A Comparative Study of Botryllid Tunicates Regeneration in the San Francisco Bay

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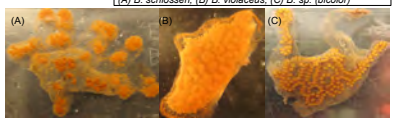
Abstract

Regeneration of body parts is a remarkable phenomenon that is shared by phylogenetically diverse organisms, including some chordates. Lizards can regenerate their tails and sea stars their arms when injured by predators. Humans can even regenerate small portions of the liver. However, colonial botryllid tunicates, basal chordates, have the incredible ability to regenerate the entire functioning body (zooids) from only a small sample of vasculature in a process called vascular budding.

Figure 1: Three species used in regeneration experiments: *(A) B. schlosseri, (B) B. violaceus, (C) B. sp. (bicolor)*



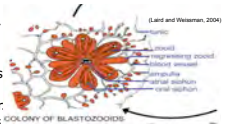
Regeneration processes for various botryllid species show some characteristic features in laboratory studies using application of retinoic acid as a developmental initiator. The three botryllid species, *Botrylloides violaceus (n = 6), Botrylloides sp. (bicolor)(n = 4), and Botryllus schlosseri (n = 3)* were collected from the San Francisco Bay, and settled onto glass plates in the laboratory. Zooids were removed and the remaining vasculature (ampullae and connecting vessels) were cut into fragments (mean = 9/ genotype) and observed for 8 - 14 days. Retinoic Acid (RA), a natural product of Vitamin A that accelerates regeneration (in botryllids, Rinkevich, 2007) was also used as a variable in this study to test regeneration ability with decreased artificial RA exposure. Regeneration in the field was also tested by deploying vascular fragments at a marina in Fort Baker after 2 day laboratory RA exposure.

The purpose of this study is to characterize and compare regeneration variation between botryllid species under standardized conditions and to determine methods for studying regeneration in the field.

Introduction

Tunicate regeneration results in the growth of an entire functioning body from a small sample of the blood. When the zooid, the part of the animal that contains the heart, feeding and excretion system and gonads, are removed, all that remains is the finger-like ampullae which attach the animal to a substrate along with the vasculature that connects them all within a clear tunic. After several days of rearrangement of the ampullae and vasculature, new buds appear which in turn grow into a new, fully functioning zooid.

Colonial tunicates are filter feeding marine invertebrates that attach to substrates such as rocks dock sides and boats that are submerged in seawater. They produce new colonies through sexual reproduction which results in a mobile larvae. This larvae has several embryological features which place them in the phylum Chordata as human's closest living invertebrate relatives.



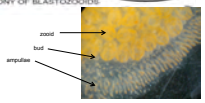


Figure 2 & 3. Anatomy of *B. schlosseri*

These features include a notochord, hollow dorsal nerve cord, and pharyngeal gill slits, however the first two are reabsorbed upon settlement and metamorphosis into the sessile adult form. At this stage, the tunicate asexually buds numerous identical buds within the same colony, meaning that all parts of a colony are genetically identical.

While regeneration has been shown to occur in laboratory settings with the application of retinoic acid in several botryllid species in separate studies, there is little information about the role of regeneration in wild populations. Here, we focus on comparing regeneration in three species using conditions more similar to conditions outside the lab. Ampullae from field collected colonies were surgically separated from adult colonies and allowed to regenerate in laboratory and field settings with routine, low, and zero levels of RA exposure.

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Methods

Regeneration in the laboratory: Samples were taken from tunicates found on various docks of marinas in the San Francisco Bay. Tunicates were settled on glass slides immediately and stored in a tank with bay water circulation. The zooids were cut from the vascular ampullae in fragments which ranged from 2mm – 7mm in length, 2-10 d after settlement within a span of 7 days. Species used in the experiment include *B. violaceus* (n=6 samples, n = 42 fragments), *B. sp. Bicolored* (n = 4 samples, n = 52 fragments), and *B. schlosseri* (n=3 samples, n = 25 fragments). Samples were placed into 10 gallon tanks with bay water which was changed out every other day. Salinity was maintained between 32 - 33 ppt. A chiller was placed in the water surrounding the tanks maintained the at 18°C.

Sample tunicates were exposed with RA (0.1 mM in aliquots of 100µL) for various lengths of time. Four samples were treated for 1 day, while six samples were exposed for 7 - 10 days (note: RA was added each time the water was changed from the tank) and two samples served as a control where no RA was added. The tanks with RA were covered in black plastic in order to protect the RA from degrading from UV light.

Pictures were taken of each section using a dissecting microscope and a Fire-i camera in order to observe the process of regeneration over the span of 8 – 14 d. Each day it was noted which stage of regeneration each fragment was in each day, any unusual formations, and the status of blood flow.

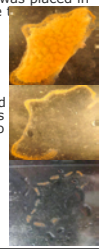

Figure 3. (above) Before (A) and after (B) of removal of ampullae and vasculature from *B. violaceus*. (C) Show sectioning of ampullar regions

Figure 4. (left) Scoring guide for regeneration growth (*B. violaceus*).
(A) Day 1- Post removal of zooids: Ampullae connected by vessels extend out from the tunicate body in an structured manner.
(B) Day 2- Reorganization of vasculature within the tunic
(C) Day 9- Ampullae enlarge. Semitransparent gelatinous tunic matrix indicated growth of bud.
(D) Day 11- Ampullae coalesced and migrate to one area of the tunic, leaving blood and a primary vessel behind.

Regeneration in the field:

In addition, to the lab experiment, additional samples of *B. violaceus* that settled on glass slides in the laboratory were divided into two treatments for a field experiment: those exposed to RA for two days (n = 2) and those that were not exposed to RA (n = 2).

These samples were placed in a device that allows water to flow through it when submerged in the bay. After 6 days of exposure at Fort Baker, the slides were observed and compared with lab samples at Day 8.

Figure 5. Field Device to hold glass slide settled tunicates in a Bay water setting at Fort Baker, CA.

Results

Phase Completion by Botryllid Tunicates

Treatment	Genotype ID (# fragments)	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
B. violaceus (n = 6)						
No RA	T4 (x = 3)	Day 4	0.00%	Days 4-6	Day 6	Day 11
	T5 (x = 11)	Day 5	0.00%	Days 4-10	Day 6-8	Days 7-11
	T8 (Field)			Day 10**		
1 Day RA	T1 (x = 10)	Days 2-4	Days 5-6	Days 5-10	Days 6-7	Days 10-11
	T6 (x = 6)	Day 4	0.00%	Days 5-6	Day 9	TBD*
RA	T7 (Field)			Day 11**		
	T11 (Field)			Day 11**		
	T9 (x = 8)	Day 2	0.00%	Day 3	Day 5	TBD*
	T13 (x = 4)	0.00%	0.00%	Day 3	TBD*	TBD*
Botrylloides sp. (bicolor) (n = 4)						
No RA	T16 (x = 6)	Day 3	0.00%	Day 5	TBD	TBD
1 Day RA	T2 (x = 10)	Day 3-5	Day 3	Day 5-7	Days11-12	Days 11-13
RA	T15 (x = 14)	Day 3	Day 3-4	Day 8	TBD*	TBD*
	T17 (x = 12)	Day 2	0.00%	Day 3-5	TBD*	TBD*
B. schlosseri (n = 3)						
1 Day RA	T3 (x = 4)	Days 2-4	0.00%	Day 5-7	TBD*	Day 10
	T14 (x = 4)	0.00%	100% Day 3	0.00%	TBD*	TBD*
RA	T10 (x = 17)	Day 3	0.00%	Day 3	TBD*	TBD*

Key
100%
76 - 99%
51 – 75%
26 – 50%
1 – 25%
0%
TBD

# of Completed Regeneration Days	
T1-2	13
T3-6	12
T7-10	9
T11-15	8
T16-17	7

Table 1. Representation of phase(s) complete by three botryllid tunicates. Day # indicates first observed characteristic of the described phase. Color indicates percentage of fragments that completed the given stage.
* Indicates samples that have completed necessary phase to generate buds, but have not reached estimated days needed to do so.
**Day indicates phase observed after retrieval from the field.

Conclusion:

Tunicate regeneration is well studied and explored, but the comparison of different species along with its function in wild populations is not well documented. After removing the zooids from the tunicate, regeneration performed in the lab generally matched the regeneration as described in literature (as Rinkevich, 2007). A comparison of regeneration including the exposure to RA is still in progress, so at this point in time no conclusion could be made. It is ideal to observe regeneration in a field setting, in which tunicates could not be constantly exposed to RA. Because progression through the various phases was observed, it is likely that experimentation in a field setting is possible. In addition, samples placed at Fort Baker for six days progressed through typical phases, but potential for bud growth is unknown. Future studies are needed to answer this question, and would be key in studying botryllid tunicates in a more natural setting.

Results

Below is the description of the typical phase completion progress by each of the three studied botryllid tunicates including those found in the field study:

B.sp. (bicolor): Soon after cutting, the ampullae slightly enlarge and rearrange. By day 3 – 4 all ampullae are slightly repositioned toward the center before fully migrating toward the center or a particular region of the matrix (day 5). It typically stays in this condensed form for several days (6-8) before a semi-translucent mass appears underneath the ampullae.

B. schlosseri: Soon after cutting the ampullae condense together in several sections within the tunic. All fragments were cut in sections small in width yet long in length, so it is hard to tell what would occur if the ampullar section was cut in a different format. The ampullae remained fairly unchanged for the remainder of the recorded observations. Ten days was needed for one fragment to regenerate a bud. More time and observations would be needed to determine typical growth.

B. violaceus:
Ampullae may slightly enlarge shortly after separation from the zooid colony, but then spread out and become thin. After 2-5 days ampullae may migrate toward one side of the tunic, in which a bud is likely to develop. Buds may develop for 4-5 days before becoming a fully functional zooid

Field study:
Samples placed out in a device at Fort Baker, CA were checked after 11 days of growth and 6 days of exposure in the field. Samples were coalescing as described in Phase 3, but may have been regressing more than lab samples. At this point it is unknown as to the potential of bud growth, but more research can help answer this question.

References

References:

Developmental Biology, Volume 273, Issue 2 15 September 2004, Pages 185 - 194

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