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Post-larval development of the commercial sponge *Spongia officinalis* L. (Porifera, Demospongiae)

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Abstract

This study investigated the development of the larvae of *Spongia officinalis* in experimental conditions, after settlement on plastic substrates, using electron and light microscopy. The released larvae show a dark pigmented ring distinguishes the posterior larval pole. The youngest larvae, covered with a flagellate epithelium, move onwards by rotating on their longitudinal axis. Over time a creeping-like motion prevails, probably linked to the need for settlement. After a free-swimming period of 24–48 h, larvae settle on the artificial substrate by the anterior pole. At settlement, the flagellate epithelium is substituted by flattened cells, which delimit the outermost surface. Post-larvae were reared to about three months. The early phase of post-larval differentiation shows a solid interior mainly consisting of granular cells varying in shape and size. They are included in a dense collagen matrix that contains a conspicuous amount of bacteria. Lacunae are already evident in the initial phase of metamorphosis. In several of them, cell debris and nucleate cells are visible. This feature is consistent with a progressive reduction of the cell mass (autolysis). Neither choanocyte chambers nor canals differentiate. The morphogenetic process leads to a metamorph only consisting of vacuolated cells and collagen fibrils included in a thin fibrous coat.

Keywords: Porifera; Larvae; Ultrastructure; Metamorphosis

1. Introduction

Sponge reproduction, sexuality, larval development and ensuing release represent research fields useful to understanding how these sessile organisms can colonize different environments and to what extent population genetic structure is affected by dispersal potential (Whalan et al., 2005).

Larval organization has attracted the interest of many researchers aiming to analyse larval development and further metamorphosis, also in consideration of the relationships within Porifera (Boury-Esnault et al., 1999; Leys et al., 2006), and between these basic Metazoa and higher animals (Eerkes-Medrano and Leys, 2006). In addition, data on sequential events occurring in the brief life of a released larva are essential to understand specific ecological needs (Maldonado, 2006) and interpret sponge population distribution (Kaye and Reiswig, 1991).

In this regard, several articles have focused on larval behaviour, settlement and metamorphosis by considering larvae under two different perspectives: (i) to provide a better definition of their architecture by means of ultrastructural analysis (Woollacott, 1990; Boury-Esnault et al., 2003; Maldonado et al., 2003; Ereskovsky and Tokina, 2004; Usher and Ereskovsky, 2005; Eerkes-Medrano and Leys, 2006; Leys and Ereskovsky, 2006; Leys et al., 2006); (ii) to test, in experimental conditions, the larval response to some parameters in order to make predictions on their possible response when exposed to the natural environment (Kaye, 1990; Woollacott and Hadfield, 1996; Maldonado et al., 2003). In addition, the study of sponge larvae represents a source of diagnostic characters at the order level (Ereskovsky and Tokina, 2004).

So far, nine larval types have been identified in Porifera (Maldonado and Bergquist, 2002) even though in demo-

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sponges, a group including the largest taxonomic class, the production of parenchymella larvae represents the most common pattern of development. Parenchymellae are often retained by the parent in a brooding chamber (Kaye, 1990; Woollacott, 1993; Leys and Degnan, 2002; Maldonado et al., 2003; Whalan et al., 2005), a feature that offers a better opportunity to investigate their organization before and after release.

Spongia officinalis L. is a worldwide known sponge, widely utilized for cosmetic purposes. Its over-exploitation and repeated epidemics have caused its disappearance from wide Mediterranean areas (Gaino et al., 1992; Pronzato et al., 1996; Cerrano et al., 2000), thus determining the inclusion of this bath sponge among the marine endangered species requiring specific management measures (Annex 3 of the Bern Convention on the Protection of Wildlife). In this context, knowledge on the reproductive pattern and larval behaviour of this species are essential for understanding the biological and ecological needs of the sponge populations (Kaye and Reiswig, 1991), and may yield information useful for its proper management, here including conservation planes. In this species the progressive development of embryos and larvae was the subject of a previous investigation carried out on post-fertilized eggs up to the newly released parenchymellae (Baldacconi et al., in press).

The present study was undertaken as a second phase of the investigation on the larvae of *S. officinalis* in order to analyse larval behaviour in experimental conditions and the basic anatomy of the post-larvae after their settlement on an artificial substrate. The aim was to add some information to the matrix of knowledge on the metamorphosis of the parenchymella in this economically relevant species.

2. Materials and methods

The current study is mainly based on the swimming behaviour of newly released parenchymellae of *S. officinalis* and on the ultrastructural aspects of their organization during metamorphosis.

2.1. Collection of larvae

Several hundred newly released larvae were collected by SCUBA divers after having removed them from a plastic trap overlapping sponge specimens in the month of their main reproductive period (June) from Ionian coasts (Porto Cesareo (LE) SE Italy—N40°16′312″ E17°51′593″). Larvae were immediately returned to the University of Bari laboratory where they were placed in glass aerated containers with natural filtered sea water. The bottom of the containers was covered with thin plastic fragments (a few millimetres in thickness) to allow free-swimming larvae to settle and attach to themselves. Each container was enriched with natural sea water daily, after having removed a certain quantity of water. This procedure reduced the risk of natural resource deprivation and bacterial pollution due to the experimental conditions. In order to test the exact age of the post-larvae, they were considered actually settled if they did not resume swimming when disturbed by moving the plastic fragments.

2.2. Observation on general swimming patterns

Laboratory observations on the larval movement were detected by viewing larvae in glass Petri dishes filled with filtered seawater collected from the habitat of *S. officinalis*, and maintained at room temperature (20–21 °C). Larval swimming ($n = 30$ specimens) was monitored and recorded with a digital video camera connected to a dissecting and light Leica stereomicroscope.

2.3. Ultrastructural analysis

Post-larvae were both partially detached from the substrate with a razor blade without disruption and partially placed with their substrate into 2.5% glutaraldehyde in artificial sea water used as buffer (pH 7.8) for five hours. For transmission electron microscopy (TEM), samples were rinsed in artificial sea water used as buffer and post-fixed for thirty minutes in osmium tetroxide (1% in sea water). Afterwards they were repeatedly rinsed in the same buffer, dehydrated in a graded ethanol series and embedded in an epon-araldyte mixture. Ultra-thin sections were obtained with an LKB ultratome, contrasted with uranyl acetate and lead citrate, and examined with a Philips EM 208 transmission electron microscope.

Preparation for scanning electron microscopy (SEM) were carried out on the glutaraldehyde fixed material, then dehydrated in a graded ethanol series and critical point dried using a CO_2 Pabish CPD 750 apparatus, mounted on stubs with silver conducting paint, and coated with gold palladium (20 nm) in a Balzers Union Evaporator. Specimens were observed under a Philips XL 30 scanning electron microscope at the accelerating voltage of 18 kV.

3. Results

Brooded larvae are easily detectable inside the mother sponge, where they tend to gather together to form clumps (Fig. 1a). Released larvae (Fig. 1b) show a black pigmented ring located at one of the larval poles, surrounded by long flagella which greatly protrude outside.

The larvae moved up and down in the Petri dish. The swimming takes place by a rotation movement on its longitudinal axis, with the posterior dark-ringed pole closer to the substrate and the anterior one uplifted. Larvae tended to direct towards the shadowed border of the dish where the light was less intense. The locomotion alternates with a short period of non-translocation. At intervals, the larva seems to explore the substrate with its anterior pole and move on fairly parallel to and almost in contact with it. This creeping-like motion becomes more frequent as time goes by and it seems to be linked to the need for settlement.

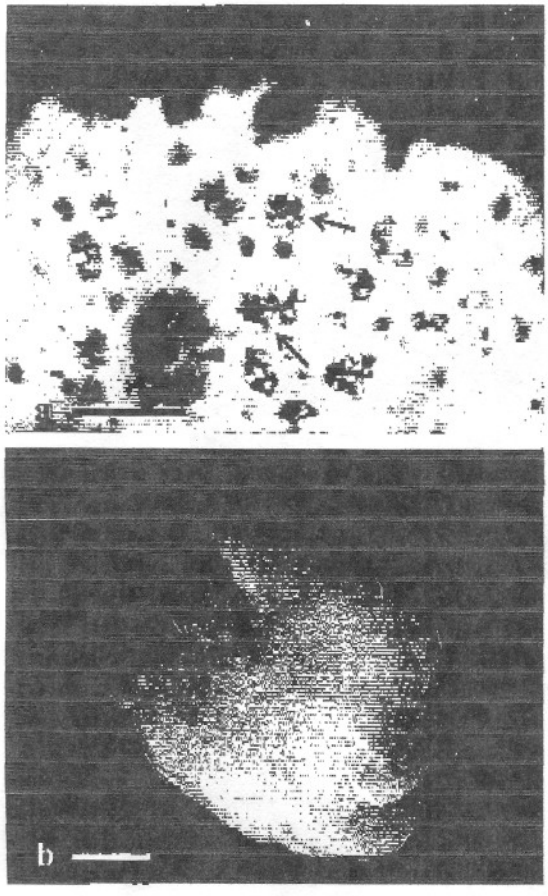

Fig. 1. View of the larvae of *Spongia officinalis*. (a) Brooded parenchymellae gathered within the mother sponge (arrows) (bar: 1 cm). (b) Parenchymella observed under SEM (bar: 100 μm). Note the black pigmented ring at one of the larval poles (arrow).

Larval settlement takes place 24–48 h after release from the parent when larvae rest fairly vertically and show the anterior pole very close to the substrate.

The onset of metamorphosis is marked by the spreading out of the cells of the contact region and the ensuing progressive flattening of the larval body that assumes a round shape in about 6–8 h from the first substrate contact. The main cell mass is centrally located and the peripheral border lacks the typical flagellated cover of the free-swimming larva, and gradually extends on the surface.

One-day-old post-larvae, observed under SEM, present a flat shape (Fig. 2a) and adhere to the substrate with their irregular surface (Fig. 2b). Semi-thin sections reveal the presence of a dense population of cells, varying in shape and size (Fig. 2c).

Electron micrographs show that along the border in contact with the substrate (Fig. 2d) a thin fibrous coat covers the post-larva. This coat is occasionally uplifted from the substrate (Fig. 2e), even though the metamorph is stabilized by the outermost fibrils that link it to the plastic substrate (Fig. 2f). Collagen filaments enter the fibrous coat (Fig. 2g), a feature supporting their involvement in its formation.

The upper surface of the post-larva is delimited by flat cells that give rise to a uniform layer (Fig. 3a). Some SEM images show that, at this stage of metamorphosis, the metamorph possesses short uplifts scattered on the surface and layered over collagen filaments (Fig. 3b).

Transmission electron micrographs confirm the occurrence of epithelial exopinacocyte-like cells delimiting the outermost sponge surface (Fig. 3c). Cavities are already evident beneath this epithelial layer interspersed among cells whose cytoplasm is filled up by electron-dense granules (Fig. 3c). These cells constitute a rich population and vary in size, shape and granular content. Some cells show intracytoplasmic vacuoles (Fig. 3d). Moving towards the central region of the post-larvae, the lacunae become more numerous and are scattered among the cells that are here present with high density (Fig. 3e). Symbiotic bacteria fill the collagen matrix (Fig. 3f).

In *five-day-old post-larvae*, semi-thin sections show a certain regionalisation of the cells that have a different morphology and arrangement in relation to their position in the metamorphosing larva. Some regions consist of cells elongated in shape and arranged in rows (Fig. 4a), whereas in others the cells have a round-shaped morphology (Fig. 4a). The collagen matrix below the epithelial layer is crossed by cytoplasmic extensions of the granular cells (Fig. 4b), which with the symbiotic bacteria form the main component of the metamorph (Fig. 4c). On occasion, cells with a large nucleus and electron-translucent inclusions are associated with the granular ones (Fig. 4d). Their peripheral border shows short cytoplasmic protrusions, a feature consistent with motile activity. Larger-sized lacunae persist, bordered by bacteria and cells. Likewise the youngest post-larvae, cells with large intra-cytoplasmic vacuoles persist (Fig. 4e).

The main aspect evident in *nine-day-old post-larvae* is the increasing number of lacunae (Fig. 5a), occasionally delimited by cells arranged along their border, as evident in semi-thin sections (Fig. 5a). Transmission electron microscopy shows the presence of cell debris inside these lacunae (Fig. 5b), along with nucleate cells with no apparent signs of degeneration (Fig. 5c).

The same feature is evident in *15-day-old post-larvae* where the dense cellular mass (Fig. 5d) results from granular cells (Fig. 5e). In this phase of development, lacunae appear delimited by a denser fibrillar ring, and include dotted material and nucleolate cells (Fig. 5f).

Moving towards the central region, the size and the number of lacunae tend to increase. Their dotted content can be associated with cell debris (Fig. 6a). In proximity of one of these lacunae, two adjacent cells show the presence of long cytoplasmic extensions emerging from their apical region (Fig. 6b). These extensions cross the collagen matrix and for half of their length are located just above the cavity border.

No further differentiation was observed in the reared post-larvae, and about three months (100 *days*) after their

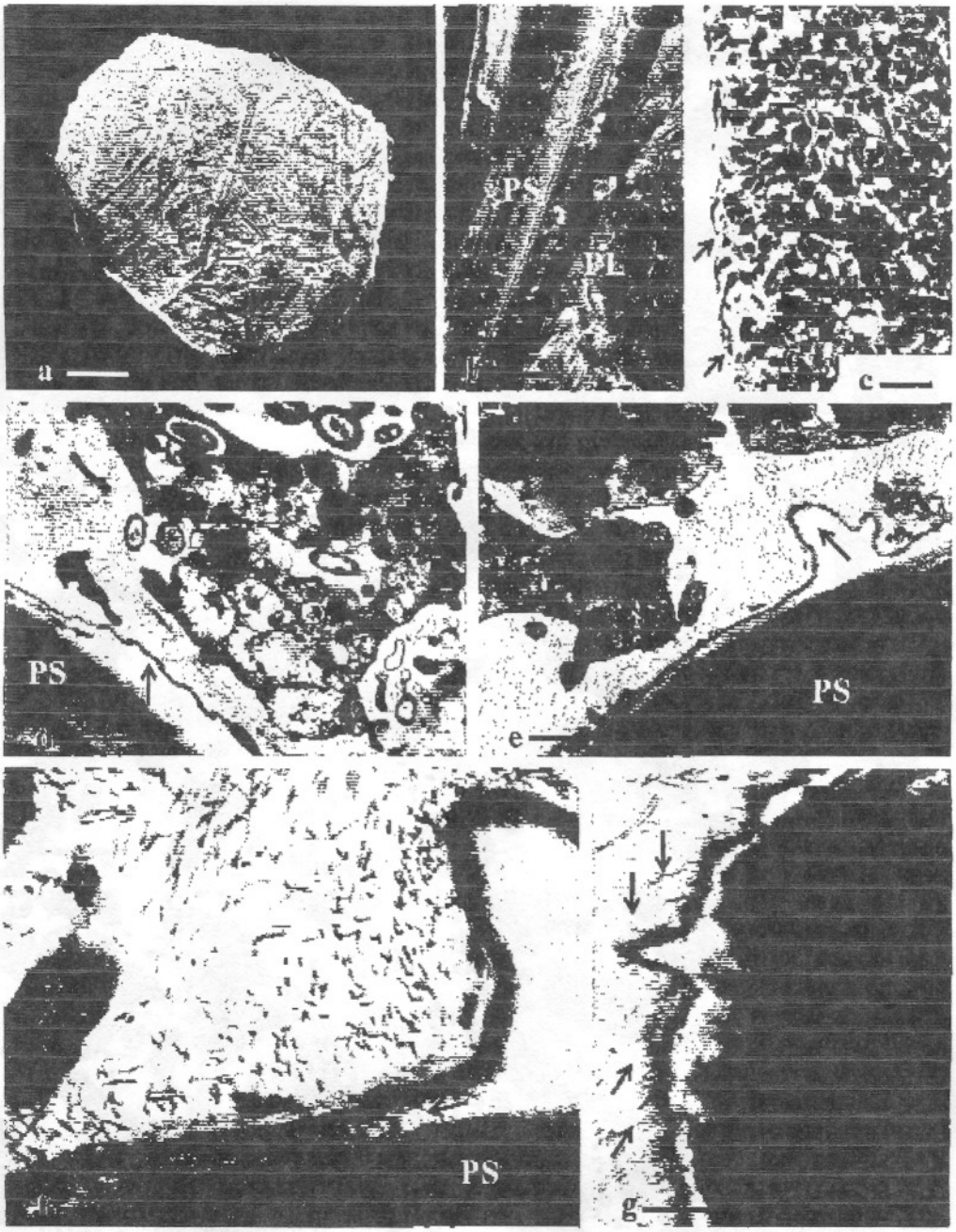

Fig. 2. Larval settlement. (a) SEM view of a one-day-old settled larva (bar: 100 μm). (b) The interface between parenchymella larva (PL) and the plastic substrate (PS) (bar: 20 μm). (c) Semi-thin section showing the upper settler surface (arrows) and the dense cell component (bar: 20 μm). (d) Electron micrograph showing the settler border (arrow) in contact with the plastic substrate (PS) (bar: 2 μm). (e) The settler border (arrow) uplifts from the plastic substrate (PS) (bar: 1 μm). (f) The metamorph is linked to the plastic substrate by outermost fibrils (arrow) (bar: 0.4 μm). (g) Collagen filaments enter the fibrous coat (arrows) (bar: 1 μm).

settlement, they are delimited by a thin fibrous coat that substitutes the initial epithelial coat (Fig. 6c). Bacteria tend to accumulate along the external fibrous coat. Cells with electron-translucent vacuoles are scattered in the collagen matrix and are almost the only cell type present (Fig. 6c). Neither choanocyte chambers nor canals differentiate.

4. Discussion

Investigations on the behavioural pattern of sponge larvae are limited to artificial conditions, a feature that may alter the response that larvae could have in the natural environment (Kaye and Reiswig, 1991). Nevertheless, even though a certain caution can be suggested in extending lab-

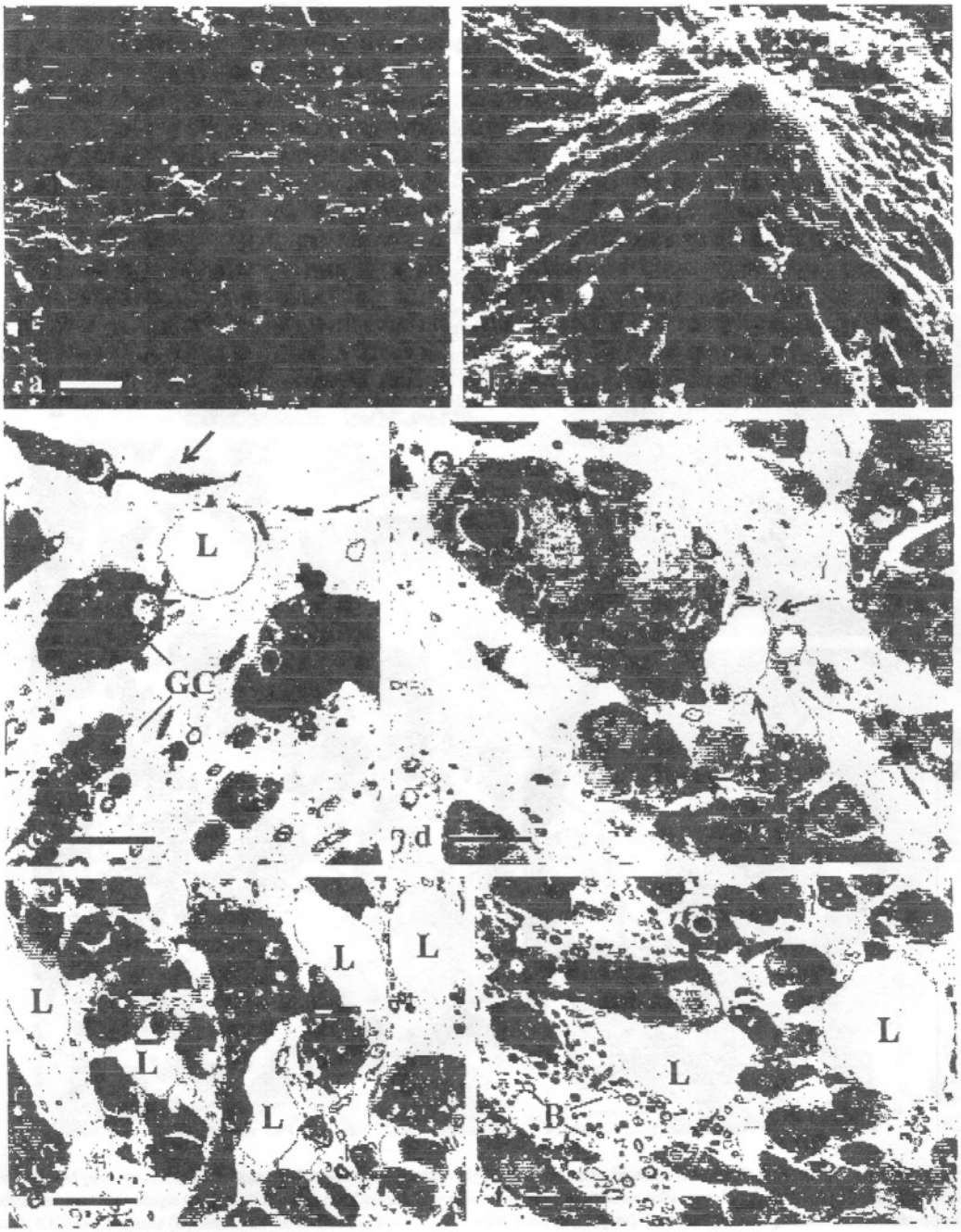

Fig. 3. SEM (a, b) and TEM (c, f) view of one-day-old post-larva. (a) The upper surface by a cover of flat cells (bar: 25 μm). (b) A short uplift delimited by flat cells. Note the collagen filaments underneath (arrows) (bar: 20 μm). (c) Pinacocyte-like cells delimit the outermost surface (arrow). Note the presence of a lacuna (L) beneath the epithelial layer and adjacent to granular cells (GC) (bar: 4 μm). (d) Granular cell showing intra-cytoplasmic vacuoles (arrows) (bar: 4 μm). (e) Several lacunae (L) in the inner post-larval region (bar: 3 μm). (f) Symbiotic bacteria (B) fill the collagen matrix. L, lacunae (bar: 6 μm).

oratory results to the larval metamorphosis taking place in natural conditions, the progressive transformation occurring after settlement provides data useful to understand the larval biology. This is particularly relevant for *S. officinalis*, in consideration of its commercial importance and its drastic decrease and disappearance from large areas (Cerrano et al., 2000). The survival of patched populations (Pronzato et al., 1996) fosters sponge-culture projects to re-populate the spoiled benthic substrata (Corriero et al., 2004; Baldacconi et al., 2006). In this regard, beyond the biological interest that larval swimming behaviour, settlement and the duration of metamorphosis can have, these studies deserve attention

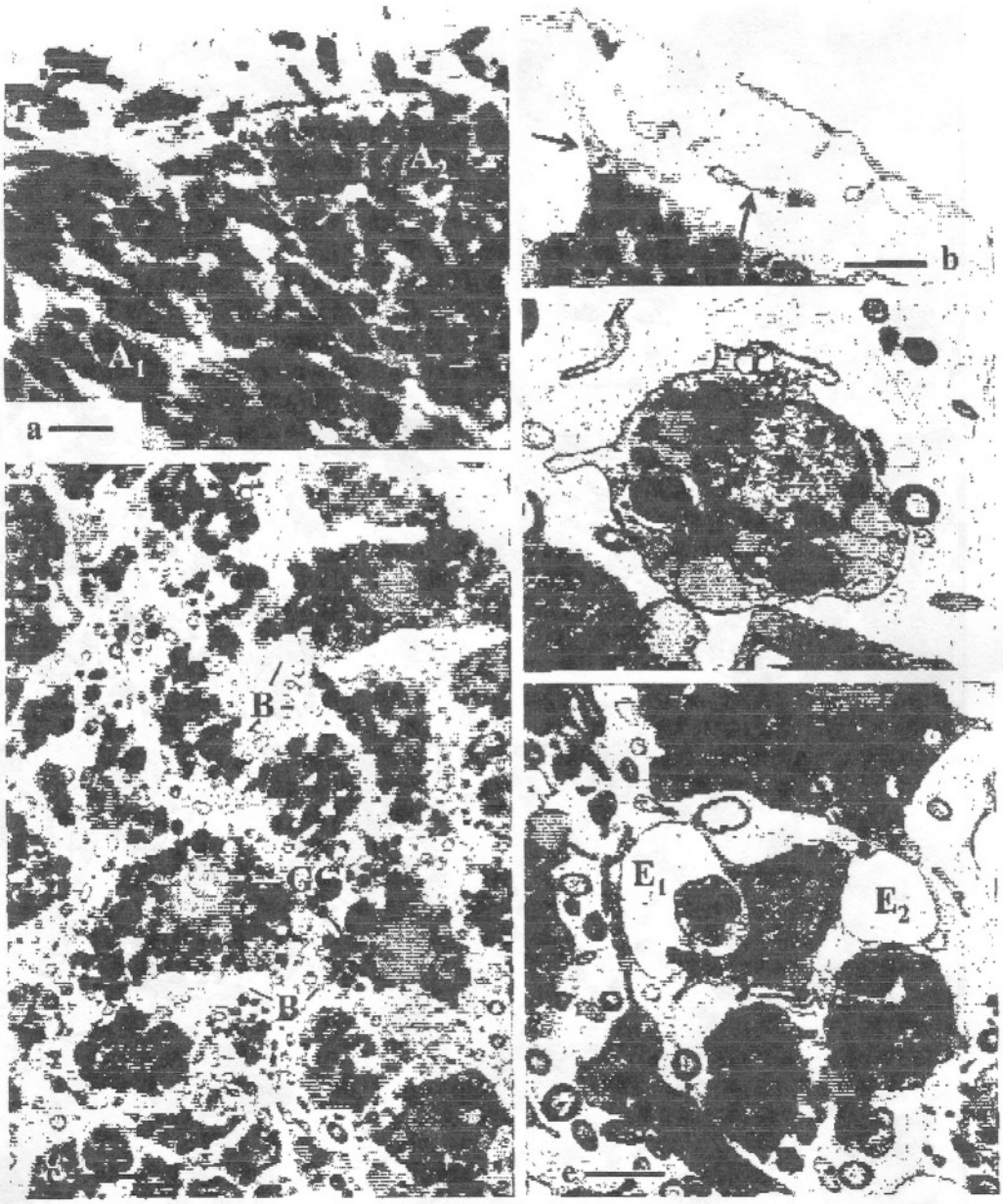

Fig. 4. Five-day-old post-larva. (a) Semi-thin section showing a regionalisation of the cells: in some areas cells are elongated in shape and disposed in row (A_1), in others are round in shape (A_2) (bar: 15 μm). (b) Cytoplasmic extensions of the granular cells crossing the collagen matrix (arrows) (bar: 2 μm). (c) Granular cells (GC) and bacteria (B) constitute the main component of the metamorph (bar: 5 μm). (d) Cell with a large nucleus, electron-translucent inclusions and short cytoplasmic protrusions (bar: 1 μm). (e) Cells with large intra-cytoplasmic vacuoles (E_1; E_2) (bar: 2 μm).

because they supply data that can be used to direct interventions to safeguard biodiversity.

The rotation and creeping behaviour of the free-swimming larvae has been observed in other commercial sponges (Kaye, 1990; Kaye and Reiswig, 1991; Maldonado and Young, 1996). The presence of a pigmented ring has also been observed in an haplosclerid parenchimella (Leys and Degnan, 2002). This feature can be considered a marker allowing the posterior larval pole to be distinguished from the anterior one. As observed in some Haplosclerida and in all the known Dictyoceratida (Ereskovsky and Tokina, 2004; Maldonado, 2006), in *S. officinalis* the posterior region also possesses a distinct ring of long flagella, the tuft. It is thought that the force for forward movement is due to the short flagella of the cells uniformly covering the larval surface whereas directionality is conferred by irregular beating of the long flagella (Leys and Degnan, 2001; Maldonado et al., 2003; Leys and Meech, 2006). Larvae of *S. officinalis* rotate for the

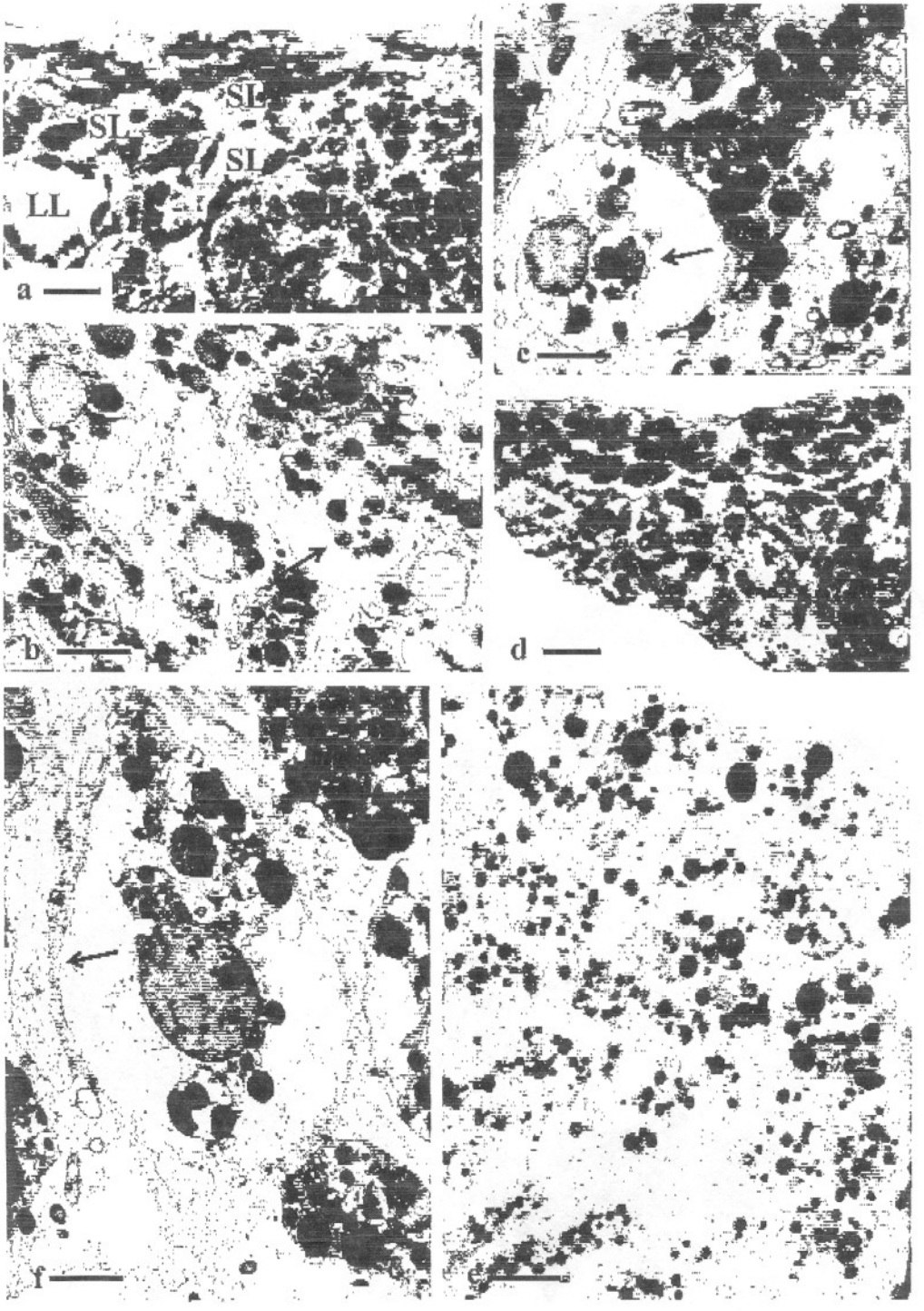

Fig. 5. Nine-day-old (a–c) and 15-day-old (d–e) post larva. (a) Semi-thin section showing a large lacuna (LL) and others smaller (SL) (bar: 20 μm). (b) A vacuole including cell debris (arrow) (bar: 5 μm). (c) A nucleate cell (arrow) inside a lacuna (bar: 2 μm). (d) Semi-thin section showing the dense cell component (bar: 20 μm). (e) Granular cells represent the main cell fraction (bar: 5 μm). (f) Lacuna including dotted material and a nucleolate cell. Note the dense fibrillar ring (arrow) (bar: 2 μm).

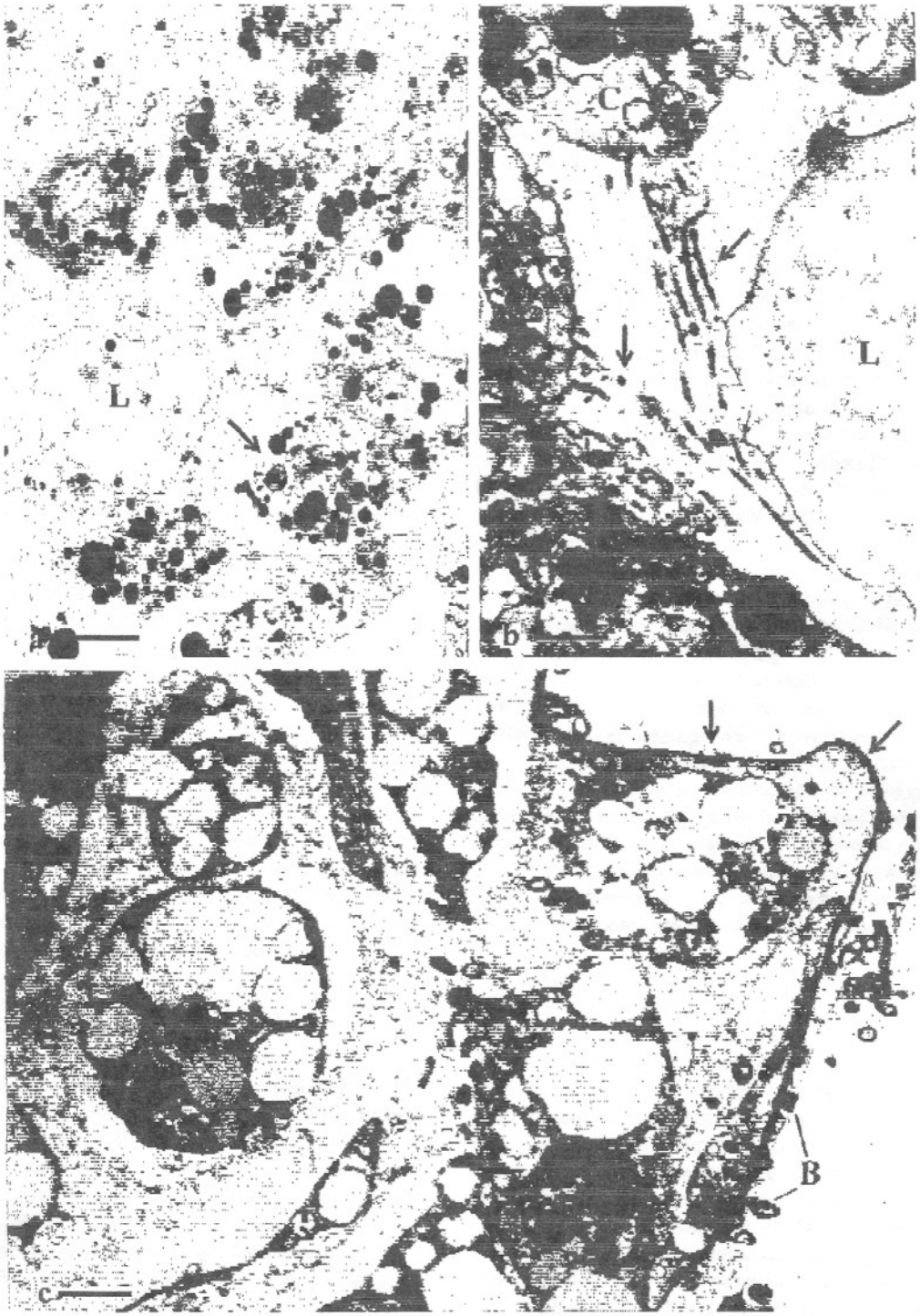

Fig. 6. Fifteen-day-old (a, b) and 100-day-old (c) post-larva. (a) Central region of the metamorph showing an increasing number of lacunae (L) with dotted content and cell debris (arrow) (bar: 4 μm). (b) Two adjacent cells (C_1, C_2) whose long cytoplasmic extensions (arrows) emerge from their apical region. Note their location in proximity to a lacuna (L) (bar: 1 μm). (c) Cells with electron-translucent vacuoles are the prevalent component of the metamorph delimited by a thin fibrous coat (arrows). Note the presence of bacteria (B) along the external fibrous coat (bar: 2 μm).

main swimming period (24–48 h), during which they prefer shaded areas of the Petri dish, thereby suggesting a negative phototaxis, likewise observed in some dictyoceratids (Kaye, 1990; Maldonado et al., 2003; Ereskovsky and Tokina, 2004) and haplosclerids (Leys and Degnan, 2001; Maldonado et al., 2003). In contrast, the larva of *Cacospongia mollior* was described as photopositive upon release, but subsequently shifting to photonegative after a few hours (Maldonado et al., 2003). In the haplosclerid *Reniera* sp. the posterior region is responsible for a coordinated response of the parenchymella to the variations in light intensity (Leys and Meech, 2006). Two distinct rodopsin peaks obtained for *Reniera* sp. are consistent with the involvement of the cells of this region in light perception (Leys and Meech, 2006). It is well known that rhodopsin acts as a photosensitive pigment not only in Metazoa, but also in fungus zoospores (Saranak and Foster, 1997) and green algae (Foster et al., 1984). Maldonado et al. (2003) in their exhaustive investigation on the mechanism by which light affects the beating pattern of parenchymellae, correlate larval phototaxis and ultrastructure by stressing that the pigment-filled protrusions of the tuft cells act as simple eyecup photoreceptors of many lower invertebrates in discriminating the direction of light. The long flagella of some adjacent cells seem to group together to beat as a cohesive unit. This arrangement giving rise to a compound flagella could enhance the beat as a unit.

In *S. officinalis* the larval rotation is gradually substituted by a creeping-like motion, which becomes more and more relevant at the end of the free-swimming period, probably in relation to the ensuing settlement.

In the newly released parenchymella of *S. officinalis* the posterior ring of long-flagellate cells have distal protrusions and numerous electron-lucent vacuoles filled with a homogeneous matrix (Baldacconi et al., in press). Their morphology is similar to that described by Leys and Degnan (2001).

The first adhesion of the parenchymella of *S. officinalis* to the plastic substrate is assured by a groundmat that forms discontinuous adhesion points. The formation of an adhesive lamella secreted by basopinacocytes has been observed in metamorphosing demosponges (Borojevic and Lévi, 1965; Boury-Esnault, 1976; Bergquist and Green, 1977; Evans, 1977; Kaye and Reiswig, 1991).

Additional data in this field could help understanding of the larval ability to interact with the substrate and select it, a process that could be at the basis of the complex mechanism of self/non-self recognition in sponges (see review in Gaino et al., 1999). A collagen layer, giving rise to a narrow spongin layer, has been also observed in the encrusting sponge *Scopalina lophyropoda*, in which the secretion of this matrix by the basal pinacocytes realises a sponge/substrate connection (Maldonado and Uriz, 1999).

The first event in the one-day-old post-larvae is the recession of the flagellate cover, in such a way that an actual flagellate epithelial covering is limited to the brooded and free-swimming larvae (Baldacconi et al., in press), in agreement with previous observations on commercial sponges (Kaye and Reiswig, 1991) and *Reniera* sp. (Leys and Degnan, 2002). The fate of these cells is still controversial but in *Reniera* sp. it was first demonstrated using a fluorescent cell tracker that the outer epithelial cells of the parenchymella transform into the flagellate choanocytes and in an elongated cell type scattered throughout the juvenile sponge (Leys and Degnan, 2002), thereby confirming observations on the poecilosclerid *Hamigera hamigera* (Boury-Esnault, 1976).

In one-day-old post-larvae of *S. officinalis* the outermost surface, opposite the basal one, is delimited by a covering of flat cells whose morphology recalls that of the exopinacocytes. The metamorphosing larvae have a solid central mass mainly constituted of cells with electron-dense inclusions immersed in a collagenous matrix enriched by a marked amount of bacteria. These bacteria, presumably derived from those already present in the free-swimming larva, can contribute to the feeding activity of the metamorph by phagocytosis, a process already evident in the course of larval development and in newly released larvae (Baldacconi et al., in press).

Post-larvae show numerous lacunae both in their superficial and inner core. Likewise observed in other Caribbean commercial sponges (Kaye and Reiswig, 1991), their development beneath the epithelial layer and within the central mass of cells can be regarded as early stages in the formation of the canal system. As the metamorphosis proceeds, cell debris and/or cells appear within these lacunae, thereby suggesting possible autolysis. This basic organization lasts for a long period and remains unchanged in 100-day-old post-larvae, which are the last phase we obtained in experimental conditions. In these specimens, it is interesting to note the occurrence of a uniform thin fibrous coat delimiting the metamorph, which seems to act as a barrier to prevent exogenous bacteria from penetrating inwards. The metamorph seems to turn into a resistant body.

In conclusion, in the larvae of *S. officinalis*, as well as in the above mentioned Caribbean commercial sponges, a completion of metamorphosis was not attained. Presumably, the laboratory conditions could delay activation of the filter-feeding system of post-settlers and affect the process of cell differentiation. On the other hand, according to literature, the time required for the completion of metamorphosis seems to vary considerably (Fell, 1989; Leys and Degnan, 2002). Indeed, whereas in many sponges metamorphosis is completed within about 24 h following larval settlement, sometimes this process proceeds much more slowly. In *Hamigera hamigera* inhalent openings and osculum develop after 9–10 days, but the flagellated chambers do not appear until after one month (Boury-Esnault, 1976).

In sponges, the term "parenchymella" has been attributed to larvae that share only general features. In this regard, some authors use the terminology "parenchymella-type", just to stress the variability of the reference model. Consequently, we cannot exclude that the duration of the morphogenetic processes can differ in "parenchymella" belonging to different groups. In experimental conditions, rapid metamorphosis

is crucial for reducing the risks of contamination and tissue damage, thereby allowing the organisational events leading to a new functional sponge to be traced.

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