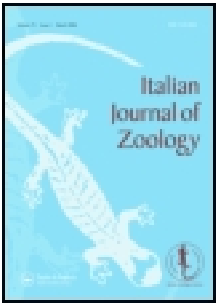

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Hibernation of the Colonial Ascidian Botrylloides Leachi (Savigny): Histological Observations

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HIBERNATION OF THE COLONIAL ASCIDIAN *BOTRYLLOIDES LEACHI* (SAVIGNY): HISTOLOGICAL OBSERVATIONS

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INTRODUCTION

In a previous paper the biological cycle of the colonial ascidian *Botrylloides leachi,* in the Laguna Veneta, was described (BRUNETTI, 1976). Such research indicated that during the winter this ascidian undergoes a process of hibernation, which resembles, in some respects, that described by BANCROFT (1903) as « aestivation » in a colony of *Botrylloides gascoi* (synonymous with *Botrylloides leachi,* see SALFI, 1931).

During hibernation the colony lacks filtering zooids and resembles a homogeneous carpet of ampullae which is usually covered by diatoms and detritus. In spring, with a rise in temperature new zooids are formed, these emerge from the tunic, and gradually reconstruct new « ladder systems ».

Due to the opacity resulting from the high density of pigmented cells, the events occuring within the colony during hibernation and vernal reactivation are not easily observed « in vivo ».

To overcome this difficulty we have performed a histological study on colonies collected at various periods in order to determine the more characteristic features of this process and follow them in greater detail than is possible with living material.

MATERIAL AND METHODS

B. leachi is a colonial ascidian composed of a large number of zooids, arranged in « ladder systems », interconnected by a vascular network that extends through the tunic. This network has numerous branches terminating in ampullae which are particularly abundant near the periphery of the tunic.

In each colony the adult zooids have one or more buds arising from their mantle; these in turn have daughter buds at earlier stages of development. Under normal conditions their development is well controlled and synchronous for all the buds of any particular generation (BERRILL, 1947). Successive « changes of generation » regularly occur in the colony and are characterized by: regression and resorption of all adult zooids; progression to functional maturity of the most developed buds with initiation of filtering activity; production of a new generation of buds which arise from the youngest buds.

The mechanism of development of the buds and the correlation of their stages appears to be similar to that occuring in the closely related genus *Botryllus* (BERRILL, 1947); indeed, it is possible to apply to *B. leachi* the sequence of developmental stages proposed by BERRILL (1941) and modified by SABBADIN (1955 a) for *Botryllus schlosseri.*

Our histological observations were performed using colonies that had developed on asbestos-cement panels, from which we periodically removed fragments from autumn through spring. Some colonies were fixed whole at given intervals.

The samples, fixed in Bouin's fluid or Davidson's fixative (seawater modification of SHAW and BATTLE, 1957), after inclusion in paraffin, were serially sectioned and stained using standard histological techniques.

RESULTS

Initiation of hibernation

The colonies that are entering into hibernation have some features that distinguish them from the colonies living under « normal » condition: reduction in rate of bud development; progressive reduction in the number of filtering zooids and reduction of their sensitivity to mechanical stimuli; increase in the duration of change of generation; and increase in the number of ampullae.

At the histological level it appears that large quantities of food residues are present in the branchial chamber or in the gut of the adults in regression and that, unlike the normal change of generation, the processes of involution involve both the adult zooids and a large number of buds (fig. 1 and 2).

Regression is evidenced by a general contraction of the individual followed by involution of the tissues, with involvement of a progressively larger number of macrophages. The latter assume a primary role since they invade the tissues of individuals undergoing regression, resulting in dissociation and eventual phagocytosis of the cells.

The synchronization between the different blastogenic generations is so altered that in a same colony the buds are found to be at various stages of development. With time, the most developed are attacked by macrophages and undergo degeneration.

The very young buds (stages 1-4 of SABBADIN, 1955 a) usually regress with the parent, but sometimes, especially if already detached from it, are capable of surviving (fig. 3).

At the beginning of hibernation the gonads, absent in the adults, are still recognizable within the buds, though poorly developed and undifferentiated. In addition, small oocytes, having evident nuclei and surrounded by follicular cells, are recognizable in the blood lacunae of the zooids and in the vascular network of the tunic, mixed with the hemocytes (fig. 4).

The histological preparations nicely document the progressive enlargement and increase in number of the ampullae that evaginate from the walls of the tunic vessels. The ampullae are roundish, delimited by a thin layer of cells and contain various types of blood cells, predominantly large macrophages.

The hibernating colony

As hibernation progresses the involution extends to almost all the zooids that are then eventually reabsorbed by the colony. The hibernating colony appears *in vivo* to be composed of a thin layer of tunic, completely filled with roundish ampullae containing a large number of blood cells. Only in sections it is possible to recognize rare regressing zooids contained in ampullae whose walls correspond to the original epidermis of the zooids and are in continuity with the remaining circulatory system of the tunic (fig. 5 and 6). The ampullae contain a diffuse and heterogeneous material in which are entangled blood cells (fig. 7), predominantly macrophages, and a few oocytes. Most macrophages have a few large heterophagous vacuoles, but some contain a single large vacuole in which undigested whole cells can be recognized.

A very characteristic aspect of the hibernating colony consists in the presence of small roundish vesicles and clumps of undifferentiated cells, which are scattered among the hemocytes. They are contained in ampullae, in tunic vessels or in residual lacunae of zooids, isolated or in groups of two to four (fig. 6, 8-10). These structures, mostly vesicular in appearance, represent bud primordia. Their various aspect might represent different stages of bud development, the possible sequence of which is shown in figs 11 to 13, beginning from aggregates of cells characterized by large roundish nuclei with prominent nucleoli and with relatively thin basophilic cytoplasm.

In hibernating colonies the buds appear to cease development at the vesicle stage before beginning organogenesis. There are present two types of vesicular buds: 1) hollow spheres with homogeneous single- or multi-layered walls (fig. 8); 2) vesicles with one considerably thicker pole having a single- or multilayered epithelium which gradually becomes thinner toward the opposite pole; the thickened area is always oriented toward the vessel wall (fig. 10 and 13). In neither type of bud gonad blastemata are recognizable, nor do migrating oocytes tend to accumulate against its walls.

Reactivation of colonies

At the time of vernal warming of the water the hibernating colonies of *B. leachi* initiate the formation of a few zooids that arise from the tunic and become organized into small systems.

At first, in the colony still filled with ampullae, are found occasional individuals (fig. 14); some are in regression, whereas others, apparently in good conditions, do not show infiltration by macrophages. They are in various stages of development and seem to originate from the small buds observed in the hibernating colonies. In fact, some of the quiescent buds initiate the formation of organ anlagen (branchial and peribranchial chambers, gut, etc.) (fig. 15). When the anlagen of the majority of the organs have been formed, daughter buds are produced by palleal budding, and at the same time the production of buds of different origin is interrupted. Not all buds present in the hibernating colony develop; most of them, in fact, regress and are resorbed. Thus in the reactivated colony only a few buds reach the adult stage in spite of their initial large number.

The general blood circulation, at first very slow, becomes considerably more rapid. The macrophages progressively decrease in number, while the reserve material they contained is made available for the reconstruction of the colony.

Some of the ampullae are conspicuous for the absence of the internal heterogeneous matte and for the presence, toward the free margin of the mantle, of a thickened region (fig. 16). This consists of high cylindrical cells with round nuclei and mucous secretory granules, both PAS and alcian blue positive, crowded near the apex. These growing ampullae emerge from the old tunic and, apparently by means of their secretion, adhere to the substrate thus permitting the expansion of the colony. This coincides with the phase of intense colonial growth, that probably at first depends on the reserve material released by the macrophages and only at a latter time is supported by the food gathered by new zooids. The oocytes migrating in the hibernating colony, now settle in the gonads of the new buds and resume their development, interrupted at the initiation of hibernation.

DISCUSSION

The process of hibernation of colonies of *B. leachi,* as has been noted in the Laguna Veneta (BRUNETTI, 1976), appears to have no previous observations with respect to its ecological aspects. At the morphological level, however, it seems to correspond in several ways with the phenomenon observed in aestivating colonies (BANCROFT, 1903), or in colonies held in the dark (PIZON, 1899) or subjected to some experimental manipulations (unpubl. obser.).

The hypothesis, proposed by several authors (CAULLERY, 1895; BANCROFT, 1903; SALFI, 1928) concerning the similarity of aestivation and hibernation in ascidians is so confirmed by the numerous histophysiological similarities that the colonies of *Botrylloides* display under different unfavourable conditions.

Generally, in ascidians hibernation and aestivation progress by successive steps of regression that are obviously related to structural characteristics of individuals (see BERRILL, 1951). In the case of *B. leachi,* which has a highly organized ripetitive structure (i.e. numerous perfectly coordinated zooids), the steps of the hibernation can be thus outlined:

1 - « *normal condition* »: correlation in developmental stages between filtering zooids and the buds; rare macrophages; gonads developed;

2 - *beginning of hibernation*: regression of the adults and progressive reduction in number of buds entering the filtering phase; loss of correlation in developmental stages; increase in number of ampullae and macrophages; gonads undifferentiated; numerous migrating oocytes; fast blood circulation;

3 - *hibernating colony*: filtering zooids absent; regression of most palleal buds; new production of vascular buds; great number of macrophages; gonads absent; migrating oocytes; slow blood circulation;

4 - *reactivation of the colony*: development of some buds into filtering zooids; stop of vascular budding; decrease in number of macrophages; settling of the migrating oocytes in the developing gonads; fast blood circulation.

In the case of only slightly unfavourable conditions (e.g. mild winters) the process can stop at different levels and the colony can thus regain the normal state by maturation of those zooids that have survived.

In its early phases the process of hibernation apparently corresponds to the normal « change of generation ». In both cases the colony, by eliminating its filtering zooids, drastically reduces its exchange with the environment and at the same time recovers the materials produced by the degeneration of the adults. In the normal « change of generation » in Botryllids, according to BERRILL (1951) and SABBADIN (1958) the adults regress since they are in an unfavourable competitive position for nutrients in comparison with their daughter buds that are in a phase of maximum growth.

In the case of aestivation or hibernation it seems that the phenomenon can not be explained completely in terms of insufficient nutrition, nor even in terms of senescence as suggested by BANCROFT (1903).

We believe instead that hibernation represents a direct response to various unfavourable environmental fluctuations and that the characteristics of the phenomenon consists in the capacity of the stressed ascidian to regress to a general state of undifferentiation. In this state numerous bud primordia are produced by means of budding modalities which may be also different from the normal palleal budding. Only a few of these bud primordia will develop into zooids during the spring revival, apparently in accord with a competitive mechanism like that demonstrated for the buds of other Botryllidae (OKA and WATANABE, 1957, 1959; SABBADIN, 1958).

As regards the small buds present in the aestivating colony, BANCROFT holds that these originate by normal palleal budding and after having lost their connection with the parent, migrate into the circulating blood. On the basis of our histological observations, we believe that this may indeed sometimes occur. In fact, we have found young buds, recently segregated, that are able to survive their regressed parents. It can not be excluded that these buds, carried away by the circulation, reach other sites where they eventually become implanted. However, in the colonies having very few zooids, the histological pictures, as well as the great increase in number of bud primordia, are a strong argument in favour of their vascular origin. Such vascular buds seem to form according to this sequence: a group of hemocytes aggregates on the wall of a vessel or ampulla, in the contact zone the epithelium becomes higher in comparison with the adjacent pavement type; next the hemoblasts become organized into a vesicle and the wall of the vessel evaginates to form a pocket in which the enlarging vesicle is nested.

The initial hemocytes are undifferentiated cells with the same cytological characteristics of the cells that give rise to the vascular buds in other Botryllidae (OKA and WATANABE, 1957, 1959; SABBADIN *et al.*, 1975) and, except for the fact that they are aggregated, they resemble the hemoblasts of *Botryllus* (SABBADIN, 1955 b) and the lymphocytes of other asci-

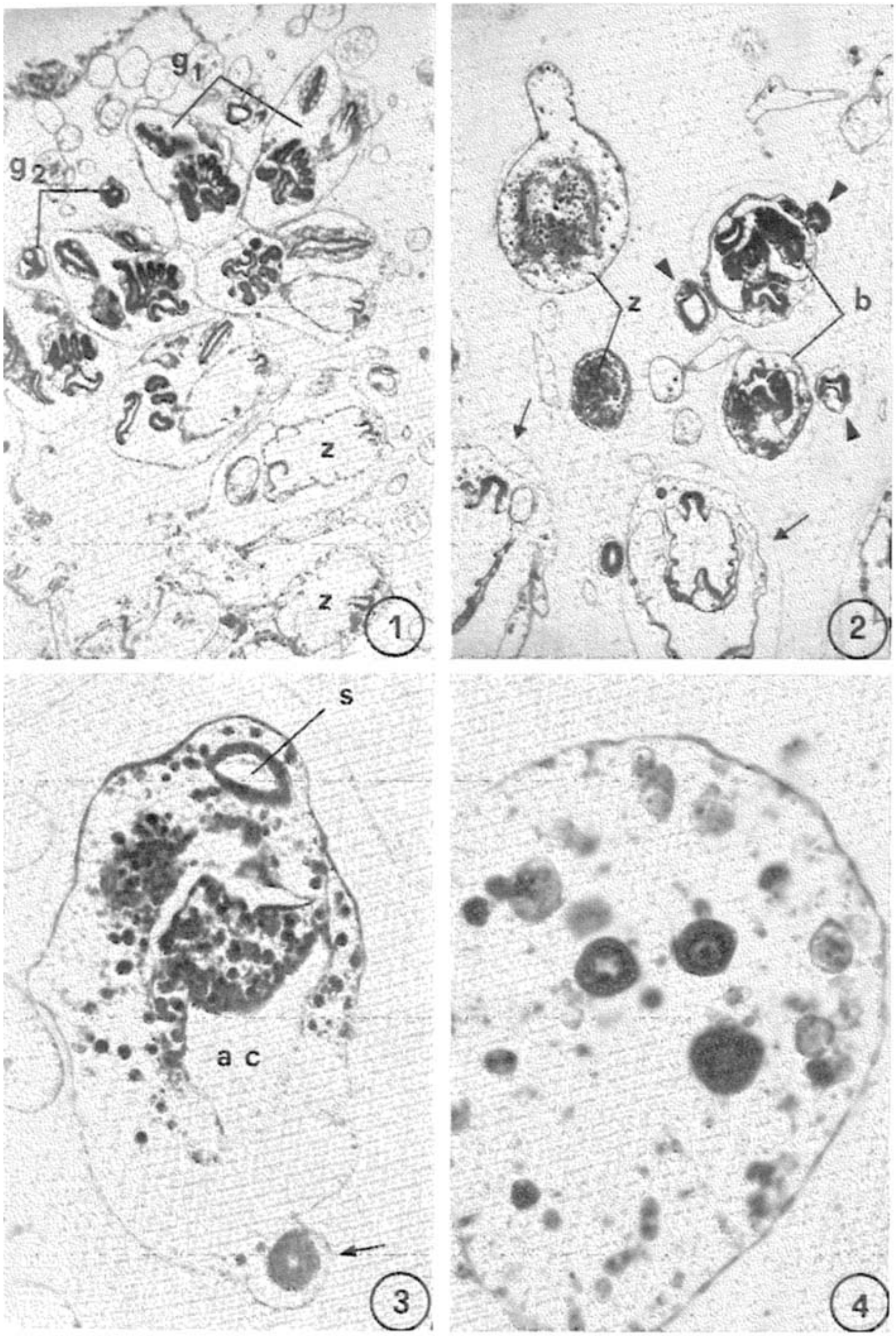
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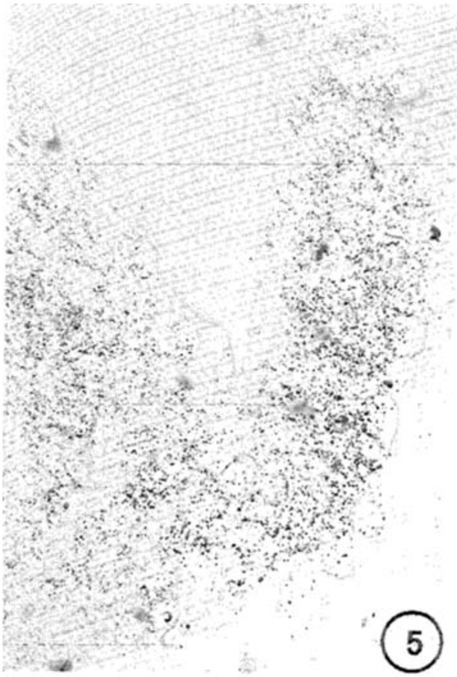
Fig. 1 — Section of a colony of *B. leachi* at the end of autumn. Both adult zooids (z) and the buds (g 1, g 2) appear in good conditions. × 30

Fig. 2 — Detail of a colony in the early phase of hibernation. In addition to remnants of adults that have regressed (z) there are several buds, some of which have been infiltrated by macrophages (b) and others (arrows) that are in good conditions. Young buds of the last generation are also visible (arrowheads). × 75

Fig. 3 — Oblique section of a regressing bud at stage 8 being attacked by macrophages. At the bottom (arrow) the internal vesicle of a young bud appears to be separated from the parent. The atrial chamber (ac) and the oral siphon (s) are also recognizable. × 200

Fig. 4 — Ampulla of a colony at the beginning of hibernation. It is delimited by a squamous epithelium and contains hemocytes. Three oocytes are recognizable among them. × 480



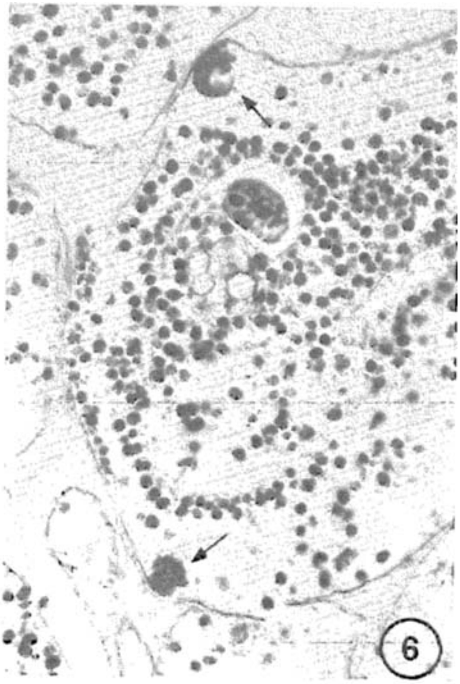


Fig. 5 — Colony of *B. leachi* in an advanced stage of hibernation at low magnification. The colony surrounded by a thin tunic has the aspect of a mass of ampullae filled with macrophages. × 30

Fig. 6 — Detail of colony in fig. 5, showing a regressed zooid in the lacunae of which are recognizable two bud primordia (arrows). An enormous quantity of macrophages is present in the lacunae of the zooid and in the surrounding ampullae. × 200

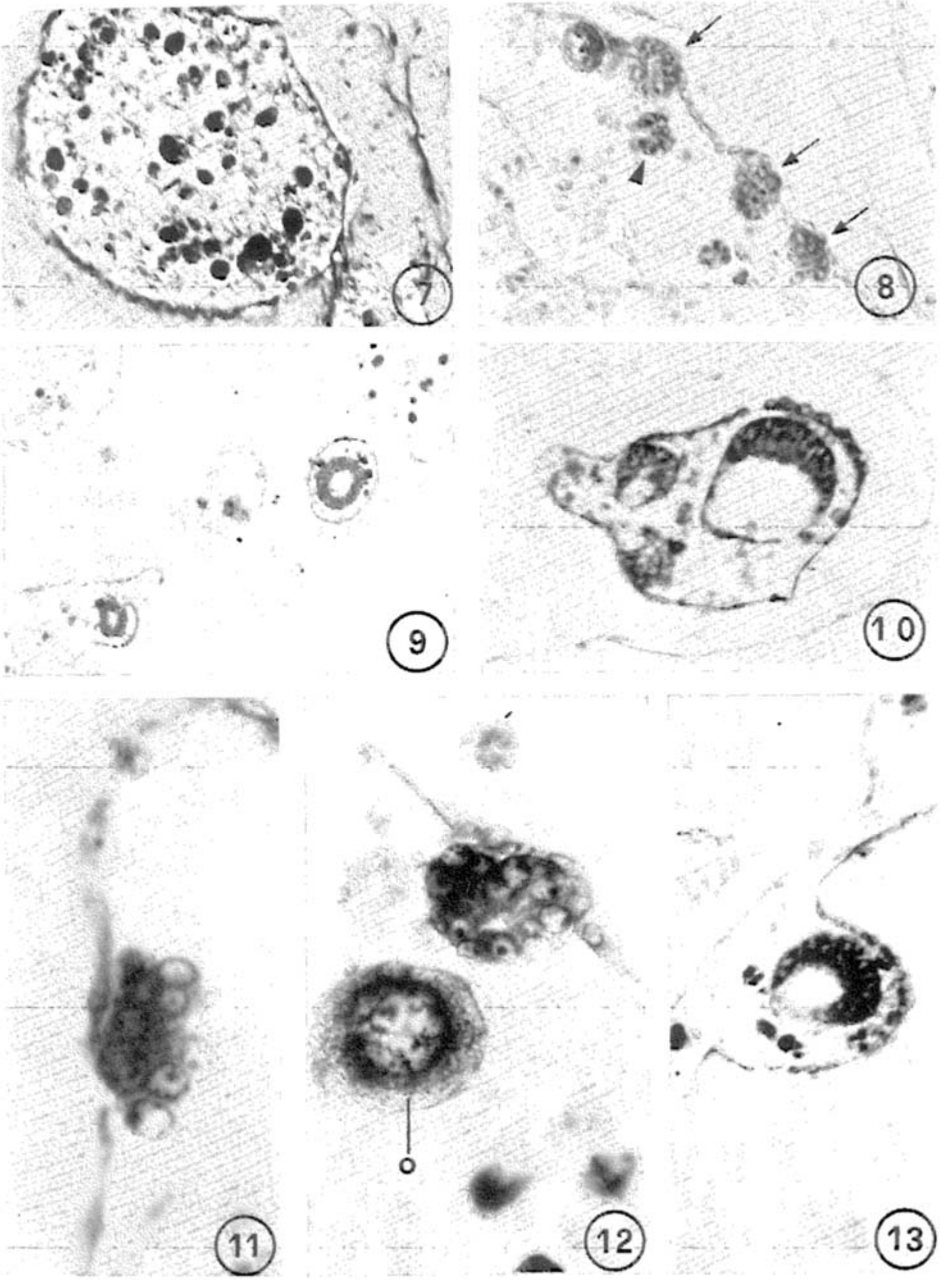

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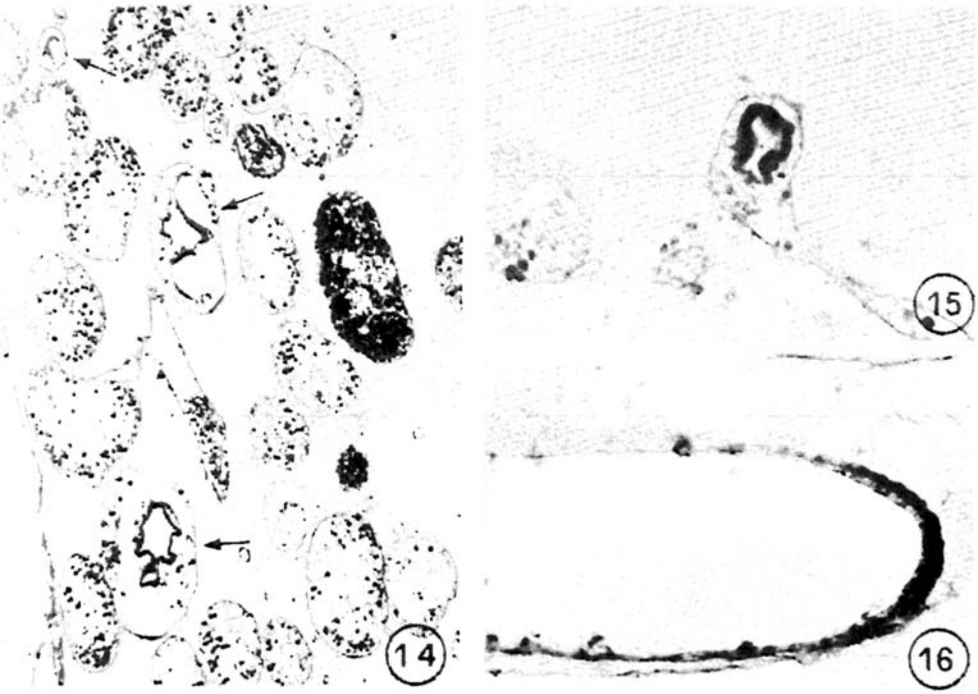
Fig. 7 — Section of ampullae in a hibernating colony showing well developed tenuous internal matte in which are entangled various types of hemocytes. Alcian blue-PAS-haemalun. × 480

Fig. 8 — Section of a hibernating colony of *B. leachi* showing three bud primordia (arrows) attached to the lacuna wall of a regressed zooid. An aggregate of hemocytes is also recognizable (arrowhead). × 480

Figs. 9 and 10 — Photomicrographs of bud primordia with homogeneous walls (fig. 9) or with thickened cap next to the vessel wall (fig. 10) in a hibernating colony of *B. leachi*. In fig. 10 three bud primordia are recognizable in the same pocket of epithelium. Fig. 9, × 200; Fig. 10, × 480

Figs. 11, 12 and 13 — These figures illustrate a sequence of three probable steps in the formation of vascular buds, beginning with an aggregation of hemocytes (fig. 11), which become organized into a vesicle (fig. 12), which is then enclosed in a pocket evaginated from the vessel wall. In fig. 12 and 13 the vessel epithelium appears thickened adjacent to bud primordia. (o = oocyte). Figs. 11 and 12 × 1,200; Fig. 13 × 480



Figs. 14, 15 and 16 — Details of colonies of *B. leachi* in the phase of reactivation at the end of the winter. In fig. 14 among the ampullae filled with macrophages, three isolated buds can be seen (arrows) at various stages of development. In fig. 15 young isolated bud is seen in section; organogenesis has just began in this bud. Fig. 16 shows a longitudinal section of an active ampulla, with a cap of high secretory cells, emerging from the tunic. Note the difference in contents as compared with the ampullae shown in fig. 7 and 14. Fig. 14 and 16 Alcian blue-PAS-haemalun.
Fig. 14 × 75; Fig. 15 × 200; Fig. 16 × 500.

dians that play a prominent role in the processes of asexual reproduction and regeneration (FREEMAN, 1964; BRIEN, 1971).

The possibility of a production of vascular buds in Botryllids, first hypothesized by GIARD (1872) and by HERDMAN (1886), and refuted by BANCROFT in the case of the aestivating colonies of *Botrylloides,* has been more recently demonstrated. In some cases, vascular budding appears under normal conditions together with palleal budding (*Botryllus primigenus,* OKA and WATANABE, 1957) while in other cases (*Botrylloides violaceum,* OKA and WATANABE, 1959 and *Botryllus schlosseri,* MILKMAN, 1967; SABBADIN *et al.,* 1975) it occurs only in colonies lacking zooids. *B. leachi* seems to occupy an intermediate position, inasmuch as the vascular buds appear only under particular natural conditions when, due to hibernation, the number of zooids has been drastically reduced.

In the hibernating colonies of *Botrylloides leachi* we have encountered two forms of vesicular bud primordia: 1) hollow spherical buds with homogeneous wall; 2) vesicular buds that are markedly polarized due to the presence of a thickened cap oriented toward the wall of the vessel. Difficulties of sampling and interpreting material collected under natural conditions do not allow to establish confidently whether these two distinct features are different fuctional phases of the same type of bud or they are features of buds of different origin (i.e. palleal buds that have survived the parents and vascular buds). Experimental investigation on this and other related problems are in progress.

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SUMMARY

Fragments and whole colonies of *B. leachi* collected during different phases of hibernation have been studied histologically. Hibernation occurs in progressive phases and involves: regression of all the adult zooids; loss of coordination of development in the buds; regression of the buds beginning with those most highly developed; production of a large populations of undifferentiated buds. These events are accompanied by a notable development of the circulatory system of the tunic and an enormous increase in the number of macrophages, which also accumulate reserve materials.

In severe winters, the hibernating colonies become reduced to a carpet of ampullae filled with macrophages; among these ampullae are numerous buds that have been blocked at the earliest stages of development. The histological picture suggests a probable

vascular origin for these buds, which begin to form as aggregates of hemocytes. However, it is not possible to exclude that at least some of the buds seen are of palleal origin, these having precociously separated from their parents and thus escaped degeneration.

At the time of vernal reactivation, some of the bud primordia probably selected by competitive mechanism, give rise to the new zooids in the expanding colony.

RIASSUNTO

Frammenti e colonie intere di *B. leachi* raccolte durante le diverse fasi di ibernazione sono state studiate a livello istologico. L'ibernazione si svolge in fasi progressive e comporta: regressione di tutti gli zooidi adulti, perdita della coordinazione tra le gemme e loro regressione a partire dalle più sviluppate, produzione di una numerosa popolazione di nuove gemmule. Questi avvenimenti sono accompagnati da un grande sviluppo del sistema circolatorio della tunica e da un enorme aumento dei macrofagi nei quali vengono accumulate le sostanze di riserva.

In inverni molto rigidi le colonie ibernanti si riducono ad un tappeto di ampolle riempite di macrofagi, nelle quali sono sparse le gemmule ferme ai primissimi stadi di sviluppo. Alla ripresa primaverile alcune di esse, selezionate mediante un probabile meccanismo di competizione si sviluppano in nuovi zooidi. Le immagini istologiche suggeriscono una probabile origine vascolare delle gemmule nelle colonie ibernanti, a partire da aggregati di cellule del sangue; tuttavia non può venir completamente escluso che almeno alcune rappresentino gemme palleali segregatesi molto precocemente e sopravissute al genitore.

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