Developmental Biology 428 (2017) 101–117

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Insights into the unique torpor of *Botrylloides leachi*, a colonial urochordate

Yosef Hyams[a,b,*], Guy Paz[a], Claudette Rabinowitz[a], Baruch Rinkevich[a]

[a] *Israel Oceanographic and Limnological Research, National Institute of Oceanography, P.O. Box 8030, Tel Shikmona, Haifa 31080, Israel*

[b] *Marine Biology Department, Leon H. Charney School of Marine Sciences, University of Haifa, Haifa 31905, Israel*

ARTICLE INFO

Keywords:
Botrylloides leachi
Hibernation
Multinucleate cells
Regeneration
Stem cell

ABSTRACT

Rough environmental conditions make the survival of many multi-cellular organisms almost impossible, enforcing behavioral, morphological, physiological and reproductive rejoinders that can cope with harsh times and hostile environments, frequently through down-regulation of metabolism into basal states of dormancy, or torpor. This study examines one of the most unique torpor strategies seen within the phylum Chordata, exhibited by the colonial urochordate *Botrylloides leachi,* which enters a state of hibernation or aestivation in response to thermal stress, during which all of its functional colonial units (zooids) are entirely absorbed and the colony survives as small remnants of the vasculature, lacking both feeding and reproduction organs. Tissue vestiges then regenerate fully functional colony when re-exposed to milder environmental conditions. The whole metamorphic cycle of hibernation and arousal was studied here and divided into seven major stages, during which the anatomical characteristics of the zooids, the blood cell populations and the expression patterns of some "stem cell" markers were monitored. The first two phases are associated with the shortening of the blastogenic cycles from the typical 7-day cycle to 3–5 day long cycles and with the significant diminution of zooids, leaving a carpet of vasculature. During hibernation this colonial carpet is made of a twisted, opaque and condensed mass of vasculature, loaded with condensed masses of blood cells that possess two types of multicellular structures, the 20–50 µm "morula-like" opaque balls of cells, and small single-layer epithelial spheres, "blastula-like" structures (50–80 µm). Arousal from hibernation starts with the emergence of several clear tunic areas among the vasculature lacunae, which then turn into transparent buds that become progressively larger and opaque. This is followed by sluggish, newfangled cell movement within the vasculature, which increases in intensity and rate over time. A closer examination of the vasculature revealed dramatic vicissitudes in the blood cell constituency as hibernation progressed, which is manifested by the appearance of two novel cell types not recorded in regular colonies, the multinucleate cells (*MNC*) and storage cells, each with 2–3 distinct cell morphs. Using mixtures of pre-labeled where half stained with a florescent marker for membranes and half stained for DNA we recorded within 2–3 days from onset new *MNC* stained by both staining, attesting for the de novo formation of *MNC* through cells fusion.

At the outset of hibernation we documented high expression levels of *PIWI, PL-10* and *PCNA* in cells residing in cell islands (*CIs*), which are the specific stem cell niches found along the endostyle at the ventral side of the zooids. During hibernation, most of the *PIWI*$^+$/ *PL-10*$^+$/*PCNA*$^+$ cells were the *MNCs*, now located in the newly shaped and dilated vasculature, where they increased in numbers. Also, most of the *PCNA*$^+$ cells were identified as *MNCs*. We further documented that the *Bl–PIWI* RNA (*in situ* hybridization) and protein (immunohistochemistry) expressions documented during the hibernation/arousal processes diverged significantly from normal blastogenesis expressions. Counting *PIWI*$^+$ blood cells at various blastogenic stages revealed a significant increase as the hibernation progressed, peaking in aroused colonies at an average of 30 *PIWI*$^+$ cells/ampulla. The *Pl-10* protein expression patterns in the zooids and buds changed as the hibernation progressed, similar to the *PIWI* and *PCNA* expressions. Considering the evolutionary perspectives to hibernation we propose linkages to the disposable-soma theory.

---

[*] Corresponding author at: Israel Oceanographic and Limnological Research, National Institute of Oceanography, P.O. Box 8030, Tel Shikmona, Haifa 31080, Israel.
E-mail addresses: yo-chyams@hotmail.com (Y. Hyams), guy@ocean.org.il (G. Paz), buki@ocean.org.il (B. Rinkevich).

http://dx.doi.org/10.1016/j.ydbio.2017.05.020
Received 20 September 2016; Received in revised form 18 January 2017; Accepted 17 May 2017

1. Introduction

The ability of numerous ectothermic and endothermic vertebrates, as well as many invertebrate taxa to enter prolonged dormancy states (generally termed “torpor”) is well-documented in terrestrial and marine organisms (Cáceres, 1997; Hand and Hardewig, 1996; Storey, 2002; Storey and Storey, 2011). Under torpor conditions, organisms embrace distinct physiological activities that enable them to tolerate harsh environmental conditions by entering periods of metabolic depression, arrested development and phenotypic plasticity, all characteristic of caloric restriction (Storey and Storey, 2010). Some taxa exhibit daily torpor bouts, whereas others display seasonal torpor, termed “hibernation” when occurring during winter, and “aestivation” when occurring during summer (Storey and Storey, 2011). From a phylogenetic perspective, the phenomena of hibernation and aestivation have developed repeatedly in unrelated animal taxa, while each specific hibernation strategy was probably initiated as the compromised interplay of various biological and environmental factors (Morin and Storey, 2009; Storey and Storey, 1996).

Hibernation and aestivation events are also widely recorded in various colonial forms within the subphylum Tunicata, and are commonly associated with natural (biological and environmental) drivers. The list of tunicates that go through hibernation states includes *Clavelina lepadiformis* (De Caralt et al., 2002), species of *Perophora* (Mukai et al., 1983), *Polysyncraton lacazei* (Turon, 1992), *Diazona* and *Aplidium* (Nakauchi, 1982), *Ecteinascidia turbinata* (Carballo, 2000), *Didemnum vexillum* (Valentine et al., 2009), *Pseudodistoma crucisgaster* (Tarjuelo et al., 2004), botryllid ascidians (Bancroft, 1903; Burighel et al., 1976) and more. Most remarkable are the hibernation/aestivation phenomena in *Botrylloides leachi* (Fig. 1a,b), a common Mediterranean sea-squirt species that has spread ubiquitously (Berrill, 1950, described in Rinkevich et al. (1993)). Colonies of this species encrust in the shallow waters on natural and man-made hard substrates like stones, algae, pilings and floats. Each colony is composed of up to thousands of genetically identical modules (zooids; each 2–3 mm in length), which are embedded within a semi-translucent gelatinous organic matrix, the tunic. Zooids are arranged in systems of two parallel, elongated and often serpentine-like rows, with long common cloacal cavities between them. All zooids within a colony are connected to each other via a ramified vasculature from which pear-shaped vascular termini (ampullae) extend towards the colony's margins (Rinkevich et al., 1993). Once the planktonic larva metamorphoses into the founder individual (the oozooid), a colony of multiple zooids develops by repeated cycles (each lasts one week at temperature condition of 20 °C) of budding processes, zooidal development and the resorption of old zooids; an astogenic route called blastogenesis (Manni et al., 2007; Rinkevich et al., 2007b; Fig. 1c). Blastogenesis may be divided into four successive stages (A-D, sensu Mukai and Watanabe, 1976) during which new zooids bud from the thoracic body wall of the oozooid and the subsequent zooids, a phenomenon also called palleal budding (Berrill, 1951). Each blastogenic cycle ends in a massive apoptotic event (Phase D; also called the “takeover” phase) in which parental zooids are resorbed concurrently with the maturation of buds into adult, functional zooids (Ballarin et al., 2008a; Lauzon et al., 1993).

It has been more than a century since the discovery (Bancroft, 1903) that *Botrylloides leachi* colonies have the ability to enter aestivation/hibernation torpor states following an increase/decrease in seawater temperatures. Hibernation/aestivation processes in this species start with an arrested blastogenesis that is followed by the morphological absorption of all functional zooids and all developing buds, regardless of the arrested blastogenesis stage. At the end of this absorption process only diminutive rudiments of the former colonies remain. Colonial rudiments have widened blood vessels (lacunae) loaded with a high concentration of cells, all embedded within tunic fragments (Fig. 1b) that may stay in this dormant state for several months (based on laboratory observations). Following temperature amendment, buds regenerate from the blood vessels and grow into new zooids that form new colonies with a normal life cycle (Bancroft, 1903; Burighel et al., 1976; Rinkevich et al., 1995). Morphological changes and phenotypic plasticity similar to the hibernation/aestivation changes observed in *Botrylloides leachi* were reported in response to antioxidant chemical exposure (Voskoboynik et al., 2001), and were further documented in other botryllid ascidians, for example in the violet tunicate *B. violaceus* (Epelbaum et al., 2009), in *Botryllus schlosseri* (Voskoboynik et al., 2007) and also during allogeneic responses between interacting *B. leachi* genotypes (Rinkevich et al., 1994). Laboratory observations (unpublished) further revealed that *B. leachi* colonies may enter the hibernation state when exposed to general stress conditions, like a harsh cleaning, or when transferred from the field to the laboratory. In other botryllid ascidians, like *Botryllus schlosseri*, a ‘light’ hibernation state has been recorded in which colonies slumber in a relatively inactive condition, in some cases with their morphologies reducing to a mass of dormant buds, and then when conditions improve they display a vascular budding that generates a new colony (Millar, 1971).

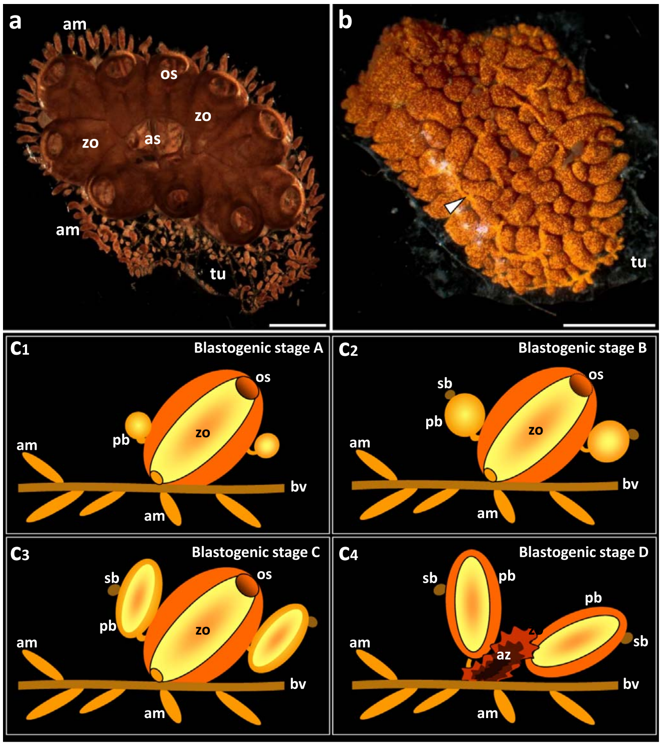

Fig. 1. *Botrylloides leachi* colonial structures. **a.** A colony growing in the laboratory on a glass slide. The zooids are peripherally surrounded by ampullae, the blind termini of blood vessels and loaded with blood cells. Each zooid is approximately 2 mm in length and contains an oral siphon, whereas a system of several zooids shares a common atrial siphon. The whole colony is embedded in a gelatinous matrix - the tunic. Bar = 2.0 mm. **b.** A colony in full hibernation state, following exposure to 15 °C water temperature for 10 days (ambient seawater- 20 °C). The resorbed zooids are replaced by a ‘carpet’ of opaque lacunae and dilated vasculature (arrowhead) loaded with pigment cells. Bar = 1.0 mm. **c.** A schematic illustration of blastogenesis, the cyclical astogeny phenomenon, typical to botryllid ascidians. Each blastogenic cycle is composed of four major stages (A–D) during which new generations of palleal buds (primary and secondary buds) emerge from the body wall of the parental zooids. Thus, a blastogenic cycle starts with the opening of the siphons of new adult zooids and ends in a massive apoptotic and phagocytic event of all the parental zooids (stage D, also called “takeover”), Simultaneous to with the swift development of primary buds into the next generation of the adult (functional) zooid stage. When blastogenic stage D is over, the colony goes back to blastogenic stage A. am = ampulla, az = apoptotic zooid, bv = blood vessel, os = oral siphon, pb = primary bud, sb = secondary bud, zo = zooid.

Cumulatively, the literature (Bancroft, 1903; Burighel et al., 1976; Rinkevich et al., 1993) insinuates that the hibernation/aestivation