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Seasonal modifications and morphogenesis of the hypercalcified sponge *Petrobiona massiliana* (Calcarea, Calcaronea)

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Abstract. The periodicity of sexual elements and soft tissue modifications during the life cycle of the hypercalcified sponge *Petrobiona massiliana* was investigated monthly from June 2006 to November 2007. Sexual reproduction, likely regulated by seawater temperatures, occurred during more than half of the year (from early April to late October); 70% of the samples appeared reproductively active. Specimens of *P. massiliana* displayed a high plasticity of tissue organization, allowing modulation and rearrangement of their aquiferous systems in response to life cycle phases and environmental changes. Permanent changes were observed in the basal region of the choanosome in non-reproductive specimens, such as disorganization/restructuring events leading to remodeling of the aquiferous system. Periodic modifications occurring during sexual reproduction included the transformation of choanocytes from a typical form to hourglass and vespiform shapes, and more global disorganization of the basal region of the choanosome during provisioning of oocytes and embryos, followed by restructuring after release of the larvae. Finally, episodic disorganization/reorganization phenomena occurred in a few specimens after unfavorable environmental conditions (e.g., decreasing seawater temperatures). Histological and ultrastructural observations of storage cells, located in peculiar trabecular tracts, suggest a transdifferentiation capacity that allows such soft tissue dynamics.

Additional key words: calcareous sponge, coralline sponge, Porifera, reproductive cycle

While many studies have focused on the life cycle and reproduction of marine and freshwater demosponges (reviewed in Maldonado & Riesgo 2008), only a few detailed studies have been dedicated to the reproductive biology of calcareous sponges (Dendy 1915; Duboscq & Tuzet 1944; Johnson 1978; Lanna et al. 2007), with most of them focused on the reproductive processes of gametogenesis, fertilization, and embryogenesis (Dendy 1915; Tuzet 1947; Vacelet 1964; Anakina 1981, 1988, 1999; Gallissian 1983; Gaino et al. 1987; Franzen 1988; Gallissian & Vacelet 1990, 1992; Nakamura et al. 1998; Watanabe & Okada 1998; Anakina & Drozdov 2000, 2001; Amano & Hori 2001; Eerkes-Medrano & Leys 2006; Lanna & Klautau 2010). Moreover, while some works have described morphological modifications of soft tissues during the life cycles of demosponges (Lévi 1956; Simpson 1968; Van de Vyver & Willenz 1975; Chen 1976; Ivanova 1978, 1981; Diaz 1979; Barthel 1986; Witte & Barthel 1994; Ereskovsky 2000; Gugel 2001), for Calcarea such descriptions are scarce (Vacelet 1964). Most studies on members of both classes have reported that some stages of the life cycle, like reproduction, might lead to local disorganization or even destruction of the soft tissue, principally concerning important reductions of choanocyte chambers and canals.

Members of *Petrobiona massiliana* Vacelet & Lévi 1958 are relatively small calcareous sponges dwelling in dark submarine caves at shallow depths in the Mediterranean. They are considered "hypercalcified" sponges due to their massive basal skeleton composed of high-magnesium calcite. The soft tissue is made of a superficial cortex and an underlying choanosome extending between large spiky crests of the skeleton (Vacelet 1964; Fig. 1a). The choanosome extends basally into narrow canals of the basal skeleton, forming trabecular tracts (Vacelet 1964; Fig. 1a). These tracts contain aggregates of archaeocyte-like cells that are rich in storage granules and resemble gemmular thesocytes (Vacelet 1964, 1990).

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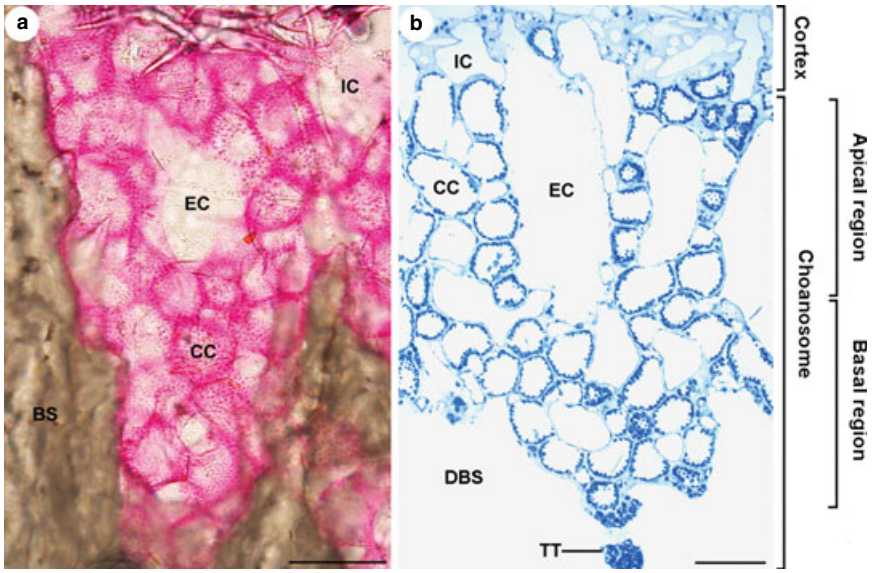

Fig. 1. Light micrographs showing the general morphology of adults of *Petrobiona massiliana*. **a.** Transverse ground section showing the relation between soft tissue (acid fuchsin stained) and the basal skeleton. Scalebar=200 μm. **b.** Transverse semi-thin section of a decalcified specimen showing internal organization of soft tissue (methylene blue stained). Scalebar=100 μm. BS, basal skeleton; CC, choanocyte chamber; DBS, decalcified basal skeleton; EC, excurrent canal; IC, incurrent canal; TT, trabecular tract.

Because of their cryptic location in narrow canals of the skeleton and their content of storage cells, trabecular tracts have been proposed to be totipotent dormant bodies able to regenerate the sponge after the death of superficial soft tissue during adverse environmental conditions (Vacelet 1964, 1990). This possible ability, which needs to be further investigated, could explain the exceptional survival of this relict hypercalcified sponge through geological time (Vacelet 1990; Vacelet et al. 2002; Manconi et al. 2009). Oogenesis and embryogenesis of *P. massiliana* have already been extensively described with light microscopy (LM) by Vacelet (1964), and at the ultrastructural level by Gallissian & Vacelet (1990, 1992). These authors demonstrated that an unusually large number of nurse cells are used in the development of oocytes and embryos; the developing oocytes and embryos absorb all the choanocytes of a choanocyte chamber during their formation, an exceptional phenomenon among Porifera (Ereskovsky 2010). In this species, reproduction is likely to cause tissue modifications with important disruptions of the aquiferous system and alterations in filtration rate. The goal of this study was to measure seasonal modifications and morphogenesis of the soft tissue in reproductive and non-reproductive specimens of *P. massiliana*. For that purpose, we focused on the life cycle of this species and completed (1) a description of the periodicity of reproductive elements in parallel with the seasonal temperature cycle, (2) an investigation of the soft tissue modifications occurring permanently (remodeling), periodically (due to reproduction), and episodically (after adverse environmental conditions) over the annual cycle, and (3) an ultrastructural study of storage cells forming trabecular tracts with an evaluation of their putative function in tissue dynamics during the life cycle.

Methods

Three entire specimens of *Petrobiona massiliana* were collected each month from June 2006 to November 2007 (except for December 2006 and August 2007) from an underwater cave at 14 m depth in the Calanque of La Vesse, Marseille (France). The seawater temperature in the cave was recorded every 6 h from October 2004 to November 2007 with a data logger (Tidbit Onset Temperature Data Logger) anchored to the substratum. The sponges were fixed immediately after collection in a solution of 4% glutaraldehyde in 0.2 mol L^{-1} sodium cacodylate, 0.1 mol L^{-1} NaCl, and 12% sucrose (pH 7.4, 1700 mOsM) for ~36 h. Specimens were then decalcified in a 4.1% EDTA solution adjusted to pH 6.8 with NaOH and supplemented with 5% polyvinyl pyrolidone (Fullmer 1966) and 12% sucrose to give a final osmotic pressure of 1142 mOsM. Decalcification solutions were renewed every other day for 1–2 months. All specimens were then rinsed in cacodylate buffer, postfixed in 1% osmium tetroxide in

0.2 mol L^{-1} sodium cacodylate and 0.3 mol L^{-1} NaCl (pH 7.4, 1040 mOsM), rinsed again in cacodylate buffer, dehydrated in a graded ethanol series, and stored in 95% ethanol at 4°C before further treatment. For LM and transmission electron microscopy (TEM), decalcified samples were embedded in a low viscosity epoxy resin (Spurr Low Viscosity Embedding Media, Polysciences Europe GmbH, Eppelheim, Germany) and sectioned with a Leica Ultracut ultramicrotome (Leica Microsystems Belgium BVBA, Grand-Bigard, Belgium). Semi-thin sections stained with methylene blue were observed on a Nikon Optiphot II light microscope (Nikon Instruments Europe B.B., Amstelveen, The Netherlands). For each of the three specimens sampled monthly, a volume of ~3.5 mm^3 of soft tissue was investigated using LM. In addition, thin sections, double contrasted with uranyl acetate and lead citrate (Reynolds 1963) were examined by TEM using a LEO 906 E (Leo Electron Microscopy Ltd, Cambridge, UK) at 80 kV. For scanning electron microscopy (SEM), fixed samples were cryofractured in liquid nitrogen using a razor blade, critical point dried (Balzers CPD 030, Balzers, BAL-TEC GmbH, Schalksmühle, Germany) using carbon dioxide as the transitional fluid, mounted on aluminum stubs, sputter-coated with gold (Balzers SCD 50), and observed with a Philips/FEI XL30 ESEM TMP (Philips/FEI Europe, Eindhoven, The Netherlands) at 30 kV. Ground sections for LM were prepared from fixed samples stained in absolute ethanol saturated with acid fuchsin for 1h and embedded in epoxy resin (Spurr Low Viscosity Embedding Media, Polysciences, Inc). Thick sections (1–2 mm) were obtained with a low speed diamond saw (Bennet Labcut 1010, Ernest Bennett Co. Ltd, Darlington, UK) prior to grinding. Digital photomicrographs were taken with a Nikon Coolpix and a Moticam camera mounted on a Nikon Optishot II microscope.

Results

Periodicity of sexual reproduction

Reproductive elements were principally observed in the basal region of the choanosome, above the trabecular tracts, from April to October of both studied years (Table 1). The percentage of reproductive specimens (those undergoing oogenesis or embryogenesis) and the seawater temperature recorded in the cave from June 2006 to November 2007 are shown in Fig. 2.

Oogenesis was recorded from early April (when the minimum water temperature reached 15°C) to October. During this period, oogonia were observed each month in at least two of three individuals. Oogonia occurred in the mesohyl, often underneath the choanoderm. Young oocytes were first observed in May, when the water temperature ranged from 15 to 21°C, and were still present in early October 2006 and September 2007 (Fig. 2). Like oogonia, they were located

Table 1. The number of specimens of *Petrobiona massiliana* in which reproductive elements were observed in soft tissues and cave water temperature (monthly minimum, maximum) each month from June 2006 to November 2007. Dashes (–) indicate that no sponge samples were collected in December 2006 and August 2007.

	Temperature (°C)		Oogonia	Oocyte	Stomoblastula	Amphiblastula
June	15.3	22.7	3	3	0	0
July	21.3	27.3	3	3	2	2
Aug.	14	20.4	2	2	0	0
Sept.	14.8	22.5	2	2	0	0
Oct.	17.5	21.6	2	2	0	0
Nov.	17.6	19.7	0	0	0	0
Dec.	15.6	17.7	–	–	–	–
Jan.	13.2	15.5	0	0	0	0
Feb.	13.4	13.9	0	0	0	0
Mar.	13	14	0	0	0	0
Apr.	13.5	18.6	2	0	0	0
May	14.7	20.3	2	1	0	0
June	14.4	22.1	2	2	0	0
July	15	21.7	3	2	0	1
Aug.	15.8	27.9	–	–	–	–
Sept.	15.6	25.8	2	1	1	0
Oct.	16.8	20.4	3	0	0	0
Nov.	13.3	18.3	0	0	0	0

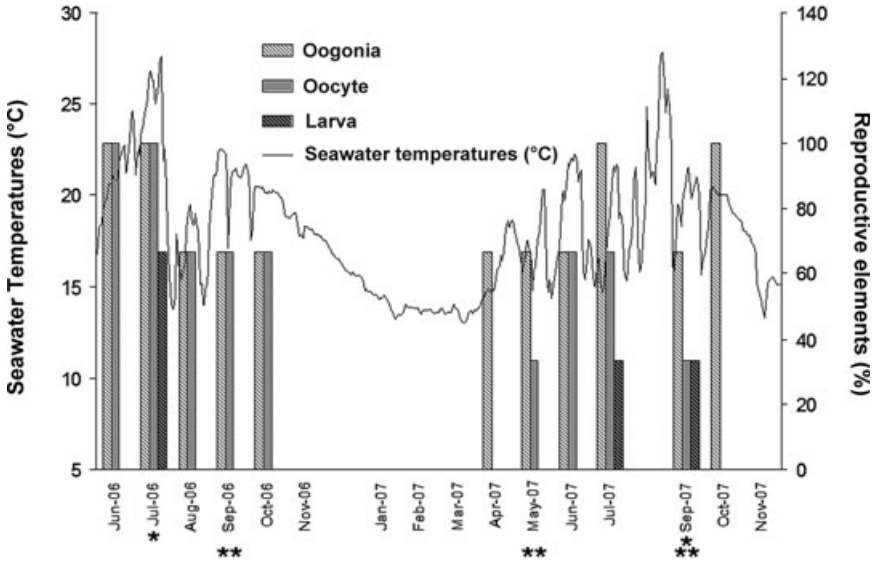

Fig. 2. Percentage of specimens of *Petrobiona massiliana* containing reproductive elements (oogonia, oocytes, or larvae; n = 3 individuals per month) and seawater temperature in the cave from June 2006 to November 2007. Single and double stars indicate the observation of (respectively) hourglass-shape and vespiform choanocytes in the choanosome of at least one specimen.

in the mesohyl, underneath the choanoderm. At this stage, oocytes were engaged in active feeding, phagocytosing differentiated choanocytes of the adjacent chamber (Vacelet 1964; Gallissian & Vacelet 1990).

Although fertilization could not be detected, embryo cleavage was observed in June 2006 and June 2007, in one sample each time, suggesting that fertilization occurred between May and June. Only four-cell stages were observed, in the mesohyl near a choanocyte chamber.

Further embryogenesis was observed in July 2006 and 2007, and in September 2007. All stages were observed mainly in the basal region of the choanosome: developing stomoblastulae in the mesohyl (Table 1), stomoblastula inversion, young amphiblastulae phagocytosing modified choanocytes, and mature larvae in the host choanocyte chamber (Table 1) (see Vacelet 1964 for details). Oogonia and unfertilized oocytes were also observed in all specimens that contained later stage embryos. In July 2006, when water temperature reached its maximum (21–27°C), two specimens contained all embryos at all stages of embryogenesis. In July 2007, water temperatures were lower (15–22°C) and only one specimen showed well-differentiated amphiblastulae. The highest temperatures were measured in August 2007, but unfortunately no sample was collected at that time. In September 2007, stomoblastulae were still observed in the mesohyl of one sample.

Modifications of the soft tissue

Comparative study of specimens sampled between June 2006 and November 2007 showed several modifications of the soft tissue over time. In reproductive specimens, changes mainly concerned the basal region of the choanosome, where reproductive elements developed. Nonetheless, the soft tissue of non-reproductive specimens also showed local changes that became even more substantial during the coldest period.

Choanosome of reproductive specimens. As soon as the first oogonia, oocytes, and modified chambers appeared in early May, choanocyte chambers were reduced in size and number, leaving a more extended mesohyl (Fig. 3a,b). During maturation of larvae (e.g., in July: Fig. 3c), the choanosome became even more disorganized, reaching its maximum disorganization after the release of amphiblastulae. For example, in August 2006, disintegration of choanocyte chambers as well as excurrent canals led to large gaps in the mesohyl (Fig. 3d). The choanosome progressively returned to its normal organization in September (Fig. 3e) and by October had regained a classical leuconoid structure with numerous large, homogenous choanocyte chambers separated by a thin mesohyl (Fig. 3f), although few oogonia and oocytes were still observed.

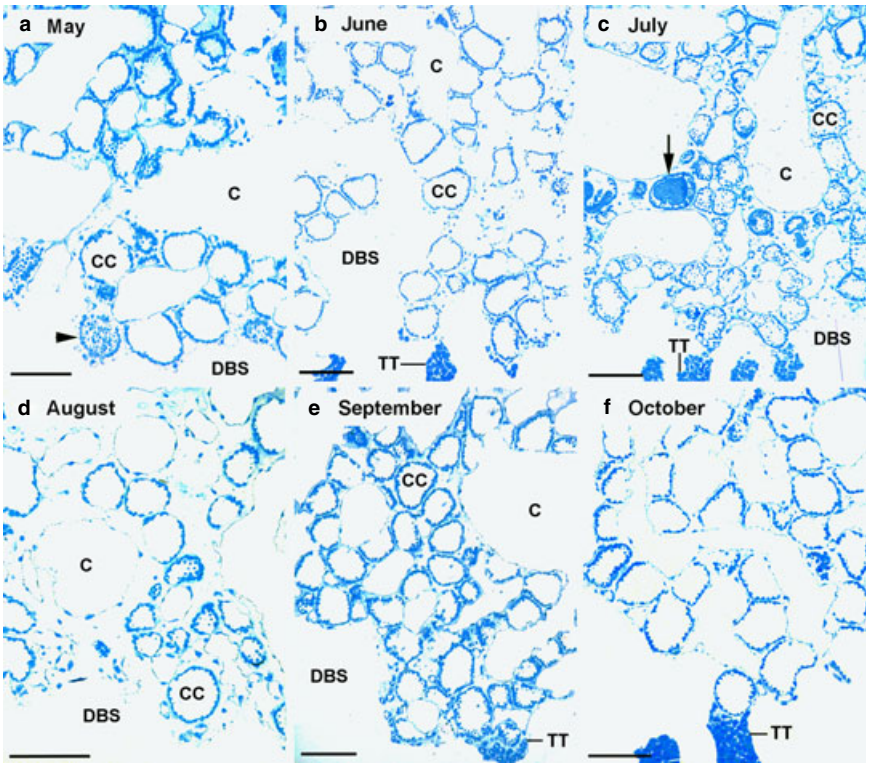

Fig. 3. Variation in the organization of the choanosome in reproductive specimens of *Petrobiona massiliana* over time. **a-f.** Light micrographs of transverse semi-thin sections in samples from different months. Sections are all oriented with cortex upwards. The arrow indicates an amphiblastula in host chamber and the arrowhead shows a modified chamber with vespiform choanocytes. All scalebars = 100 μm. C, canal; CC, choanocyte chamber; DBS, decalcified basal skeleton; TT, trabecular tract.

Choanosome of non-reproductive specimens. Specimens not involved in reproduction usually had a well-organized choanosome with numerous large, homogeneous choanocyte chambers and canals, similar to the reorganized reproductive specimens sampled from October (Fig. 3f). Nonetheless, minor disorganizations or reorganizations occurred locally in the basal regions of all non-reproductive specimens investigated. The extent of those basal disorganization/reorganization events varied within the same specimen, but also between specimens from the same monthly sampling. These zones were characterized by a slightly thicker mesohyl resulting from some smaller choanocyte chambers and narrower canals. In these areas, endopinacocytes were larger than elsewhere in the choanosome and contained many cytoplasmic vacuoles.

Finally, non-reproductive specimens showed more significant disorganization of the choanosome when seawater temperature decreased. In two samples from November 2006, in addition to the local basal modifications related above, the aquiferous system was reduced, leading to a thickened mesohyl in the whole choanosome (Fig. 4a). Choanocyte chambers and canals were disrupted and often appeared detached from the surrounding mesohyl (Fig. 4b, double arrowhead). In one sample from January 2007, these changes principally occurred basally in different parts of the sponge (Fig. 4c). These basal changes were similar but more dramatic than those observed permanently in all non-reproductive specimens. As mentioned above, endopinacocytes of the reduced canals contained numerous intracytoplasmic vacuoles (Fig. 4d).

Choanocyte chambers. Typical choanocyte chambers had a diameter ranging 40–100 μm and were observed year-round (Fig. 5a,b). Typical choanocytes were cylindrical (6 × 10 μm) with a 15-μm long flagellum surrounded by a collar of microvilli (10 μm in length) and they were apinucleate, which is typical of Calcaronea.

As previously described by Vacelet (1964) using LM, classical choanocytes of *Petrobiona massiliana* presented two modified morphologies during the life cycle, known as "hourglass-shape" and "vespiform."

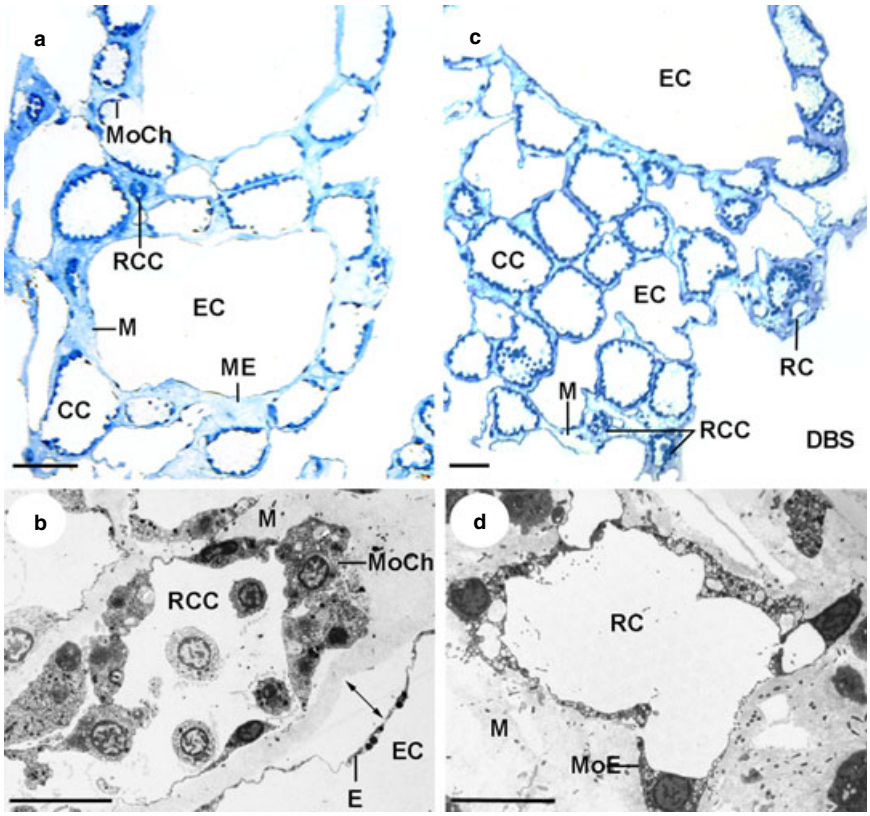

Fig. 4. Variation in the organization of the choanosome in non-reproductive specimens of *Petrobiona massiliana* after episodic unfavorable environmental conditions. **a.** Light micrograph of a section of a specimen collected in November 2006. Scalebar=50 μm. **b.** Transmission electron micrograph of a reduced choanocyte chamber in a specimen from November 2006. Double arrowheads indicate a detached pinacocyte. Scalebar=10 μm. **c.** Light micrograph of a section of a specimen collected in January 2007. Scalebar=50 μm. **d.** Transmission electron micrograph of a modified excurrent canal in a specimen from January 2007. Scalebar=10 μm. CC, choanocyte chamber; DBS, decalcified basal skeleton; MoCh, modified choanocyte; MoE, modified endopinacocyte; E, endopinacocyte; EC, excurrent canal; M, mesohyl; ME, missing endopinacocyte; RC, reduced canal; RCC, reduced choanocyte chamber.

In specimens with developing embryos, some choanocyte chambers contained "hourglass-shape" choanocytes (Table 1, Fig. 2). In these choanocytes, the cell body was elongated, reaching about 15 μm in length with a diameter of about 6 μm, while the basal part was wider, reaching 9 μm in diameter (Fig. 5c,d). A constriction of the cell frequently appeared below the nucleus. The flagellum was generally present, while microvilli of the collar were shorter (about 5 μm long) (Fig. 5d) or absent. These transformations appeared principally in the choanocyte chambers of the central and basal regions of the choanosome and concerned almost all choanocytes of those chambers (Fig. 3c).

The chambers with "vespiform" choanocytes, observed only in May and September (Fig. 2), were always contracted and therefore smaller (Fig. 5e). The apical part of the cells appeared either still continuous with the basal part through a constriction (Figs. 5f, 6a) or free in the lumen of the contracted chamber (Figs. 5f, 6b). In both cases, the apical parts of the cells were ovoid, with shorter microvilli than typical choanocytes (Fig. 6b–d). The thin cytoplasm surrounded a large nucleus and contained mitochondria and a large Golgi apparatus (Fig. 6b,c). Where apical and basal parts of vespiform choanocytes were separated, the remaining quadrangular-shaped basal parts formed a capsule-like compartment closed by prosopylar and apopylar cells, surrounding the free apical parts enclosed in the lumen (Figs. 5f and 6d). The cytoplasm of basal parts contained phagosomes, few mitochondria (Fig. 6a), and often a linear rough endoplasmic reticulum along the cell membrane (Fig. 6a). The lumen of the contracted chambers always contained a large central cell (Figs. 5f and 6b). These transformations affected only a small

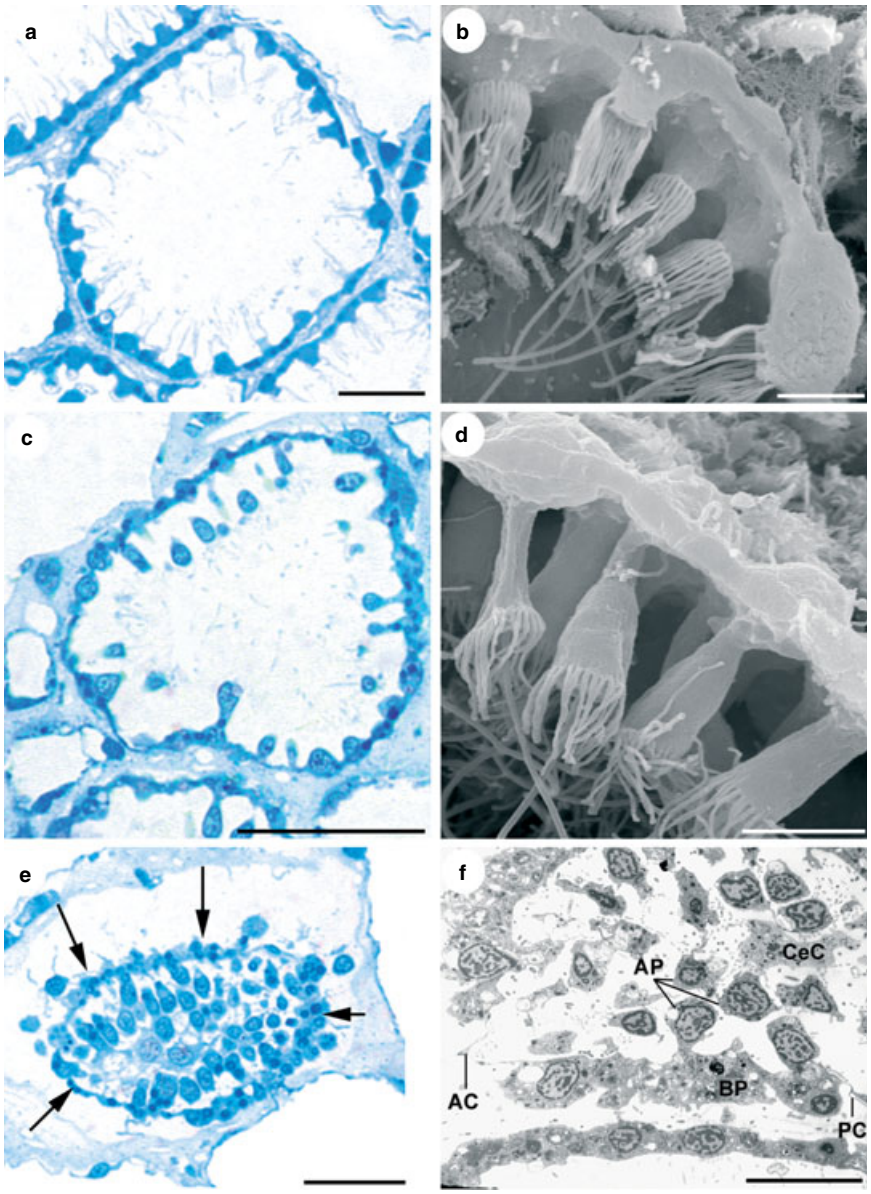

Fig. 5. Morphology of choanocyte chambers during the reproductive cycle of *Petrobiona massiliana*. **a.** Light micrograph of chamber containing typical choanocytes. Scalebar=20 μm. **b.** Scanning electron micrograph of chamber containing typical choanocytes. Scalebar=5 μm. **c.** Light micrograph of chamber containing hourglass-shape choanocytes. Scalebar=50 μm. **d.** Scanning electron micrograph of chamber containing hourglass-shape choanocytes. Scalebar=5 μm. **e.** Light micrograph of chamber containing vespiform choanocytes. Arrows indicate characteristic constriction of the chamber. Scalebar=20 μm. **f.** Transmission electron micrograph of chamber containing vespiform choanocytes. Scalebar=10 μm. AP, apical part of choanocyte; AC, apopylar cell; BP, basal part of choanocyte; CeC, central cell; PC, prosopylar cell.

number of chambers, but all choanocytes in affected chambers were modified to the vespiform morphology (Fig. 3a).

Storage cells of the trabecular tracts

Trabecular tracts, as defined by Vacelet (1964, 1990), are elongated (sometimes >100 μm long) basal portions of the choanosome containing storage cells and extending into narrow canals of the basal skeleton (Fig. 7a–c). They are often adjacent to choanocyte chambers (Fig. 7a,b). Trabecular tracts also contain numerous microdiactine spicules and a few eosinophilic archaeocytes with small, electron-dense granules.

Storage cells presented three different morphologies depending on their position in the trabecular tracts. They are designated here in an ascending sequence

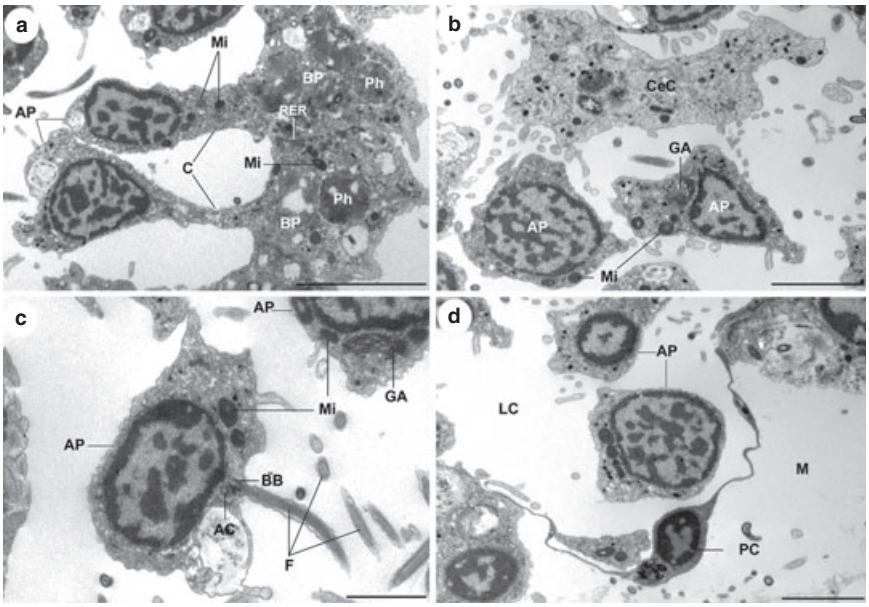

Fig. 6. Transmission electron micrographs of vespiform choanocytes of *Petrobiona massiliana*. **a.** Two vespiform choanocytes with apical parts still attached to basal parts through a cell constriction. Scalebar=5 μm. **b.** Central cell and "migrated" apical parts of two choanocytes. Scalebar=2 μm. **c.** "Migrated" apical part of a choanocyte still bearing a flagellum. Scalebar=2 μm. **d.** Prosopylar cell closing the contracted chamber. Scalebar=2 μm. AC, accessory centriole; AP, apical part of choanocyte; BB, basal body; BP, basal part of choanocyte; C, constriction; CeC, central cell; F, flagella; GA, Golgi apparatus; LC, lumen of the choanocyte chamber; M, mesohyl; Mi, mitochondria; Ph, phagosome; PC, prosopylar cell.

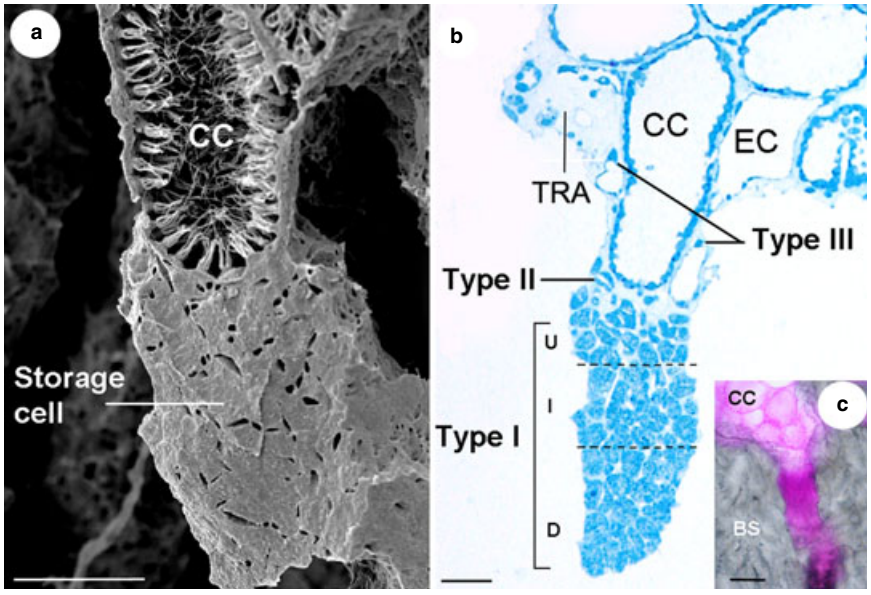

Fig. 7. General morphology of trabecular tracts of *Petrobiona massiliana*. **a.** Scanning electron micrograph of a cryofractured specimen showing a trabecular tract just below a choanocyte chamber. Scalebar=20 μm. **b.** Light micrograph of a semi-thin section of a trabecular tract from a non-reproductive specimen showing type I, II and III storage cells, and a small area with tissue remodeling. Scalebar=20 μm. **c.** Light micrograph of a thin polished section of a trabecular tract in a non-decalcified specimen. Scalebar=50 μm. BS, basal skeleton; CC, choanocyte chamber; D, deeper portion; EC, excurrent canal; I, intermediate portion; TRA, tissue remodeling area; U, upper portion.

from the bottom of the tracts toward the apical choanocyte chambers as storage cells type I, II, and III (Fig. 7b).

Type I storage cells. Storage cells of the first type were closely packed together in the trabecular tracts, mostly in their deepest parts (Fig. 7b), associated in pairs with a thin central space, or lacuna, separating the two cells of a pair (Fig. 8a,b). All cells were about 10 μm in diameter and had a nucleolated nucleus (3–5 μm in diameter), a small Golgi apparatus, rough and smooth endoplasmic reticulum, phagosomes or secondary lysosomes (1–2 μm), few lipid droplets (1 μm), and numerous small dense inclusions (400–800 nm, Fig. 8a,b). Cells of this compact zone of the tract varied in their content of other cytoplasmic inclusions and were therefore further divided into deep, intermediate, and upper cells (Fig. 7b). The deepest cells contained numerous large granular inclusions (1–2 μm in diameter) completely or partially filled by a mass of electron-opaque grains sometimes assembled into a polygonal shape, and some other large granular inclusions with light or electron-opaque crystal shapes (Fig. 8a). The latter were more common in the cytoplasm of intermediate cells, which showed fewer inclusions with electron-opaque polygonal masses (Fig. 8b). Intermediate cells also contained several phagosomes or secondary lysosomes, some containing one to three large granular inclusions (Fig. 8b). Finally, in the upper type I storage cells, large granular inclusions included more electron-lucent granular inclusions with crystal shapes. These cells rarely included electron-dense polygonal or crystal-shaped inclusions (Fig. 8c).

Type II storage cells. Type II storage cells could be distinguished from the other types according to three criteria. First, they were observed in less compact portions of trabecular tracts (Fig. 7b), principally in their upper part (Fig. 8d). Second, paired cells were less tightly bound, in comparison with type I storage cells, leaving a larger intercellular central space (Fig. 8d). Third, their cytoplasm showed several small, dense inclusions, while large electron-dense granular inclusions were rarely observed (Fig. 8e). Type II storage cells contained only large, lightly granular or empty vacuoles, a wider vesicular smooth endoplasmic reticulum network, phagosomes or secondary lysosomes, few lipid droplets, and a large nucleolated nucleus (Fig. 8d,e).

Type III storage cells. The third type of storage cell was found in the uppermost part of trabecular tracts and also in the basal region of the choanosome near trabecular tracts (Figs. 7b, 8f–h). They typically formed a much larger space between members of pairs (Figs. 7b, 8f) and were similar in morphology to endopinacocytes of the aquiferous canals. Moreover, they were often observed connected to excurrent canals or choanocyte chambers (Fig. 8g). In addition to a large nucleolated nucleus, their cytoplasm was loaded with a highly developed vesicular smooth endoplasmic reticulum, small and large empty vacuoles, small dense inclusions, and a few small phagosomes or secondary lysosomes (Fig. 8f–h).

The three types of storage cells were observed throughout the sponge life cycle, but with different degrees of development. In non-reproductive specimens (Fig. 9a), the choanosome was well organized with long trabecular tracts. In these specimens, some tracts contained an abundance of type I storage cells with only rare type II and no type III storage cells. Other tracts contained more type II storage cells at the very top, with some type III storage cells outside tracts (Fig. 9a). Nonetheless, in two non-reproductive specimens from November 2006 and one from January 2007, all three of which had partly disorganized choanosome, most tracts seemed almost empty in their uppermost part, while numerous type II and III storage cells were observed in the basal region of the choanosome in the vicinity of tracts.

In both years, type II and mostly type III storage cells occurred more abundantly in sponges undergoing reproduction and its associated soft tissue disorganization. In July, the basal region of the choanosome of specimens containing growing larvae included numerous intercellular spaces formed by type II and III storage cells (Fig. 9b), resulting in less dense tracts in comparison with non-reproductive specimens from the same collections. When choanosome disorganization reached its maximum after larval release (e.g., August 2006), trabecular tracts were almost empty of storage cells. Type III storage cells were abundant in the whole basal region of the choanosome (outside of trabecular tracts) and delineated large spaces (Fig. 9c). They often appeared in electron-lucent "hollows" left by disintegrated canals and choanocyte chambers. After embryogenesis (e.g., September 2006), reproductive specimens showed trabecular tracts with closely packed storage cells, although there were still numerous type II and III storage cells in basal parts of the choanosome (Fig. 9d). These features were similar to those observed in some basal regions of non-reproductive samples.

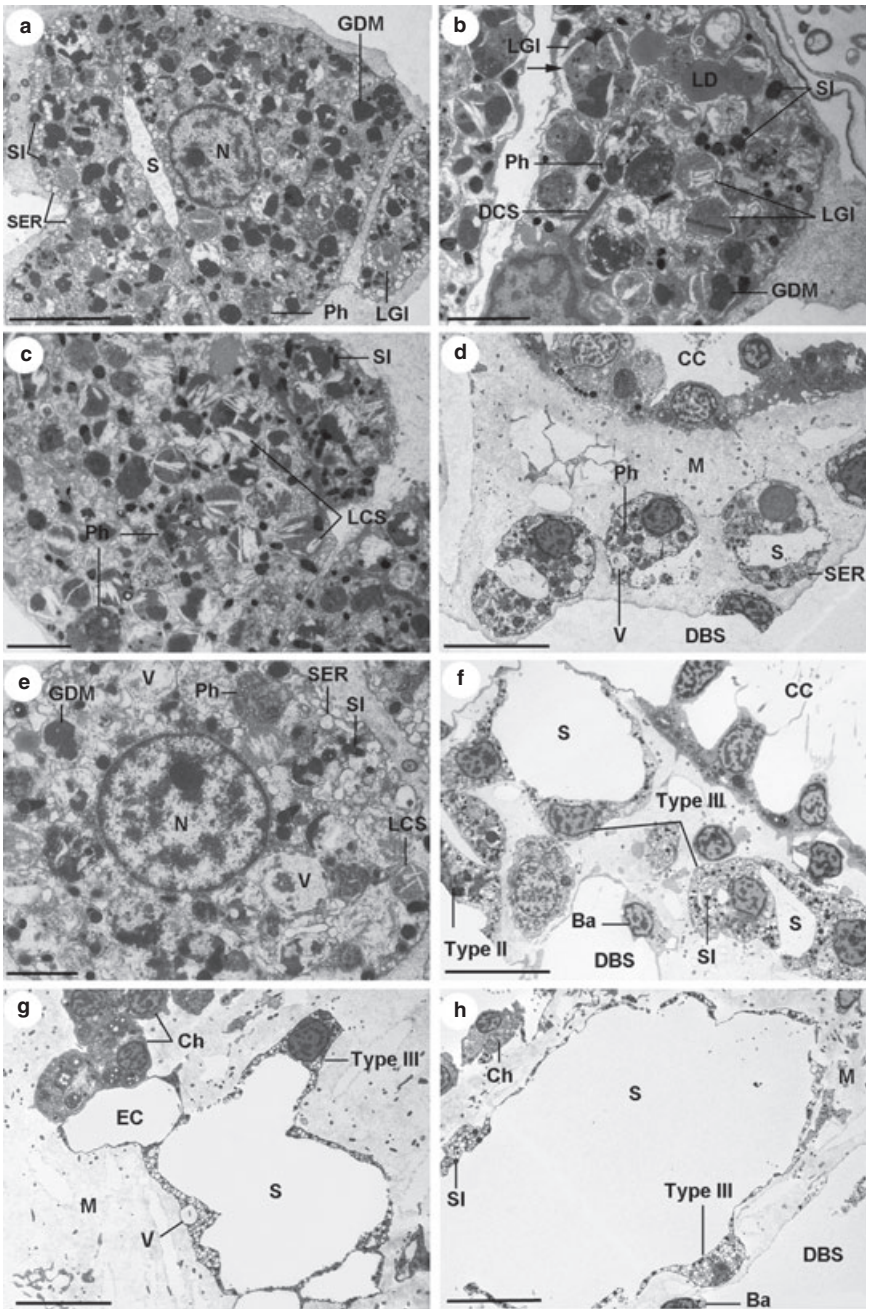

Fig. 8. Transmission electron micrographs showing cytological modifications of storage cells from trabecular tracts of *Petrobiona massiliana*. **a.** Pair of type I storage cells from the deeper portion of a trabecular tract. Scalebar=5 μm. **b.** Detail of cytoplasmic inclusions in a type I storage cell from the intermediate portion of a tract. Scalebar=2 μm. **c.** Detail of cytoplasmic inclusions in a type I storage cell from the upper portion of a tract. Scalebar=2 μm. **d.** Type II storage cells in the upper portion of a trabecular tract. Scalebar=2 μm. **e.** Detail of a type II storage cell. Scalebar=10 μm. **f.** View of the upper portion of a trabecular tract with type II and III storage cells and basopinacocyte. Scalebar=10 μm. **g.** View of type III storage cells forming a large space connected to a choanocyte chamber. Scalebar=10 μm. **h.** View of type III storage cells forming a larger space. Scalebar=10 μm. Ba, basopinacocyte; Ch, choanocyte; CC, choanocyte chamber; DBS, decalcified basal skeleton; DCS, dense crystal shape; EC, excurrent canal; GDM, granular dense mass; LCS, light crystal shape; LD, lipidic droplet; LGI, large granular inclusion; M, mesohyl; N, nucleus; Ph, phagosome or secondary lysosome; S, space; SER, smooth endoplasmic reticulum; SI, small dense inclusion; V, vacuole.

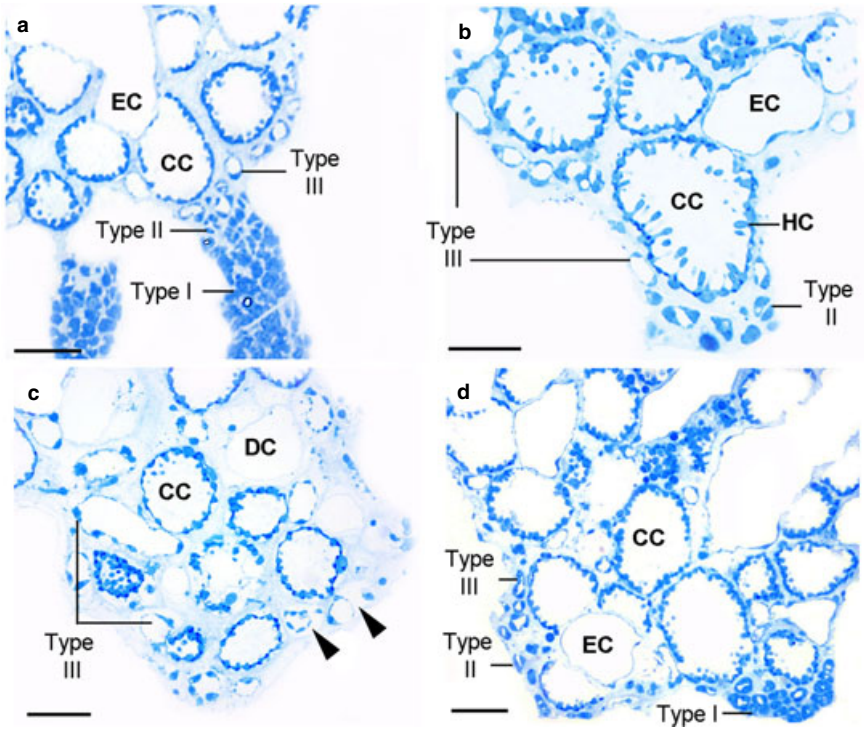

Fig. 9. Light micrographs of semi-thin sections of the basal choanosome and trabecular tracts of *Petrobiona massiliana* showing modifications of storage cells during the life cycle. **a.** A non-reproductive specimen from January 2007. **b.** A specimen from July 2006 undergoing embryogenesis. **c.** A reproductive specimen after release of larvae (August 2006). Arrowheads indicate a disintegrating canal and choanocyte chamber. **d.** A reproductive specimen at the end of the reproduction period (September 2006). All scalebars=50 μm. CC, choanocyte chamber; EC, excurrent canal; HC, hourglass-shape choanocyte; DC, disintegrated (i.e., hollow) canal or choanocyte chamber.

Discussion

In temperate regions, the breeding season of sponges is generally regulated by seasonally contrasting water temperatures (Simpson 1968; Sarà & Vacelet 1973; Elvin 1976; Andronikov 1975; Johnson 1978; Fell 1983; Simpson 1984; Kaye & Reiswig 1991; Fromont 1994; Ereskovsky 2000; Lepore et al. 2000; Usher et al. 2004; Ettinger-Epstein et al. 2007; Mercurio et al. 2007; Whalan et al. 2007; Riesgo & Maldonado 2008). The reproduction of adults of *Petrobiona massiliana* from Marseille, occurring from April to October (as observed by Vacelet [1964] and in this study) was related to water temperature, despite large unpredictable water cooling episodes caused by the mistral. During two consecutive years, oogenesis was initiated in early April when water temperature in the cave reached 15–16° C, after the low of 13°C measured during winter. In 2006, more reproductive specimens undergoing both oogenesis and embryogenesis were observed in early summer during warmer months June (22.7°C max.) and July (27.3°C max.). However, in 2007, similar high reproductive activity was observed later, from July to September. This difference may be partially explained by the delay in seawater warming that year, with maxima recorded later in August (27.9°C) and September (25.8°C).

Diaz (1979) proposed a distinction between permanent tissue remodeling and periodic or episodic tissue regression/restructuring in the sponge *Suberites massa* Nardo 1847. Periodic changes occurred after the reproductive period, and episodic changes after unfavorable environmental conditions were experienced. This terminology is used here to differentiate modifications of cells and tissues observed in light and electron microscopy during the life cycle of *P. massiliana*.

During the reproduction of adults of *P. massiliana*, choanocytes occurred in two modified morphologies that might correspond to changes linked to reproduction, adverse environmental conditions, or a synergy of both. Hourglass-shape choanocytes were observed only in specimens undergoing embryogenesis. Duboscq & Tuzet (1939) first suggested that such variability of choanocyte shape could be linked to

the growth of oocytes and embryos. However, Vacelet (1964) considered this modification in specimens of *P. massiliana* as a degeneration related to anoxia obtained, for example, by experimental warming of the water to 26°C. Nonetheless, as our samples were fixed immediately after collecting and as normal choanocytes were observed in non-reproductive sponges sampled during the warmest months (July 2006 and September 2007), the modification cannot be explained only by anoxia due to high temperatures. Eerkes-Medrano & Leys (2006) also noted the presence of hourglass-shape choanocytes in healthy specimens of the calcareous sponge *Sycon coactum* (Urban 1906) directly fixed after sampling, and presenting well-opened ostia. According to these authors, anoxia alone could not explain these changes; instead, they argued that choanocyte elongation and the shortening of the microvillar collar might result from a reduction of water circulation in the sponge caused by the presence of large embryos underneath the choanoderm. Nonetheless, specimens of *P. massiliana* undergoing embryogenesis during the colder July of 2006 (22°C maximum temperature) did not show hourglass-shape choanocytes, contradicting this interpretation. Therefore, we suggest that the increased prevalence of hourglass choanocytes is a periodic change occurring when reproductive activity (reduced and/or obstructed chambers during vitellogenesis of oocytes and embryos) and unfavorable environmental conditions (high seawater temperature) act in synergy.

While the choanocytic origin of spermatogonia is commonly accepted for demosponges (Simpson 1968; Tuzet et al. 1970; Diaz et al. 1973; Diaz & Connes 1980; Gaino et al. 1984; Paulus & Weissenfels 1986; Efremova et al. 1987; Barthel & Detmer 1990; Kaye & Reiswig 1991; Riesgo et al. 2007, 2008), in Calcarea, spermatogenesis has only been described in a few controversial works, which showed that spermatogonia were derived from mesohyl cells (Schulze 1878; Poléjaeff 1882; Görich 1904) or from choanocytes (Haeckel 1872; Dendy 1915; Gatenby 1920; Sarà & Relini-Orsi 1975; Anakina & Korotkova 1989; Lanna & Klautau 2010). Our observations as well as previous descriptions of the transformation of normal into vespiform choanocytes in Calcarea (Bidder 1892; Dendy 1893; Duboscq & Tuzet 1939; Vacelet 1964) are similar to the described transformation of choanocytes into spermatogonia in demosponges. Therefore, we hypothesized that the vespiform choanocytes we observed could correspond to a stage in the formation of spermatogonia. According to this assumption, the nucleated apical parts of vesperiform choanocytes would have "migrated" into the lumen of the contracted chambers and would correspond to newly differentiated spermatogonia. The basal parts remaining in place would form a kind of envelope closed by prosopylar and apopylar cells. Central cells, almost always observed in the lumen of the chambers formed by vespiform choanocytes, could then be implicated in the interruption of the water circulation, restraining the expulsion of spermatic elements during their differentiation. This water current discontinuity might then explain why these chambers were always contracted. However, although Vacelet (1964) pointed out the strong resemblance between vespiform shape modifications of choanocytes in calcareous sponges and some stages of spermatogenesis in demosponges, he linked the former phenomenon to cellular degeneration. Nonetheless, in his 4-year analysis of the life cycle of *P. massiliana*, Vacelet (1964) always noticed vespiform choanocytes only during the typical reproductive period, except for January 1962, when this modification was present in four specimens. Interestingly, conditions during the winter of 1961–1962 were exceptional and apparently initiated premature reproduction, as he observed mature amphiblastula in some of these samples (Vacelet, pers. comm.). Moreover, the large intact nucleus, Golgi apparatus, and mitochondria of the "migrated" apical part did not appear to degenerate. Finally, Lanna & Klautau (2010) have recently demonstrated the choanocytic origin of spermatogonia in *Paraleucilla magna* Klautau, Monteiro & Borojevic 2004, another member of Calcaronea. They showed that spermatogenesis occurred in modified choanocyte chambers containing hourglass-shape or vespiform choanocytes. The first stages of spermatogenesis they describe are very similar to the vespiform choanocytes that we observed in *P. massiliana,* except that chambers were not contracted. Further studies to elucidate the next steps of spermatogenesis in members of *P. massiliana* are still necessary. Nonetheless, the vespiform choanocyte transformation is likely related to the reproductive period, and we will consider it a periodical change.

Small basal changes observed in non-reproductive specimens during the life cycle of *P. massiliana* could be related to permanent local disorganization/reorganization events allowing remodeling or growth of the aquiferous system of the sponge. They correspond to degeneration of some canals and chambers while new ones are built up. In these areas, endopinacocytes were commonly larger than usual with many cytoplasmic vacuoles and vesicles, becoming very similar to type III storage cells. Nonetheless,

these cytological features could represent either degeneration of endopinacocytes or their regeneration to form new canals. Diaz (1979) also detected spontaneous disorganization and reorganization events in the tissue of *S. massa*, interpreting them as, respectively, remodeling of the canal system and growth.

A higher level of disorganization of the choanosome was observed using LM in specimens showing a high level of reproductive activity, as well as in a few sponges sampled in November 2006 and January 2007 after a decrease in seawater temperature. Histological observations of reproductive specimens clearly identified structural disorganization, principally localized in the basal region of the choanosome of sponges with growing oocytes and larvae, and after the spawning of the latter. Such disorganized features may be predictable considering given that choanocytes probably gave rise to both germ cells and nurse cells, \ the intense phagocytosis of the latter (i.e., the production of one larva required the content of two chambers) (Vacelet 1964; Gallissian & Vacelet 1990, 1992), and \ that larvae, hosted in chambers, locally obstruct water flow. These modifications of the aquiferous system corresponded to periodical changes during the reproductive phase. Nonetheless, choanosomal regression observed in the samples from November and January could be an episodic modification in response to adverse winter conditions.

Previous reports of such soft tissue disorganization with substantial reduction of choanocyte chambers and canals, referred as tissue regression (for reviews see Simpson 1984 and Ereskovsky 2010), have only been reported in demosponges. These are often related to temperature decreases occurring in fall or winter, and are regarded as overwintering-related changes induced by a change of environmental conditions (Simpson 1968; Van de Vyver & Willenz 1975; Diaz 1979; Fell & Jacob 1979). In other cases, soft tissue modifications have been observed during or directly after the reproductive period, as in the demosponges *Halisarca nahantensis* Chen 1976, *Myxilla incrustans* (Johnston 1842), *Iophon piceus* (Vosmaer 1882), *Halichondria panicea* (Pallas 1766), *Ephydatia fluviatilis* (Linnaeus 1759), and *Halisarca dujardini* Johnston 1842, where complete regression of both central and basal regions of the choanosome occurs after each period of reproduction (Chen 1976; Ivanova 1978, 1981; Barthel 1986; Pronzato & Manconi 1994; Witte & Barthel 1994; Ereskovsky 2000, 2010). In members of *S. massa*, Diaz (1979) demonstrated that tissue regression could be correlated both with increases in temperature and sexual reproduction (i.e., periodic changes) and to a decrease of temperature in the winter (i.e., episodic changes). During regression, choanocytes and endopinacocytes progressively disappeared, while archeocytes increased in number. In this species, as in *H. nahantensis*, tissue regression is followed by a reorganization of functional structures through the transdifferentiation of totipotent cells like archeocytes (Chen 1976; Diaz 1979). In samples of *P. massiliana*, permanent remodeling occurred and functional tissue was rapidly recovered after release of larvae or after the end of unfavorable environmental conditions. Toti- or pluripotent cells, or transdifferentiating somatic cells like storage cells, might be implicated in those local or global remodeling/restructuring morphogenetic processes.

In this study, we also characterized cytological differences in the three types of storage cells from trabecular tracts. Type I storage cells are closely packed in the deeper portions of tracts and contain numerous inclusions with lipidic and glucidic content; type II storage cells with less electron-dense cytoplasmic content are located in the apical part of the tracts; and type III storage cells, which are similar in ultrastructure to endopinacocytes from excurrent canals, are found in the upper portion of tracts and in the basal region of the choanosome. All three storage cell types might correspond to different stages in the transformation of the same cell lineage. On one hand, during tissue regression, old degenerating cells or injured ones, like endopinacocytes, might lead to new storage cells (Fig. 10: pathway 1). Pinacocytes would transform into type III storage cells with a wide smooth endoplasmic reticulum and phagosomes or secondary lysosomes. Large type III storage cells progressively intertwine while they accumulate lipidic and glucidic storage and move toward the apical portion of the tract where they form type II storage cells. The production of storage inclusions is carried out until the morphology of fully compacted type I storage cells is reached in the deepest portion of the tracts. On the other hand, storage cells might also contribute to restructuring of the aquiferous system (Fig. 10: pathway 2). Type I storage cells associated in pairs might progressively digest their storage content during their ascension toward the top of the tracts, becoming type II cells. Type II storage cells progressively spread out from each other, enlarging the space separating them. As lipidic and glucidic inclusions are replaced by vacuoles and large smooth endoplasmic reticulum, these become type III storage cells, that then form larger canals, perhaps by union with other paired type III storage cells. The developing canals would then

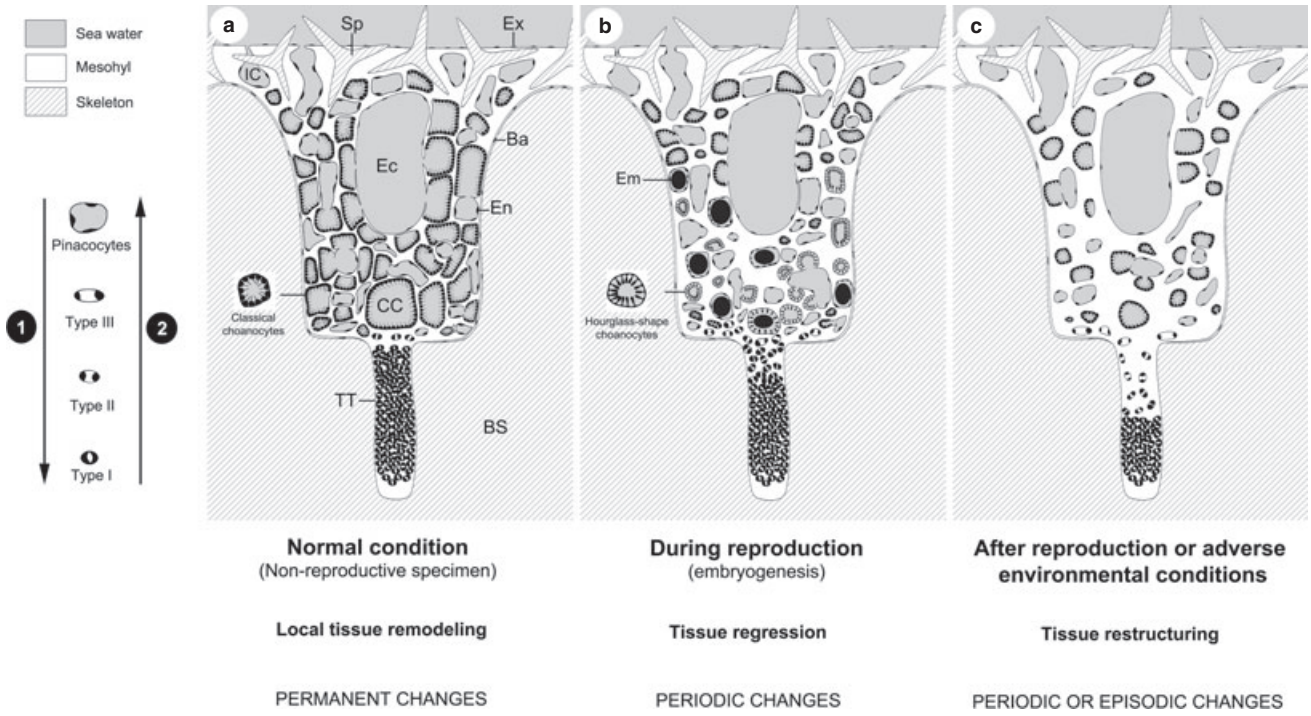

Fig. 10. Schematic representation of soft tissue modifications occurring permanently, periodically, and episodically during the life cycle of *Petrobiona massiliana*. **a.** Normal condition of non-reproductive specimens. **b.** Condition of reproductive specimens. **c.** Condition of specimens after reproduction, or in response to adverse environmental conditions. Pathway 1 shows trans- or dedifferentiation of old or injured cells, like endopinacocytes, into new storage cells. Pathway 2 indicates trans- or dedifferentiation of storage cells into pinacocytes, contributing to the remodeling or restructuring of the aquiferous system. Ba, basopinacocyte; BS, basal skeleton; CC, choanocyte chamber; EC, excurrent canal; Em, embryo; En, endopinacocyte; Ex, exopinacocyte; IC, incurrent canal; Sp, spicule; TT, trabecular tract.

connect to choanocyte chambers or other canals. These two pathways of transdifferentiation might occur, sometimes simultaneously, during permanent remodeling in normal conditions (non-reproductive specimens without unfavorable environmental conditions, Fig. 10a), during tissue regression (i.e., leading to new storage cells) when sponges are undergoing intense provisioning of oocytes and embryos (Fig. 10b), and during tissue restructuring (i.e., leading to new endopinacocytes) after reproduction (e.g., August 2006) or after an adverse environmental episode (e.g., November 2006) (Fig. 10c).

Vacelet (1964, 1990) first described the peculiar trabecular tracts, filled with thesocyte-like cells that he interpreted as storage cells according to their lipidic content, in members of *P. massiliana*. He did not notice the nucleolus and Golgi apparatus. Archaeocytes with inclusions ("eosinophilic amoebocytes": Vacelet 1964) that we found in these tracts were reported to transfer their storage granules to other cell types like choanocytes (Gallissian & Vacelet 1985). As Gallissian & Vacelet (1985) also observed eosinophilic cells in trabecular tracts in contact with storage cells (type I in the present study), these highly mobile cells probably play an important role in the transfer of supplies accumulated by storage cells to other cells in the choanosome. This supplementary nutrient-providing pathway may be important during vitellogenesis.

Vacelet (1964, 1990) also observed morphological changes among storage cells during the life cycle of *P. massiliana*, but described cells (type III storage cells) migrating into the choanosome as single cells cytologically similar to those found in the tracts. He did not observe their association in canals. Furthermore, Vacelet (1964) noticed dispersed storage cells throughout the choanosome in winter or after adverse environmental conditions, as in aquarium experiments, and interpreted them as cells migrating into the tissue to provide choanocytes with their storage content, or to differentiate into other cell types. While we are also suggesting such a differentiation capacity, we did not find single storage cells in the choanosome transferring their inclusions to choanocytes. In addition, Vacelet (1964, 1990) described recruitment of those cells as a regenerative process

after soft tissue damage due to temporary unfavorable environmental conditions in autumn and winter. However, we additionally noticed subtle mobilization of storage cells from tracts in non-reproductive specimens (permanent remodeling process), and more extensive migrations during restructuring of soft tissue after embryogenesis.

Vacelet (1990) compared the storage cells of members of *P. massiliana* with those of two other living hypercalcified sponges, *Merlia normani* KIRKPATRICK 1908 and *Acanthochaetetes wellsi* HARTMAN & GOREAU 1975, both belonging to Demospongiae. He noted striking similarities between crypt cells or storage cells of members of these three species (all three descendants of ancient reef-building sponges) with the thesocytes of the gemmules of *S. massa*, based on features of storage cells and their function as reduction bodies able to regenerate a complete sponge after unfavorable conditions. Vacelet (1990) also noted, in these three living hypercalcified sponges, the occurrence of dormant masses of cells near the massive basal skeleton. The skeleton might provide isolated spaces that trigger the differentiation of choanocytes or archeocytes into storage cells (Vacelet 1990). To maintain their transdifferentiation capacity, sponge stem cells (e.g., the thesocytes/archeocytes of gemmules) must be in a physical and molecular microenvironment with cell–cell contacts (the "stem cell niche": Müller et al. 1999; Müller 2006). The high compaction of storage cells in thin canals of the skeleton might provide such a "niche" to preserve their differentiation capacity.

Our morphological investigation of specimens of *P. massiliana* showed permanent, periodic, and episodic soft tissue modifications during their life cycle. Remodeling and restructuring processes rapidly followed tissue regression. Therefore, as in most poriferans (Pavans de Ceccatty 1979; Korotkova 1981, 1997; Bond & Harris 1988; Gaino & Burlando 1990; Bond 1992; Gaino et al. 1995; Plotkin et al. 1999; Ereskovsky 2003, 2010), specimens of *P. massiliana* have a high plasticity of tissue organization, allowing modulation and rearrangement of the aquiferous system in response to environmental changes and life cycle phases. This soft tissue dynamism could be conferred by transdifferentiation ability and mobility of cells. Storage cells accumulated in trabecular tracts during disorganization events of the choanosome could act as such stem cells for this calcareous sponge, being able to transdifferentiate into at least endopinacocytes to trigger remodeling and restructuring of the canal system during permanent, periodic, and episodic modifications. Although such a transdifferentiation capacity of storage cells is still highly speculative, it could be tested in future investigations by using *in situ* hybridizations with stem cell markers such as PIWI (Funayama 2008) or pinacocytic lineage-specific molecular markers as *Iroquois* (Perović et al. 2003).

In conclusion, this investigation of the life cycle of *P. massiliana* showed (1) that sexual reproduction induced important choanosomal disorganizations, (2) that permanent remodeling of the aquiferous system occurred in the basal region of the choanosome, (3) that unfavorable environmental conditions caused episodic tissue regression, and that (4) storage cells may provide energy and a pool of toti- or pluripotent cells to the rest of the sponge, allowing such activities as rapid restructuring of the aquiferous system (and possibly replenishment of other cell types, like skeletogenetic cells).

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References

Amano S & Hori I 2001. Metamorphosis of coeloblastula performed by multipotential larval flagellated cells in the calcareous sponge *Leucosolenia laxa*. Biol. Bull. 200: 1–20.

Anakina RP 1981. The embryological development of Barents Sea sponge *Leucosolenia complicata* Mont. (Calcarea). In: Morphogenesis in Sponges. Korotkova GP, ed., pp. 52–58. Publ. Leningrad University, Leningrad.

——— 1988. The early stages of fertilization of sponge *Leucosolenia complicata* Montagu (Calcispongia, Leucosoleniida) from the Barents Sea. In: Gubki i knidarii. Sovremennoe sostoyanie i perspektivy issledovanii. Porifera and Cnidaria. Modern and perspective investigations. Starobogatov Y, ed., pp. 8–12. Acad. Sci. USSR, Zool. Inst., Leningrad.

——— 1999. Peculiarities of fertilization process in the sponge *Leucosolenia complicata* Montagu (Calcispongiae: Calcaronea) from the Barents Sea. Mem. Qd. Mus. 44: 44.

Anakina RP & Drozdov AL 2000. Peculiarities of ovogenesis in the Barents Sea sponge *Leucosolenia complicata* Montagu (Calcispongiae, Calcaronea). Tsitologiya 42(2): 128–135.

Anakina RP & Drozdov AL 2001. Gamete structure and fertilization in the sponge *Leucosolenia complicata* from the Barents Sea. Biol. Morya/Mar. Biol. 27(3): 179–186.

Anakina RP & Korotkova GP 1989. Spermatogenesis in *Leucosolenia complicata* Mont., Barents Sea sponge. Ontogenesis. 20: 77–86.

Andronikov VB 1975. Heat resistance of gametes of marine invertebrates in relation to temperature conditions under which species exist. Mar. Biol. 30: 1–11.

Barthel D 1986. On the ecophysiology of the sponge *Halichondria panicea* in Kiel Bight. I. Substrate specificity, growth and reproduction. Mar. Ecol. Prog. Ser. 32: 291–298.

Barthel D & Detmer A 1990. The spermatogenesis of *Halichondria panicea* (Porifera, Demospongiae). Zoomorphology 110: 9–15.

Bidder GP 1892. Note on excretion in sponges. Proc. R. Soc. (B) 51(313): 474–485.

Bond C 1992. Continuous cell movements rearrange anatomical structures in intact sponges. J. Exp. Zool. 263: 284–302.

Bond C & Harris A 1988. Locomotion of sponges and its physical mechanism. J. Exp. Zool. 246: 271–284.

Chen WT 1976. Reproduction and speciation in *Halisarca*. In: Aspects of Sponge Biology. Harrison FW & Cowden RR, eds., pp. 113–139. Academic Press,New York & London.

Dendy A 1893. Studies on the comparative anatomy of sponges. V: observations on the structure and classification of the Calcarea Heterocoela. Quart. J. Micr. Sc. 35(2): 159–257.

——— 1915. Observations on the gametogenesis of *Grantia compressa*. Quart. J. Microsc. Sci. 60: 313–376.

Diaz JP 1979. Variations, différenciations et fonctions des catégories cellulaires de la démosponge d'eaux saumâtres, *Suberites massa*, Nardo, au cours du cycle biologique annuel et dans des conditions expérimentales. Thèse de doctorat d'Etat. Université des Sciences et Techniques du Languedoc.

Diaz JP & Connes R 1980. Étude ultrastructurale de la spermatogenèse d'une Démosponge. Biol. Cell 38(3): 225–230.

Diaz JP, Connes R, & Paris J 1973. Origine de la lignée germinale chez une démosponge de l'étang de Thau: *Suberites massa* Nardo. CR Acad. Sci. Paris. 277(7): 661–663.

Duboscq O & Tuzet O 1939. Les diverses formes des choanocytes des éponges calcaires hétérocoeles et leur signification. Archs. Zool. Exp. Gén. 80(5): 353–388.

Duboscq O & Tuzet O 1944. L'ovogénèse, la fécondation et les premiers stades du développement de *Sycon elegans* Bower. Archs. Zool. Exp. Gén. 83: 445–459.

Eerkes-Medrano DI & Leys SP 2006. Ultrastructure and embryonic development of a syconoid calcareous sponge. Invertebr. Biol. 125(3): 177–194.

Efremova SM, Ereskovsky AV, & Tokina DB 1987. Gametogenesis in sponges of the family Myxillidae from the White Sea. 2. Spermatogenesis in *Myxilla incrustans* and *Iophon piceus* (Demospongiae, Poecilosclerida). Ontogenez. 18(3): 263–268.

Elvin DW 1976. Seasonal growth and reproduction of an intertidal sponge. *Haliclona permollis* (Bowerbank). Biol. Bull. 151: 108–125.

Ereskovsky AV 2000. Reproduction cycles and strategies of the cold-water sponges *Halisarca dujardini* (Demospongiae, Halisarcida), *Myxilla incrustans* and *Iophon piceus* (Demospongiae, Poecilosclerida) from the White Sea. Biol. Bull. 198(1): 77–87.

——— 2003. Problems of coloniality, modularity, and individuality in sponges and special features of their morphogeneses during growth and asexual reproduction. Russ. J. Mar. Biol. 19: 46–56.

——— 2010. The Comparative Embryology of Sponges. Springer, New York.

Ettinger-Epstein P, Whalan SW, Battershill CN, & De Nys R 2007. Temperature cues gametogenesis and larval release in a tropical sponge. Mar. Biol. 153(2): 171–178.

Fell PE 1983. Porifera. In: Reproductive Biology of Invertebrates. Oogenesis, Oviposition, and Oosorption. Vol 1. Adiyodi KG & Adiyodi RG, eds., pp. 29. John Wiley & Sons, New York.

Fell PE & Jacob WF 1979. Reproduction and development of *Halichondria sp*. in the Mystic Estuary, Connecticut. Biol. Bull. 156(1): 62–75.

Franzen W 1988. Oogenesis and larval development of *Scypha ciliata* (Porifera, Calcarea). Zoomorphology (Berl.) 107: 349–357.

Fromont J 1994. Reproductive development and timing of tropical sponges (Order Haplosclerida) from the Great Barrier Reef, Australia. Coral Reefs 13(2): 127–133.

Fullmer HM 1966. Histochemical studies of mineralized tissues. Ann. Histochim 11: 369–374.

Funayama N 2008. Stem cell system of sponge. In: Stem Cells from Hydra to Man. Bosch T. C. Ged., pp: 17–35. Springer, Berlin, Heidelberg.

Gaino E & Burlando B 1990. Sponge cell motility: a model system for the study of morphogenetic processes. Bull. Zool. 57: 109–118.

Gaino E, Burlando B, Zunino L, Pansini M, & Buffa P 1984. Origin of male gametes from choanocytes in *Spongia officinalis* (Porifera, Demospongiae). Int. J. Invert. Reprod. 7: 83–93.

Gaino E, Burlando B, & Buffa P 1987. Ultrastructural study of oogenesis and fertilization in *Sycon ciliatum* (Porifera, Calcispongiae). Int J Invertebr Reprod Dev. 11: 73–82.

Gaino E, Manconi R, & Pronzato R 1995. Organizational plasticity as a successful conservative tactics in sponges. Anim. Biol. 4: 31–43.

Gallissian M-F 1983. Étude ultrastructurale du développement embryonnaire chez *Grantia compressa* F. (Porifera, Calcarea). Arch Anat Microsc 72: 59–75.

Gallissian M-F & Vacelet J 1985. Ultrastructure des amoebocytes éosinophiles d'éponges calcaires calcaronées. C. R. Acad. Sci. 300(4): 151–156.

Gallissian M-F & Vacelet J 1990. Fertilization and nutrition of the oocyte in the Calcified Sponge *Petrobiona massiliana*. In: New Perspectives in Sponge Biology. Smithson. Rützler K, ed., pp. 175–181. Smithsonian Inst. Press, Washington, D.C.

Gallissian M-F & Vacelet J 1992. Ultrastructure of the oocyte and embryo of the calcified sponge *Petrobiona massiliana* (Porifera, Calcarea). Zoomorphology 112(3): 133–141.

Gatenby JB 1920.The germ-cells, fertilization and early development of *Grantia* (*Sycon*) *compressa*. J. Linn. Soc. Zool. 34: 261–297, pls xix–xxiii.

Görich W 1904. Zur Kenntniss der Spermatogenese bei den Poriferen und Coelenteraten nebst Bemerkungen uber die Oogenese der ersteren. Z. Wiss. Zool. 76: 522–543.

Gugel J 2001. Life cycles and ecological interactions of freshwater sponges (Porifera, Spongillidae) in the River Rhine in Germany. Limnologica. 31: 185–198.

Haeckel E 1872. Vols: 1–3. Die Kalkschwämme, Eine Monographie. Verlag von Georg Reimer, Berlin.

Ivanova LV 1978. Morphogenetic processes and seasonal changes in the anatomic and histological organisation of the Barents sea sponge *Halichondria panicea* (Pallas). Arch. Anat. Hist. Emb. 75: 62–72.

——— 1981. The life cycle of *Halichondria panicea* (Pallas). In: Morphogenesis in Sponges. Korotkova GP, ed., pp. 59–73. Leningrad University Press, Leningrad.

Johnson MF 1978. Studies on the reproductive cycles of the calcareous sponges *Clathrina coriacea* and *C. blanca*. Mar. Biol. 50(1): 73–79.

Kaye HR & Reiswig HM 1991. Sexual reproduction in four Caribbean commercial sponges. I. Reproductive cycles and spermatogenesis. Invertebr. Reprod. Dev. 19 (1): 1–11.

Korotkova GP 1981. Origin and Evolution of Ontogenesis. Leningrad University Press, Leningrad.

——— 1997. Regeneration in Animals. St. Petersburg University Press, St. Petersburg.

Lanna E & Klautau M 2010. Oogenesis and spermatogenesis in *Paraleucilla magna* (Porifera, Calcarea). Zoomorphology 129(4): 249–261.

Lanna E, Monteiro LC, & Klautau M 2007. Life cycle of *Paraleucilla magna* Klautau, Monteiro and Borojevic, 2004 (Porifera, Calcarea). In: Porifera Research: biodiversity, innovation and sustainability. Custódio MR, Lôbo-Hajdu G, Hajdu E, & Muricy G, eds., pp. 413–418. Museu Nacional Universidade Federal, Rio de Janeiro.

Lepore E, Sciscioli M, Scalera-Liaci L, Santarelli G, & Gaino E 2000. Sexual reproduction of Cinachyra tarentina (Porifera, Demo- spongiae). Ital. J. Zool. 67: 153–158.

Lévi C 1956. Etude des *Haliscarca* de Roscoff. Embryologie et systématique des Démosponges. Archs Zool. Exp. Gén. 93: 1–181.

Maldonado M & Riesgo A 2008. Reproduction in the Phylum Porifera: a synoptic overview. Treb. Soc. Cat. Biol. 59: 29–49.

Manconi R, Ledda FD, Serusi A, Corso G, & Stocchino GA 2009. Sponges of marine caves: notes on the status of the Mediterranean palaeoendemic *Petrobiona massiliana* (Porifera: Calcarea: Lithonida) with new records from Sardinia. Ital. J. Zool. 76(3): 306–315.

Mercurio M, Corriero G, & Gaino E 2007. A 3-year investigation of sexual reproduction in *Geodia cydonium* (Jameson 1811) (Porifera, Demospongiae) from a semi-enclosed Mediterranean bay. Mar. Biol. 151(4): 1491–1500.

Müller WEG 2006. The stem cell concept in sponges (Porifera): Metazoan traits. Semin. Cell Dev. Biol. 17: 481–491.

Müller WEG, Wiens M, Batel R, Steffen R, Borojevic R, & Custodio MR 1999. Establishment of a primary cell culture from a sponge: primmorphs from *Suberites domuncula*. Marine Ecol. Prog. Ser. 178: 205–219.

Nakamura Y, Okada K, & Watanabe Y 1998. The ultrastructure of spermatozoa and its structural change in the Choanocyte of *Sycon calcaravis* Hozawa. In: Sponge Sciences. Multidisciplinary Perspectives. Watanabe Y & Fusetani N, eds., pp. 179–191. Springer, Tokyo.

Paulus W & Weissenfels N 1986. The spermatogenesis of *Ephydatia fluviatilis* (Porifera). Zoomorphology 106: 155–162.

Pavans de Ceccatty M1979. Cell correlation and integration in sponges. In: Biologie des Spongiaires. Lévi C & Boury-Esnault N, eds., pp. 123–136. Colloques Internationaux, Paris.

Perović S, Schröder HC, Sudek S, Grebenjuk VA, Batel R, Maurotifani IMM, & Müller WEG 2003. Expression of one sponge *Iroquois* homeobox gene in primmorphs from *Suberites domuncula* during canal formation. Evol. Dev. 5: 240–250.

Plotkin AS, Ereskovsky AV, & Khalaman VV 1999. The analysis of modular organization of Porifera using *Polymastia mammillaris* (Müller, 1806) as a model. Zh. Obshchei. Biol. 60: 18–28.

Poléjaeff N 1882. Über das Sperma und die Spermatogenese bei *Sycandra raphanus*. Haeck. Sitzungsb. Akad. Wiss. Wien. LXXXVI: 276–298.

Pronzato R, & Manconi R 1994. Life history of *Ephydatia fluviatilis* a model for adaptive strategies in discontinuous habitats. In: Sponges in Time and Space. van Soest RWM, van Kempen TMG, & Braekman JC, eds., pp. 327–331. Balkema, Rotterdam/Amsterdam.

Reynolds ES 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17(1): 208–212.

Riesgo A & Maldonado M 2008. Differences in reproductive timing among sponges sharing habitat and thermal regime. Inv. Biol. 127(4): 357–367.

Riesgo A, Maldonado M, & Durfort M 2007. Dynamics of gametogenesis, embryogenesis, and larval release in a Mediterranean homosclerophorid demosponge. Mar. Fresh. Res. 58: 398–417.

Riesgo A, Maldonado M, & Durfort M 2008. Occurrence of somatic cells within the spermatic cysts of demosponges: a discussion of their role. Tissue Cell 40: 387–496.

Sarà M & Rellini-Orsi L 1975. Sex differentiation in *Sycon* (Porifera Calcispongiae). Pubbl. Staz. Zool. Napoli 39(Suppl.): 618–634.

Sarà M & Vacelet J 1973. Écologie des Demosponges. In: Traité de Zoologie. Grassé PP, ed. Masson et Cie, Paris.

Schulze FE 1878. Recherches sur l'anatomie et le développement des éponges. Les métamorphoses de *Sycandra raphanus*. Arch Zool Exp Gén 1: XXI–XXV.

Simpson TL 1968. The Biology of the Marine Sponge *Microciona prolifera* (Ellis and Sollander). II. Temperature- related, annual changes in functional and reproductive elements with a description of larval metamorphosis. J. Exp. Mar. Biol. Ecol.. 2: 252–277.

——— 1984. The Cell Biology of Sponges. Springer, New York.

Tuzet O 1947. L'ovogénèse et la fécondation de l'eponge calcaire *Leucosolenia* (*Clathrina*) *coriacea* Mont. et de l'Eponge siliceuse *Reniera elegans* Bow. Archs. Zool. Exp. Gén. 85(2): 127–148, pls I–II.

Tuzet O, Garrone R, & Pavans de Ceccatty M 1970. Observations ultrastructurales sur la spermatogenèse chez la démosponge *Aplysilla rosea* Schulze (Dendroceratides): une métaplasie exemplaire. Ann. Sci. nat. (Zool.) 12: 27–50.

Usher KM, Sutton DC, Toze S, Kuoc J, & Fromont J 2004. Sexual reproduction in *Chondrilla australiensis* (Porifera: Demospongiae). Mar. Fresh. Res. 55: 123–134.

Vacelet J 1964. Étude monographique de l'Eponge Calcaire Pharétronide de Méditerranée, *Petrobiona massiliana* Vacelet et Lévi. Les Pharétronides actuelles et fossiles. Rec. Trav. Stn. Mar Endoume. 34(50): 1–125, pls I–III.

——— 1990. Storage cells of calcified relict sponges. In: New Perspectives in Sponge Biology. Rützler Ked., Smithsonian Institution Press, Washington, DC.

Vacelet J, Borojevic R, Boury-Esnault N, & Manuel M 2002. Order Lithonida. In: Systema Porifera. A Guide to the Classification of Sponges. Vol. 2. Hooper JNA, & van Soest RWM, eds., pp. 1185–1192. Kluwer Academic/Plenum Publishers, New York.

Van de Vyver G & Willenz Ph 1975. An experimental study of the life-Cycle of the fresh-water sponge *Ephydatia fluviatilis* in its natural surroundings. Wilhelm Roux's Arch. Dev. Biol. 177: 41–52.

Watanabe Y & Okada K 1998. The involvement of two carrier cells in fertilization and the ultrastructure of the spermiocyst in *Sycon calcaravis*. In: Sponge Sciences. Multidisciplinary Perspectives. Watanabe Y & Fusetani N, eds., pp. 193–202. Springer, Tokyo.

Whalan SW, Battershill CN, & de Nys R 2007. Sexual reproduction of the brooding sponge *Rhopaloeides odorabile*. Coral Reefs 26(3): 655–663.

Witte U & Barthel D 1994. Reproductive cycle and oogenesis of *Halichondria panicea* (Pallas) in Kiel Bight. In: Sponges in Time and Space. van Soest RWM, van Kempen TMG, & Braekman JC, eds., pp. 297–306. Balkema, Rotterdam/Amsterdam.