. 2014) of *B. schlosseri* – are priceless for investigating the molecular processes of gonad formation, maintenance, resorption, and regeneration of gonads. Recent improvements in techniques for *B. schlosseri*, such as whole mount in situ hybridization, allow an unbiased analysis of both spatial and temporal gene expression (Langenbacher et al. 2015). Indeed, a strength of this technique is its ability to detect gene expression throughout blastogenesis for both infertile and fertile colonies on several tissues, including the germ line, gonads, zooids, and primary and secondary buds (Rodriguez et al. 2014; Langenbacher et al. 2015). Moreover, this technique allows for the analysis of co-expression of multiple transcripts.

The lack of transgenic colonial ascidians has made understanding the molecular pathways regulating their sexual and asexual reproduction difficult. Nevertheless, new approaches for labeling tissues and/or cell populations – such as vascular labeling (Braden et
al. 2014), blood phagocyte labeling (Lauzon et al. 2013) – combined with well-established techniques – such as flow cytometric cell sorting and molecular biology – make this non-conventional model organism great for studying a variety of biological processes. The CRISPR/Cas9 system was recently used to mutate endogenous genes in the solitary ascidian *Ciona intestinalis* (Sasaki et al. 2014; Stolfi et al. 2014), thus opening the door to perform forward- and reverse-genetic experiments to test hypotheses based on bioinformatics data. In time, this and other molecular approaches will be applied to fully understand the unique characteristics of colonial ascidians.

**Acknowledgements**

The authors would like to acknowledge Snjezana Rendulic for the micrographs of the hatching larvae. The authors would also like to acknowledge Dr. Adam D Langenbacher for his input for this review.
References


This article is protected by copyright. All rights reserved


Figure Legends

Figure 1. *B. schlosseri* colonies. **A-B**: Colonies from the Santa Barbara Harbor attach to mussels, revealing different color morphs – including blue, orange, and black. **C-F**: *B. schlosseri* colonies are also readily reared in laboratory conditions, with many colonies maintained in a single tank (C), reared on individual glass slides (D). Hatches and a colony of hundreds of zooids clustered into star-shaped systems (E) can be obtained using this method. Two-system *B. schlosseri* colonies can also be established (F). Scale bars, 1 cm (A, B, D, E); 8 cm (C), or 1 mm (F).

Figure 2. Structure of the colony and body plan of *B. schlosseri*. **A**: Dorsal view of a colony of individual adult animals (orange box and arrow), each of which is connected to asexually propagating primary buds (blue box and arrow) and secondary buds (yellow box and arrow). Dashed arrow points at the extracorporeal network of vessels that connect all individuals and extends to the periphery of the colony, to structures known as ampullae (solid arrow). Scale bar, 1 mm. **B**: Body plan of zooid, primary bud, and secondary bud.

Figure 3. The blastogenetic cycle of a fertile adult. At stage A1 a secondary bud appears as a thickening of the epidermis and the peribranchial chamber leaflet of the primary bud, which evaginates into a closed vesicle by stage B2, followed by organogenesis during stages C1-D. Gonadogenesis occurs in the secondary bud from mobile precursors (blue; stages B1-C2). During takeover (stage D to A1), the primary bud opens its siphons and becomes a functional adult (zooid) while the secondary bud becomes the primary bud; a new blastogenetic cycle begins thereafter for the next secondary bud. Orange circles, gonad progenitors; pink circles.
(large and small), developing oocytes and mature egg; blue circle, testes precursors; blue lobes, developing and mature testes. The comparable staging by Sabbadin (1955a) is denoted in parentheses for each stage (see also Table S1).

Figure 4. *B. schlosseri* embryogenesis and metamorphosis. Brightfield micrographs of early embryogenesis: (A) two-cell stage; (B) gastrula; (C) late gastrula; (D): tail-wrap stage. Larval stages: (E) free swimming larva; (F) close-up of the head of a larva; (G) notochord/tail. Metamorphosis into oozooid: (H) tail resorption; (I) close-up of the tail resorption; (J) bell shape, tail is almost completely reabsorbed and the ampullae start extending out. Oozooid: (K) lateral view of an oozooid; (L) ventral view of an oozooid. Scale bars, 100 μm.

Figure 5. Hatching larvae, at the transition between stage C2 and D. Most larvae hatch out of the mother and swim away. A: Two larvae can be seen inside the oral siphons with their tails wrapped around just prior to hatching. B: A larva swimming out of the oral siphon. Scale bars, 750 μm.

Figure 6. Fertility stages of *B. schlosseri*. A-B: Juvenile stage with no apparent gonads. (A, A’) Juvenile oozooid with no gonads. (B, B’) Juvenile primary bud with no gonads. C-D: Male colony. (C, C’) Male colony with mature testes in the zooid and developing testes in the primary bud. (D, D’) Male primary bud with developing testes. E-F: Hermaphrodite colony. (E-E’) Hermaphrodite colony with a mature egg and two testes (on each side) in the zooid and primary bud with both developing testes and oocytes. (F-F’) Hermaphrodite primary buds with both developing testes and oocytes. G-I: Infertile colony (adult without gonads). (G-G’) Infertile colony with no apparent gonads. (I-I’) Infertile colony primary bud with no apparent
developing gonads. Dashed white line encompasses a zooid; yellow dashed line surrounds a primary bud; light blue line marks the developing testes; dark blue line indicates the mature testes; cyan line surrounds oocytes; magenta line surrounds a mature egg. Herma, hermaphrodite; Infert, infertile (adult without gonads); Juv, juvenile; pb, primary bud. Scale bar indicates 200 µm.

Figure 7. Sperm discharge of *B. schlosseri*. Images from a 48-hour time-lapse video (ventral view). **A**: Testes are fully loaded at stage B1. **B**: Testes reduce their volume as sperm is being released at stage B2. **C**: Most sperm has been released by stage C1, and the overall size of the testis is reduced. Scale bar, 150 µm.

Figure 8. Fluorescence in situ hybridization of genes expressed by gonads, gonad precursors, and follicle cells. **A**: Expression of *vitellogenin-1 (vtg-1)* on follicle cells surrounding mature testes. **B**: Stage-2 oocyte expressing *testis-specific serine/threonine protein kinase 2 (tsk2)*. **C**: Oocyte developing on the primary bud expressing *low-density lipoprotein receptor (ldlr)*. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), shown in blue, while riboprobes are shown in green. Scale bars, 50 µm.

Figure 9. Ovulation of *B. schlosseri*. Images from a 24-hour time-lapse video (ventral view) of the ovulation of eggs into the branchial chamber of the new adult zooid. **A**: All eggs are still outside of the primary bud before completion of stage D. **B**: The left side is the first to ovulate (shown on the right side on the ventral view). **C**: The second egg to ovulate is on the lower left of the image. **D**: After the left side has completed ovulation, then the right side ovulates the last egg. All eggs are now inside of the branchial basket of the new zooid. Solid white line,
mature eggs before ovulation. Dashed white line, ovulated eggs in the branchial basket. Scale bars, 300 μm.
Table 1. Overview of the terms used throughout this review.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastogenesis</td>
<td>Asexual development of an individual from a blastema.</td>
</tr>
<tr>
<td>Colonial ascidian</td>
<td>A number of genetically identical individuals (zooids) that are permanently associated.</td>
</tr>
<tr>
<td>Genotype</td>
<td>Genetically identical individuals from the same colony.</td>
</tr>
<tr>
<td>Gonad resorption</td>
<td>The process by which the gonads are completely reabsorbed.</td>
</tr>
<tr>
<td>Gonadogenetic potential</td>
<td>The ability to give rise to new gonads.</td>
</tr>
<tr>
<td>Oozooid</td>
<td>The first zooid derived from the larva metamorphosis.</td>
</tr>
<tr>
<td>Primary bud</td>
<td>Asexually derived bud directly linked to the zooid.</td>
</tr>
<tr>
<td>Secondary bud</td>
<td>Asexually derived bud directly linked to the primary bud.</td>
</tr>
<tr>
<td>Systems</td>
<td>Groups of 2 and up to 20 zooids assembled into star-shaped structures.</td>
</tr>
<tr>
<td>Takeover</td>
<td>The process of replacement of the zooid(s) by new, asexually derived ones as the original zooid dies and is resorbed.</td>
</tr>
<tr>
<td>Tunic</td>
<td>A tissue composed of proteins, carbohydrates, and cellulose.</td>
</tr>
<tr>
<td>Zooid</td>
<td>An animal or individual of a colonial organism.</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3
Figure 7
Figure 8
Figure 9