

# Cell Proliferation and Apoptosis in Gill Filaments of the Lucinid *Codakia orbiculata* (Montagu, 1808) (Mollusca: Bivalvia) During Bacterial Decolonization and Recolonization

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**ABSTRACT** The shallow-water bivalve *Codakia orbiculata* which harbors gill-endosymbiotic sulfur-oxidizing  $\gamma$ -proteobacteria can lose and acquire its endosymbionts throughout its life. Long-term starvation and recolonization experiments led to changes in the organization of cells in the lateral zone of gill filaments. This plasticity is linked to the presence or absence of gill-endosymbionts. Herein, we propose that this reorganization can be explained by three hypotheses: (a) a variation in the number of bacteriocytes and granule cells due to proliferation or apoptosis processes, (b) a variation of the volume of these two cell types without modification in the number, and (c) a combination of both number and cell volume variation. To test these hypotheses, we analyzed cell reorganization in terms of proliferation and apoptosis in adults submitted to starvation and returned to the field using catalyzed reporter deposition fluorescence in situ hybridization, immunohistochemistry, and structural analyses. We observed that cell and tissue reorganization in gill filaments is due to a variation in cell relative abundance that maybe associated with a variation in cell apparent volume and depends on the environment. In fact, bacteriocytes mostly multiply in freshly collected and newly recolonized individuals, and excess bacteriocytes are eliminated in later recolonization stages. We highlight that host tissue regeneration in gill filaments of this symbiotic bivalve can occur by both replication of existing cells and division of undifferentiated cells localized in tissular bridges, which might be a tissue-specific multipotent stem cell zone. *Microsc. Res. Tech.* 75:1136–1146, 2012. © 2012 Wiley Periodicals, Inc.

## INTRODUCTION

The symbiotic relationship binding marine invertebrates and chemoautotrophic bacteria was first described in hydrothermal vents (Cavanaugh et al., 1981; Felbeck et al., 1981) and later was discovered in various environments such as shallow sheltered sediments (Berg and Alatalo, 1984; Ott et al., 2004) and organic falls (Deming et al., 1997; Feldman et al., 1998; Lorion et al., 2008). These environments are characterized by the presence of reduced chemical compounds such as hydrogen sulfide and a redox potential discontinuity. Sustainability of these symbiotic relations relies on symbiont transmission between generations by: (i) vertical transmission in solemyid (Cary, 1994; Krueger et al., 1996) and vesicomid (Cary and Giovannoni, 1993; Endow and Ohta, 1990) bivalves and (ii) environmental transmission as reported in vestimentiferans, siboglinids (Nussbaumer et al., 2006), and the Lucinidae (Gros et al., 1996, 1998, 2003). In the latter species, environmental transmission has been proposed due to the possible infection of aposymbiotic larvae from free-living symbiosis competent bacteria. This ability to acquire symbionts is not restricted to larval stages but persists throughout the entire life of the bivalve as it has also been described in adult stages (Gros et al., submitted). In the Lucinidae, the intracellular sulfur-oxidizing bacteria are exclusively hosted in the gill filaments. As described for *Codakia orbicularis* (Frenkiel and Mouëza, 1995), gills of

freshly collected individuals of *Codakia orbiculata* consist in an arrangement of filaments organized in three zones (from frontal to abfrontal): (i) the ciliated and (ii) the short intermediary zones are composed of cells totally lacking bacteria, and (iii) the lateral zone which constitutes the main part of the filament. Four cell types are found in the latter zone: intercalary cells, mucocytes, and two main cells. The first one called bacteriocyte is the only one that harbors symbiotic bacteria. It covers the upper two-thirds of the lateral zone. This cell type has been widely described in all chemo-symbiotic bivalves. The second one, mostly localized in the lower third of the lateral zone, is called granule cell. This cell type has been described in *C. orbicularis* (Frenkiel and Mouëza, 1995) and *Divaricella quadrisulcata* (Gros et al., 2000) but is not present in all Lucinidae. It has also been described in Thyasiridae (Passos et al., 2007). Their role remains unknown. Many experiments conducted on Lucinidae have reported that the cellular organization within the lateral zone is highly dynamic (Brissac et al., 2009; Caro et al., 2009; Gros et al., 1998, submitted). In both larval

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and adult stages, cell organization is related to the presence or absence of symbiotic bacteria in the environment of the bivalves. In the larval stages, Gros et al. (1998) have shown that the ability of larvae cultivated in sterile sand to acquire bacteria is strongly related to the development and expansion of bacteriocytes. In other species (vestimentiferans and siboglinids), Nussbaumer et al. (2006) have shown that environmental transmission of symbionts is made through the integument. This ability is exclusively restricted to larval stages and produces changes in tissue organization characterized by massive apoptosis of integument cells to prevent further infection.

In the Lucinidae, long-term starvation experiments (no food, no sulfide added) led on adults induce a strong decrease in symbiotic bacteria, and consequently, produce changes in cell organization in the lateral zone of gill filaments (Brissac et al., 2009; Caro et al., 2009; Gros et al., submitted).

Granule cells become the predominant cell type in the lateral zone and eventually totally replace bacteriocytes. This latter area becomes more voluminous, invades the bacteriocyte area, and finally totally replaces it. Such tissue reorganization also occurs when starved adults are returned to their natural environment and can acquire new bacteria (Gros et al., submitted). In this case, the initial cell organization which involves a dominance of the bacteriocyte area is restored. As it is quite difficult for a differentiated cell to turn into another cell type, it is unlikely that bacteriocytes can become granule cells and vice versa. The ability of larvae to gain symbionts and adults to gain or lose symbionts demonstrates the great plasticity of the lateral zone within the gill filament during the larval development or in adults.

Until now, no studies have elucidated the cellular mechanisms underlying the plasticity of the lateral zone. Herein, we will analyze cell reorganization in adults submitted to starvation experiments and returned to the field. We propose three hypotheses to explain this cell reorganization: (i) a variation in the number of bacteriocytes and granule cells due to proliferation or apoptosis processes, (ii) a variation of the volume (reduction and increase) of both cells without modification in the number, and (iii) a combination of both number and cell volume variation. As measurements of cell size, volume, or number are difficult under light microscope due to the limit of resolution capacity of such microscopes, we here report qualitative changes in the relative size and abundance of different cell types. To show cell proliferation in our experiments, we used both 5-bromo-2'-deoxyuridine (BrdU) techniques and phosphohistone H3 (PH3) detection. To assess the apoptotic processes, we used Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) completed by the detection of caspase 3 (CASP3).

## MATERIALS AND METHODS

### Collection of *C. orbiculata*

*C. orbiculata* (Montagu, 1802) were collected by hand from low-sulfide *Thalassia testudinum* seagrass beds, on îlet Cochon (16°12'53"N; 61°32'05"W) in Guadeloupe (FWI, Caribbean). Individuals caught were between 15 and 22 mm shell length. Twenty freshly

collected bivalves were used for the entire experiment, and four animals were kept for controls (no starvation, no recolonization experiments).

### Starvation Experiment

Sixteen individuals were kept in 50 L plastic tanks containing 0.22 µm-filtered seawater. No food or sulfur was added to the seawater, which was changed every week and continuously oxygenated with aquarium pumps. To inhibit algal development, tanks and shells were washed each week. This starvation lasted 5 months. Among the 24 starved animals, 20 animals were subjected to the recolonization experiment.

### Bacterial Colonization of Starved Individuals

Twenty individuals of the 5-month starved clams ( $T_5$ ) were painted in green before being placed back in their natural environment. Then, four individuals were collected randomly after 4, 8, and 15 days ( $T_{5+4d}$ ,  $T_{5+8d}$ , and  $T_{5+15d}$ ). On each of those days, one individual served as a control, while the three others were incubated in BrdU (Sigma®).

### BrdU Incubation

BrdU is an analogue of thymidine which is incorporated in the DNA of proliferative cells during replication. The presence of this particular base in the newly formed strands of DNA allows its detection by immunohistochemical techniques and is therefore a good marker for cell division.

Three freshly collected individuals ( $T_0$ ), three starved individuals ( $T_5$ ), and three individuals of each period ( $T_{5+4d}$ ,  $T_{5+8d}$ , and  $T_{5+15d}$ ) from the bacteria-recolonizing group were incubated with a BrdU solution at a concentration of 200 µg/mL ( $65 \times 10^{-5}$ M) in seawater for 2 and 6 h before being sacrificed. One control at  $T_0$ , one at  $T_5$ , and one at each delay of recolonization were exclusively incubated in 0.22 µm-filtered seawater.

### Fixation, Dehydration, Embedding, and Sectioning

Gills were fixed in 2% paraformaldehyde in 0.22 µm-filtered seawater at room temperature (RT). After 2 h, they were rinsed three times, before dehydration in successive ethanol baths of increasing concentration. Samples were stored at 4°C in absolute ethanol until embedding. For each sample, the entire gill was embedded in paraffin or polyester wax, except for a small part of the gill (1 mm<sup>2</sup>) which was embedded in LR White resin.

For histology, immunohistochemistry (IHC) and catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) experiments samples were embedded in paraffin or in polyester wax (Steedman, 1957). For polyester wax, dehydrated gills were transferred into baths of: 50% wax–50% absolute ethanol for 2 h, 100% wax for 2 h, and 100% wax overnight. All these steps were performed at 37°C, the melting point of Steedman's wax.

Successive slides were used for different types of staining, to allow direct comparison. Serial, 5 µm thin sections were made with a Leitz® 1516 microtome, and placed on silane-coated glass slides. Before IHC and CARD-FISH, embedded medium was removed by etha-

anol (96%) or toluene, and hydration was done by routine procedures.

For ultrastructural observations, samples were embedded in LR White resin. Dehydrated gills were transferred into 100% LR White resin (two successive 2 h baths, then overnight). All these steps were performed at RT. Polymerization was performed at 48°C in an oxygen free box using generators for culture in jars of anaerobic bacteria (GENbox anaer, Biomerieux®). Semithin sections were made using a Leica® Ultracut E ultramicrotome. The semithin sections were directly stained with toluidine blue in 1% sodium borate buffer.

### Immunohistochemistry (IHC)

**Cell Proliferation Detection.** Cell proliferation detection with anti BrdU (mouse IgG, monoclonal PRB-1; Alexa Fluor 488 conjugate; Molecular probes®) was done as described by Pflugfelder et al. (2009) on Steedman's wax embedded sections. First, incubation in blocking solution was made for 5 h at RT before 20 min digestion in proteinase K (20 µg/mL in Tris-HCl 20 mM). Slides were rinsed with 0.2 N HCl on ice, and then, DNA was denatured with 2 N HCl for 1 h at RT. Slides were rinsed twice with phosphate buffered saline (PBS).

The second incubation in blocking solution was made for 30 min at RT. Then, sections were incubated with anti-BrdU (20-fold dilution factor in blocking solution) at RT for 2–3 h and at 4°C overnight. BrdU exposed samples were compared with nonexposed controls.

Cell mitosis was detecting on Steedman's wax using antiphosphohistone H3 (rabbit IgG polyclonal; Millipore®; diluted 1:200 in blocking solution) for 2 h and a rabbit IgG polyclonal secondary antibody (GenTex®; diluted 1:200 in blocking solution) was applied for 1 h at RT.

**Apoptosis Detection.** TUNEL assay is based on the detection of DNA fragments. It allows visualization of 3'-OH end groups by labeling them with labeled nucleotides (Gavrieli et al., 1992). However, DNA fragmentation is not restricted to apoptosis and can occur in necrosis or DNA damage. For this reason, this technique has to be strengthened by other methods. In healthy cells, the DNase responsible for DNA fragmentation during apoptosis is complexed with its inhibitor (ICAD). During apoptosis, caspase 3 and 7 release CAD, leading to DNA fragmentation (Chang and Yang, 2000).

To highlight apoptosis by TUNEL assay, we used in situ Cell Death Detection Kit, POD (Roche®). Hydrated paraffin sections were digested in Proteinase-K (20 µg/mL in Tris-HCl 20 mM) 20 min at 37°C. Then, they were rinsed twice in PBS for 10 min. The sections were then incubated in 50 µL of TUNEL mixture containing Terminal deoxynucleotidyl transferase (TdT) from calf thymus and dUTP nucleotide mixture in TdT buffer for 60 min at 37°C in a humidified chamber. Positive controls were obtained by incubating sections in DNase I recombinant, grade I (3,000 U/mL–3 U/mL from Roche® in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mg/mL BSA) for 10 min at 15–25°C to induce DNA strand breaks, before labeling procedures. Negative controls were obtained by incubated sections without TdT.

Apoptosis detection was also performed with anti-Caspase 3. Paraffin sections were incubated in blocking solution (PBS containing Bovine Serum Albumin or BSA) for 2 h at RT. Then, slide were covered with a rabbit polyclonal anti-Caspase 3 primary antibody (R&D system®; dilution 1:1,000) for 2 h at RT. Slides were rinsed twice in PBS. As secondary antibody an anti-rabbit IgG polyclonal antibody, horseradish peroxidase conjugate (Stressgen®; dilution 1:200) was used for 1 h at RT.

For all IHC experiments, negative controls were performed by omitting the primary antibody.

Signals were amplified using a buffer containing fluorescein isothiocyanate (FITC) for TUNEL assay, BrdU, CASP3, and phosphohistone 3 detection. Slides were mounted with Cytomation fluorescent mounting medium (Dakocytomation®) and viewed under an epifluorescence microscope, Eclipse 80i (Nikon®).

### Fluorescent In Situ Hybridization Experiments (CARD-FISH)

CARD-FISH was performed according to Pernthaler et al. (2002). Dewaxed and hydrated sections were permeabilized using HCl 0.2 M, Tris-HCl 20 mM at RT, 0.5 µg/mL Proteinase K at 37°C. The endogenous peroxidases were inhibited using HCl 0.01 M at RT. Enzymatic digestions were performed with 10 mg/mL lysozyme at RT for 1 h. Hybridizations were performed using horseradish peroxidase-labeled probes EUB 338III (5'-GCT GCC ACC CGT AGG TGT-3') at 46°C for 3 h. Negative controls were performed by omitting probes. Amplification was done using a buffer containing Alexa fluor 546. Slides were mounted with Cytomation fluorescent mounting medium (Dakocytomation®) and viewed under an epifluorescence microscope, Eclipse 80i (Nikon®).

### Histological Staining

We identified mucosubstances with Trichrome staining (alcian blue, pH 3). Nuclei were stained with Groat's hematoxylin and granule cells were stained with Orange G.

### Transmission Electron Microscopy

Pieces of gills were prefixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer adjusted to 900 mOsM with NaCl and CaCl<sub>2</sub> to improve membrane preservation. After a brief rinse, tissues were stored in the same buffer at 4°C. Gills were dissected, fixed for 45 min at RT in 1% osmium tetroxide in the same buffer, then rinsed in distilled water and postfixated with 2% aqueous uranyl acetate for 1 h before embedding and observation as described previously (Gros et al., 1998).

## RESULTS

### Organization of Gill Filaments

**Freshly Collected Individuals.** Each ctenidium of *C. orbiculata* is constituted of two lamellae, composed of parallel ciliated gill filaments, regularly joined together by tissue bridges (Fig. 1). These tissue bridges are inter-lamellar junctions. Adjacent filaments are fused to form bacteriocyte channels (Fig. 2a) lined with bacteriocytes and intercalary cells as previously described by Distel and Felbeck (1987) in *Lucinoma*

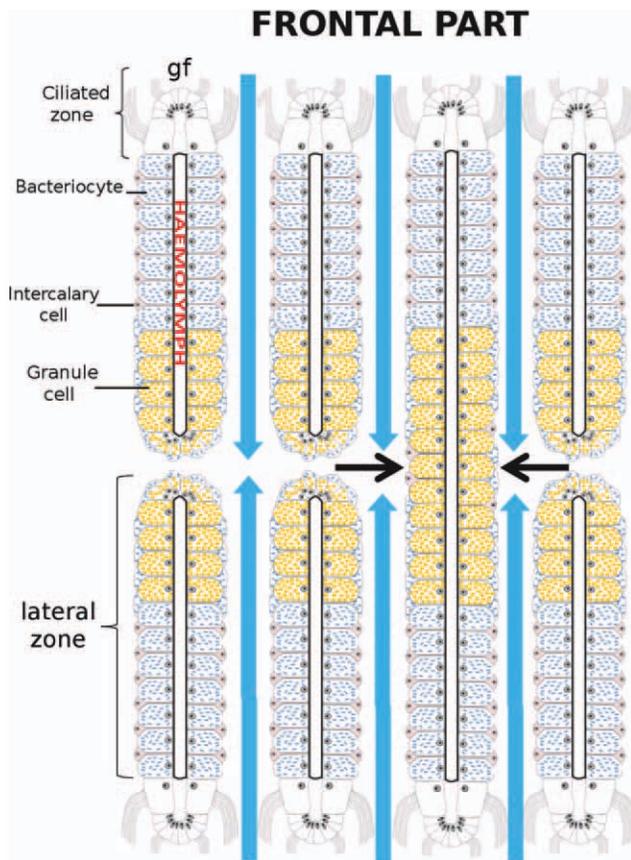


Fig. 1. Gill organization in a freshly collected individual of *C. orbiculata*. Schematic representation of the organization of gill filaments in a transverse section. The section plane passes through three bacteriocyte channels through which seawater flows (blue arrows). Each filament consists of two main areas: the ciliated zone, devoid of symbionts and the lateral zone. The lateral zone is composed of four cell types: bacteriocytes bearing symbionts, intercalary cells recognizable by their apical nucleus, granule cells mainly confined to the abfrontal part of the filament, and scattered mucocytes (not shown here). This pseudostratified epithelium is organized around a blood lacuna through which hemolymph containing hemocytes circulate. Some continuous gill filaments connect the two layers of the gill by tissue bridges (inter-lamellar junctions) (black arrows). gf: gill filament. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

*aequizonata*. Each channel is delimited by the blood lacuna. In a transverse section, each gill filament is constituted of a pseudostratified epithelium composed of three zones. The short ciliated and intermediary zones (not shown) are totally free of symbiotic bacteria. The lateral zone which occupies the main part of the filament is crossed by a blood lacuna containing few hemocytes. The lateral zone is composed of four cell types (Figs. 2a–2d). The first main cell type, the bacteriocytes, occupies the main part of the filament and is situated in the frontal part of the lateral zone. As observed from semithin sections (Figs. 2b and 2c), thin sections (Fig. 2d), and CARD-FISH data (Fig. 3a), they are characterized by a basal nucleus and the presence of symbiotic sulfur-oxidizing bacteria occupying most of the cell volume. The second main cell type, granule cells, is encountered in the abfrontal part of the lateral zone. This cell type which also possesses a basal

nucleus is free of bacteria and full of homogenous granules stained in orange with Orange G (Figs. 3d–3f, 3i, and 3j). Few intercalary cells, typically characterized by their apical nucleus and their trumpet shape are interspersed between bacteriocytes (Fig. 2d). A few mucocytes (stained in blue by alcian blue) are also obvious in the filaments (Figs. 2a and 3d).

**Starved Individuals.** After 5 months of starvation ( $T_5$ ), the cell organization encountered in freshly collected individuals is modified (Fig. 3e). The main part of the filament is now occupied by granule cells. Bacteriocytes remain in the frontal part of the lateral zone, but their area is clearly reduced. This reduction runs parallel with the decrease on the relative abundance of bacteria detectable by CARD-FISH compared with  $T_0$  (Fig. 3b).

**Recolonized Individuals.** When individuals are returned to their natural environment, bacterial colonization of gill filaments is progressive. After 4 days ( $T_{5+4d}$ ), few bacteria are detectable in gill filaments (Fig. 3c) and this relative abundance will progressively increase as supported by CARD-FISH data showing that the fluorescence labeling is stronger after 15 days ( $T_{5+15d}$ ) exposure animals than in controls ( $T_0$ ) (Figs. 3g and 3h). This pattern is also confirmed by histological staining within gill filaments displayed by 15 days ( $T_{5+15d}$ ) exposure animals (Fig. 3j) and controls (Fig. 3d). In fact, from 8 ( $T_{5+8d}$ ) to 15 days ( $T_{5+15d}$ ) of exposure, the bacteriocyte area regains the upper one-third of the lateral zone and increases again until it reaches levels observed in controls ( $T_0$ ). Ultimately, bacteriocytes invade almost of the lateral zone reducing the granule cell area to the abfrontal part.

### Cell Proliferation

In vivo incorporation of BrdU followed by fluorescence-immunohistochemical detection was used to monitor cell proliferation in gills of freshly collected, starved, and recolonized individuals of *C. orbiculata*, and results are reported in Table 1. No major differences have been detected between animals incubated 2 h or 6 h with BrdU, and 2 h incubation were used for all the experiments.

BrdU-positive cells (BrdU<sup>+</sup>) were identified in all samples and were systematically present in the ciliated zone. This latter zone is a good control for the BrdU labeling within gill filaments as it is well known that it is characterized by a high turnover of cell proliferation. Strong labeling was also detected in hemocyte nuclei. For all examined delays, BrdU<sup>+</sup> cells were always more numerous than PH3-positive cells (PH3<sup>+</sup>). No significant immunoreactivity was detected in the granule cells.

Nuclei located in the ciliated zone were labeled with anti-BrdU in freshly collected individuals ( $T_0$ ). In the lateral zone however, labeled cells were mainly intercalary cells (bacteria-free cells) and some scattered bacteriocytes and hemocytes. No PH3<sup>+</sup> cells were observed in these samples.

As a general rule, in starved individuals ( $T_5$ ) cell proliferation decreased in the entire gill filament compared with  $T_0$ . Only a low BrdU immunoreactivity was found in the ciliated zone and in hemocytes of the blood lacuna. No PH3<sup>+</sup> cells were observed in these samples.

In recolonized individuals, only BrdU-negative bacteriocytes (BrdU<sup>-</sup> bacteriocytes) have been observed at any delays examined. The  $T_{5+4d}$ – $T_{5+8d}$  period is characterized by a general increase of BrdU and PH3 immunoreactivity in some cells of the lateral zone. Few BrdU<sup>+</sup> nuclei were observed in the ciliated cells (Figs. 4a and 4b) and a high proliferative zone in tissue bridges (inter-lamellar junctions) of the dorsal part of the gill has been detected, characterized by a strong

immunoreactivity for both BrdU (Fig. 4c) and PH3 (Figs. 4e–4f) antisera. Further analysis using CARD-FISH technique (Fig. 4d) shows that the immunopositive cells were totally lacking bacteria. According to the apical position of these nuclei, most cells constituting these proliferative zones are intercalary cells. Interestingly, particular labeled cells with basal nuclei have been observed and might be undifferentiated cells. These cells are totally free of bacteria, mucus, and granules and are observed exclusively in this stage of recolonization.

After 15 days of exposure, cell proliferation was similar to  $T_0$ .

### Cell Death

Cell death was detected in freshly collected, starved, and recolonized individuals of *C. orbiculata*, and results are summarized in Table 2.

For all examined delays, results obtained by TUNEL technique and by CASP3 seem to be correlated. Nevertheless, TUNEL labeled positive cells were always more numerous than CASP3 labeled ones (Figs. 5a–5d). As a general rule, the level of cell death is high in the ciliated zone and in the blood lacuna at any sampling time.

In freshly collected individuals ( $T_0$ ), TUNEL and caspase labeling has been detected in all cell types.

In starved individuals ( $T_5$ ) (Figs. 5a–5d), cell death increases in all cell types of the lateral zone except for bacteriocytes compared with  $T_0$  individuals. Labeling of granule cells (Figs. 5e–5f) is greater at this delay than after 10 months of starvation (author's observations, not shown).

In recolonized individuals, cell death in the lateral zone decreases gradually compared with  $T_5$ . In  $T_{5+8d}$ , apoptosis particularly concerns hemocytes and granule cells. Scattered bacteriocytes and intercalary cells were affected by cell death. In  $T_{5+15d}$ , apoptosis decreases

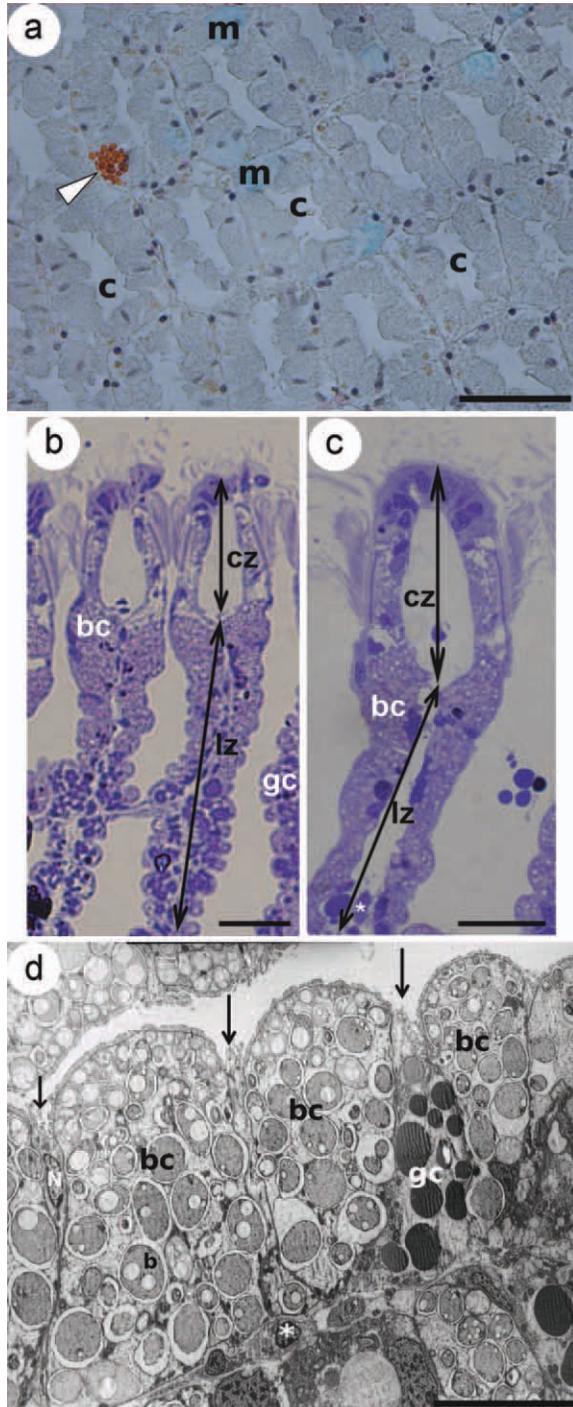


Fig. 2. Gill filament structure in a freshly collected individual of *C. orbiculata*. **a**: Light micrograph showing the bacteriocyte channels (c). The walls of these channels are essentially formed by bacteriocytes. This organization allows communication between all cells of the gill. m: mucocytes; arrowhead: granule cell bar 40  $\mu$ m. **b**: Light micrograph of semithin sections from a freshly collected individual showing cell organization of gill filaments. The ciliated zone (cz) is organized around a collagen axis, and the lateral zone (lz) seems to be composed of four types of cells. Two of the latter cells are obvious on this micrograph: bacteriocytes (bc) which contain bacterial endosymbionts and granule cells (gc). Bacteriocytes are present in the frontal part of the lateral zone, whereas granule cells are located in the abfrontal part bar 20  $\mu$ m. **c**: Higher magnification focusing on the ciliated zone (cz) which is characterized by bacteria free cells with numerous cilia. In the frontal part of the lateral zone (lz), bacteriocytes (bc) contain chemo-autotrophic bacteria characterized by sulfur granules that appear as empty vesicles due to alcohol treatment during the embedding process. The axis of the lateral zone is occupied by a blood lacunar space containing hemocytes (\*) bar 20  $\mu$ m. **d**: Transmission electron micrograph of the lateral zone of gill filament. Bacteriocytes (bc) have a rounded apical pole with microvilli. They are full of envacuolated bacteria (b) containing sulfur granules. The basal pole of bacteriocytes is in contact with the blood lacuna through which hemocytes circulate (\*). Intercalary cells (arrows) are interspersed between bacteriocytes. They have a trumpet shape with a thin basal pole and a larger apical pole containing the nucleus (N). The apical pole of granule cells (gc) is coated by intercalary cells, and their basal pole is in contact with the blood lacuna bar 10  $\mu$ m. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

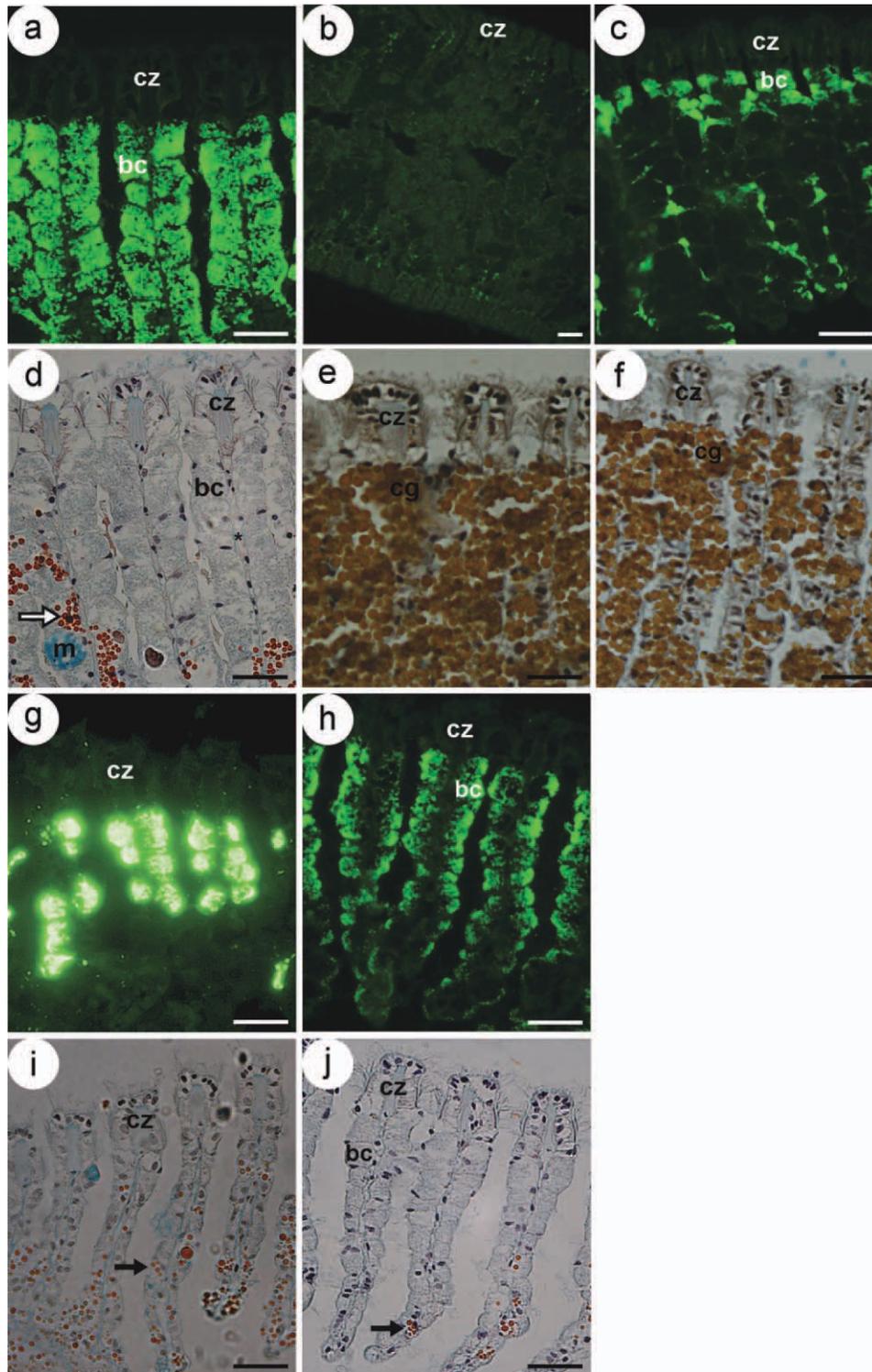


Fig. 3. Plasticity of gill filament of *C. orbiculata* during starvation and bacterial colonization according to CARD-FISH experiments and histological staining. **a**: In a  $T_0$  individual, the eubacterial probe (EUBIII) positively hybridizes gill-endosymbionts, which are stained in green with FITC. Throughout the lateral zone, the endosymbionts occupy the entire volume of the cytoplasm of bacteriocytes (bc). The ciliated zone (cz), free of bacteria, is not hybridized bar 40  $\mu$ m. **d**: Histological staining of gill filaments from a  $T_0$  individual. The ciliated zone (zc) possesses numerous cilia and the lateral zone is occupied by a blood lacuna (asterisk) containing hemocytes with elongated nuclei. Bacteriocytes (bc) hosting symbiotic bacteria occupy most of the lateral zone. The rest is mostly occupied by granule cells (arrow) stained in orange. m: mucocyte. Bar 40  $\mu$ m. **b,e**: In a  $T_5$  individual, weak positive hybridization is observed in the lateral zone indicating that gills, i.e., bacteriocytes, are nearly devoid of bacteria (b). Granule cells (gc) occupy most

of the lateral zone (paraffin embedded sample) (e). cz: ciliated zone bar 40  $\mu$ m. **c,f**: In a  $T_{5+4d}$  individual, symbiont acquisition from the environment has already begun and bacteriocytes (bc) (mostly in the frontal part of the lateral zone) are positively labeled in CARD-FISH experiments (c). Granule cells (gc) still occupy most of the filament (paraffin embedded sample) (f). cz: ciliated zone bar 40  $\mu$ m. **g,i**: In  $T_{5+8d}$  individuals, CARD-FISH signal increases and more bacteriocytes are labeled (g). The area occupied by granule cells (arrow) has clearly decreased (i). cz: ciliated zone bar 40  $\mu$ m. **h,j**: In a  $T_{5+15d}$  individual, bacteriocytes occupy most of the lateral zone, and granule cells are restricted to the lower abfrontal part of the filament. The area occupied by bacteriocytes is more important at  $T_{5+15d}$  than at  $T_0$ . bc: bacteriocyte, cz: ciliated zone, arrow: granule cells bar 40  $\mu$ m. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

again for all cell types but surprisingly increases for bacteriocytes. As a consequence, after 1 month of recolonization, the relative abundance of bacteriocytes remains fewer than in  $T_{5+15d}$  (authors' observation, not shown).

## DISCUSSION

According to the previous description of gill filaments in bivalves belonging to the genus *Codakia* (Frenkiel and Mouëza, 1995), *C. orbiculata* gill filaments are classically composed of three zones: a ciliated zone, a

TABLE 1. Synthesis of cell proliferation results obtained by immunohistochemistry performed on *Codakia orbiculata* sections (5  $\mu$ m)

Cell type	Delay							
	$T_0$		$T_5$		$T_{5+8d}$		$T_{5+15d}$	
	BrdU	PH3	BrdU	PH3	BrdU	PH3	BrdU	PH3
Cells of the ciliated zone	++	-	+	-	++	+	++	-
Bacteriocytes	+	-	-	-	-	-	-	-
Undifferentiated cells	$\emptyset$	$\emptyset$	$\emptyset$	$\emptyset$	+++	++	$\emptyset$	$\emptyset$
Granule cells	-	-	-	-	-	-	-	-
Intercalary cells	+	-	-	-	+++	++	+	-
Haemocytes	+	-	++	-	+	+	+	+

Results are given in a semiquantitative way: (-) is an absence of labeling, and labeling increases from (+) to (+++).  $\emptyset$  corresponds to the absence of observation of a cell type.

short intermediary zone, and a lateral zone constituted of four cell types (bacteriocytes, granule cells, mucocytes, and intercalary cells). Bacteriocyte channels are also present as described in other lucinids (Ball et al., 2009; Distel and Felbeck, 1987; Frenkiel and Mouëza, 1995; Johnson and Fernandez, 2001). This organization provides a constant bathing of exposed cells by sea water. As each channel is delimited by the blood lacuna, the resulting network allows communication in the entire gill. Interestingly, we also evidenced a new type of cell in the lateral zone of starved individuals, the undifferentiated cells (free of bacteria), never reported in this genus, but previously described in Vestimentiferans (Pflugfelder et al., 2009). These particular cells with basal nuclei were localized in tissue bridges (inter-lamellar junctions) of gill filaments.

As described in *C. orbicularis* (Caro et al., 2009) and more recently in *C. orbiculata* (Gros et al., submitted), we observed a progressive decrease of the area occupied by bacteriocytes in the lateral zone and also of intracellular bacteria relative abundance during starvation. But, what happens to bacteriocytes which have lost completely their intracellular bacterial symbionts? Do they die in absence of symbionts, or do they just decrease their cell volume?

In agreement with Gros et al. (submitted), we also observed cellular plasticity in the lateral zone in both starvation and recolonization stages. When starved

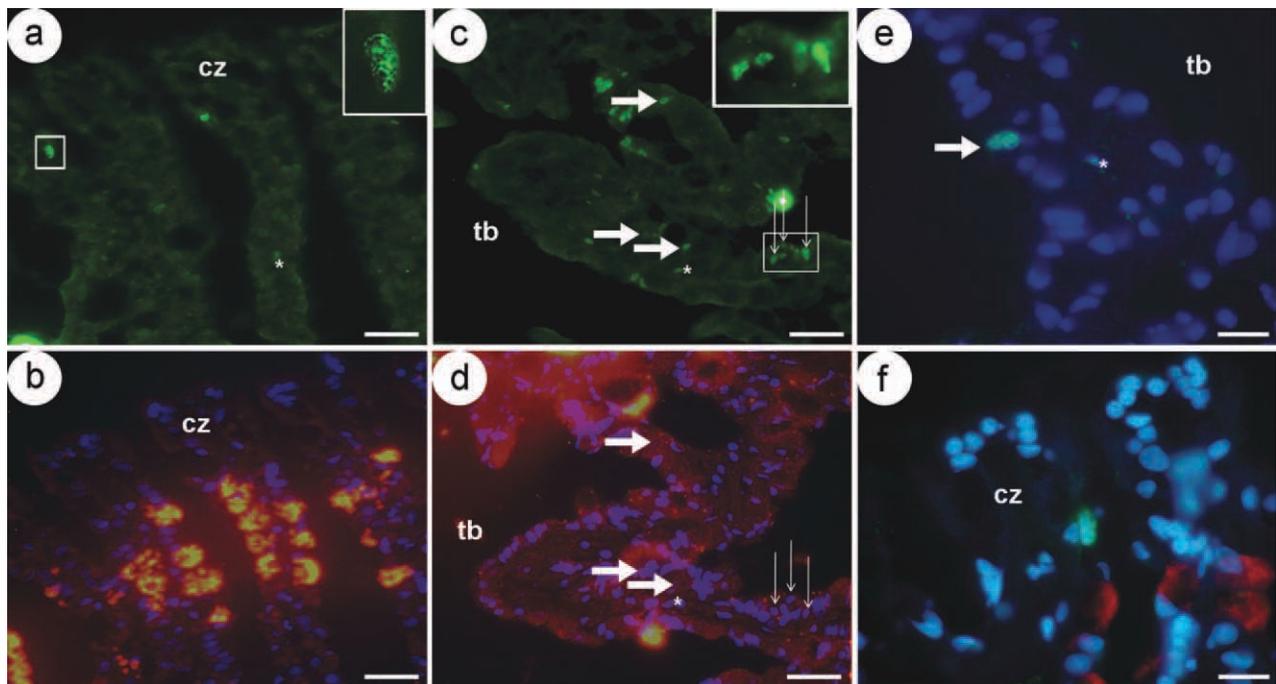


Fig. 4. Gill renewal in recolonized individuals of *C. orbiculata*. Fluorescent micrographs of sections of BrdU and PH3 treated individuals at  $T_{5+8d}$ . Few labeled nuclei are observed in the ciliated zone (a). Double labeling of CARD-FISH (bright yellow) and DAPI (bright blue nuclei) shows that these cells are nonsymbiotic (b). Numerous intercalary cells, characterized by their apical nuclei (inserts, fine arrows) are labeled with BrdU in tissular bridges (inter-lamellar junctions). Undifferentiated cells with basal nuclei are also observable (full arrows) (c). Note that double labeling of CARD-FISH (yellow) and DAPI (blue) shows that these cells, localized in tissular bridges, are

devoid of bacteria. Yellow dots are due to background autofluorescence identified by the lack of corresponding DAPI signal from bacteria (d). Double labeling (arrow) of anti-PH3 (bright green nucleus) and DAPI (bright blue nuclei) also allow visualization of stained intercalary cells in tissular bridges (e). Finally, using a longpass emission filter, triple labeling of CARD-FISH (bright red), anti-PH3 (bright green nucleus), and DAPI (bright blue nuclei) is shown in the ciliated zone (f). cz: ciliated zone, tb: tissue bridge, \*: labeled hemocytes bars 40  $\mu$ m (a, b, c, d), 20  $\mu$ m (e, f). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

TABLE 2. Synthesis of cell death results obtained by immunohistochemistry performed on *Codakia orbiculata* sections (5  $\mu\text{m}$ )

Cell type	Delay							
	$T_0$		$T_5$		$T_{5+8d}$		$T_{5+15d}$	
	TUNEL	CASP3	TUNEL	CASP3	TUNEL	CASP3	TUNEL	CASP3
Cells of the ciliated zone	++	+	++	+	++	+	++	+
Bacteriocytes	++	+	++	+	+	+	+++	++
Undifferentiated cells	$\emptyset$							
Granule cells	++	-	+++	++	+++	++	++	-
Intercalary cells	++	+	+++	++	++	+	++	+
Haemocytes	++	+	+++	++	++	+	++	+

Results are given in a semiquantitative way: (-) is an absence of labeling and labeling increases from (+) to (+++).  $\emptyset$  corresponds to the absence of observation of a cell type.

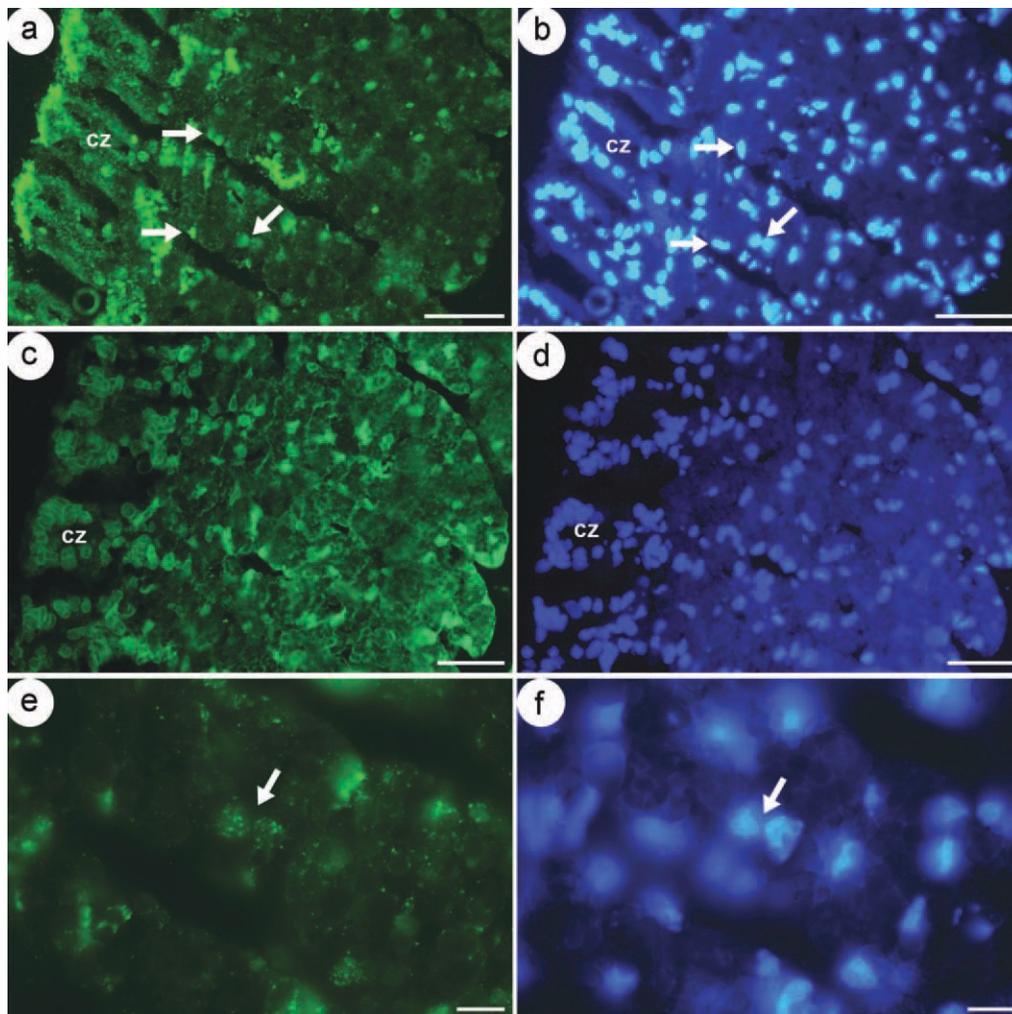


Fig. 5. Cell death in gill of 5-month starved individuals of *C. orbiculata*. Fluorescent micrographs of CASP3<sup>+</sup> nuclei (bright green nuclei, arrows) (a) counterstained by DAPI (bright blue nuclei, arrows) (b) shows important death in granule cells and intercalary cells. Signal obtained for CASP3 is lower than signal obtained by TUNEL assay (bright green nuclei) (c) and counterstained with DAPI

(bright blue nuclei) cz: ciliated zone (d). Higher magnification of the lateral zone showing CASP3<sup>+</sup> (bright green nuclei) granule cells free of bacteria (arrow) (e) as confirmed by DAPI staining (bright blue nuclei, arrows) (f). Bars 40  $\mu\text{m}$  (a, b, c, d), 10  $\mu\text{m}$  (e, f). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

adults are exposed to their natural environment, individuals might gradually acquire new symbiotic bacteria by phagocytosis of symbiosis competent bacteria, at the apical pole of bacteriocytes. Does bacterial recruit-

ment lead to bacteriocyte proliferation or do they increase their cell volume? Results obtained in this study give us indications of the answers to these questions.

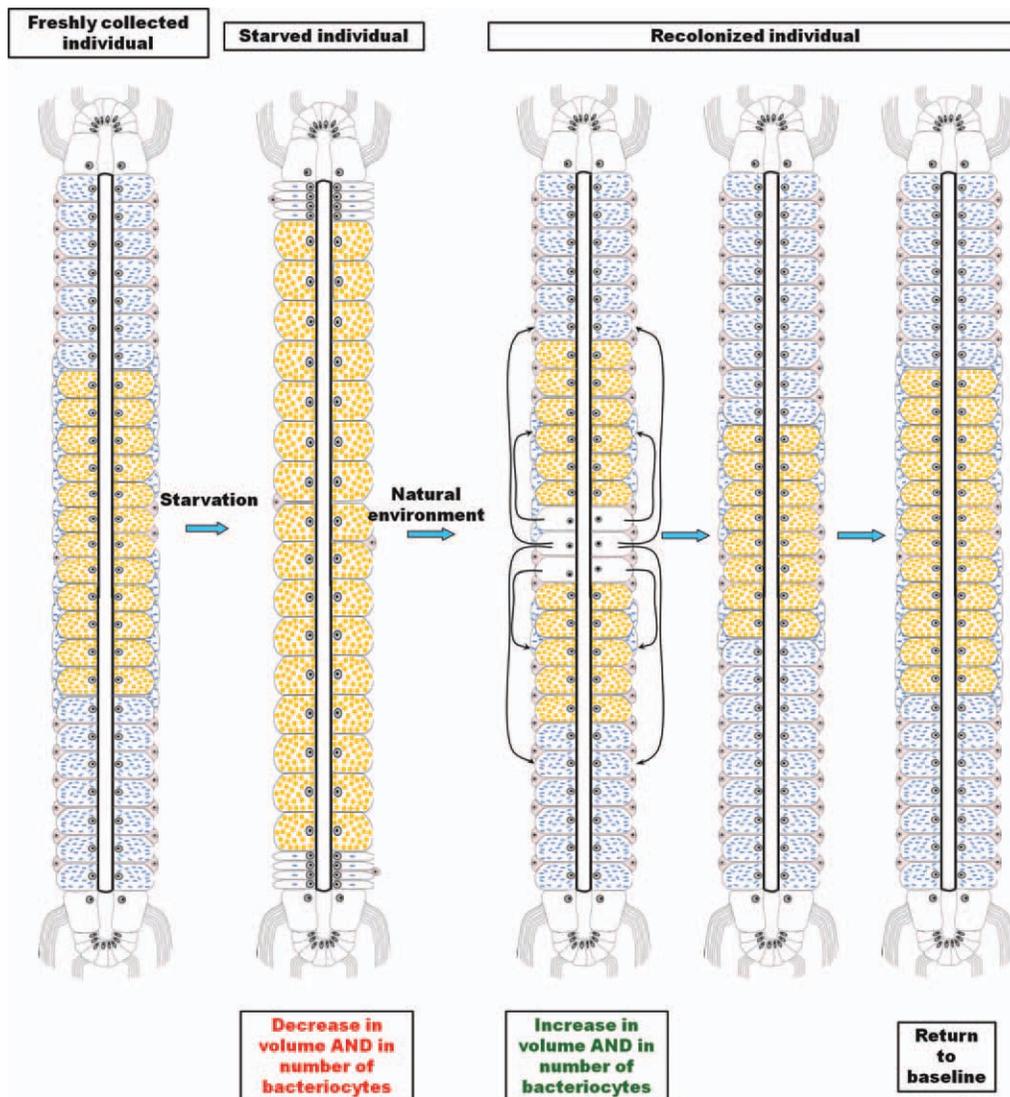


Fig. 6. Diagram representing the most likely hypothesis explaining tissue plasticity in gills of a *C. orbiculata*. Plasticity can be explained by a combination of both relative abundance and cell apparent volume variation. In this case, the origin of new cells would be

mainly “undifferentiated cells” in tissue bridges (inter-lamellar junctions), but division of differentiated bacteriocytes (not shown) can also occur. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

First, we highlighted that sparse simple division of differentiated cells can occur in the entire gill filaments. This mechanism of division is more or less important depending on cell type and delay analyzed. In fact, we only observed bacteriocytes labeled with anti-BrdU at  $T_0$ , and no significant immunoreactivity has been detected in granule cells. In the ciliated zone, cells were always labeled regardless of the delay analyzed reflecting their high turnover. As in previous studies (Gómez-Mendikute et al., 2005; Hanselmann et al., 2000; Matozzo et al., 2008; Mayrand et al., 2005; Zaldibar et al., 2004), we found that hemocyte division can occur in the hemolymph suggesting that it is not restricted to a specific organ or area as described in crustaceans (Johansson et al., 2000).

Second, in a more surprising and major result, we have shown high proliferative zones in tissue bridges of gills between 4 and 8 days of recolonization and

exclusively at this time. These seem to be preferential sites of division in *Codakia* gills. Two types of cells were highly labeled: (i) intercalary cells recognizable by their shape and their apical nucleus. Their function has not yet been clearly identified. They were proposed to replace old bacteriocytes (Reid and Brand, 1986) or to modulate the contact of bacteriocytes with seawater depending on water parameters (Frenkiel and Mouëza, 1995; Frenkiel et al., 1996; Gros et al., 2000). (ii) undifferentiated cells free of bacteria, granules, or mucus, with basal nuclei. We can hypothesize that these cells might be undifferentiated precursors of cell types with basal nuclei that later differentiate into bacteriocytes, granules cells, or mucocytes, depending on environmental conditions. They would migrate in the upper lateral zone later in a similar manner to cells described in mammal intestines for example (Sancho et al., 2003). Furthermore, a recent study on cell proliferation

in symbiotic vestimentiferan tubeworms proposed the existence of a “tissue-specific unipotent bacteriocyte stem cell” in the central region of the trophosome (Pflugfelder et al., 2009). An analogy could be made between this latter study and our results, suggesting the tissue bridges (inter-lamellar junctions) of gills might be a tissue-specific multipotent stem cell zone.

Third, we did not observe massive apoptosis of granule cells in individuals returned to the sediment. For bacteriocytes, no massive death occurred in 5 month starved individuals but we suggest that this process might have occurred previously. Further studies should be conducted during the first months of starvation to confirm this. Cell death can occur by nonapoptotic (necrosis, autophagy, senescence, and mitotic catastrophe) and apoptotic pathways: programmed cell death in which terminal events are termed apoptosis (Okada and Mak, 2004). In this study, we visualized DNA fragmented nuclei using TUNEL assay and apoptotic nuclei with CASP3 staining. As a general rule, TUNEL-positive nuclei (TUNEL<sup>+</sup>) were more numerous than CASP3-positive nuclei (CASP3<sup>+</sup>). This might either be due to environmental stress induced by starvation or being suddenly returned to its natural environment. It also can be explained by the fact that DNA fragments are detectable for a longer time than activated CASP3 (D’haeseleer et al., 2006).

According to the three points developed above, the scenario of tissue and cell plasticity in gill filaments of *C. orbiculata* can be reconstituted. As a general rule, existing bacteriocytes can multiply sporadically related to symbiont acquisition that is possible throughout adult life. After long-term starvation, and as a consequence to the absence of bacteria, a part of these cells may have reduced their apparent volume (they are no longer visible under photonic microscope), and the rest may have been eliminated to recover energy. When bacteria are once again available, pre-existing bacteriocytes with supposed reduced volume, might expand again, rapidly acquire symbionts in the first days of recolonization (between 1 to 4 days) and restore their initial apparent volume. Between 4 and 8 days, undifferentiated cells, which might have proliferated before, may now be able to differentiate in new bacteriocytes. Production of these cells is so important that, after 15 days, bacteriocytes are more numerous than after 8 days recolonization. Excess cells are eliminated by apoptosis between 15 and 30 days. According to this, the most likely scenario can be represented by Figure 6.

In the hard clam *Mercenaria mercenaria*, Hanselmann et al. (2000) have shown abundant proliferating cells in the epithelium of the gill base and lower numbers in gill plical epithelium, using proliferating cell nuclear antigen. Scattered proliferating cells were also observed throughout the gills of *Mytilus galloprovincialis* (Gómez-Mendikute et al., 2005). However, studies on bivalves have never clearly demonstrated the existence of several cell sources for tissue regeneration in gills. Our study is the first reporting that tissue regeneration can occur by both bacteriocytes and undifferentiated cells in gill filaments of symbiotic bivalves. Coexistence of several regeneration mechanisms has been demonstrated in several other tissues. Regeneration by both pre-existing endothelial cells and endothelial progenitors has been demonstrated in blood vessels

(Tepper et al., 2003). Sources of hepatocytes in liver growth processes are also multiple, implying existing hepatocytes, progenitor cells, and stem cells (Fausto, 2004).

The elucidation of cell mechanisms underlying plasticity in gill filaments of lucinids demonstrates the complexity of such a phenomenon. It involves a combination of cell proliferation, death, and possible apparent volume variation to maintain tissue homeostasis and so contributes to sulfur-oxidizing symbiont population management.

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