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Abstract. Despite the common assumption that most Haplosclerida are viviparous sponges, this study of the reproductive cycle of *Haliclona fulva* demonstrates that this species is actually oviparous and gonochoric. Intriguingly, not a single male was recorded in 15 months of sampling. Oogenesis is synchronous, starting in late April and terminating in September. Asexual reproduction is represented by cyclic budding, which occurs from late November to early March. During the season of asexual reproduction, the reproductive effort represents from 0.21% to 1.49% of the parental tissue, with the highest values being recorded in winter. During the season of sexual reproduction, the female reproductive effort ranges 0.05–1.15%, with the highest effort appearing in early summer. However, no significant correlation between reproductive efforts and seawater temperature fluctuations could be detected. We describe the ultrastructural morphogenesis of the buds for the first time in this species. This process is asynchronous, with buds of variable size being attached to the maternal apical surface via a short stalk. Young buds lack any particular anatomical organization, whereas bud maturity is characterized by the development of mesohyl and by the appearance of an increasing number and volume of lacunae in the central part of each bud. At this stage, buds harbor numerous small choanocyte chambers scattered throughout the inner region, and all cell types known from the mesohyl of parental sponges: microgranular cells, granular cells, archaeocytes, endopinacocytes and exopinacocytes, central cells, and sclerocytes.

Additional key words: asexual reproduction, budding, Haplosclerida, oviparity, reproductive cycle

Knowledge of the life history and reproductive cycles of sponges is important for understanding their evolution, role in marine ecosystems, and to the understanding of marine population dynamics. In the past, investigations of the reproductive cycles of marine sponges have mainly dealt with the period of sexual reproduction. Although the dispersion and development of sponges in natural habitats is also supported by asexual reproduction, very few studies have considered the overall sponge life cycle and the alternation of sexual and asexual reproduction (Ayling 1980; Battershill & Bergquist 1990; Wulff 1991; Corriero et al. 1996; Corriero et al. 1998; Plotkin & Ereskovsky 1997). Indeed, asexual reproduction is an important

reproductive strategy for sessile organisms such as sponges, and represents a significant evolutionary advantage that can compensate for the fact that mature adults are unable to move and search for mates. Sponge asexual reproduction may proceed by fragmentation, gemmulogenesis, and budding (Fell 1993; Simpson 1984; Ereskovsky 2010). For instance, budding occurs occasionally in almost all sponge clades and in any type of habitat (Fell 1993; Simpson 1984; Gaino et al. 2006; Ereskovsky & Tokina 2007; Ereskovsky 2010; Teixido et al. 2006), but it is observed regularly among marine representatives of Porifera. For many Demospongiae of the orders Tethyida, Polymastiida, and Tetractinellida, budding represents a suitable reproductive strategy that enhances dispersal and colonization of the habitat (Sarà 1988; Bergquist & Kelly-Borges 1991; Sarà et al. 1993; Gaino et al. 2006; Cardone et al. 2010). Some cases of obligatory asexual reproduction have been recorded in Spongillida, Tethyida, Clionaida, Suberitida, Polymastiida and Tetractinellida. For instance, gemmulogenesis occurs in all families of Spongillida (Ereskovskii 1999), except in Lubomirskiidae, and in the families Clionaidae (Clionaida) and Suberitidae (Suberitida) (Topsent 1888; Herlant-Meewis 1948; Hartman 1958; Connes 1977; Connes et al. 1978). Budding has been recorded in the families Polymastiidae and Tethyidae (Merejkowsky 1878, 1879; Connes 1967; Battershill & Bergquist 1990; Chen et al. 1997; Plotkin & Ereskovsky 1997; McDonald 2002; Gaino et al. 2006, 2009; Singh & Thakur 2015).

When asexual reproduction exists in Demospongiae, the life cycle often presents a regular alternation of sexual and asexual phases (Ayling 1980; Pomponi & Meritt 1990; Fell 1993; Corriero et al. 1996; Plotkin & Ereskovsky 1997). Potential explanations for this alternation rely on the competition for cells between the two reproduction modes, especially for archaeocytes, which are likely polypotent cells (Korotkova 1988a). Archaeocytes are greatly involved in both oogenesis and embryogenesis, so a very low incidence or absence of asexual reproduction occurs during the season of sexual reproduction. This alternation can be seen as a trade-off between the two processes, and is thus in line with the hypothesis formulated by Korotkova (1988a,b) concerning the incompatibility of sexual and somatic morphogenesis, because they involve the same cell types.

In general, the initial stages of bud development in Demospongiae are represented by a dense conglomerate of cells at the parental sponge surface. At this stage, buds lack an aquiferous system, choanocyte chambers, canals, and oscula (Fell 1993). When the bud settles on the substrate, the aquiferous system starts to develop and the separated post-bud grows. Few studies have provided detailed information on the fine morphology and cytology of sponge buds (see Connes 1967; Ereskovsky & Tokina 2007; Gaino et al. 2006, 2009), resulting in very incomplete knowledge of their cell composition. We hypothesized that further study would improve our understanding of the roles played by different cells in the formation of these asexual propagules.

Viviparity (brooding) and oviparity are widespread reproductive modes in Demosponges (Riesgo et al. 2013). Some orders are completely oviparous, like Polymastiida, Clionaida, Tethyida, and Verongida, while other orders have only viviparity, for example, Spongillida, Dendroceratida, and Dictyoceratida (Ereskovsky 2010). It is generally accepted that the families of order Haplosclerida (Morrow & Cardenas 2015) display characteristic viviparity, with some oviparous species found in family Petrosiidae (Fromont & Bergquist 1994).

In the Mediterranean Sea, information on sponge reproductive cycles and reproductive modes is already available for a number of Demospongiae from different clades (Siribelli 1962; Scalera Liaci et al. 1971; Corriero et al. 1996, 1998; Lepore et al. 2000; Meroz-Fine et al. 2005; Baldacconi et al. 2007; Mercurio et al. 2007; Riesgo & Maldonado 2008; Piscitelli et al. 2011;

Pérez-Porro et al. 2012; Di Camillo et al. 2012; Mercurio et al. 2013; Zarrouk et al. 2013; Reverter et al. 2016). However, there is less knowledge about the sponges from order Haplosclerida, which are relatively common and abundant in the Mediterranean Sea. Up to now, we have data on reproductive cycles for only four species of Haplosclerida (Scalera Liaci et al. 1973; Maldonado & Riesgo 2009).

In this work, we investigate *Haliclona fulva* (TOPSENT 1893), a common shallow-water Mediterranean demosponge of the order Haplosclerida, family Chalinidae. We chose *H. fulva* for this study because this species regularly uses budding in its life cycle, unlike other haplosclerids, which have never been described to possess this mode of asexual reproduction. Moreover, as we show here, it is the only oviparous species in the family Chalinidae. We evaluate the incidence of sexual and asexual reproduction in the life cycle of *Haliclona fulva* and describe budding in *H. fulva* thoroughly, detailing the cellular composition of buds at different stages of development and comparing them with parental tissue.

Methods

Biological model

Haliclona (*Halichoclona*) *fulva* is a common Mediterranean sponge species, individuals of which form thick crusts (5–15 mm thickness) that vary in color from orange to red and which inhabit shaded benthic communities, such as semi-dark submarine caves or coralligenous formations, at depths of 5–50 m (Fig. 1A).

Sampling site

Sponges were collected by SCUBA diving at depths of between 14 and 16 m at a site called Grotte à Corail, located at Maire Island (Marseilles Bay). This sampling site was equipped with a permanent temperature recorder (HOBO Tidbit Data Logger). From September–March 2007 to December 2008, between four and nine individuals were collected monthly (during June and August 2008, the sponges were sampled twice), which represents a total of 103 individuals studied (Table 1). Only one month (October 2007) could not be sampled due to bad weather conditions. In order to pinpoint the period of budding, we also examined a great number of underwater photographs taken by scientific divers at different sites in the Marseille region from 1999 to 2009.

Morphological and ultrastructural analysis

To characterize the life cycle and assess the reproductive effort, samples were preserved in Bouin's fixative. Tissue fragments were then dehydrated through an ethanol series and embedded in paraffin. Serial sections of 6 µm thickness were mounted on glass slides and stained with Masson-Goldner's trichrome hematoxylin, and then observed under a WILD M20 light microscope.

For semithin sections and for transmission electron microscopy (TEM) investigations, sponges were fixed in a solution composed of one volume of 25% glutaraldehyde, four volumes of 0.2 M cacodylate buffer, and five volumes of filtered seawater for 2 h before being post-fixed in 2% OsO_4 in seawater at room temperature for 2 h. After fixation, samples were washed in 0.2 M cacodylate buffer and distilled water successively, and finally dehydrated through a graded ethanol series. Specimens were embedded in Araldite resin. Semithin sections (1 µm in thickness) were cut on a Reichert Jung ultramicrotome equipped with a "Micro Star" 45° diamond knife before being stained with toluidine blue, and observed under a WILD M20

microscope. Digital photos were taken with a Leica DMLB microscope using the Evolution LC color photo capture system. Ultrathin sections (60–80 nm) were cut with a Leica UCT ultramicrotome equipped with a Drukkert 45° diamond knife. Ultrathin sections, contrasted with uranyl acetate, were observed under a Zeiss-1000 transmission electron microscope (TEM).

Data analysis

Calculations of sexual reproductive effort (sRE) were carried out on serial histological sections. For each specimen, digital photographs of 16 histological sections were analyzed. Four photographs per serial section were taken. To avoid the overlapping of reproductive products that would lead to over-estimation, photographs of tissue were taken at least 200 µm from each other. The four photographs provided a total surveyed area of 1 mm^2 per sponge. We determined the number of sexually active sponges over time, counted the number of reproductive elements, and calculated the area of each reproductive element within the tissue sample, using ImageJ Software (http://rsb.info.nih.gov/ij/index.html). Reproductive elements were related to the overall surface of the section, and reproductive effort could thus be expressed as a percentage of reproductive tissue (mean ± SD).

In order to calculate the number of asexual reproductive efforts (aRE), the number of buds per individual was counted and bud area was estimated. These data allowed us to obtain the total surface of buds relative to the sponge surface, with aRE also expressed as a percentage of reproductive tissue.

The nonparametric Kruskal–Wallis test was used to determine the seasonal variability of each reproductive effort. To assess the putative influence of seawater temperature, we then applied a Spearman correlation test. Statistics and graphs were performed using RStudio (R Development Core Team, 2012).

Results

Reproductive cycle

No occurrence of gametogenesis was found between November 2007 and April 2008, nor from November to December 2008. In 2008, we observed oogenesis starting in late April and ending in September (Table 1, Fig. 2). No hermaphroditic individuals were observed; thus, *Haliclona fulva* appears to be gonochoric and oviparous, given the absence of observable embryo development. Through examination of our sponge collection and *in situ* photographs, we were able to observe the beginning of the budding period of *H. fulva* in early November and the end in April (Table 1, Fig. 2), which indicates an alternation between sexual and asexual processes.

Sexual reproduction

No male reproductive elements were recorded during this study of 103 individuals collected from this subpopulation. Of the 44 individuals observed during this period, 7 individuals lacked reproductive elements, but not a single male was recorded.

Oocytes were observed throughout the mesohyl, with the exception of the most superficial layer of the sponge tissue below the exopinacoderm. Oogenesis was synchronous within a mother sponge and in the entire population. Young oocytes were of small size, 14–24 µm in diameter. At this previtellogenic stage, oocytes (35–47 µm in diameter) had an irregular shape, a consequence of their active movement in the maternal mesohyl and phagocytosis of amoebocytes (Fig. 3A,B). During vitellogenesis, oocytes increased significantly in size to reach 50–77 µm in diameter. When the yolk started to accumulate, the oocytes amassed near exhalant

canals (Fig. 3C,D). At this stage, they were surrounded by a follicle. At the end of oogenesis, the eggs penetrated into the exhalant canals to be released into the seawater column through the aquiferous system.

Reproductive effort

The calculation of monthly mean sexual reproductive effort (sRE) was possible for females only and revealed significant variation over time (Kruskal–Wallis test: χ^2=24.40, K=24.28, df=8, p=0.002), with a minimum of 0.05% in April 2007 and a maximum of 1.15% in late August 2008 (Fig. 4A). Furthermore, the observed interannual variation is supported by a significant difference between sRE in September 2007 (2.70%) and September 2008 (0.44%) (Mann–Whitney–Wilcoxon: U=3, p=0.009).

The calculation of asexual reproductive effort (aRE) revealed a minimum of 0.21% for March 2008 and a maximum of 1.49% for January 2008 (Fig. 4B). We used the Kruskal-Wallis test to compare median aRE values for January, February and March, which showed that the apparent differences were not significant (χ^2=3.84, K=3.84, df=2, p=0.147). A Spearman correlation statistic was used to quantify the association between reproductive effort and temperature fluctuations. No significant correlation could be detected between temperature fluctuations and either sRE or aRE (Spearman rank correlation: r=0.130, p=0.843; and r=0, p=1, respectively).

Morphological observations of budding

Buds were mostly concentrated on the central part of the sponge surface (Fig. 1B). They were attached to the maternal apical surface by a short stalk (Fig. 5A), and were of different sizes and shapes due to their asynchronous development. Buds were oval or spherical, their mean surface was 0.5 mm^2 and their diameter ranged 0.5–3 mm (Fig. 5A,B). Their density also differed among specimens.

The beginning of budding was marked by the formation of small, irregular protuberances that emerged from the sponge surface. The buds then grow gradually as their apical region swelled (Fig. 5A,B). Bud skeletons consisted of oxea arranged uniformly at the periphery of the bud and in the mesohyl, but at this early stage of development the bud surface remained smooth. At the histological level, early buds did not possess any particular anatomical organization: there was no developed ectosome or choanosome (Fig. 5C). They consisted of a mass of compact cells, with a higher density of cells in the central part than at the periphery (Fig. 5C). Cells with inclusions (microgranular, granular cells), as well as cells with irregular shapes and long, thin cytoplasmic extensions (lophocytes, archaeocytes), were uniformly distributed throughout the bud tissue.

Just before their detachment, the external morphology and anatomy of mature buds changed. At this stage, buds clearly protruded from the sponge surface. They were oval in shape and had a pleated surface. Their central part slackened due to a significant increase in the number and volume of internal lacunae. Meanwhile, the mesohyl developed with a synthesis of collagen bundles and spicules (Fig. 5D). In contrast with the early stage, many small choanocyte chambers, composed of a few choanocytes and a central cell, were scattered through the inner region of the buds (Fig. 5D).

Cytological composition of the buds

Many choanocytes could be observed in aggregates in the central part of young buds, but at this stage of development, they did not yet form choanocyte chambers (Figs. 5C, 6A). These cells were spherical or oval and measured about 3.2 μm in width and 4.3 μm in height. Their nucleus was oval (~1.5 μm in diameter), lacked a nucleolus, but featured prominent heterochromatin (Fig. 6A). In mature buds, choanocyte chambers measured 15–18.5 μm in diameter (Figs. 5D, 6B).

The central cells were large amoeboid-like cells that were present inside of each choanocyte chamber (Fig. 6B,C). The central cells were irregular and branched in shape and had numerous cytoplasmic projections. Their nucleus was large (2.3–2.9 μm in diameter), spherical, and without a nucleolus. The cytoplasm of these cells was perforated by one large canal (1.8×3.5 μm in diameter) and other, smaller, canals (~0.5 μm in diameter) into which entered the flagellum of each choanocytes in the chamber (Fig. 6C).

The buds and the stalk were covered with an exopinacoderm composed of exopinacocytes, which were flat cells (~17.8 μm wide and 5.1 μm high) that harbored an oval anucleolated nucleus (5.0×2.6 μm in diameter) (Figs. 5C, 7A).

Microgranular cells with small inclusions had an amoeboid, slightly irregular shape (~8.5×4.9 μm), with a nucleus of 1.8 μm in diameter (Fig. 7B). These cells had vacuoles measuring 1–1.5 μm in diameter, with small, electron-dense inclusions. Microgranular cells were more numerous at the periphery of the buds (where they represented ~30% of total cells) than in the central part (where they represented only 8%) (Fig. 5C,D).

Granular cells had an oval or amoeboid shape (~9.5 μm in diameter) and harbored an anucleolated nucleus and large, homogenous electron-dense inclusions which were spherical in shape (1.2–2.7 μm in diameter) (Figs. 5C, 7C). These cells were less numerous than the previous cell type.

Archaeocytes were amoeboid or spheroid cells with no particular inclusions (~8.2 μm wide and 4.6 μm high). These cells were also abundant and harbored a large nucleus (2.5 μm in diameter) and a prominent nucleolus (Fig. 7D). Another amoeboid cell was the lophocyte. They were the biggest cells found in the buds (~18 μm wide and 5 μm high), with a large oval nucleus (4.5×2.5 μm) that may have had a nucleolus (Fig. 7E). The cytoplasm of lophocytes contained different sorts of inclusions such as granules and phagosomes, and these cells presented a developed, rough endoplasmic reticulum.

Sclerocytes were oval or amoeboid cells (~7.0 μm in diameter) with a large vacuole (Fig. 7F). Endopinacocytes were flat cells (~19.6 μm wide and 3.0 μm high) with an oval nucleus (4.2×2.0 μm) and no nucleolus, and the cytoplasm of these cells included numerous vacuoles (Fig. 7G). These cells formed the canals of the aquiferous system at the late stage of bud development. Collagen fibrils were quite abundant in the central part of the bud where they were sometimes arranged in fine bundles; they were, however, rare at the periphery. Finally, many extracellular symbiotic bacteria were found in the mesohyl (Figs. 6A,B; 7B,F).

Discussion

It is considered that asexual reproduction together with sexual reproduction accompanied multicellular animals throughout their entire evolution. Asexual reproduction in one form or another has repeatedly appeared and disappeared in different groups of Metazoa (Ivanova-Kazas 1977; Adiyodi & Adiyodi 1993). In some animals, such as flatworms, annelids, and holothurians (Christensen 1984; Franke 1999; Reuter & Kreshchenko 2004; Dolmatov 2014), asexual reproduction is a sporadic process. In other organisms with mixed life history strategies, such as

most cnidarians, tunicates, bryozoans and some sponges, asexual reproduction is an obligatory stage of their life cycle (Ivanova-Kazas 1977; Adiyodi & Adiyodi 1993; Brusca 2016). In the latter case, sexual reproduction can be an adaptive advantage in unstable or unpredictable environments, while asexual reproduction is the competitively superior tactic for colonization of the parental environment (Williams 1975; Maynard Smith 1978).

According to the available limited data, budding might promote the maintenance and growth of marine sponge populations (Corriero et al. 1996; Corriero et al. 1998; Cardone et al. 2010; Singh & Thakur 2015). Indeed, buds have been shown to contribute significantly to the dispersal and recruitment of new sponges (Wulf 1995), in particular under conditions of environmental stress. This strategy thus may improve the survival of individual sponge genotypes, and enhance the growth of a sponge population, as is the case for clonal organisms (Jackson & Coates 1986).

Reproductive pattern

Viviparity (brooding) is more widespread and perhaps an ancestral reproductive mode in Porifera (Riesgo et al. 2014). However, oviparity is the general rule in the Demospongiae orders Tetractinellida, Polymastiida, Suberitida, Clionaida, Tethyida, Chondrosida, Verongida, Agelasida, Biemnida, and Axinellida (Ereskovsky 2010). Viviparous representatives are found in some these orders, however; these representatives include, for example, the viviparous genera *Alectona* and *Thoosa* (order Suberitida), genus *Stylocordyla* (order Suberitida), and genus *Halisarca* (order Chondrosida) (Vacelet 1999; Sarà et al. 2002; Bautista-Guerrero et al. 2010; Ereskovsky & Gonobobleva 2000). It is largely accepted today that these two reproductive traits do not have any phylogenetic value and cannot be used for taxonomical revision of Porifera (Hoppe 1988; van Soest 1991; Ereskovsky 2010; Riesgo et al. 2014).

The studies by Fromont (1994a, b) and Fromont and Bergquist (1994) led to the general acceptance that all Haplosclerida families are characterized by viviparity, with the only exception being the oviparity in some Petrosiidae (Table 2). Although a good number of representatives of Chalinidae, especially in the *Haliclona* genus, have been shown to be viviparous, the present work demonstrates surprisingly that the Mediterranean *Haliclona fulva* is oviparous.

Another feature of the life cycle of *H. fulva* that is unusual in Haplosclerida is that this species includes budding as an obligatory phase of asexual reproduction, alternating with a sexual one. This case once more supports the observations that budding in Demospongiae is correlated with oviparity (Fell 1993; Ereskovsky 2010). Up to now there are only two exceptions to this rule: the viviparous demosponges *Mycale (Aegogropila) contarenii* (LIEBERKÜHN 1859) (Poecilosclerida) (Corrierro et al. 1998) and *Radiospongilla cerebellata* (BOWERBANK 1863) (Spongillida) (Saller 1990).

While different modes of reproduction often occur within the same genus in various marine metazoans, for instance in Echinodermata and Bivalvia (Strathmann 1978; Kasyanov 2001; Byrne et al. 2003), in sponges this phenomenon has previously been observed only in two genera of Petrosiidae (Haplosclerida). In the genus *Xestospongia*, *X. bergquistia* FROMONT 1991, *X. testudinaria* (WILSON 1925), and *X. muta* (SCHMIDT 1870) are oviparous, releasing sperm and eggs, while *X. bocatorensis* DIAZ, THACKER, RÜTZLER & PIANTONI, 2007 is viviparous, releasing brooded larvae (Fromont & Bergquist 1994; Becerro 2005; Collin et al. 2010). In the genus *Neopetrosia*, *N. exigua* (KIRKPATRICK 1900) is oviparous, while *N. proxima* (DUCHASSAING & MICHELOTTI 1864) is viviparous (Fromont & Bergquist 1994; Collin et al. 2010).

Another explanation for the phenomenon of oviparity in *Haliclona fulva* and different reproductive patterns in *Xestospongia* and *Neopetrosia* follows from the systematics of the order Haplosclerida. The phylogenetic relationships among haplosclerids are not clear yet, and suggestions of the polyphyletic nature of the various taxa within this order have appeared in various publications (Hill et al. 2013; McCormack et al. 2002; Raleigh et al. 2007; Redmond et al. 2007; Redmond et al. 2011; Redmond et al. 2013). It is important that the biggest genus *Haliclona* is distributed across the order Haplosclerida. Redmond et al. (2013) showed that members of this genus are positioned within Clade C with Niphatidae and Petrosiidae. The latter family, as mentioned above, includes both oviparous and viviparous species, particularly the viviparous *Neopetrosia proxima* (Collin et al. 2010) in Clade C. Species of *Haliclona* are also positioned within Clade B and has high support for a sister relationship with the viviparous *Xestospongia bocatorensis* (Collin et al. 2010). It should be noted that this Clade B also includes the oviparous *X. muta*. According to the results of Redmond et al. (2011), *H. fulva* is located in the cluster including the oviparous *Petrosia ficiformis* (POIRET 1789) and three other *Petrosia* species, as well as *H. mucosa* (GRIESSINGER 1971) and *Cribrochalina vascuum* (LAMARCK 1814). Therefore, it is possible that *H. fulva* might be misplaced in the genus *Haliclona*, and relationships with other oviparous clades might be the key to understanding reproductive behavior in *H. fulva*.

The general pattern of *H. fulva* oogenesis is similar to that reported for other oviparous Haplosclerida: no degeneration of mesohyl is observed during oogenesis and the majority of the eggs that develop to maturity retain an ovoid shape (Fromont 1988; Lepore et al. 1995; Maldonado & Riesgo 2009). During vitellogenesis, the oocytes have an amoeboid shape and participate in the phagocytosis of somatic cells, rich of phagosomes, like other investigated Haplosclerida (Ereskovsky 2010).

It is always a challenge to describe the reproductive cycle of oviparous sponges, but it is even more difficult when the males are hidden. Despite the large number of specimens of *H. fulva* investigated, no spermatocysts were observed. A very low ratio of male : female individuals has been reported for many marine sponges (Hogg 1967; Scalera Liaci et al. 1971; Ayling 1980; Corriero et al. 1996; Corriero et al. 1998; Mercurio et al. 2007; Ereskovsky et al. 2013). For example, after two years of fortnightly monitoring, Ayling (1980) found males of *Aaptos aaptos* (SCHMIDT 1864) in only one year of the study. No males were found in Mediterranean oviparous sponges *Tethya citrina* SARA & MELONE 1965 and *T. auranrium* (PALLAS 1766) during monthly collection over an 18-month period (Corriero et al. 1996). In a striking parallel to our study of *H. fulva*, the same author was unable to detect a single male among populations of *Raspailia topsenti* DENDY 1924 and *Polymiastia* sp. over a 2-year monitoring period (Ayling 1980).

Giesel (1972) proposed that deviations in sex ratio may internally regulate the size of a population by affecting its reproductive potential. In brooding corals, for example, female biased sex ratios may be an evolutionary adaptation caused by the physical limitations of the incubation chamber inside of polyps (Szmant 1986). Whalan et al. (2007) discussed some possible problems that may lead to biased sex ratios in sessile or slow moving marine invertebrates: proximity to mates (Sewell & Levitan 1992; Babcock & Keesing 1999), sperm limitation (Brazeau & Lasker 1992), and dilution of gametes (Oliver & Babcock 1992). Other possible factors that could influence the sex ratio are temperature, salinity, and the quantity and quality of food available (Simonini & Prevedelli 2003).

In this study of *H. fulva*, we remain perplexed as to where the males are. One explanation for the absence of males could be that the species has a very short period of spermatogenesis. In demosponges the period of spermatogenesis often is shorter compared to oogenesis (Ayling 1980; Diaz 1979; Ereskovsky 2000; Riesgo & Maldonado 2008; Mercurio et al. 2007; Whalan et al. 2007; Ereskovsky et al. 2013; Stephens et al. 2013). This trend is more pronounced in oviparous sponges, particularly in oviparous Haplosclerida. For example, in *Xestospongia bergquistia* and *X. testudinaria* from the Great Barrier Reef, the period of oogenesis lasts more than 5 months, while spermatogenesis lasts less than 5 days (Fromont & Bergquist 1994). In Mediterranean *Petrosia ficiformis,* oogenesis duration is 7–8 months, and the duration of spermatogenesis is only 2–2.3 weeks (Scalera Liaci et al. 1973; Maldonado & Riesgo 2009). Corriero et al. (1996, 1998) proposed that a very short period of spermatogenesis accounted for the absence of males during monthly collections of *Tethya citrina, T. auranrium,* and *Mycale contarenii* over 18 months of monitoring. Thus, it's possible to assume that the nine individuals of *Haliclona fulva* that lacked any reproductive elements (of the 37 individuals observed during the period of oogenesis) could be males with a short spermatogenesis period.

Reproductive cycle and effort

Numerous external factors could be responsible for induction of sexual or asexual reproduction in individuals within a population of heterogonic invertebrate species. Among the more important are the physicochemical quality of water, hydrodynamic, food availability, population density, habitat stability, and seasonal variation in temperature (for review see: Adiyodi & Adiyodi 1993). Nevertheless, it seems that water temperature and food availability play the principal roles in this process in the case of various marine invertebrates, such as sea anemones (Bucklin 1987), scyphozoans (Lucas et al. 2012; Purcell et al. 2012), echinoderms (Lawrence & Herrera 2000), and bryozoans (O'Dea 2006). In sponges, the increase in the frequency of specimens with buds and in the number of buds per sponge happens concomitantly with rapid decreases in water temperature as, for example, in the Mediterranean species *Tethya citrina T. aurantium, Mycale contarenii* (Connes 1968; Corriero et al. 1998; Cardone et al. 2010) and in White Sea species *Polymastia arctica* (MEREJKOWSKY 1878) (Plotkin & Ereskovsky 1997). In *H. fulva*, budding takes place from late November to early March, which is the coldest season. This is also the case for the two Mediterranean *Tethya citrina* and *T. aurantium* (Connes 1968; Corriero et al. 1996; Gaino et al. 2006) and *Mycale contarenii* (Corriero et al. 1998). However we were unable to demonstrate a significant correlation between the natural variations of seawater temperature and asexual reproductive effort.

Reproductive effort is an integrative indicator of resource allocation to reproductive compartments, and it can vary greatly depending on the reproductive strategy. *Haliclona fulva* allocates energy to the differentiation of gametes or buds at very different times, and as in other marine sponges, there is a clear alternation of sexual and asexual phases of reproduction (Fell et al. 1979; Corriero et al. 1996; Corriero et al. 1998). Both reproductive efforts are of roughly the same intensity, with oogenesis representing 0.7% on average over the period, and budding representing 0.9% *H. fulva* demonstrates low annual variability of both reproductive processes, whereas marine organisms can exhibit highly variable sexual or asexual reproductive efforts which are sometimes related to the natural variations of the sea water temperature (Adiyodi & Adiyodi 1993; Olive 1995; Llodra 2002). Overall, biotic and abiotic factors that regulate the proportion of asexual and sexual reproduction in the life cycle of heterogonic species are poorly understood. Unfortunately, in the case of *H. fulva* we were unable to fill this knowledge gap.

Although our sampling strategy has proven its efficiency in a number of previous studies of sponge life cycles (see for instance Pérez et al. 2011; Ivanisevic et al. 2011a, b; Ereskovsky et al. 2013; Zarrouk et al. 2013; Reveter et al. 2016), we are here confronted with a limitation with this strategy for *H. fulva*.

Bud formation, structure and cell composition

The development of buds differs among sponge species. For example, the formation of the aquiferous system can occur either during bud development (Saller 1990; Ereskovsky & Tokina 2007; Gaino et al. 2009; this work), or after detachment from the parental sponge (Ayling 1980; Battershill & Bergquist 1990; Gaino et al. 2006).

Haliclona fulva presents three stages in its bud formation: an ectosomal protuberance caused by cell migration; growth of the apical part of the bud with formation of the stalk connected to the parental sponge; and thin-stalked buds protruding noticeably from the sponge surface, followed by detachment of the bud. These stages are very similar to what was described in *Tethya aurantium* (Connes 1968; Gaino et al. 2006), *Radiospongilla cerebellata* (Saller 1990), *T. seychellensis* (WRIGHT 1881) (Gaino et al. 2009), and in *Cinachyrella cavernosa* (LAMARCK 1815) (Singh & Thakur 2015).

Cells in buds are amoeboid-like in shape and show cytoplasmic extensions. They tend to align either in parallel rows or along the spicule bundles. These features suggest that these cells may be able to migrate from the parental sponge to the newly formed buds. The migration of specialized and polypotent cells, and their subsequent differentiation into definitive cells, is a typical feature of the budding process (Ereskovsky 2003, 2010; Gaino et al. 2006). In this work we have shown that unreleased buds of *H. fulva* possess choanocyte chambers. The occurrence of choanocyte chambers is very uncommon in demosponge buds. Until now, these structures have been observed in unreleased buds of only four demosponge species: in *Mycale contarenii* (Devos 1965; Corriero et al. 1998), in the freshwater sponge *Radiospongilla cerebellata* (Saller 1990), in *Tethya seychellensis* (Gaino et al. 2009), and in *T. wilhelma* SARA, SARA, NICKEL & BRÜMMER 2001 (Hammel et al. 2009), as well as in homoscleromorph sponges of the genus *Oscarella* (Ereskovsky & Tokina 2007).

Cell composition in demosponge buds has been poorly studied. Optic microscopy has provided only two good descriptions of bud cell composition in *Mycale contarenii* and *Axinella damicornis* (ESPER 1794) (Devos 1965; Boury-Esnault 1970), and electron microscopy has illustrated this condition in *Tethya aurantium, T. citrina, T. seyshellensis, Radiospongilla cerebellata,* and *Cinachyrella australiensis* (CARTER 1886) (Connes 1967, 1968; Saller 1990; Chen et al. 1997; Gaino et al. 2006, 2009). According to these descriptions, it seems that cells with inclusions represent the main cellular components of buds, and that archaeocytes are the second most abundant cell type observed. In *A. damicornis*, *M. contarenii,* and *R. cerebellata,* all cell types of the parental sponge are present in equal proportions in the buds at their later developmental stages (Devos 1965; Boury-Esnault 1970; Saller 1990). In this respect, the buds of *H. fulva* do not differ from these sponges, as they are composed of microgranular cells, granular cells, archaeocytes, endopinacocytes (and exopinacocytes), choanocytes, central cells, and sclerocytes. On average, all these cells are similar in size to the cells of the parental sponge. Further research is needed to determine whether cell inclusions represent stored material useful in sustaining morphogenetic processes (Connes 1967), and whether they might thus be pivotal for the acquisition of complete functionality.

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Figure legends

Fig. 1. Specimen of *Haliclona fulva, in situ,* (**A**) without buds, in June 2008, and (**B**) with buds, in February 2008. Scale bars=30 mm. b, buds; o, oscula.

Fig. 2. Diagram of the reproductive cycle of *Haliclona fulva*.

Fig. 3. Oogenesis in *Haliclona fulva,* histological section (Masson-Goldner's trichrome hematoxylin staining). **A**. The mesohyl with oocytes at previtellogenic stage. **B**. Previtellogenic oocyte phagocyting the cells (arrowhead). **C**. The mesohyl with the eggs. **D**. Egg of *H. fulva*. Scale: A=50 µm; B,D=20 µm; C=100 µm. cc, choanocyte chambers; eg, egg; exc, exhalant canal; gc, granular cell; n, nucleus; oo, oocytes; s, spicules.

Fig. 4. Reproductive efforts of *Haliclona fulva.* **A**. Boxplot distribution of reproductive efforts during oogenesis and corresponding water temperatures during the study. **B**. Boxplot distribution of reproductive efforts during budding.

Fig. 5. Buds of *Haliclona fulva.* **A**. Buds *in vivo* at different stages of development. **B**. Buds *in vivo* at last stages of development. **C**. Semithin section of early bud. **D**. Semithin section of the bud at last stage. **Inset:** TEM micrograph of collagen bundles in the mesohyl of a bud. Scale: A=5 mm, B=1.5 mm, C=100 µm, D=150 µm, Inset=4 µm. b, buds; cb, collagen bundles; cc, choanocyte chambers; ch, choanocytes; ep, exopinacocytes; gc, granular cells; l, lacuna; mgc, microgranular cells; o, osculum; sb, symbiotic bacteria; ss, spongin of spicules.

Fig. 6. TEM images of the choanocytes in buds from individuals of *Haliclona fulva*. **A**. The aggregate of separated choanocytes in early bud. **B**. Choanocyte chamber with central cell in the bud at last stage. **C.** Central cell inside of a choanocyte chamber. Scale: A,B=5 µm; C=2 µm. c, canal in a central cell cytoplasm; ce, central cell; ch, choanocyte; f, flagella of choanocytes; mv, microvilli; n, nucleus; sb, symbiotic bacteria.

Fig. 7.TEM images of the cells in buds from individuals of *Haliclona fulva*. **A**. Exopinacocytes. **B**. Microgranular cells. **C**. Granular cell. **D**. Archaeocytes. **E**. Lophocyte. **F**. Sclerocytes. **G**. Endopinacocyte. Scale: A–D,F,G=5 µm; E=10 µm. af, axial filament of a spicule; ar, archaeocytes; ch, choanocyte; en, endopinacocyte; ex, exopinacocytes; g, granule; lo, lophocyte; mgc, microgranular cells; n, nucleus; nu, nucleolus; sb, symbiotic bacteria; sc, sclerocytes; ss, spongin of spicule; v, vacuole.

Table legend

Table 1. The number of individuals of *Haliclona fulva* collected during the sampling period for histological investigation.

Fig. 1

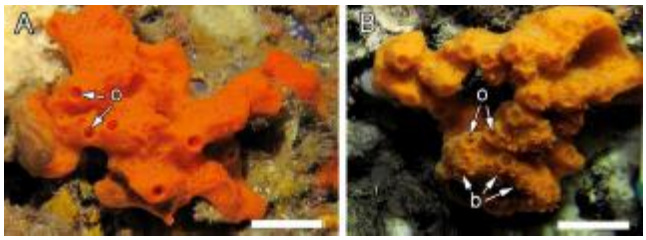

Fig. 2

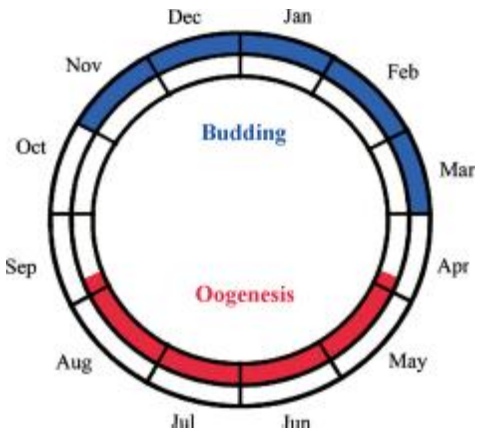
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Budding
Oogenesis

Fig. 3

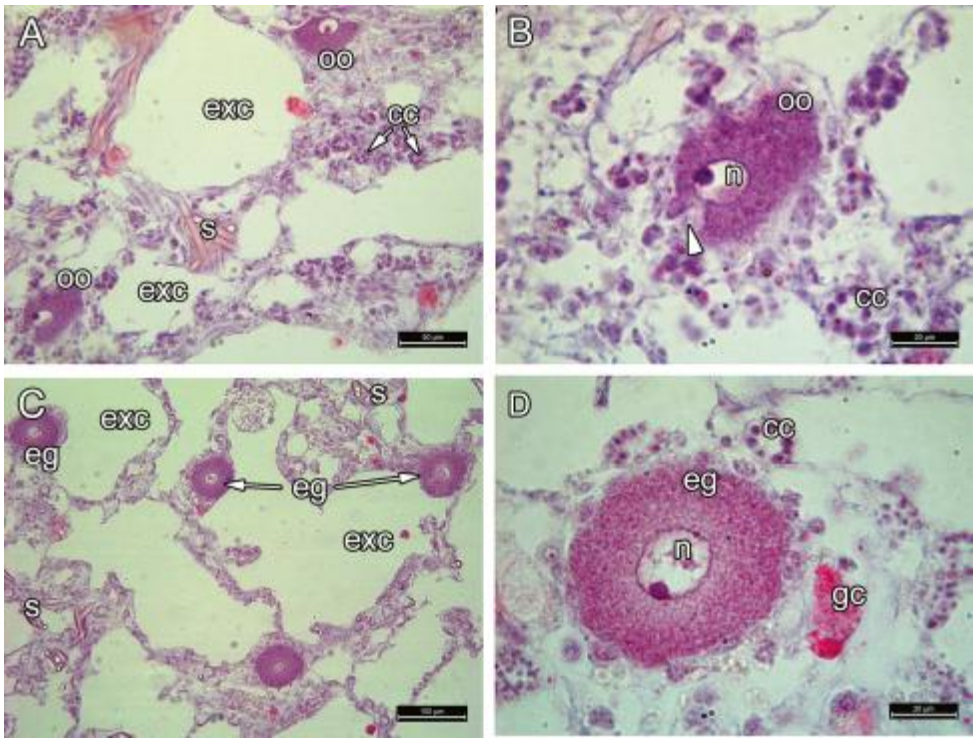

Fig. 4

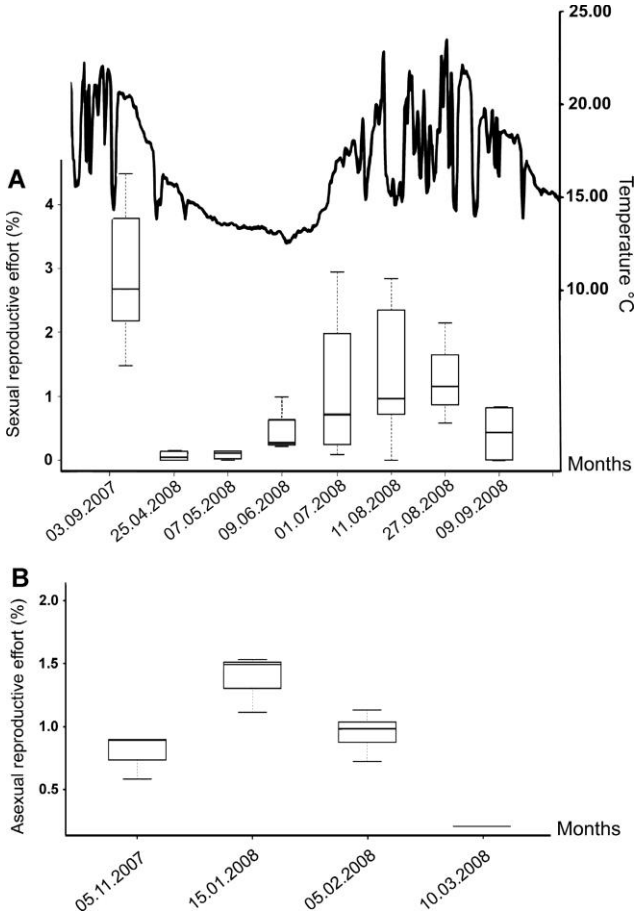
A
Sexual reproductive effort (%)
0
1
2
3
4
25.00
20.00
15.00
10.00
Temperature °C
Months
03.09.2007
25.04.2008
07.05.2008
09.06.2008
01.07.2008
11.08.2008
27.08.2008
09.09.2008
B
Asexual reproductive effort (%)
0.5
1.0
1.5
2.0
Months
05.11.2007
15.01.2008
05.02.2008
10.03.2008

Fig. 5

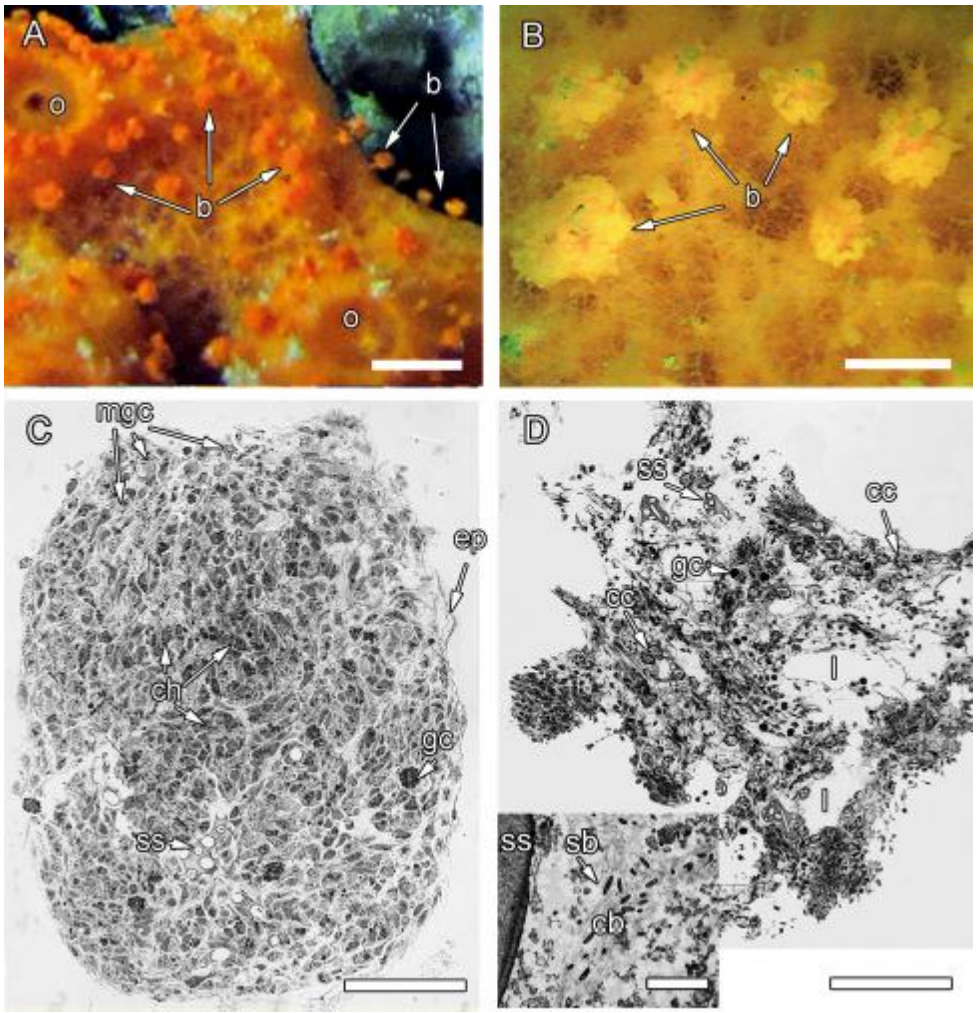

Fig. 6

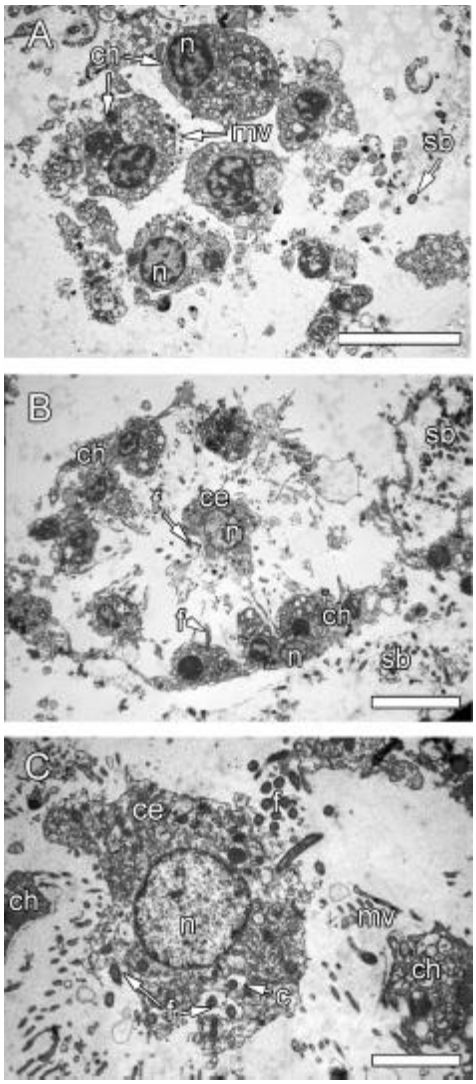

Fig. 7

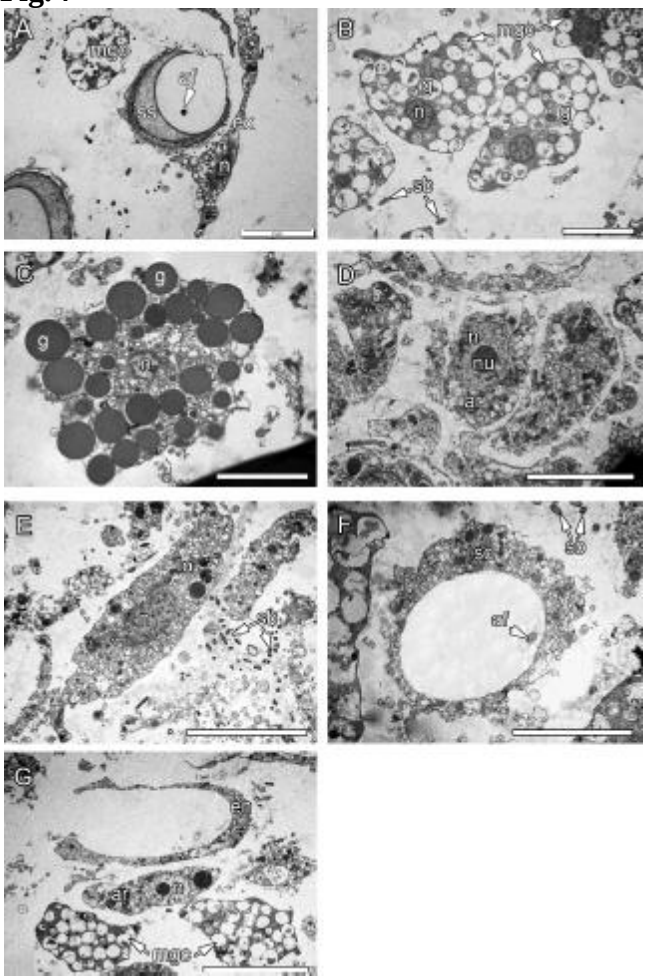

Table 1

Date	No. of sponges	Budding	Oogenesis	Embryogenesis	Spermatogenesis
September 3, 2007	7	No	7	No	No
November 5, 2007	10	No	No	No	No
December 5, 2007	9	9	No	No	No
January 15, 2008	5	5	No	No	No
February 5, 2008	6	6	No	No	No
March 10, 2008	4	No	No	No	No
April 25, 2008	6	No	4	No	No
May 7, 2008	6	No	4	No	No
June 9, 2008	5	No	5	No	No
June 19, 2008	6	No	4	No	No
July 1, 2008	6	No	6	No	No
August 11, 2008	5	No	5	No	No
August 27, 2008	5	No	5	No	No
September 9, 2008	5	No	4	No	No
October 14, 2008	4	No	No	No	No
November 18, 2008	6	6	No	No	No
December 8, 2008	4	4	No	No	No
January 15, 2009	4	4	No	No	No
Total	103	25	44	0	0