Botryllid tunicates: Culture techniques and experimental procedures

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Abstract

Botryllid tunicates have become increasingly important as model experimental organisms in a variety of biological fields, including invasion ecology. This paper summarizes existing botryllid culture methods and offers new ones for culturing Botryllus schlosseri (golden star tunicate) and Botrylloides violaceus (violet tunicate) under laboratory conditions. Techniques for maintaining adult colonies, obtaining larvae, and establishing juvenile cultures are presented. Considerations for designing laboratory experiments using botryllid tunicates are discussed.

Key words: Botrylloides, Botryllus, culture techniques, golden star tunicate, violet tunicate

Introduction

Botryllid tunicates have become increasingly important as model experimental organisms in a variety of biological fields such as immunology, physiology, ecology, genetics, developmental biology, evolutionary biology, and aging (e.g., Boyd et al. 1986; Rinkevich and Shapira 1998; Manni et al. 2007 and references therein). Golden star tunicate, Botryllus schlosseri Pallas, 1766 and violet tunicate, Botrylloides violaceus Oka, 1927 – which most likely originated in the Mediterranean Sea and the northwest Pacific, respectively (Berrill 1950) – have invaded much of the north Pacific, north Atlantic, and the Mediterranean and are becoming a growing concern in areas of expanding aquaculture (reviewed in Lambert 2001, 2005). The ability of these species to spread rapidly to new environments and become major components of fouling communities impose a need for more knowledge of their environmental tolerances and interactions with native species, as well as of the potential for controlling their spread. For each of these types of studies, maintenance of healthy laboratory cultures allows circumventing seasonal variations in colony availability, growth rate, and sexual reproduction, and makes it possible to conduct long-term experiments year round. Laboratory cultures also make it possible to directly and accurately assess the effects of various physical, chemical, and biological factors on colony survivorship, growth, and reproduction.

Several previous studies have cultured ascidians under directly-controlled laboratory conditions (Berrill 1937; Grave 1937), including botryllid tunicates (Berrill 1937; Grave 1937; Milkman 1967; Sabbadin 1971 and earlier studies cited therein; Boyd et al. 1986; Rinkevich and Weissman 1987; Grosberg 1988; Rinkevich and Shapira 1998). However, most of these studies focused on specific experimental aspects and described culture methods only briefly. Boyd et al. (1986) provided the most detailed description of laboratory methods and conditions for culturing colonies of B. schlosseri, but knowledge gaps still exist. The primary objective of this paper is to summarize existing methods and offer new techniques for culturing botryllid tunicates under controlled laboratory conditions for various experimental purposes.
Culture methods

I. General description of tunicates studied

Our study focused on two non-indigenous botryllid species (B. schlosseri and B. violaceus) now common in the Strait of Georgia, British Columbia, Canada. Both are sessile colonial tunicates that grow as thin, flat, encrusting mats or as irregular lobes, depending on the substratum and colony age. Colonies are composed of morphologically identical individuals (zooids) that are embedded in a common tunic and share a circulatory system. Both species exist in a variety of colour morphs. Colonies of B. violaceus are a solid colour (usually purple, pink, yellow, or orange), while B. schlosseri often has two-toned zooids (predominant colours being orange/brown and black/green). Colonies grow by synchronous asexual multiplication (budding of the zooids) and propagate through sexual reproduction and occasionally through colony fragmentation. Like other colonial botryllids, both species are hermaphrodites and brood embryos inside the parent colony. Their tadpole larvae settle within a day and metamorphose into the first zooid (oozooid) of a new colony. Oozooids produce a set of asexual buds that differentiate into new zooids (blastozooids), which in their turn bud synchronously, providing colony growth (Grosberg 1988). Botryllus schlosseri and B. violaceus differ in gonad arrangement, embryo/larval incubation, and some morphological aspects related to the digestive system (Brunetti 1979). Species of the genera Botryllus and Botrylloides exhibit similar growth patterns and some have been shown to have similar environmental tolerances (Brunetti et al. 1980). Therefore, culture techniques for B. schlosseri and B. violaceus are similar, specific differences being noted below.

II. Establishment and maintenance of adult (broodstock) cultures

Botryllid tunicates are often abundant on artificial structures in harbours (e.g., wharves, marinas, docks, pilings, lines, buoys, boat hulls) and at aquaculture sites (e.g., cages, trays, floats, lines, anchors). Fragments of colonies can be gently scraped from the substratum and transported to the laboratory in coolers with ambient seawater. When experimental design requires re-attachment of fragments to certain substrata (e.g., slides or tiles) it is better to collect fragments of flat colonies as they generally have better re-attachment capabilities than those forming thicker clumps or lobes (Milkman 1967; A. Epelbaum pers. obs.). Fragments can be glued to the substratum with plastic surgery glue (Dijkstra et al. 2007) or left to re-attach naturally, either by securing (Grosholz 2001; McCarthy et al. 2007) or simply placing (Bullard et al. 2007, our study) them on the substratum. We found that securing colonies with rubber bands often damaged zooids, while placing them on the substratum (glass slides) and adjusting the water flow – so that they remained in place and were not dislodged – allowed most fragments to re-attach within 24 hours, after which time they began spreading along the substratum naturally (Figure 1).

![Figure 1. Fragment of B. violaceus colony growing on a glass slide (six days following re-attachment).](image)

III. Larval and juvenile cultures

Obtaining larvae and establishing juvenile colonies: The duration of the breeding season in botryllid tunicates varies with temperature. Millar (1971) summarized breeding records for B. schlosseri from Naples, Italy (January – December); Venice, Italy (April – November); Brittany, France (June – October); and Millport, Scotland (June – September). Along the east coast of Vancouver Island, Canada, B. schlosseri and B. violaceus generally produce larvae from early June to late September (A. Epelbaum pers. obs.).

Colonial ascidians are known to release larvae in response to light stimulation (Cloney 1987). The time of release can be controlled by keeping the colonies in the dark for several hours before the larvae are needed (Watanabe and Lambert...
1973). To stimulate release of larvae, brooding colonies can also be gently torn into small fragments (~8 cm²) (Marshall et al. 2006). *Botryllus schlosseri* and *B. violaceus* that we collected along the coast of Vancouver Island in June and kept in the laboratory on a 16h light/8h dark cycle (daytime light intensity 1000 lux, ‘dawn’ at 5:30 AM) released larvae in batches every morning for approximately one week, mostly between 10:00 and 11:00 AM.

Free-swimming tadpole larvae usually remain in the water column for 2–12 hours (Grave 1937) and do not feed. Offspring size can be measured as the head length of the larva or as the settler’s branchial basket area (Marshall and Keough 2003, Marshall et al. 2006). To make larvae settle on the desired substrata, the latter can be arranged in the culture tank facing parental colonies. Newly-settled oozooids that have attached to non-target substrata may be carefully removed by hand and transferred to the desired location (Berrill 1937; Boyd et al. 1986; Rinkevich and Weissman 1987). A different method of ensuring larvae settle on the desired substratum was described by Karande and Nakauchi (1981) for *Aplidium multiplicatum*. Tadpole larvae were placed into tap or distilled water for 20 seconds, where they became motionless, and then were immediately released close to the surface of glass slides submerged in normal seawater. Larvae adhered to the glass surface immediately if the freshwater ‘shock’ was adequately timed; seven out of every 10 tadpoles were reported to successfully settle when this technique was applied (Karande and Nakauchi 1981).

We attempted to develop a technique that would make juvenile colonies grow in the precise location desired, while ensuring minimal handling of the animals. In preliminary trials, we arranged 60 glass slides horizontally in trays with seawater (2 cm deep), put a small ring of PVC pipe over the centre of each slide so that the top of the ring was above the water surface, and placed a single tadpole larva inside each ring (i.e., 60 larvae in total). This method was not efficient, however, as 32 larvae (>50%) settled on the sides of the PVC rings rather than the desired substratum. In subsequent trials, we developed another method of ensuring that larvae settle on the desired substratum. Larvae of both species are positively phototactic (Grave 1937) and thus, when held in a dark-sided tank illuminated from the top, most will settle, inverted, at the water surface and begin metamorphosis. Larvae that have settled in such a way can be gently collected by pipette, placed individually in small drops of water in the desired location on the target substratum, and arranged so that their papillae (ampullae) are facing downwards. In the current study, using this method, 536 out of 540 larvae of *B. violaceus* immediately attached to the desired substratum (glass slides), successfully metamorphosed into oozooids, and started developing blastozooids (Figure 2).

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**Figure 2.** Oozooid of *B. violaceus* (24 hours following attachment).

This method allows for minimal handling of oozooids, which favours their proper development. However, it is less effective for obtaining young colonies of *B. schlosseri* as oozooids of this species are almost transparent and much smaller than those of *B. violaceus* (branchial basket length ~0.3 mm, as opposed to ~1 mm), making their proper positioning on the desired substratum more difficult. We found that larvae of *B. schlosseri* could be easily collected by suspending glass slides vertically, parallel to each other, just below the water surface in the larval containers. In 24 hours, slides could be retrieved and inspected for the presence of oozooids, with all unwanted oozooids being removed.

**Feeding juvenile colonies:** After larval settlement and metamorphosis, the oozooid acquires atrial and branchial siphons and begins feeding within one to two days (Takeuchi 1980). Several
types of diets have been previously used in botryllid tunicate culture, with different levels of success (Table 1). In these studies, tunicates were cultured for different purposes, and thus life-history parameters and the ways they were registered differed as well. This makes direct comparison of diet performances among the studies rather difficult.

Rinkevich and Shapira (1998) demonstrated that a mixture of several diet types was superior to any monotype diet. In our culture experiments we fed animals every other day with three alternating diets: Liquifry Marine, MicroVert, and a 1:1 (by cell number) mixture of two species of microalgae (see Table 1). Use of these three diets resulted in exponen-tial growth of juvenile colonies of both species; colonies of *B. schlosseri* attained sexual maturity and released their first batch of larvae in 45 to 50 days at 20°C.

**Physical setup and culture conditions for juvenile colonies:** Artificial substrata with newly attached oozooids need to be kept horizontally in standing seawater for about two days in order to let oozooids attach firmly. After this time, the substrata can be transferred to experimental/culture containers where they may be arranged aslant, with the young colonies facing down (Milkman 1967), or vertically (e.g., in the slots of glass staining racks as done by Boyd et al.)

### Table 1. Diets used in botryllid tunicate culture.

<table>
<thead>
<tr>
<th>Food</th>
<th>Concentration</th>
<th>Tunicate species</th>
<th>Outcome of Study</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural seawater, coarsely filtered</td>
<td>N/A</td>
<td>Tunicates in general</td>
<td>N/A</td>
<td>Berrill 1937</td>
</tr>
<tr>
<td><em>Cyclotella nana</em></td>
<td>5 to 25x10⁷ cells/L</td>
<td><em>B. schlosseri</em></td>
<td>Good growth, up to three buds per zooid</td>
<td>Milkman 1967</td>
</tr>
<tr>
<td>Mixture of <em>Phaeodactylum tricornutum</em>, <em>Dunaliella</em> sp., and <em>Chlorella</em> sp.</td>
<td>Not specified</td>
<td><em>B. schlosseri</em> * <em>B. leachi</em></td>
<td>Juvenile and young adult colonies exhibited positive growth at a number of temperature and salinity combinations</td>
<td>Brunetti et al. 1980</td>
</tr>
<tr>
<td>Liquifry Marine (Interpret Ltd., Dorking, UK)</td>
<td>3.8 to 60 µL/L</td>
<td><em>B. schlosseri</em></td>
<td>Juvenile colonies exhibited positive growth at a number of temperature and salinity combinations</td>
<td>Boyd et al. 1986</td>
</tr>
<tr>
<td>1:1 mixture of <em>Dunaliella tertiolecta</em> and <em>Phaeodactylum tricornutum</em></td>
<td>2.5 to 5x10⁷ cells/L each</td>
<td><em>B. schlosseri</em></td>
<td>High mortality and poor growth</td>
<td>Boyd et al. 1986</td>
</tr>
<tr>
<td>Marine Invertebrate Diet (Aquarium Products Inc., Houston, USA)</td>
<td>0.1 to 0.3 ml per 1000 zooids</td>
<td><em>B. schlosseri</em></td>
<td>Juvenile colonies exhibited positive growth at 15–25°C</td>
<td>Grosberg 1988</td>
</tr>
<tr>
<td>Invertfood (Waterlife Research Ltd., Longford, UK)</td>
<td>Not specified</td>
<td><em>B. schlosseri</em></td>
<td>The use of all diets alone and in combinations resulted in positive growth of juvenile colonies</td>
<td>Rinkevich and Shapira 1998</td>
</tr>
<tr>
<td>Hatchfry Encapsulation 50-150 µm (Argent Chemical Lab, Redmond, USA)</td>
<td>7.5 g/L</td>
<td><em>B. schlosseri</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artificial Plankton (Argent Chemical Lab, Redmond, USA)</td>
<td>7.5 g/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dried Algae Algal 161 (Celsys, Cambridge, UK)</td>
<td>7.5x10¹⁸ g/L total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixture of <em>Nannochloropsis</em> sp., <em>Dunaliella salina</em>, and <em>Isochrysis galbana</em> Freeze-dried rotifers <em>Brachionus plicatilis</em></td>
<td>7.5 g/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquifry Marine (Interpret Ltd., Dorking, UK)</td>
<td>30 µL/L</td>
<td><em>B. schlosseri</em> * <em>B. violaceus</em></td>
<td>Diets were alternated. Juvenile colonies exhibited positive growth at 10–25°C. <em>B. schlosseri</em> released the first batch of larvae in 45 to 50 days after settlement at 20°C</td>
<td>Our study</td>
</tr>
<tr>
<td>MicroVert (Kent Marine Inc., Acworth, USA)</td>
<td>30 µL/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1 mixture of <em>Chaetoceros muelleri</em> and <em>Isochrysis</em> sp. (Tahitian strain, T-iso)</td>
<td>5x10⁷ cells/L each</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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(1986)). Such arrangement ensures that faecal pellets, debris, and fouling algae are less likely to accumulate on or around the colonies. However, regular cleaning of colonies might be required (Boyd et al. 1986; Rinkevich and Shapira 1998), especially when natural seawater is used. When algal growth on or around the colonies became considerable, we observed colonies growing away from algae-covered areas by forming buds towards the unfouled space in each asexual growth cycle (see Figure 3). Cleaning needs to be done gently with something like a soft, small paintbrush to ensure zooids do not get damaged.

In our studies, we suspended glass slides with juvenile colonies vertically from the top of the culture containers using rods and clips (e.g., all-plastic cloth pegs or badge clips) (Figure 4). This technique proved to be quite useful as it allowed unrestricted water movement, minimized the surface area to be cleaned, and simplified handling and transfer of slides to new containers.

Aeration, if employed, requires careful implementation as faecal pellets and detritus suspended in the water column might cause reflex cessation of pumping, which negatively affects feeding efficiency (Milkman 1967). In all of our experiments we used containers with static seawater and no aeration. When colonies are cultured in static seawater, evaporation needs to be controlled or compensated for. To control evaporation, Milkman (1967) suggested covering culture vessels with polyethylene covers through which oxygen and carbon dioxide can pass and water vapour can not. However, to the best of our knowledge, this option has never been experimentally tested in tunicate culture.

Both species possess broad temperature and salinity tolerances. In our experiments, young colonies of *B. schlosseri* survived environmental conditions between 10 and 25°C and 14 and 38‰, while *B. violaceus* tolerated temperatures between 5 and 25°C and salinities between 20 and 38‰. Best growth of both species was observed at 20°C and 32‰. These results are similar to those reported by Brunetti et al. (1980) for *B. schlosseri* from the Venetian Lagoon, Italy.

The frequency of water exchange largely depends on the size of the culture container, colony size, temperature, and type of food used. When juvenile colonies (1–40 zooids) were cultured in 1-L containers, with four juvenile colonies per container at 20°C and 32‰ and fed every other day with three alternating feeds as described above, changing water every 48 hours kept ammonia levels below 0.03 mg/L (Table 2), within the safe limit for marine invertebrates (Boardman et al. 2004).
Table 2. Ammonia concentration (NH$_3$-N in mg/L) in tunicate culture over time. Young colonies (1-20 zooids) were cultured in 1-L containers at 20°C, four colonies per container. For food concentrations used see Table 1. Ammonia levels were measured using a DR 2800 portable spectrophotometer (Hach, Loveland, USA) with a TNT 830 ultra low range reagent (sensitivity: 0.015 - 2 mg/L NH$_3$-N).

<table>
<thead>
<tr>
<th>Food</th>
<th>1 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquifry Marine</td>
<td>≤0.015</td>
<td>0.019</td>
<td>0.026</td>
<td>0.026</td>
</tr>
<tr>
<td>(Interpret Ltd.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MicroVert</td>
<td>≤0.015</td>
<td>≤0.015</td>
<td>0.019</td>
<td>0.021</td>
</tr>
<tr>
<td>(Kent Marine Inc.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1 mixture of Chaetoceros</td>
<td>≤0.015</td>
<td>≤0.015</td>
<td>0.023</td>
<td>0.023</td>
</tr>
<tr>
<td>muelleri and Isolechrysis sp. (Tahitian strain, T-iso)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

During our preliminary experiments some young colonies of B. violaceus were attacked by ciliate protozoans that penetrated colonies, multiplied rapidly, and consumed colony tissues. Ciliates were able to completely consume healthy juvenile colonies of B. violaceus within 24 hours, but interestingly were never observed attacking B. schlosseri cultured under the same conditions at the same time. To control ciliates in our subsequent cultures, we cartridge-filtered all seawater to 1 µm and treated with UV light (Coralife Turbo-Twist 12x, Energy Savers Unlimited Inc., Carson, USA). As a result, in subsequent experiments ciliates were found in only two out of 536 B. violaceus colonies, thereby enhancing culture success.

Monitoring colony condition and growth: About every three to ten days, depending on the water temperature, botryllid colonies pass through an asexual growth cycle, or blastogenesis. During each cycle, zooids bud bilaterally, shrink, and get replaced by the new buds. Some buds can be re-absorbed together with their parent, thus the total number of zooids in a colony can increase, remain constant, or decrease. The replacement of zooids during each cycle in Botryllus spp. was described in detail by Mukai and Watanabe (1976) and Grosberg (1988). This synchronous, successive change of generations complicates colony growth measurements. Several approaches have been developed, including measuring colony area (Millar 1952), counting the number of zooids (Boyd et al. 1986), and recording the number of primary buds per zooid in each cycle and the duration and number of cycles (Grosberg 1988). Data from Yamaguchi (1975), Grosberg (1988), and our study suggest that growth rates of botryllid colonies (in terms of changes in number of zooids per unit time) are exponential until the onset of sexual maturation (Figure 5). We believe that a modification of Grosberg’s formula (Grosberg 1988) for calculating a pre-reproductive growth rate reflects growth most accurately, as it incorporates both of its components (i.e., number of zooids and number of growth cycles):

\[
\log g = \frac{\log (z_{n+1})}{(n-1)}
\]

where \( g \) = pre-reproductive growth rate, \( z_n \) = number of zooids in a colony at asexual cycle \( n \), and \( n \) = cumulative number of asexual cycles at sexual maturity.

![Figure 5. Mean colony size of B. schlosseri and B. violaceus at 20°C and 32‰. On Day 1 colonies were 10-12 d old. Black arrow shows when the first batch of B. schlosseri larvae was released. \( n = 6 \) and error bars = SE.](image-url)

Colony condition, or general health, may be monitored using the following parameters: morphology of the zooids and ampullae (well-formed vs. shrunken; absence vs. presence of dense accumulations of pigment cells), integrity of the circulatory system, and integrity and transparency of the tunic. Overall colony condition may be assessed by assigning points to each parameter and calculating a mean value (e.g., Brunetti et al. 1980).
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Under certain unfavourable conditions, colonies may undergo reversible colonial regression or irreversible degeneration (Brunetti et al. 1980). Degenerating colonies go through distinct stages of deterioration preceding death, described by Rinkevich et al. (1992) and Chadwick-Furman and Weissman (1995) for B. schlosseri. First, blood vessels narrow and blood flow slows; then, the zooids shrink and become densely pigmented; in the third stage, groups of zooids become disorganized; in the fourth and final stage, the tunic gradually disintegrates and all of the colony tissue dies.

IV. Experimental designs

The following section contains some experimental results and considerations that may be useful when designing laboratory experiments using botryllid tunicates.

Acclimation to experimental conditions: Some experimental work requires keeping or growing colonies under environmental conditions that differ from those to which the colony would normally be exposed in nature (e.g., temperature or salinity). In these types of experiments it is important to offer an adaptation regime that is compatible with the biological properties of the test animal. When a stepwise acclimation technique is applied, salinity is usually adjusted in 1 to 5‰ steps to allow gradual attainment of osmotic equilibrium (Karpevich 1960). For our experiments, we chose a 2‰ salinity acclimation step and experimentally explored the effects of varying the acclimation rate (i.e., time intervals between salinity adjustments) on post-acclimation survival and growth of young colonies of B. violaceus. Newly established (7-10 day old) colonies, initially maintained in ambient seawater (28.5‰), were gradually brought to experimental salinities (15, 21, 27, 33, and 39‰) in 2‰ steps. Water of various salinities was prepared by addition of freshwater or artificial marine salt Coralife (Energy Savers Unlimited Inc., Carson, USA) to natural seawater. Acclimation rates were 24, 12, 6, 2, and 0 hours per 2‰ salinity step (an acclimation rate of “0” hours means that colonies were immediately exposed to experimental salinities). The experiment combined five levels of salinity and five levels of acclimation rate in a totally-crossed experimental design (25 treatments in total). There were 15 to 18 replicates per treatment, with a total of 431 colonies used for the experiment. For each colony, condition and size (number of zooids) were recorded at the start of the experiment and 15 days after the colony reached its experimental salinity (Figure 6). Statistical analyses were carried out using SYSTAT® Version 11 (SYSTAT Software Inc., Richmond, USA). A two-way ANOVA of the growth data (percent increase in number of zooids from the beginning of the experiment) revealed a significant interaction between the factors salinity and acclimation rate ($F_{16,165} = 1.98$, $P = 0.014$). Therefore, the effects of acclimation rate on colony growth were analysed at each experimental salinity using one-way ANOVAs followed by Tukey’s post-hoc tests. For most salinity levels (15, 21, 27, and 33‰), differences in growth rates among all acclimation rate treatments were insignificant ($P > 0.05$). However, at 39‰ growth was significantly higher when the salinity acclimation rate was ≥12 hours (Figure 6). For subsequent experiments we used an acclimation rate of 12 hours per 2‰ salinity step.

Experimental treatment replicates: In all of our experiments, young colonies of both species, and especially B. schlosseri, demonstrated high growth-rate variability within all treatments (Figures 5, 7). Previous studies also have shown high variability among colonies in several life-history aspects, such as survivorship, growth, longevity, and reproduction (Brunetti et al. 1980; Boyd et al. 1986; Rinkevich and Shapira 1998). Therefore, in order to obtain statistically significant results, sufficient numbers of replicates of experimental treatments is necessary. In certain cases it might be useful to conduct a preliminary growth study first and run a power analysis of the results in order to determine a sufficient number of replicates. For example, for our study on the combined effects of temperature (5, 10, 15, 20, and 25°C) and salinity (14, 20, 26, 32, and 38‰) on survival and growth of B. schlosseri, a minimum of six replicates per treatment were required (based on the results of a power analysis).

Use of colour morphs: Botryllid tunicates are known for their colour polymorphism. They exist in a variety of colour morphs, but all zooids within a colony are always the same colour or combination of colours. Sabbadini (1959) and Sabbadin and Graziani (1967) demonstrated that the presence/absence of orange, blue, and reddish pigment cells in colonies of B. schlosseri were controlled by three independent Mendelian loci, while two other loci controlled the distri-
Figure 6. The effects of salinity acclimation rates on *B. violaceus* at various salinities: A – survivorship at day 15, B – percent growth over 15 d (i.e., percent increase in number of zooids from the beginning of the experiment). *n* = 15-18 and error bars = SE. Treatments within salinities denoted by different lower case letters differ significantly (1-way ANOVAs followed by Tukey’s post-hoc tests, *P*<0.05).

Figure 7. Box plot showing mean colony size of *B. schlosserii* (30 d old) at 25ºC and various salinities. *n* = 24, boxes = middle 50% of data, horizontal lines in boxes = medians, dots = outliers.

bution of nephrocytes around and between the siphons, giving rise to a variety of colour morphs. There currently is no evidence of colour morph-specific differences in life-history patterns of botryllids; in nature, colonies of various colours often occupy the same substratum, growing right next to each other (A. Epelbaum pers. obs.). This indicates that botryllid colour morphs likely exhibit the same physiological characteristics and environmental tolerances and preferences. The pigmentation pattern can thus be used as an experimental marker that allows easy distinguishing among colonies.

Control of environmental parameters: Many types of experiments require precise control of the environmental parameters involved in colony growth, such as temperature, humidity, photoperiod, and light intensity. The use of growth chambers, or incubators, may be considered as it allows consistent reproduction of these parameters throughout the experiment. Growth chambers have been used for culturing a variety of marine organisms, such as larval sea stars and crabs, anemones, and polychaetes (e.g., Watts et al. 1982; Forward 1989; Nii and Muscatine 1997;
Lindsay et al. 2004), as well as tunicates (Grosberg 1988). However, when precise control is important we would recommend that incubators be thoroughly tested for the uniformity of conditions inside the chamber prior to starting culture experiments. Our tests on low-temperature incubators showed that temperature was not consistent throughout the chamber, especially at lower temperature ranges (≤15°C) and when photoperiod control was used. For our culture experiments we used a water-bath system with heating/cooling coils that provided temperature control of ±0.3°C (SD).

Concluding Remarks

Laboratory culture of botryllid tunicates has several important advantages. It allows maintaining genetically defined populations and producing animals or tissues continuously throughout the year for a variety of studies. Laboratory experiments in ecological research provide the means by which cause and effect can be established by allowing precise control of variables and accurate replication of trials. However, life-history traits exhibited by laboratory cultures – including patterns of growth, reproduction, and longevity – may not always reflect the evolutionary or ecological processes that act upon species in nature (Chadwick-Furman and Weissman 1995). For example, life-history patterns in field-grown colonies of *B. schlosseri* in Monterey Bay, California were found to differ from colonies raised in the laboratory: field cohorts exhibited rapid growth, short and intense reproduction, short lifespan, and senescence soon after reaching maturity, while colonies raised in the laboratory exhibited slow growth or shrank over many months, ceased reproduction long before death, and lived for more than two years (Boyd et al. 1986, Rinkevich and Weissman 1987). Similar life-history differences were observed between field- and laboratory-raised colonies in Mediterranean populations (Brunetti 1974; Brunetti and Copello 1978).

Several factors may be responsible for these differences. Under laboratory conditions water motion is slower and food might be less varied than in the field (Milkman 1967; Brunetti and Copello 1978). In addition, the absence of natural grazers in the laboratory may lead to the formation of a fouling film (Chadwick-Furman and Weissman 1995, A. Epelbaum pers. obs.). We observed juvenile colonies of *B. schlosseri* and *B. violaceus* growing away from algal-covered areas of the slides (Figure 3), indicating that fouling may inhibit colony growth. More studies comparing growth of field- and laboratory-raised botryllid colonies would be valuable. At present, growth of laboratory-raised colonies should be interpreted with caution as in some cases it might not directly reflect growth rates and survivorship observed in nature, but rather reveal the physiological capabilities of the species. Laboratory studies are still a useful means for testing environmental limits of species in a replicated controlled environment.

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