

Comparative modifications in bacterial gill-endosymbiotic populations of the two bivalves *Codakia orbiculata* and *Lucina pensylvanica* during bacterial loss and reacquisition

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Abstract

Until now, the culture of sulphur-oxidizing bacterial symbionts associated with marine invertebrates remains impossible. Therefore, few studies focused on symbiont's physiology under stress conditions. In this study, we carried out a comparative experiment based on two different species of lucinid bivalves (*Codakia orbiculata* and *Lucina pensylvanica*) under comparable stress factors. The bivalves were starved for 6 months in sulphide-free filtered seawater. For *C. orbiculata* only, starved individuals were then put back to the field, in natural sediment. We used *in situ* hybridization, flow cytometry and X-ray fluorescence to characterize the symbiont population hosted in the gills of both species. In *L. pensylvanica*, no decrease in symbiont abundance was observed throughout the starvation experiment, whereas elemental sulphur slowly decreased to zero after 3 months of starvation. Conversely, in *C. orbiculata*, symbiont abundance within bacteriocytes decreased rapidly and sulphur from symbionts disappeared during the first weeks of the experiment. The modifications of the cellular characteristics (SSC – relative cell size and FL1 – genomic content) of the symbiotic populations along starvation were not comparable between species. Return to the sediment of starved *C. orbiculata* individuals led to a rapid (2–4 weeks) recovery of symbiotic cellular characteristics, comparable with unstressed symbionts. These results suggest that endosymbiotic population regulation is host-species-dependent in lucinids.

Introduction

Characterization of symbiosis involving microorganisms can be difficult, due to the inherent interdependence of both partners, but has known a new change with the expansion of molecular biology (Orphan, 2009). This is especially true in chemosynthetic symbioses, where symbionts cannot be cultivated out of their hosts. In this type of symbiosis, the host usually provides to symbionts the chemical molecules that full symbiont's metabolism like inorganic compounds: oxygen, hydrogen (Petersen *et al.*, 2011), hydrogen sulphide (Cavanaugh *et al.*, 1981; Felbeck, 1981), nitrogen (Hirsch *et al.*, 2001) but also organic

compounds like methane (Childress *et al.*, 1986; Cavanaugh *et al.*, 1987). These compounds are converted by bacteria using chemical energy into more complex carbon molecules using dissolved CO₂ from the environmental seawater thanks to Calvin-Benson or reductive tricarboxylic acid cycle (Hügler *et al.*, 2005; Robidart *et al.*, 2008).

First descriptions of the model *Lucinidae-Gammaproteobacteria* were mostly based on light and electron microscopic studies (Berg & Alatalo, 1984; Frenkiel & Mouëza, 1995; Ball *et al.*, 2009; Brissac *et al.*, 2011a, b). Conventional light microscope investigations gave useful information about structure and ultrastructure of the gills but left some gaps in the understanding of the

relationship especially concerning physiology and metabolism. Ultrastructural studies have shown the presence of periplasmic inclusions in the gill-endosymbionts appearing as empty granules in conventional transmission electron microscopy (TEM) pictures (Vetter, 1985; Frenkiel & Mouëza, 1995; Gros *et al.*, 2000; Ball *et al.*, 2009). The use of high-pressure cryofixation and EFTEM microanalysis helped to determine the chemical form of such sulphur compounds, stored by bacteria under optimal sulphidic conditions (Lechaire *et al.*, 2006) and mobilized under 'nonsulphidic' conditions [starvation] (Lechaire *et al.*, 2008) without quantifying the decrease of these compounds. Although short-term experiments have been performed to evaluate the decrease of sulphur storage in some vesicomysids, this kind of experiment was only performed for a few days by moving adult clams from sulphidic to nonsulphidic habitats (Goffredi *et al.*, 2004). Therefore, quantitative data are missing in other models, and longer term experiments are needed to monitor sulphur up to its depletion. Thus, a quantification of endosymbionts within the gills or the bacteriocyte population remains to be performed. Recent morphological and molecular analyses (Elisabeth *et al.*, 2012; Gros *et al.*, 2012) support the conclusion that it is possible to induce complete or nearly complete loss of symbionts in adult lucinids by depriving their symbionts of their energy source (mainly in sulphide-free environment). Decreases in endosymbiont abundance within gill cells have been shown according to structural investigations of the gill tissue in other chemosynthetic bivalves such as thyasirids (Dufour & Felbeck, 2006), vesicomysids (Goffredi *et al.*, 2004), bathymodiolin (Kádár *et al.*, 2005) and two lucinids (Caro *et al.*, 2009; Gros *et al.*, 2012). However, no data are available concerning the modifications undergone by the endosymbionts themselves (at the metabolic or genomic level) during increase or decrease of their populations within the gill tissue either in the frame of starvation laboratory experiments or in their natural habitats.

The input of novel approaches based on molecular biology brought new insights about the transmission mode of bacterial symbionts in lucinids (Gros *et al.*, 1996, 2003, 2012) and in other chemoautotrophic models (Kádár *et al.*, 2005; Nussbaumer *et al.*, 2006; Harmer *et al.*, 2008; Verna *et al.*, 2010). In symbioses from chemosynthetic environments, only a couple of studies have provided some indications for symbiont uptake in adult hosts. In aquarium experiments with bathymodiolin mussels, Kádár *et al.* (2005) have shown that these bivalves might be able to take up symbionts from bacteria released by co-occurring mussels in the aquarium. For their part, Verna *et al.* (2010) hypothesized that repeated infection events occur during the life of the bone-eating worm

Osedax mucofloris from shallow-water whale falls. More recently, Gros *et al.* (2012) have shown that in the tropical shallow-water lucinid *Codakia orbiculata*, symbiont-free adult hosts (obtained after a long starvation in laboratory) can take up their bacterial symbionts from their natural environment. To date, this is the only bivalve species in which such a phenomenon has been demonstrated. However, the techniques used in these studies (i.e. FISH and/or TEM views) could not quantify for sure loss and gain of bacteria and/or sulphur storage during these conditions.

Another input came with the first use of flow cytometry (FCM) in chemoautotrophic symbiosis studies. Caro *et al.* (2007) highlighted the existence of different symbiont populations within the gills of *C. orbicularis* individuals that can be distinguished in terms of size and genomic content. Such diversity in bacterial populations hosted in the gills could be explained by the regular uptake of small free-living competent bacteria from the environment and by their evolution (growth, genome replication and/or cell division) inside the bacteriocytes. Long-term starvation experiments showed variation in the number of these symbiont subpopulations in the same lucinid host species (Caro *et al.*, 2009).

While hosts generally tend to digest their symbionts in case of food and sulphide starvation (Goffredi *et al.*, 2004; Kádár *et al.*, 2005; Dufour & Felbeck, 2006; Caro *et al.*, 2009; Gros *et al.*, 2012), data obtained according to FCM analysis are only available from a single lucinid species (*Codakia orbicularis*) and new data are necessary to better understand the pathways implemented by the hosts during the loss of their symbionts. The host regulates the bacterial population in a comparable way whatever the host species or do different ways of regulation exist depending on the host species?

The objective of this study was to compare the modifications occurring in the symbiotic populations hosted by two different host species: *C. orbiculata* and *L. pensylvanica* under starvation conditions. Both host species belong to the same family of *Lucinidae* and assumed to harbour the same gill-endosymbiont species (Durand & Gros, 1996; Durand *et al.*, 1996). The modifications at the population level were set out on gill sections using fluorescence *in situ* hybridization (CARD-FISH). The cellular characteristics of symbionts (relative cell size and genomic content) as a proxy of physiological status were investigated by FCM as well as the detailed composition of the symbiotic population (e.g. number of subpopulations). The sulphur content of symbiotic populations was also assessed by X-ray fluorescence. Through this study, the following question is addressed: are the modifications undergone by symbionts specifically controlled by the

host species, or rather induced by starvation conditions independent of the host species?

Materials and methods

Sampling of bivalves

Codakia orbiculata (Montagu, 1802) individuals (maximum shell length 25 mm [Abbott, 1974]) were collected in *Thalassia testudinum* seagrass beds on 'îlet cochon' (16°12'53"N; 61°32'05"W) in Guadeloupe (French West-Indies, Caribbean). They measured from 15 to 24 mm in length.

Lucina pensylvanica (Linné, 1758) individuals (maximum shell length 50 mm [Abbott, 1974]) were collected in sandy-muddy sediment near *Thalassia testudinum* seagrass beds in peninsula of François (14°37'09"N; 60°53'08"W) in Martinique (French West-Indies, Caribbean). The individuals collected measured from 25 to 44 mm in length.

All the individuals from the two lucinid species were adults and collected outside their reproductive period (from June to October) to minimize the differences in their behaviour and physiological responses during starvation experiments.

Induction of symbiont loss

Individuals of the two species (*C. orbiculata* [$n = 33$] + *L. pensylvanica* [$n = 21$]) were kept in 50-L plastic tanks with 0.22- μm -filtered seawater at 26 °C up to 6 months (T_1 , T_2 , T_3 , T_4 , T_5 , T_6). The water was oxygenated with an aquarium pump. No food and no sulphur were added to the water throughout the starvation experiment. The seawater was changed every week and continuously oxygenated with aquarium pumps. To inhibit algal development, tanks and shells were washed each week. This starvation lasted 6 months.

Induction of symbiont acquisition

Six-months-starved individuals of *C. orbiculata* (T_6) were painted in blue and placed back in *Thalassia testudinum* seagrass beds to induce symbiont acquisition. Three individuals were randomly taken after 4, 8, 15, 21 and 31 days ($T_6 + 4\text{d}$, $T_6 + 8\text{d}$, $T_6 + 15\text{d}$, $T_6 + 21\text{d}$, $T_6 + 31\text{d}$).

Flow cytometry

Extraction and purification of endosymbionts

For each individual, endosymbionts were extracted from $\frac{3}{4}$ of gill and purified using the Percoll cushion method

(Distel & Felbeck, 1988), with some modifications. To extract the endosymbiotic bacteria from the gill tissue, a piece of gill was homogenized in 8 mL of sterile seawater (35‰) using a handheld Dounce homogenizer. The homogenized tissue was centrifuged (30 g for 1 min). Four millilitre of the supernatant were then centrifuged at 400 g for 2 min to collect the bacteria. The bacterial pellet was resuspended in 1 mL of 0.2- μm -filtered seawater. The suspension was gently layered on a Percoll (Sigma) cushion (3 mL) diluted with imidazole-buffered saline (490 mM NaCl, 30 mM MgSO₄, 11 mM CaCl₂, 3 mM KCl, and 50 mM imidazole) and centrifuged at 1000 g for 8 min at 4 °C. As the Percoll cushion method is based on differences in density between the symbionts and the host organelles, the loss of elemental sulphur globules (S_0) in the periplasmic space of the symbionts over a period of long-term starvation prevents the separation of the endosymbionts from the host debris. The final Percoll concentration, therefore, had to be adjusted from 50% (for freshly collected specimens) to 30% (for 5- and 6-months-starved individuals) throughout this study. The symbionts were finally collected under the cushion, washed once and finally suspended in 1 mL of 0.2- μm -filtered artificial seawater. The purification was performed at 4 °C to avoid growth of marine bacteria, initially present on the gills or between the gill filaments as contaminants (Gros, personal observations). An aliquot of the purified symbiont suspension was fixed with 1% formaldehyde in 0.2- μm -filtered seawater and immediately stored in liquid nitrogen for further nucleic acid content analysis by FCM. For each time point, we analysed three individuals.

Characterization of relative nucleic acid content (FL1) and cell size (SSC) of symbionts in *C. orbiculata* and *L. pensylvanica* using FCM

Symbiont cells extracted from *C. orbiculata* and *L. pensylvanica* gills were analysed according to their nucleic acid content (FL1, green fluorescence) and their relative cell size (SSC, side scatter) using a FACS Calibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser (488 nm). The symbiont suspension was thawed and diluted (1/100) in saline water (30 ‰ NaCl). Nucleic acids were stained for 15 min in the dark at 4 °C with SYBR Green I (Molecular Probes, Eugene, Oregon) according to the method described by Marie *et al.* (1997) (1 : 10 000 v/v). As internal standard for normalization of symbiont fluorescence emission, 1- and 2- μm yellow green fluorescent cytometry beads (Polysciences, INC) were added to the samples. The sheath fluid (NaCl solution, 30‰) was filtered through 0.2- μm -pore-size membrane. Analyses were run at low speed (around

18 $\mu\text{L min}^{-1}$), and acquisition was done for 2 min corresponding to a total of 25 000–35 000 detected cells. Fluorescence of SYBR green-stained symbionts was collected in the green fluorescence channel FL1 (530 nm). SSC parameter was used as a proxy of relative symbiont size (Caro *et al.*, 2007, 2009) as the size scatter is influenced by the cellular size and the intracellular sulphur granules hosted by the lucinid symbionts. FL1 and SSC parameters were collected on a logarithmic scale. Cytometric analyses were performed on symbionts extracted from starved bivalves and from starved bivalves put back in the field.

Statistical analysis of FCM data

The effect of symbiont's loss and reacquisition on SSC and FL1 parameters was investigated by the use of nonparametric statistics. Kruskal–Wallis rank sum test was used to investigate the global effect of the two treatments (symbiont loss for both species and reacquisition for *C. orbiculata*) on SSC and FL1 values. Then, if an effect was detected, we applied Kruskal–Wallis one-tailed multiple comparison test to determine which levels of the treatment differ from the control. We used T_0 as control for the symbionts loss experiment and both T_0 and T_6 for the symbionts acquisition experiment. One-tailed multiple comparison test is described in Siegel & Castellan (1988) and implemented in R package `pgirmess` (<http://cran.r-project.org/web/packages/pgirmess/index.html>). The effect of treatments on SSC and FL1 simultaneously was investigated using non-parametric multivariate analysis of variance (PERMANOVA; Anderson, 2001). This method allows the test of a global treatments effect on both variables but also pairwise comparisons of the different levels of treatments. For all statistical analysis, the *P*-value threshold was set at 0.05.

X-ray fluorescence analysis

Sample preparation

For *C. orbiculata*, 22 mg of gill were ground in 3 mL of 0.22- μm -filtered seawater with a Dounce[®] homogenizer. The suspension was divided into two equal portions (1.5 mL) which were centrifuged at 10 000 *g* for 2 min at 4 °C. One of the two pellets was resuspended in 3 mL of absolute ethanol (#1) to dissolve elemental sulphur and the second one in 3 mL of filtered seawater (#2) for 15 min. The two suspensions were centrifuged as before, and the new pellets were, respectively, resuspended in absolute ethanol (for #1) and in filtered seawater (for #2) for 15 min before centrifugation at 10 000 *g* for 2 min at 4 °C. Then, each pellet was resuspended in 3 mL of filtered seawater and introduced into liquid cells for X-ray fluorescence spectrometry analyses.

For *L. pensylvanica*, the same sample preparation procedure was used with 100 mg of gills. For each treatment time, analyses were performed from three individuals as triplicates.

X-ray fluorescence spectrometry parameters

X-ray fluorescence spectra were collected on a Panalytical Axios PW2400 Spectrometer with an anode X-ray tube in rhodium (Rh). The spectra were collected on the liquid dispersion under helium atmosphere in the energy range 1.9–2.7 keV (germanium crystal) allowing us to record X-ray fluorescence lines of sulphur (K_{α} at 2.307 keV) and chlorine (K_{α} at 2.621 keV) used for quantitative purposes. The data were quantitatively processed using SUPER QUANT software. Because all samples had similar chlorine content, this element is used as internal standard. The amount of sulphur in each sample is given as atomic ratio (S/Cl). This allowed us to quantitatively follow the evolution of the symbionts' sulphur content over time.

It is important to note that elemental sulphur (S^0_8) stored inside bacteria is soluble in ethanol (Vetter, 1985). The sample preparation in the presence of ethanol allowed us to extract selectively elemental sulphur and then to determine the ionic sulphur concentration (ISC) remaining in the bacteria, whereas the analyses carried out on samples prepared with seawater allowed us to obtain the concentration of all forms of sulphur (TSC) including elemental and ionic sulphur species. The elemental sulphur concentration (ESC) corresponds to the difference TSC–ISC.

CARD-FISH

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 until embedding in paraffin. Five micrometer-thin sections were made with a Leitz[®] 1516 microtome and placed on silane-coated glass slides. Paraffin was removed before hybridization with toluene, and sections were rehydrated in a decreasing ethanol series. 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 $\mu\text{g mL}^{-1}$ 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^{-1} lysozyme at RT for 1 h. Hybridizations were performed using horseradish peroxidase-labelled probes EUB 338III 5'-GCT GCC TCC CGT AGG AGT-3' (Amann *et al.*, 2001) at 46 °C for 3 h. Hybridizations were performed using 50% formamide, and signals were amplified using a

buffer containing carboxyfluorescein (FITC). Positive controls were performed on gill sections obtained from freshly collected *C. orbiculata* individuals using the universal bacterial probe EUB 338III (Amann *et al.*, 2001), while negative controls were performed using the probe NON338 (Wallner *et al.*, 1993) to check for false positive signals after hybridization. Slides were mounted with Cytomation fluorescent mounting medium (Dakocytomation[®], France) and visualized under an epifluorescence microscope, Eclipse 80i (Nikon[®], France).

Total protein quantification

Twenty milligram of *C. orbiculata* and *L. pensylvanica* gills were disrupted and incubated for 30 min at 4 °C in 0.5 mL of extraction buffer made of 0.15 M NaCl, Tris-EDTA pH 7.5 (1 M Tris and 0.5 M EDTA), Protease inhibitor cocktail (dilution 1 : 1000;Sigma[®]) and 0.01% Triton X-100. Then, the lysate was centrifuged (13 000 g, 4 °C, 30 min) to recover the protein supernatant which was stored at -80 °C until analysis. For each time point, we analysed three individuals.

Bradford assay (Sigma[®] kits) was performed to measure total protein concentrations (in triplicate) according to Bradford method (1976), using bovine serum albumin (BSA) as a standard. The total protein concentration in

gill's homogenate was expressed as milligram of protein per gram of tissue.

Results

Relative cell size (SSC) and genomic content (FL1) of symbionts

Codakia orbiculata

The endosymbiotic population isolated from gills of 10 freshly collected individuals (T_0) was analysed by flow cytometry. For all analysed specimens, a unique and homogeneous symbiotic population was described, with a mean relative cell size (SSC) of 0.93 and a mean fluorescent level (FL1) of 0.24 (Fig. 1a). These cellular characteristics (mean values of SSC and FL1) were comparable with those described for the symbiotic population hosted by a closely related Lucinid, *Codakia orbicularis* (Caro *et al.*, 2009). But the detailed analysis of the symbiotic population hosted by *C. orbiculata* and *C. orbicularis* results in a unique and homogeneous population for *C. orbiculata*, whereas several subpopulations were described for *C. orbicularis*.

During starvation, the analysis of three individuals' replicates at T_1 , T_2 , T_3 , T_5 and T_6 showed that the relative size (SSC) and the genomic content (FL1) of the symbi-

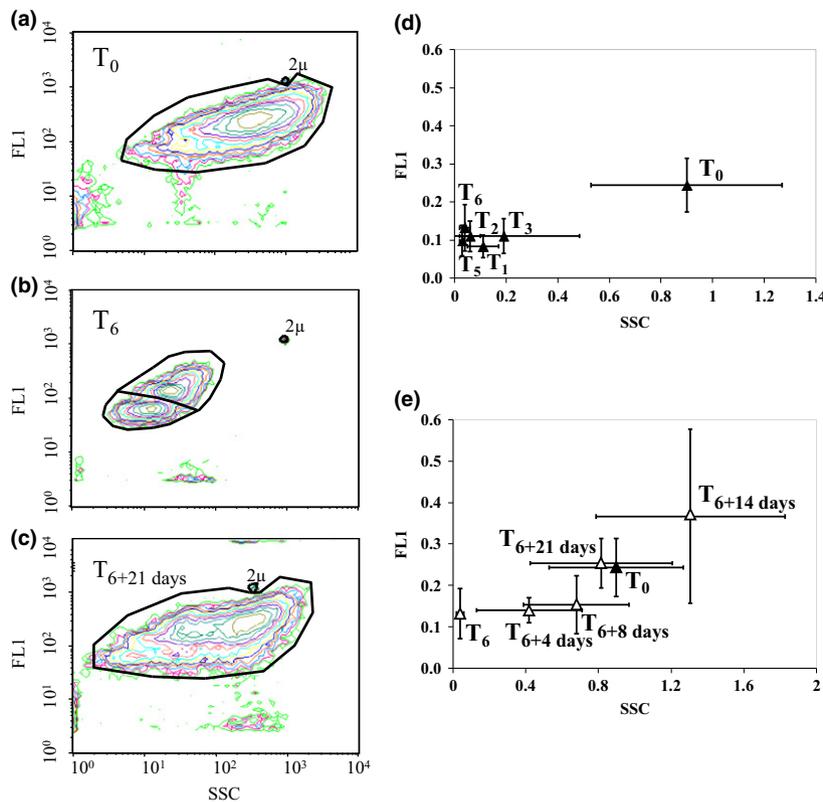


Fig. 1. FCM analyses of sulphur-oxidizing bacterial-endosymbionts hosted by *Codakia orbiculata* during starvation in laboratory and after return in the field. Contour plot presentations in a freshly collected individual (a), in a 6-months-starved individual (b) and in an individual starved for 6 months and returned to the field for 21 days (c). SSC and FL1 values were normalized with respect to 2- μ m green fluorescent cytometry beads. In graphs (d) and (e), each point represents the mean value of SSC and FL1 (discriminated by FCM after SYBR green I staining) obtained from several populations replicates associated with confidence intervals. For each replicate, the symbiotic population is characterized by a value of SSC and FL1 (barycenter) calculated from a minimum of 10 000 cells in the whole symbiotic population. The values of graphs d and e correspond to the mean of 10 replicates for T_0 and three replicates for other times, after 6 months of starvation (d) and return to the field for 21 days (e). T_1 , T_2 ... T_6 : starvation time in month (d). T_{6+4d} , T_{6+8d} ... T_{6+21d} : incubation time in the field (4 days, 8 days ... 21 days, respectively) after 6 months of starvation (e).

otic populations were greatly reduced (Fig. 1d). During the first month of starvation, the cellular characteristics of symbionts dropped from 0.9 to 0.11 for SSC and from 0.24 to 0.08 for FL1. Then, both parameters decreased slowly over the 6 months of starvation to reach 0.04 for SSC and 0.13 for FL1, decreased by a factor 22 and 1.7, respectively, compared with T_0 (Fig. 1d). Statistical analysis (Kruskal–Wallis test) showed a significant effect of the starvation time on both SSC (P -value = 0.00319) and FL1 (P -value = 0.010); pairwise one-tailed multiple comparisons also showed that all SSC and FL1 values were significantly different from T_0 (Supporting Information, Fig. S1). Moreover, the symbiotic population became heterogeneous during the course of experiment; two subpopulations were clearly identified after 6 months of starvation (Fig. 1b). Long-term starvation seemed to affect mainly the relative cell size and slightly the mean genomic content. After 6 months of starvation, symbionts were not detectable anymore by CARD-FISH (Fig. 4b) and did not have elemental sulphur (Fig. 3a).

The reintroduction of bivalves in their natural environment resulted in a significant increase in the relative cell size of symbionts from the fourth day till the 21th compared with T_6 (Kruskal–Wallis test, P -value = 0.017; Fig. 1c and e, Fig. S2A). At T_{6+14d} , the increase of SSC was such that it exceeded the value obtained at T_0 (1.32 against 0.93; Fig. 1e). Four days after their reintroduction in the seagrass bed, the relative cell size (SSC) of symbionts, taking into account both cell size and sulphur content, was comparable from that of freshly collected bivalves [T_0] (Fig. S2A). But, unlike T_0 , the symbiotic population remained heterogeneous, showing that all cells of the symbiotic population did not totally recover their initial relative sizes (Fig. 1c).

Moreover, we also observed a significant effect of reintroduction of hosts in the field on the bacterial genomic content (FL1) compared with freshly collected bivalves (T_0) (Kruskal–Wallis test, P -value = 0.012; Figs 1e and S2b). But the bivalves need a reintroduction of at least 14 days in the sediment to get a genomic content level comparable with freshly collected individuals (0.24 at T_0) (Kruskal–Wallis test, one-tailed multiple comparisons) (Fig. S2B). As for SSC, the genomic value of FL1 at T_{6+14d} exceeds that of T_0 (0.37 vs. 0.24, respectively).

Lucina pensylvanica

Purified symbionts obtained at T_0 from 10 individuals of *L. pensylvanica* (Fig. 2d) had significant higher levels of SSC and FL1 (2.4 ± 0.4 and 0.4 ± 0.1 , respectively) compared with *C. orbiculata* (0.9 ± 0.4 for SSC and 0.24 ± 0.07 for FL1). Moreover, the symbiotic population

of *L. pensylvanica* was characterized by a heterogeneous population; two subpopulations one with high genomic content (HGC) and one with low genomic content (LGC) could be distinguished based on FL1 differences (Fig. 2a). During starvation, the symbiotic population showed a significant decrease of the mean SSC (P -value = 0.0034) till the sixth month (Figs 2c and S1c). At T_6 , the mean SSC value was decreased by a factor of 8.3 compared with T_0 , indicating a reduction in relative cell size of the whole symbiotic population (linear tendency $R^2 = 0.6169$; P -value 2.73×10^{-7}). From the second month of starvation, all the SSC values were significantly different from T_0 (One-tailed multiple comparison test) (Fig. S1C). Conversely to SSC, the symbionts' mean genomic content (FL1) increased during the first 4 months of starvation (except for T_3), from 0.4 at T_0 to 0.7 at T_4 . The increase in FL1 corresponds to the disappearance of LGC subpopulation, modified in HGC-LS (high genomic content and probably low sulphur content in regards with the decrease of SSC) (Fig. 2b). Beyond T_4 , FL1 decreased till 6 months of starvation to reach a mean FL1 level slightly lower than T_0 (Fig. 2d). Statistically, all the FL1 values were significantly different from T_0 (except T_3) (Fig. S1D). The cytogram obtained at T_6 (Fig. 2c), in comparison with T_0 , showed a single population with heterogeneous levels of SSC and FL1, showing effects of starvation on the cellular characteristics after 6 months of starvation. Moreover, the comparison between both bivalve species after long-term starvation (6 months) revealed that the symbionts' characteristics remained higher for *L. pensylvanica* (2.5 times for SSC and 2.5 times for FL1) than for *C. orbiculata*.

Elemental sulphur concentration in gill tissue

Codakia orbiculata

In freshly collected individuals, the concentration of elemental sulphur in gills expressed as S/Cl atomic ratio is 0.023. The evolution curve of sulphur S_0 concentration vs. time, showed a rapid decrease at the beginning of the starvation experiment to reach zero after 1.5 months (Fig. 3a). This value is reached, while the symbionts were still detectable in the gill by CARD-FISH. The elemental sulphur stayed at zero until 6 months of starvation. When individuals were returned in the field, a very slight increase in elemental sulphur was observable between 0 and 15 days (Fig. 3a), while bacteria were already present in the tissue according to CARD-FISH (Fig. 4c). Most of the elemental sulphur was acquired between 15 and 31 days in *Thalassia testudinum* seagrass bed, where the ratio S/Cl finally reaches 0.071, three times higher than the initial value (0.023).

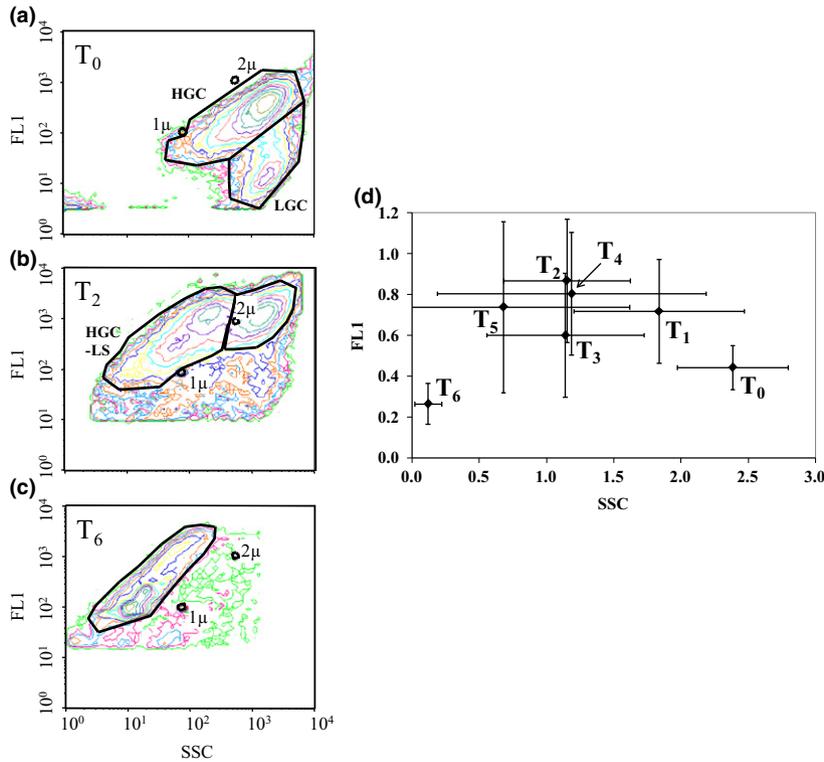


Fig. 2. FCM analyses of sulphur-oxidizing bacterial-endosymbionts hosted by *Lucina pensylvanica* during starvation in laboratory. Contour plot presentations in a freshly collected individual (a) and in a 2- and 6-months-starved individual (b and c, respectively). SSC and FL1 values were normalized with respect to 2- μ m green fluorescent cytometry beads. In graph (d), each point represents the mean value of SSC and FL1 (discriminated by FCM after SYBR green I staining) obtained from several population replicates associated with confidence intervals. For each replicate, the symbiotic population is characterized by a value of SSC and FL1 (barycenter) calculated from a minimum of 10 000 cells in the whole symbiotic population. The values of graph d correspond to the average of 10 replicates for T_0 and three replicates for other times after 6 months of starvation (T_1 , T_2 ... T_6).

Lucina pensylvanica

In a freshly collected individual, the concentration of elemental sulphur S/Cl is 0.013, that is, half of concentration observed in *C. orbiculata* (Fig. 3b) but both values were not significantly different. The evolution curve of S concentration over time shows a rapid decrease after 3 months of starvation, where the concentration of sulphur reached 0.001. This value remains constant up to 8 months of starvation (Fig. 3b) although many symbionts were still detectable in gills by CARD-FISH after 6 months of starvation (Fig. 4e).

CARD-FISH

As recently shown (Elisabeth *et al.*, 2012; Gros *et al.*, 2012), the amount of endocellular bacteria detected by CARD-FISH in *C. orbiculata*, decreased gradually during starvation (Fig. 4a and b). After 6 months of starvation in the laboratory, no bacteria were detectable in bacteriocytes by CARD-FISH (Fig. 4b). When 6 months-starved individuals were returned to their natural habitat, symbionts were again detectable inside the bacteriocytes (Fig. 4c). In *L. pensylvanica* (Figs. 4d and e), symbiotic bacteria were still present in the gill after 6 months of starvation. Their numbers did not appear to decrease below this level.

Total protein quantification in gills

A decrease of total protein concentration was observed from gills of *C. orbiculata* during starvation period (Table 1). A loss accounting for 29% of total protein occurred over the first 4 months and reached up to 46.7% at the end of the starvation period.

Conversely, the total protein concentration in *L. pensylvanica* gills was relatively stable during starvation period (Table 1). Only a small decrease accounting for 4.6% was observed between 0 and 6 months.

Discussion

While thioautotrophic symbioses involving sulphur-oxidizing bacteria and marine invertebrates have been known for over 40 years, there are only few studies that had focused on the bacterial population control by hosts. The more adapted models for such investigations are represented by shallow-water invertebrates that can be collected without damage and too much stress and that can be easily maintained in the laboratory. Tropical lucinids represent easily accessible and easy to handle models. These bivalves live slightly burrowed in low sulphidic sediment of *Thalassia testudinum* seagrass beds (HS^- concentrations measured from 10 to 30 μM [Maurin, personal communication]). In such environmental sulphi-

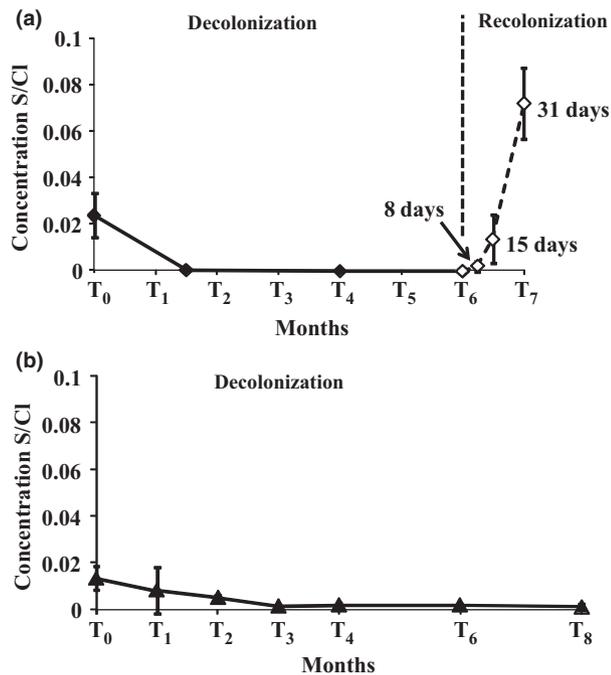


Fig. 3. Elemental sulphur concentration in gill tissues in *Codakia orbiculata* (a) and in *Lucina pensylvanica* (b) during starvation. Modifications in elemental sulphur concentrations during starvation in laboratory ('Decolonization') for several months in *C. orbiculata* (a) and in *L. pensylvanica* (b) and after return in the field ('Recolonization') during 8, 15 and 31 days in *C. orbiculata* (a). Concentration of sulphur is expressed as a ratio S/Cl, as chlorine of the sea water is used as internal standard for quantitative comparison (constant concentration of chlorine in the different suspensions).

dic conditions, numerous symbionts are individually enclosed in each bacteriocyte of the gill filaments of lucinids investigated to date (Reid, 1990; Frenkiel *et al.*, 1996; Gros *et al.*, 2000; Ball *et al.*, 2009; Brissac *et al.*, 2011a, b), including *C. orbiculata* (Elisabeth *et al.*, 2012) and *L. pensylvanica* (Gros *et al.*, 1996) according to ultrastructural analyses. Histological observations from the gills of both species had already revealed important differences, especially in the lateral zones (Gros *et al.*, 1996; Elisabeth *et al.*, 2012). The most important feature concerns the presence of numerous granule cells filled with proteinic granules in the lateral zone of the gill filaments of *C. orbiculata* (as described for *C. orbicularis* by Frenkiel & Mouëza, 1995), while in *L. pensylvanica*, no granule cells could be observed through the lateral zone which only possesses bacteriocytes, intercalary cells, and mucocytes. Moreover, below the ciliated zone, there are two types of secretory cells in *L. pensylvanica* that have never been observed in *C. orbiculata*. However, despite these structural differences, *C. orbiculata* and *L. pensylvanica* harbour the same bacterial species according to the

16S rRNA gene phylogenetic analyses (Durand & Gros, 1996; Durand *et al.*, 1996). However, to better discriminate the two endosymbionts while belonging to the same bacterial species, one 'core metabolic gene' as *aprA* (involved in the sulphur metabolism) and few 'house keeping genes' as *cbbL*, *gyrB* and *dnaE* were sequenced (Brissac, 2009a). Using these genes, Brissac (2009a) had demonstrated that the two gill-endosymbionts represent in fact two different haplotypes within the same species. Thus, the differences concerning the bacterial populations could not be due to the bacteria but more likely to different strategies developed by the bivalve host. Although lucinids could supplement their nutrition (20–30%) through filter-feeding in natural environments (Cary *et al.*, 1989; Duplessis *et al.*, 2004; Rossi *et al.*, 2013), no food was added in the aquaria in this study, so that the intracellular digestion of symbionts was supposed to be the only source of nutrition available for the host during starvation.

Characterization of the symbiotic populations hosted by freshly collected bivalves

The symbiotic population isolated from freshly collected individuals of *C. orbiculata* appeared to be homogenous according to FCM analysis (Fig. 1a). These cellular characteristics (means of relative cell size and genomic content) of the whole populations were comparable with those described for *C. orbicularis* gill-endosymbionts, which highlight a high genomic content (Caro *et al.*, 2009). But the detailed analysis of the symbiotic populations revealed a single, that is, homogeneous, population for *C. orbiculata*, whereas up to seven different subpopulations were described for *C. orbicularis* (Caro *et al.*, 2007). For *L. pensylvanica*, the symbionts presented higher mean values of relative cell size (SSC) and genomic content (FL1) compared with *C. orbiculata*. As bacterial cells with high genomic content were demonstrated to be highly active (Lebaron *et al.*, 2001; Servais *et al.*, 2003), we suggest that the symbionts of *L. pensylvanica* support a greater activity in the symbiotic relation with host than those of *C. orbiculata*. Accordingly, although the part of carbon brought by the symbionts in the bivalve nutrition remains variable vs. filtration part (Cary *et al.*, 1989; Duplessis *et al.*, 2004; Rossi *et al.*, 2013), it is likely that *C. orbiculata* relies more on particulate feeding than *L. pensylvanica*, which relies preferentially on 'symbiont' nutrition. Moreover, the detailed composition of the symbiotic population of *L. pensylvanica* corroborates this hypothesis as 70–90% of the symbiont population belong to the HGC (high genomic content), rather considered as active cells (Caro *et al.*, 2007). From freshly collected individuals, the S₀

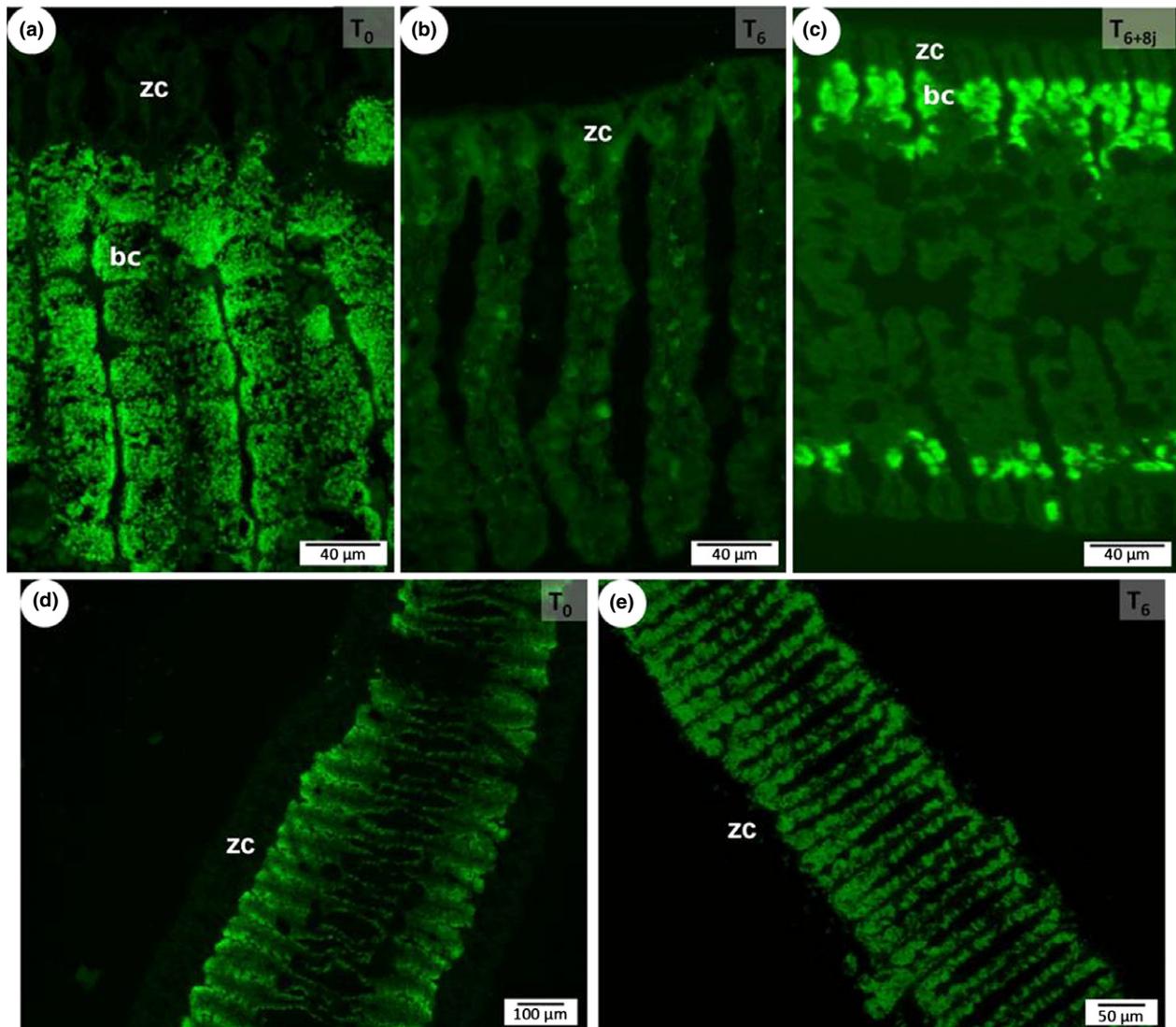


Fig. 4. Modifications occurring at the symbiotic population level in *Codakia orbiculata* and in *Lucina pensylvanica* according to CARD-FISH analyses. In a freshly collected individual of *C. orbiculata* (T_0), the eubacterial probe (EUBIII) strongly hybridized the gill-endosymbionts that appear stained green with FITC (a). Symbiotic bacteria occupied the whole bacteriocyte cytoplasmic volumes (bc). Ciliated zone (zc) is not hybridized. In a 6-months-starved individual of *C. orbiculata*, there is no signal and the bacteriocytes seem devoid of symbionts (b). In a 6-months-starved individual of *C. orbiculata* returned 4 days in the field (c), symbionts are again present in the bacteriocytes (bc). In a freshly collected individual *L. pensylvanica*, hybridization of endosymbionts with the same probe showed the presence of many symbionts along the lateral zone of gill filaments (d) as in a 6-months-starved individual (e).

Table 1. Total protein concentration in gill tissues in *C. orbiculata* and *L. pensylvanica* at T_0 and after 3, 4 and 6 months of starvation. Total protein concentration is expressed in milligram of protein per gram of tissue \pm standard deviation

	<i>Codakia orbiculata</i>		<i>Lucina pensylvanica</i>
T_0	157.5 \pm 43.6	T_0	164.2 \pm 26.9
T_4	111.8 \pm 22.4	T_3	160.8 \pm 20.8
T_6	78.8 \pm 12.1	T_6	156.6 \pm 15.2

concentration measured in endosymbionts was comparable for both host species. As sulphur content was shown to greatly influence the relative cell size (SSC) measured by flow cytometry (Caro *et al.*, 2007), then the differences between SSC mean values of *C. orbiculata* (0.9) and *L. pensylvanica* (2.4) should be attributed mainly to different cell size of symbionts. *Lucina pensylvanica* would host larger symbionts than *C. orbiculata*.

Impact of starvation conditions on symbiotic population and host survival strategy

For *L. pensylvanica*, the maintenance of host in starvation during 6 months had several consequences on the symbiotic population. While the bacterial abundance of symbionts appeared stable according to FISH throughout the experiment, suggesting the absence of symbiont digestion by the host, sulphur content decreased regularly till the third month of starvation. This is consistent with the progressive decrease of SSC parameter measured by flow cytometry which takes into account both sulphur content and cell size of symbionts. During the first 3 months of starvation, the symbionts rely on their sulphur granule storage to sustain an efficiency metabolism and subsequent host nutrition while stocks last. At the same time, this symbiotic activity to sustain host nutrition found expression in the genome content/cell which increases till the fourth month. Similar increases in the nucleic acid content of *C. orbicularis*-symbiont were already observed in the same starvation conditions but in a different time scale (Caro *et al.*, 2009).

The increase in the genomic content of symbionts consecutive to host starvation could correspond to an increase in the genome copy number, to counterbalance the starvation conditions of the host. As the host is supposed to be nourished by its symbionts by translocation of organic compounds, the more the symbionts have copies of genomes, the more active they are. As a consequence, the nutrition of the host is supposed to be better. Indeed, variation in the genome copy number was demonstrated with endosymbionts of aphids in relation with the physiological conditions of the host (Komaki & Ishikawa, 2000). In the present study, during the first months of starvation, the 'Low Genomic Content' subpopulation of *L. pensylvanica*'s symbionts observed at T_0 was progressively replaced by a new subpopulation, with high genomic content and assumed as low sulphur content HGC-LS (Caro *et al.*, 2007). Yet, Lebaron *et al.* (2001) demonstrated that bacteria with high genomic content corresponded to active cells vs. low genomic content bacteria, considered as inactive cells. In our case, the increase in FL1 signal till the fourth month (while SSC decreased) can be interpreted as a substitution of the inactive subpopulation (Low Genomic Content) by an active subpopulation (High Genomic Content-Low Sulphur), by increasing the number of genome copies. After 6 months of starvation, as no more sulphur was available in the symbiont cells as energy source, the symbionts exhibited some physiological alterations, in particular at the genomic level which decreased from T_4 (0.8) to T_6 (0.3). The decrease in sulphur content had a clear impact on the SSC signal in cytometric analysis as SSC dropped from

2.4 at T_0 to 0.1 at T_6 in spite of a constant abundance of symbionts in bacteriocytes. We suggest that for *L. pensylvanica*, activation of symbiont metabolism through chromosome replication in response to host starvation could result from a modification of the host control on the symbiotic population. The increase of the bacterial chromosome replication probably strengthens symbiont metabolism and finally ensures nutritional requirements of the bivalve host. In the *Rhizobium*-leguminous symbiosis, the DNA replication of the endosymbiont bacteroid is controlled by the host-plant (Mergaert *et al.*, 2006). One could suggest a similar regulation in the symbiotic relationships between *L. pensylvanica* and its symbionts, although the molecular regulation driven by the host remains unknown. The activation of symbionts under stress conditions could represent a survival strategy developed by the host. Moreover, the gills did not lose weight during starvation of the bivalves in the laboratory, suggesting that the host itself is not really impacted by the lack of food in its environment during the first 4 months of starvation. This is probably due to a quite normal metabolism of its symbionts, relying on its sulphur storage. Thus, *L. pensylvanica* favours the protection of its symbionts compared with *C. orbiculata* which digests its symbionts once they are not productive enough. Beyond the period where elemental sulphur is available in the gills of *L. pensylvanica*, the survival strategy of the host remains unclear.

For *C. orbiculata*, the response of the symbiont population was totally different during the 6 months starvation period. FISH data revealed that the number of symbionts decreased through the starvation as already shown in *C. orbicularis* (Caro *et al.*, 2009). As symbionts are not released into the environment during starvation (Brissac, 2009b), this decrease is presumably a consequence of symbionts' digestion by host bacteriocytes using lysosomal enzymes. In addition, the stock of sulphur in gills disappeared after 1 month of starvation. Concomitantly, the symbiont characteristics (relative cell size and genomic content) changed but in a different way compared with *L. pensylvanica*. The main modification at the symbiotic level for *C. orbiculata* occurred during the first month of starvation and consisted in an decrease in relative cell size (SSC). This was consistent with the decrease in sulphur content measured through X-ray fluorescence analyses, probably related to the consumption of elemental sulphur by symbionts as the only energy source available. However, a reduction of the cell size cannot be excluded. The increase of the genomic content in bacterial cells during starvation was not observed for *C. orbiculata*, suggesting that host would not trigger symbiont activation under sulphide starvation as it was observed for *L. pensylvanica*. Between the first

and sixth month of experiment, no important changes were observed in terms of relative cell size and genomic content of the whole symbiont population. However, the cytometric pattern of the population was modified as two subpopulations appeared after long-term starvation, indicating progressive degradation of genome or reduction in the number of genome copies as observed with *C. orbicularis* (Caro *et al.*, 2009). Thus, we propose the following survival strategy: during the first month of starvation, *C. orbiculata* nutrition relies on metabolically active thioautotrophic symbionts that synthesize organic compounds via carbon fixation according to the elemental sulphur available from their sulphur granule storage. Then, when symbionts failed to transfer enough carbonates to the host cells due to lack of elemental sulphur that fuel carbon fixation, the bivalve considers the bacterial symbionts themselves as a putative carbon storage. Thus, bacteriocytes begin to digest its intracellular symbionts using classical lysosomal activity (Fiala-Médioni *et al.*, 1994; Liberge *et al.*, 2001) to sustain host nutrition and survival. A similar strategy was demonstrated for *C. orbicularis* (Caro *et al.*, 2009) that digest its symbiotic population to face long-term starvation. Moreover, during starvation, *C. orbiculata* individuals lost weight ($\sim -50\%$ for gills within 6 months) suggesting that digestion of the symbionts is even not sufficient for the bivalve in such drastic conditions. So, this host species needs to acquire new symbiosis-competent bacteria from its environment as soon as possible to recover a normal physiology based on efficiency bacterial metabolism.

Impact of the reintroduction of starved *C. orbiculata* in their natural environment on symbiotic population and host strategy

The reacquisition of gill-endosymbionts by starved *C. orbiculata* individuals after return to the field was already shown along with a reorganization of the gill epithelium (Elisabeth *et al.*, 2012; Gros *et al.*, 2012), demonstrating a real plasticity of eukaryotic cells in the gill tissue. Nevertheless in this study, we bring new insights about the modifications occurring at the symbiont population level. When individuals starved for 6 months were put back to the field, that is, sulphidic environment, symbionts rapidly get in bacteriocytes. During the first 2 weeks after their reintroduction into the field, the cellular characteristics of bacterial populations showed an important increase in relative cell size (SSC), larger than that at T_0 , along with an increase in the genomic content (higher than T_0) and a weak increase in sulphur content. These data evidenced mainly an initial increase in cell size during the first 2 weeks. Beyond, symbiont cells restore the

elemental sulphur storage, as the sulphur amount increased greatly till the 30th day in the field. At the same time, the relative cell size (SSC) returns to values close to T_0 in spite of sulphur content increase, indicating that the symbiont population recovers its initial structure in terms of cell size and genomic content; this was confirmed by the cytometric pattern. The interval time necessary to recover a structural and functional symbiotic population was approximately 3 weeks for a classical colonization of gills.

These data suggest that lucinids can control their symbionts completely differently depending on the host species in case of food and/or sulphide depletion. Apparently, *L. pensylvanica* uses other nutrition pathways than symbionts digestion during long-term starvation and prefers to preserve its bacterial symbionts as long as possible. The strategy seems different for *C. orbiculata* which digests quickly its bacterial population to supply the deficiency of carbon and nitrogen sources and remade new gill-endosymbiont stocks once in contact with free-living symbiont forms present in the environment. Further studies are needed to understand the metabolic pathway, different from symbiont digestion, used by *L. pensylvanica* to face starvation conditions.

Author's contribution

N.H.E. and A.C. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Mean values of SSC and FL1 during a 6-months-starvation period for *Codakia orbiculata* (A, B, respectively) and *Lucina pensylvanica* (C, D, respectively).

Fig. S2. Mean values of SSC (A) and FL1 (B) after *Codakia orbiculata*, starved during 6 months (T₆), were put back to the field during 4, 8, 14 and 21 days.