Mar Biol (2010) 157:1577–1590
DOI 10.1007/s00227-010-1431-7

ORIGINAL PAPER

# Recurrent disease outbreaks in corneous demosponges of the genus *Ircinia*: epidemic incidence and defense mechanisms

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Received: 14 January 2010 / Accepted: 16 March 2010 / Published online: 7 April 2010


**Abstract** During 2008 and 2009, an epidemic affected sponges of the genus *Ircinia* in the Western Mediterranean. Investigations at a site on the European coast (6°43′08.80″N; 3°43′52.20″W) and another on the African coast (35°10′51.00″N; 2°25′33.00″W) revealed healthier African populations. The disease started with small pustules on the sponge surface, which subsequently coalesced forming larger, extensive lesions. An ultrastructural study suggested that a twisted rod is the etiological agent. It infected the sponges from the outside, initially killing the cells below the ectosome and then penetrating deeper into the body. The sponges responded to the bacterial progression by secreting concentric barriers of collagen and concentrating phagocytic cells at the diseased zones. This primitive immune system successfully resisted the disease in many instances, although mortality reached 27% in the studied populations. Epidemic outbreaks recur each year in September through November, arguably favored by abnormally high seawater temperatures in August.

Communicated by J. P. Grassle.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00227-010-1431-7) contains supplementary material, which is available to authorized users.

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## Introduction

During the twentieth century, episodic disease outbreaks have devastated Mediterranean and Caribbean populations of both commercial sponges and non-commercial related species, mostly belonging to the taxonomic order Dictyoceratida (e.g., Galtsoff et al. 1939; Smith 1941; Lauckner 1980; Vacelet et al. 1994; Pronzato et al. 1999; Perez et al. 2000). The dictyoceratids are characterized by a skeleton consisting of an elastic network of proteinaceous (collagen-derived) fibers instead of the rigid mineral (calcareous and/or siliceous) pieces occurring in most other demosponges. Episodes of extensive mortality among sponges with mineral skeletons have also been noticed (e.g., Lauckner 1980; Rützler 1988; Nagelkerken et al. 2000; Cerrano et al. 2001), with the global incidence of sponge diseases increasing dramatically in recent years (Wulff 2006; Webster 2007).

One of the first reports in the scientific literature on sponge diseases (Carter 1878) described the attack on Indian populations of the dictyoceratid *Ircinia* sp. by a filamentous fungus. Ever since, little attention has been paid to epidemics in this genus, which unlike other dictyoceratids, lacks commercial value. It is now accepted that the putative filaments of the "pathogenic fungus" originally described by Carter as *Spongiophaga communis* were not "hyphae", but spongin filaments, which along with the spongin fibers compose the natural proteinaceous skeleton of sponges in the genus *Ircinia* (see Poléjaeff 1884; Vacelet et al. 1994). The infection that devastated the commercial dictyoceratids in the Caribbean in 1939, and that was tentatively attributed to a filamentous fungus, did not affect non-commercial dictyoceratids, such as *Ircinia* spp. and *Sarcotragus* spp. (Galtsoff et al. 1939). Therefore, there is no evidence for any previous fungal infection in *Ircinia*

spp. In contrast, a disease that wiped out the Mediterranean populations of commercial dictyoceratids from 1985 to 1989, and that was attributed to a bacterial infection, did affect their non-marketable relatives *Ircinia* spp. and *Sarcotragus* spp. (Vacelet et al. 1994). However, *Ircinia* spp. and *Sarcotragus* spp. populations were affected later and to a lesser extent than their commercial relatives, suggesting a more efficient defensive system in these genera. Therefore, in addition to a reliable identification of the etiological agents, another aspect that requires further investigation is the ability of sponges to defend themselves from microbial infections. Herein we report on a newly detected disease outbreak in populations of dictyoceratids in the genus *Ircinia*. More specifically, we document the incidence of the epidemic and assess its potential relationship with the annual cycle of seawater temperature. By using transmission electron microscopy (TEM), we also document the cytology of the disease process and the immune response.

## Materials and methods

### Studied material and study site

We investigated a disease outbreak in *Ircinia variabilis* and *Ircinia fasciculata*, which are closely related species in the Family Irciniidae, Order Dictyoceratida. Members of the Irciniidae (i.e., *Ircinia*, *Sarcotragus*, *Psammocina*) are distinct from the remaining dictyoceratids because of the presence of collagen filaments that supplement their skeletons of spongin fibers (e.g., Cook and Bergquist 2002). We studied sponge populations on both the European (coast of Granada) and the African (Chafarinas Islands) side of the Alboran Sea (Western Mediterranean; Online Resource: Supplementary Figs. 1, 2, 3). Both study sites are marine protected areas, with no declared impact by fishing trawls and relatively low levels of pollution from urban and industrial discharges. Nevertheless, the coast of Granada hosts extensive plantations of tropical fruit trees, so that inputs from agricultural activities are more likely than in the Chafarinas Islands, which are ~1.5 km from a shore with less urban and agricultural development. The sponges occurred at similar densities at both study sites (although densities were not actually measured prior to the study), in similar wave-exposed, rocky-bottom communities (5–30 m deep), which were mostly of two types: (1) sloping, well-illuminated bottoms dominated by green macroalgae, and (2) shaded walls and overhangs dominated by red algae, large filter feeders, and the scleractinian *Astroides calycularis*. All sponge populations included many massive individuals, up to 35 cm in width and/or height, and with a variable body shape depending on habitat.

### Temperature data

To assess the possibility that unusually warm periods were associated with the disease outbreak, we obtained sea temperatures from the coast of Granada (Gr) and Chafarinas Islands (Ch) for the past 5 years (January 2004 to December 2009) using satellite measurements by the MODIS (aqua) sensor system, made available as "Ocean Level-2" HDF data by the NASA Goddard Space Flight Center (http://oceancolor.gsfc.nasa.gov/). HDF files were read and processed using Mathlab R2009a software. In the analysis, we only considered high-quality sensor readings of temperature (flag values of 0 or 1), discarding less-reliable readings (flag values of 2 or 3). Suitable surface temperature readings ($n_{Gr} = 5053$; $n_{Ch} = 14382$) used in our analyses corresponded to daily means in a 9-km$^2$ area centered at the following coordinates: 36°43′08.80″N–3°43′52.20″W (Punta de la Mona, Granada) and 35°10′51.00″N–2°25′33.00″W (Chafarinas Islands). By selecting temperature readings ($n = 45$) corresponding to the warmest period of summer (i.e., July 15 to September 15) for each year at each site, we examined differences in sea surface temperature as a function of year (05, 06, 07, 08, 09) and site (Gr, Ch) using a 2-way ANOVA of square-transformed temperature data. A posteriori pairwise comparison to identify the groups responsible for the main significant factors was made using the Student–Newman–Keuls (SNK) test.

### Disease incidence

During weekly routine dives along the Granada coasts in August 2008, we did not notice any disease in *I. fasciculata* and *I. variabilis*, so we assumed that all sponges were healthy at that time (time = 0). After observing an extensive disease outbreak in the same sponge populations in October 2008, a random dive track was performed on SCUBA to obtain a first preliminary estimate (%) of diseased and dead individuals. After confirming persistence of the disease in the sponge populations, we undertook random tracks fortnightly or monthly, starting in February 2009. We used underwater scooter vehicles, which allowed us to cover large areas (Online Resource: Supplementary Figs. 2, 3) and to record the "status" of 100 to 250 sponge individuals per track. Some sponges were difficult to identify as either *I. variabilis* or *I. fasciculata,* and there is also some controversy about these two species being synonyms (Pronzato et al. 2004), so we pooled individuals from both putative species in our counts of this "*variabilis-fasciculata*" complex (hereafter referred to as *Ircinia* spp.). During our tracks, we surveyed large rocky-bottom areas (Online Resource: Supplementary Fig. 2), including boulder bottoms, shaded and illuminated rocky walls,

overhangs, cave entrances, and *Posidonia oceanica* beds at depths of 5–30 m. During these surveys, we assigned each individual a numeric value (0 to 4) referring to how much the sponge was affected by the disease (Fig. 1): "0" for sponges with no evidence of disease; "1" for the presence of a few (one to several), small pustules; "2" for few (one to several) large pustules, usually arising by coalescence of smaller adjacent pustules; "3" for extensive lesions, with coalescent pustules taking over large areas; "4" for dead individuals or sponges with evident signs of imminent death (see Fig. 1e). In this latter case, the entire sponge became discolored, crumbly if handled, and with decaying tissues. As the disease often progressed from basal zones toward the sponge apex, small and apparently healthy (i.e., still pigmented) areas were visible at the top of the body of some seriously affected individuals. Whenever such a healthy portion became "disconnected" from the substratum because it was completely surrounded by unhealthy tissue, the sponge was classified as level 4, since there was no further possibility of re-attachment.

After realizing that some affected individuals had recovered from the disease, an initial set of 20 randomly selected individuals were tagged (effective $n = 19$; one label lost). These sponges were used to evaluate the rate of new contagia and the expansion of pustules within an individual, as well as the incidence of mortality and eventual recovery. This sponge set was monitored either weekly or fortnightly from mid-November 2008 to June 2009, when most signs of disease had subsided. On June 8, 2009 a set of 36 healthy individuals was tagged in addition to the 19 previously marked, making a total of 55 tagged sponges that were monitored periodically in 2009. During all surveys of tagged sponges (November 2008 to December 2009), we assigned each individual a numeric code (0 to 4) identical to that used for the untagged individuals to refer to the level of disease. However, for tagged individuals, we added a new category, "level 5", to refer to sponges that showed clear symptoms of recovery, including healing pustules and regenerating ectopinacoderm (Fig. 1). Diseased sponges in 2008 that progressively healed their lesions in early 2009 and subsequently showed no external sign of previous lesions were finally transferred from the "recovered" category (level 5) to the "healthy" category (level 0).

In October 2009, when the disease epidemic was peaking in Granada populations, we surveyed the sponge populations of the Chafarinas Islands from 6 to 21 October for comparative purposes (Online Resource: Supplementary Figs. 1, 3). We surveyed a total of 26 random tracks (at depths of 5–30 m), using the methods described earlier.

### Histology of disease

To investigate the cytology of the disease process, small portions of ectosomal and endosomal tissues were excised from diseased individuals, from individuals that were recovering, and from healthy individuals during SCUBA dives. Immediately upon collection, tissue samples were fixed in 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer (MPB) and 1.4 M NaCl for 5 d. Samples were then rinsed with MPB for 30 min, post-fixed in 2% osmium tetroxide in MPB, dehydrated in a graded acetone series, and embedded in Spur's resin. Ultrathin sections obtained with an Ultracut Reichert-Jung ultramicrotome were mounted on gold grids and stained with 2% uranyl acetate for 30 min, then with lead citrate for 10 min. Observations were conducted with a JEOL 1010 transmission electron microscope (TEM) operating at 80 kV and provided with a Gatan module for acquisition of digital images.

## Results

### Temperature data

Analysis of sea surface temperature records indicated that the summer temperature maximum progressively increased at the Granada coast during the past 5 years, ranging from 25.22°C in 2005 to 27.59°C in 2009 (Fig. 2). Surface waters around the Chafarinas Islands did not experience such an increase, with maxima staying around 26°C (Fig. 2). However, although during most years the highest temperatures were recorded on some summer days at the Granada coast (Fig. 2), the 2-way ANOVA clearly indicated that summer averages (15 July–15 September) at the Chafarinas islands were significantly higher than those at the coast of Granada ($P < 0.001$; Fig. 3). The SNK test revealed that such a pattern was true for years 2005, 2006, and 2008, but that there were no significant between-site differences in summer temperature in 2007 and 2009 (test power >90%). The ANOVA also indicated that there were significant between-year differences ($P < 0.001$), and that the magnitude of such differences strongly depended on site (significant "site * year" interaction term: $P < 0.001$; Fig. 3). The SNK tests corroborated a progressive increase in summer average temperature over the past 5 years at the coast of Granada: 2005 being the least warm summer; 2006, 2007, and 2008 being not significantly different from each other but all being warmer than 2005; and 2009 being significantly warmer than all previous summers (see SNK tests in Fig. 3). Such a progressive summer warming over

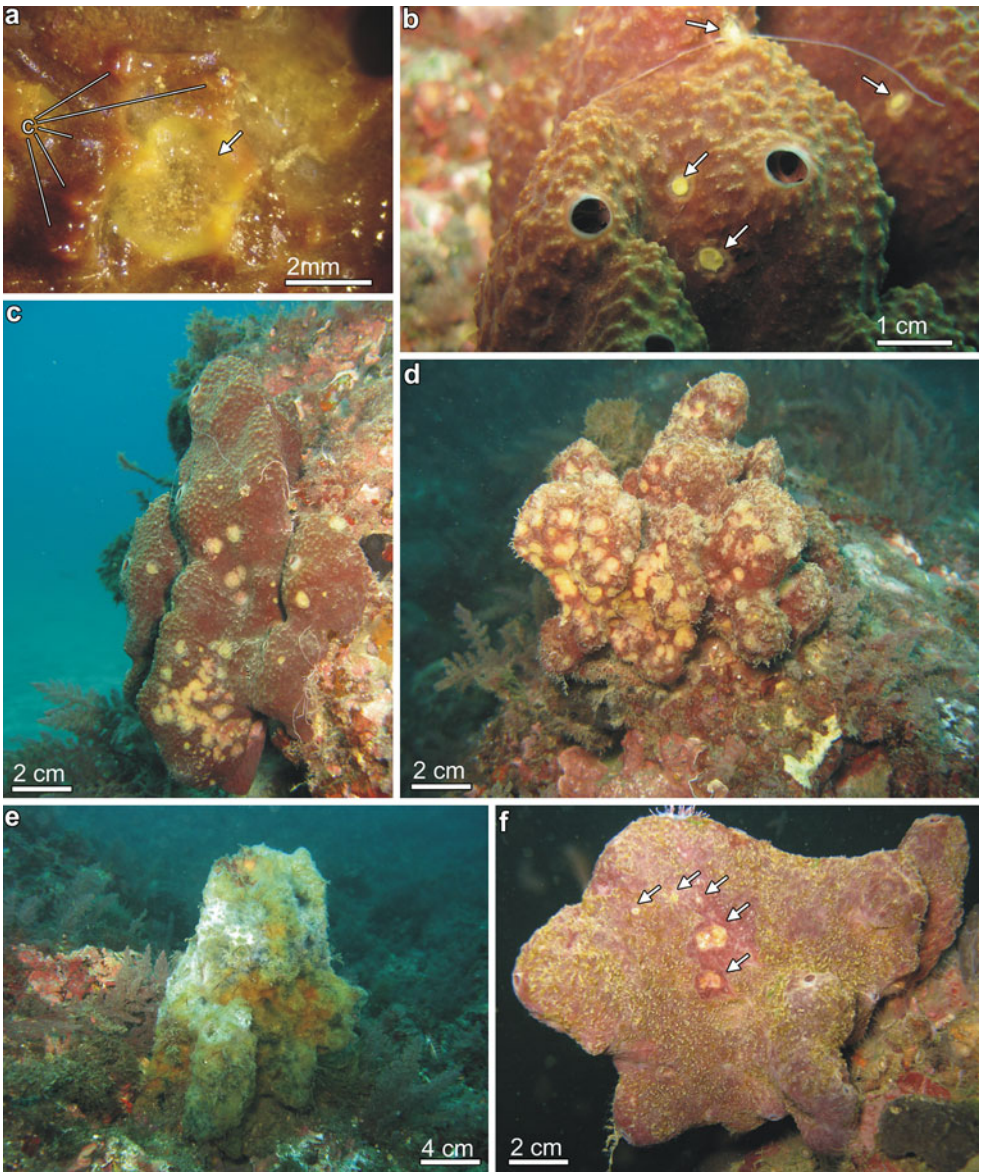


**Fig. 1** Sequence of disease stages in *Ircinia* spp. **a** Detail of a pustule (*arrow*) surrounded by conules (*c*). **b** Disease level #1: initial symptoms characterized by few, small, subcircular pustules (*arrows*). **c** Disease level #2: large, coalescing pustules restricted to some sponge areas. **d** Disease level #3: large, coalescent pustules extended over large sponge areas. **e** Disease level #4: dying sponge with necrotic ectosome. **f** Disease level #5: individual recovering from disease by healing its lesions (*arrows*)

the years did not occur at the Chafarinas Islands, with 2007 being the least warm summer and all remaining years having summers that were not significantly different from each other (see SNK tests at Fig. 3).

## Disease incidence

Our first observation of *Ircinia* spp. individuals showing small pustules (about 2% of the population) was on April 18, 2006, i.e., two and a half years before the present study. The first extensive occurrence of pustules at the population level was not noticed until mid-October 2008 (Fig. 4a). An initial random survey involving 50 sponges indicated that 25% had serious lesions or were dying. On November 24, 2008, the first inspection of 19 previously tagged individuals revealed that 10 (i.e., 52.6%) had pustules on the body surface. Interestingly, none of those diseased individuals died in subsequent months in 2008 and 2009. In contrast, they all had progressively healed ectosomal lesions and were recovering (Figs. 4b, 5). Random transects in late February 2009 and early March, 2009 ($n = 198$ individuals) confirmed this slow recovery process at the population level: 57% had no recognizable sign of disease, and 8.1% had healing lesions; the percentage of sponges showing

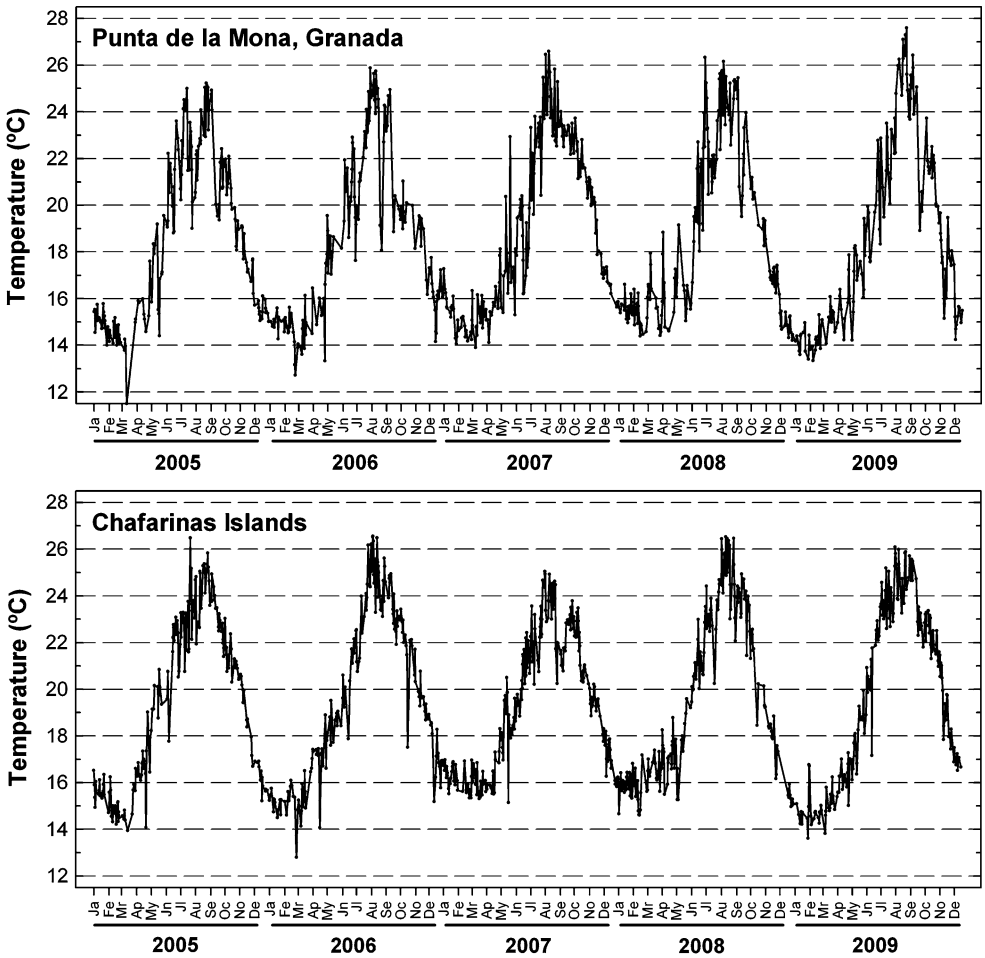


**Fig. 2** Daily average of sea surface temperatures at Punta de la Mona (Granada coast) and Chafarinas Islands in 2005–2009

pustules (31.3%) and dying (3.6%) had also decreased (Fig. 4a). During spring 2009, the occurrence of pustules progressively subsided and lesions had virtually disappeared from the sponges by May 2009 (Fig. 4a, b). Sponges showed no sign of disease in June and August. However, a severe disease outbreak was detected again by mid-September 2009 (Fig. 4a, b). Random tracks ($n = 214$ sponges) confirmed a widespread epidemic, with <10% of the population having no sign of disease and up to 27% of individuals dying during that month (Fig. 4a). Most of the affected individuals in the population (~60%) showed extensive lesions due to coalescing pustules. The situation was similar among the group of tagged individuals, in which only about 11% of sponges remained free of pustules (Fig. 4b). Most of the tagged sponges showed serious lesions (Figs. 4b, 6a–f) and about 3.5% died in September 2009 (Fig. 6g–j). Surprisingly, many of the sponges showing serious lesions did not die during subsequent months (Fig. 4b). Rather, they experienced partial necrosis, which involved a reduction in biomass of 15–80% of the body (Fig. 6a–f), and lesions slowly healed during October 2009 (Figs. 4, 5). By late October, about 20% of the population was still diseased, and remains of fiber skeletons attached to the rocks indicated ~22% mortality (Fig. 4a). In the group of tagged individuals, an additional 1.5% died in October, but the bulk of the group recovered in terms of symptom intensity (Fig. 4b). Data from both the random transects and the tagged sponges indicated that disease subsided rapidly in November 2009. Diseased individuals healed most of their lesions so rapidly that pustules had virtually disappeared from the surviving sponges by December 2009 (Figs. 4b, 5). It is worth noting that some tagged individuals controlled progression of the disease in both 2008 and 2009, recovering successfully each year (Fig. 5).

During October 2009, a time when the epidemic outbreak was peaking on the Granada coast, we conducted random transects over large areas on the platform of the Chafarinas Islands examining more than 600 *Ircinia* spp. individuals (Online Resource: Supplementary Figs. 1, 3). We detected no epidemic outbreak at the population level, and only three individuals with small, localized pustules (disease level = 1) and one showing extensive lesions (disease level = 3; Fig. 1c). Additionally, we found two dead individuals, although their appearance suggested that causes other than pustule disease (e.g., accidental breakage,

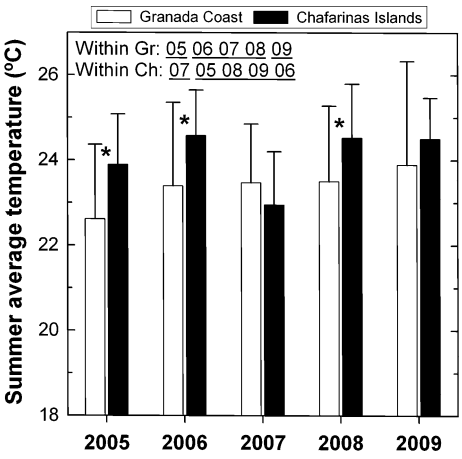


**Fig. 3** Summary of sea surface temperature averaged for hottest period (i.e., 15 July to 15 September) in 2005 to 2009. SNK tests following a 2-way ANOVA analyzing differences in mean temperature as a function of "site" (Gr = Granada coast; Ch = Chafarinas Islands) and "year" (05 = 2005, 06 = 2006, 07 = 2007, 08 = 2008, 09 = 2009) are also summarized. In upper left corner, summer temperature means for years within each "site" level (Gr, Ch) are ordered by increasing magnitude; years sharing an underline were not significantly different from each other ($P > 0.05$) according to SNK tests. Significant ($P < 0.05$) between-site differences for each year are indicated by an asterisk (*) between column pairs

predation, aging, etc.) were more likely responsible for those deaths.

### Histology of disease

Light microscopy and TEM examination indicated that the pustules were slightly concave portions of ectosome that had been damaged by microbial attack and thickened because of a "defensive" collagen deposit by the sponge. Even when most cellular elements were destroyed, pustules did not detach from the sponge surface because numerous spongin filaments emerging from the inner tissue held them in place. Microscopical examination suggested that the disease was caused by an external bacterium that invades the sponge body. It was a relatively elongated (2.50–3.00 μm × 0.20–0.31 μm), slightly twisted rod (Fig. 7a–b). In the bacterial cytoplasm, there was a distinct central nucleoplasmic area, which consisted of "fibrous" or partially condensed material. The riboplasm was peripheral (Fig. 7b–d), limited by a cytoplasmic membrane enclosed by a thin wall with typical Gram-positive organization with no obvious glycocalyx (Fig. 7c–d). The bacterium showed no internal polarization. Neither was a flagellum noticed in our TEM sections.

Upon penetration into the sponge body, rods proliferated surrounding the sponge cells that occurred immediately below the ectosome (Fig. 8a) and began to digest them (Fig. 8b). Such an attack produced large, ulcer-like cavities under the ectosome (Fig. 8c). While the tissue layer immediately below the ectosome was being digested, the sponges produced an underlying collagen barrier, in an apparent attempt to stop bacteria progressing deeper into the body (Fig. 9a, b). This protective barrier was about 0.2 μm thick and consisted of densely compacted collagen fibrils. Up to four concentric collagen barriers were observed in microscopic examinations of diseased sponges (Fig. 9a–c), but it is likely that more collagen layers are laid down by the sponges. Outside the first collagen barrier, there were still some cells that originally formed the sponge ectosome (ectopinacocytes), but they were isolated and scattered, rather than forming a continuous epithelium (Fig. 9d). Cell diversity drastically decreased around these defensive collagen barriers, with amoebocyte-like cells being very abundant (Fig. 10) and other cells typical of the peripheral mesohyl in healthy conditions being scarce (Fig. 8d). Amoebocytes were characterized by the presence of pseudopodia and phagosomes at various digestion stages, as well as by a large nucleolate nucleus, a well-developed Golgi apparatus, and abundant mitochondria and glycogen granules (Fig. 10a–c). These amoebocytes engulfed infective bacteria (Fig. 10a), but also regular microbial symbionts of the sponge (data not shown). These glycogen-filled amoebocytes were also abundant in the peripheral tissue of sponges that were recovering from the disease. It is worth noting that the sponge tissue immediately around and below the pustules contained few microbes other than the infective rods (Fig. 11a). In contrast, the tissue of healthy "control" sponges showed a high diversity of microbial symbionts, including a wide variety of bacteria (Fig. 11b) and several cyanobacteria, such as *Aphanocapsa raspaigellae*, *Aphanocapsa feldmannii*, and *Synechococcus spongiarum* (data not shown).

## Discussion

Our study has revealed that the pustule disease in *Ircinia fasciculata* and *Ircinia variabilis* is a recurrent epidemic, reappearing each year, typically after the hottest months. The appearance of pustules and the subsequent necrosis of the sponge tissue appear to be related to proliferation of an external bacterium within the peripheral mesohyl. Although up to 30% of affected individuals died, the sponge immune response stopped the disease in many instances, allowing regeneration of necrotic mesohyl areas and epithelia.

Agents causing disease outbreaks in sponges have rarely been identified. A viral infection, known from only one sponge individual (Vacelet and Gallissian 1978), was suggested to cause localized tissue lesions, but never epidemic mortality. Symbiotic cyanobacteria growing out of

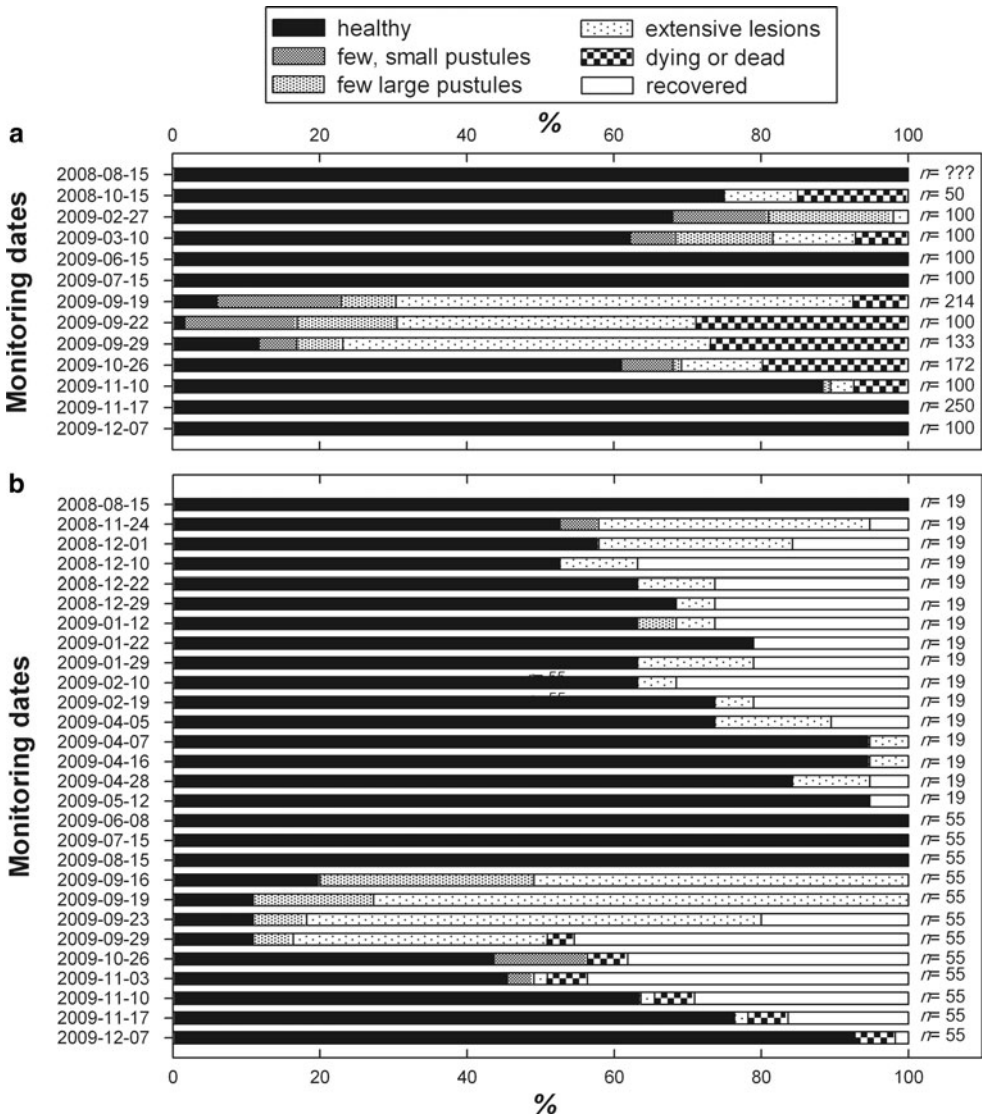


**Fig. 4** Summary of disease incidence on the coast of Granada, as estimated from random dive tracks (**a**) and from a group of tagged individuals (**b**). Total number of sponges ("*n*") per sampling date indicated on the side

control have been suggested to cause extensive mortality in populations of the non-commercial mangrove sponge *Geodia papyracea* (Rützler 1988). A filamentous fungus was tentatively suggested as the etiological agent that devastated Caribbean populations of commercial dictyoceratids (genera *Spongia* and *Hippospongia*) from 1938 to 1944 (Smith 1939, 1941; Galtsoff et al. 1939; Osorio-Tafall and Cardenas 1945). Bacteria have also been blamed as the cause of several episodes of epidemic mortality (reviewed by Lauckner 1980; Gaino and Pronzato 1989; Vacelet et al. 1994). Because most sponges harbor enormous numbers of symbiotic and/or associated bacteria, isolating and identifying a putative disease-causing organism has proved to be complicated and challenging. Only the advent of molecular techniques has allowed the first reliable identifications of the etiological agents involved in some newly detected cases of epidemic mortality. In these cases, the bacteria that emerged as responsible had very diverse phylogenetic affiliations (e.g., Webster et al. 2002; Cervino et al. 2006; Webster 2007, Webster et al. 2008). Our ultrastructural study strongly suggests that the microbe responsible for the pustule disease is a twisted rod of external origin. Nevertheless, we are aware that a definitive demonstration of the etiological agent would require isolation of the microbe from the pustules, experimental infection of healthy individuals using laboratory isolates, and subsequent recovery of the rod from the pustules experimentally induced. Interestingly, the twisted rod that we found abundantly in the pustules and adjacent tissues seems to be morphologically similar to a bacterium that affected populations of the ircinid *Sarcotragus spinulosum* in 1989 and caused excavation of their spongin filaments (Vacelet et al. 1994). Because the most serious damage in the *Ircinia* spp. occurred in the cellular elements of the sponge, we did not check for potential damage to its collagenous skeletal structures. Further evidence supporting the twisted rod as

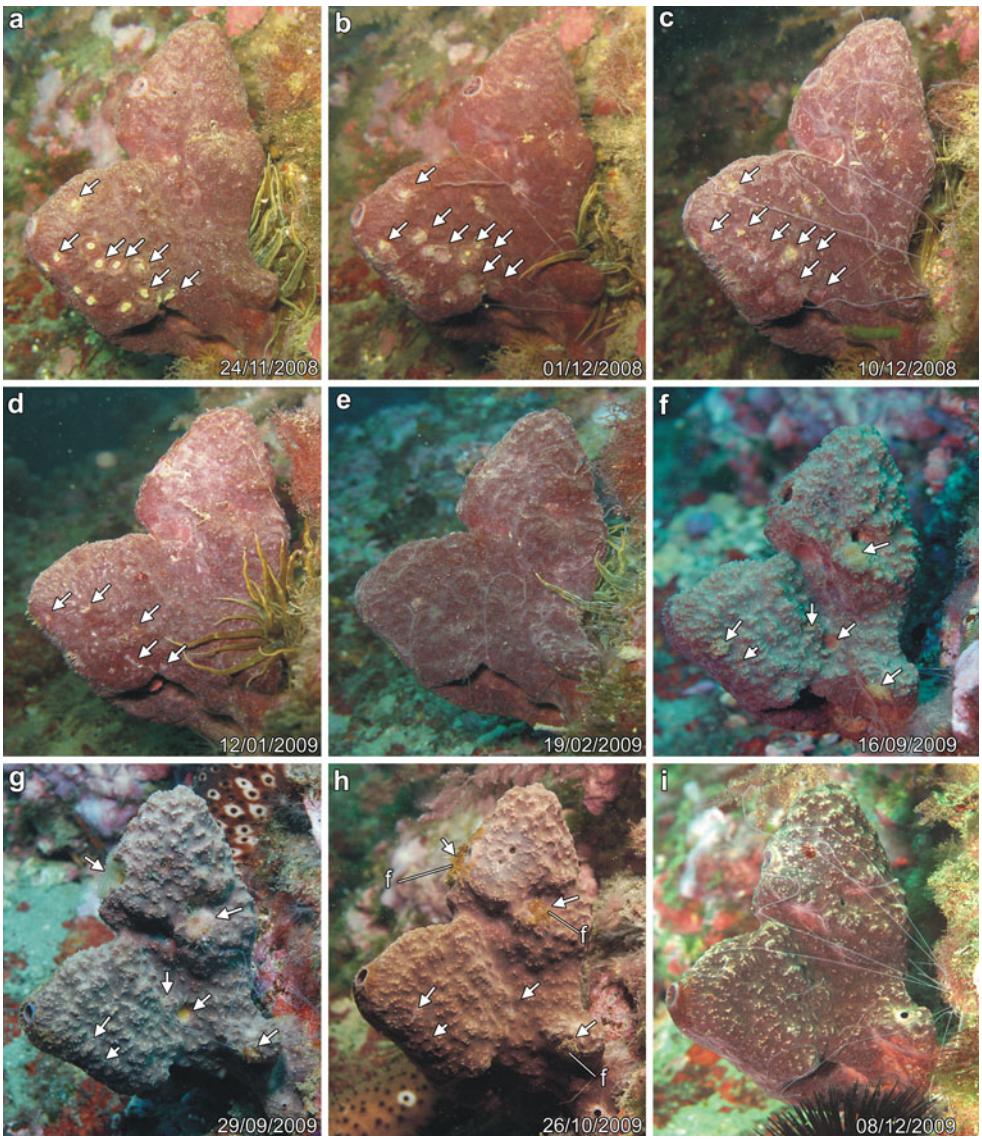


**Fig. 5** Course of disease in *Ircinia* spp. #6 in 2008 and 2009. **a** November 24, 2008, small whitish pustules (*arrows*). **b** December 1, 2008, initiation of pustule healing (*arrows*). **c–d** December 10, 2008 and January 12, 2009, progressive recovery stages showing fewer and fewer pustules (*arrows*) over time. **e** February 19, 2009, completely recovered stage with no obvious sign of previous pustules. **f** September 16, 2009, second disease event, with appearance of few large pustules (*arrows*). **g** September 29, 2009, initiation of pustule (*arrows*) healing. **h** October 26, 2009, subsequent recovery stage in which small pustules have healed; large pustules caused necrotic surface areas where underlying skeletal fibers (*f*) were visible. **i** December 8, 2009, stage showing an entirely recovered ectosome and no obvious sign of previous lesions

the etiological agent is that all other sponge-associated microbes disappeared from diseased tissues. Such a decline in microbial diversity in the diseased tissues is noteworthy, since microbial diversity has been shown not only to change but also to increase during tissue decay processes in other sponge species (e.g., Webster et al. 2008).

The factors triggering the disease outbreak in *Ircinia* spp. populations remain unclear. Recent outbreaks in invertebrates of tropical and temperate seas have been attributed to climatic anomalies characterized by elevated seawater temperatures. Some bacteria appear to be thermo-dependent pathogens. This is the case for *Vibrio coralliilyticus*, which becomes virulent against Caribbean, Red-Sea and Indo-Pacific corals (Ben-Haim et al. 2003; Sussman et al. 2008; Rypien et al. 2010) and Mediterranean gorgonians (Bally and Garrabou 2007) when seawater temperatures are abnormally elevated. The lengthened, twisted morphology of the bacterium found in *Ircinia* spp. differs from the short-stout cell morphology of the YB1$^{T}$ strain reported in the original description of *V. coralliilyticus* by Ben-Haim et al. (2003). However, *V. coralliilyticus* YB2 strain, as depicted by Meron et al. (2009), is a lengthened, twisted rod, with

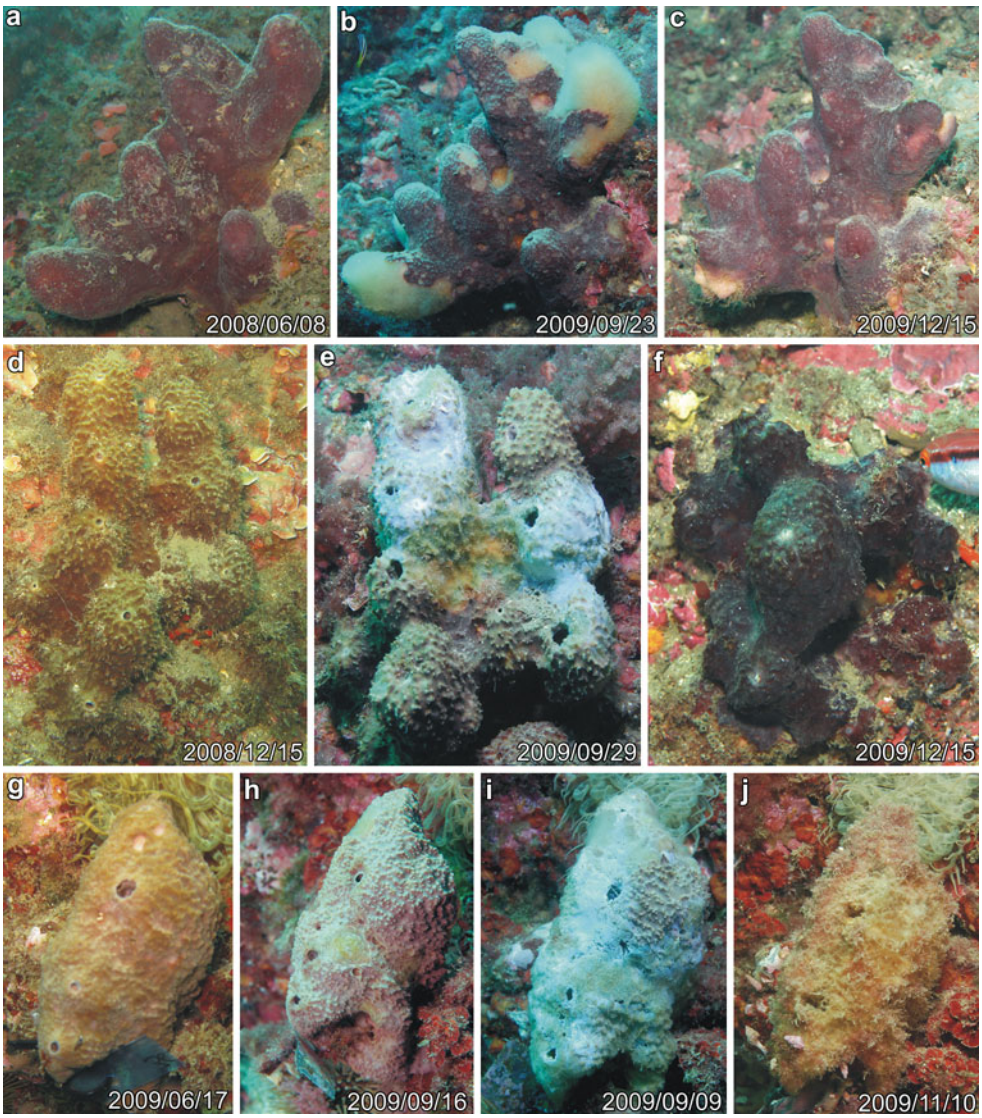


**Fig. 6** Disease effects on 3 tagged *Ircinia* spp. that were seriously affected. **a–c** Healthy individual #15 experienced severe localized lesions, but survived after losing ~20% of its biomass. **d–f** Healthy individual #1 experienced extensive lesions, but survived after losing about half of its body mass. **g–j** Healthy individual #36 experienced localized lesions initially, which subsequently expanded leading to sponge death (**j**)

dimensions similar to those of the putative etiological agent of *Ircinia* spp. At first sight, the absence of an obvious flagellum in the twisted rod found in *Ircinia* spp. suggests that it does not belong to the genus *Vibrio.* However, we cannot discard the possibility that the insertion of a thin flagellum in the bacterial cell was never caught in our non-serial sections. Furthermore, a non-flagellated mutant strain of *V. coralliilyticus* has been recently described (Meron et al. 2009), although it has shown limited ability to cause infections, at least in corals. The ability of *Vibrio* spp. to infect sponges remains little investigated, but experimental assays with a flagellated strain of *Vibrio anguillarum* that is pathogenic to many marine animals have shown that it is unable to infect a demosponge (Maldonado et al. 2010). Therefore, efforts are now focused on identifying the twisted bacterium found in *Ircinia* spp."

Reappearance of disease outbreaks on the Granada coast from mid-September through November suggests that previous short periods (days) of abnormally high temperatures during August may be the triggering factor in disease establishment. Indeed, a progressive warming of surface water during the summer of the past 5 years has clearly occurred along the Granada coast. However, the much lower incidence of disease in *Ircinia* spp. populations of the Chafarinas Islands, typically characterized by either similar or higher average temperatures during summer, complicates assessment of the role of temperature in the disease epidemic. Given than our detailed sponge monitoring in 2008

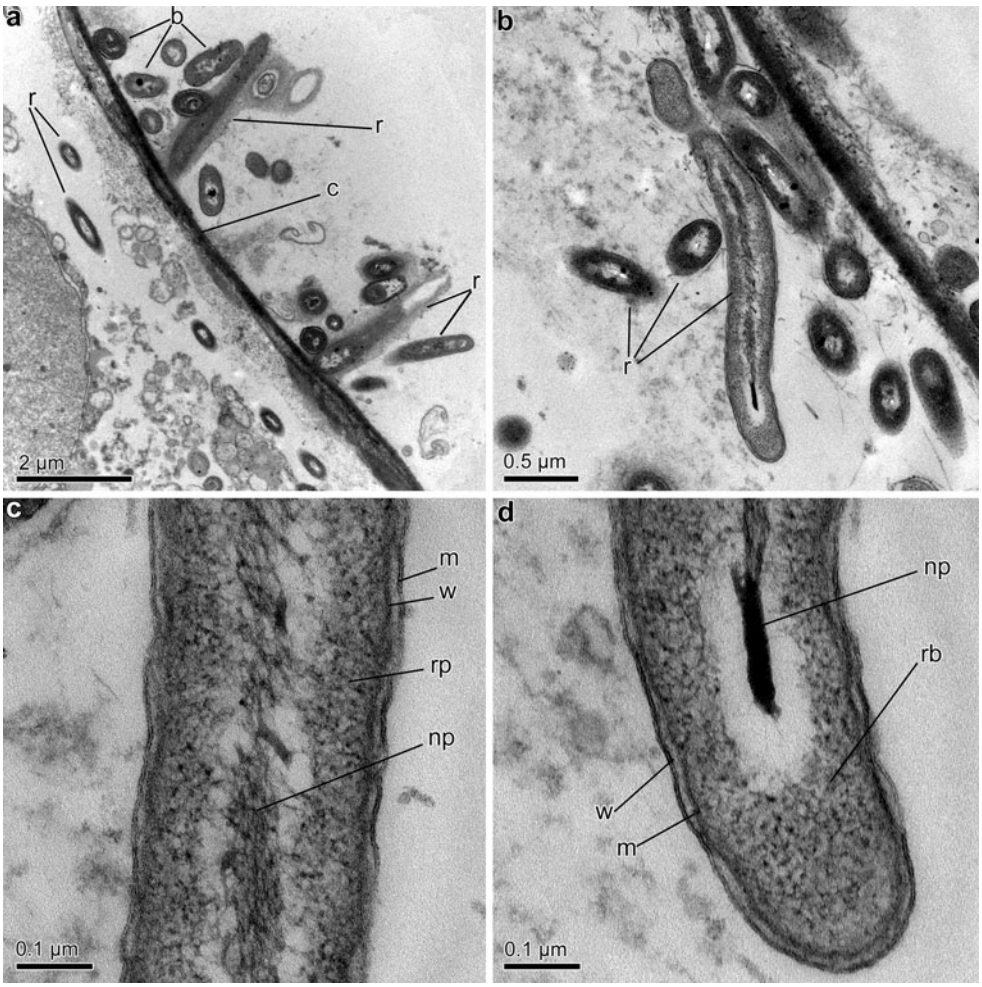


**Fig. 7** Cytology of putative etiological agent and disease progression in *Ircinia* spp. **a** Peripheral section of sponge, showing external cuticle (*c*) and twisted rods (*r*) apparently attempting to penetrate it; several other bacteria (*b*) on sponge cuticle and several transverse-sectioned rods (*r*) have already invaded sponge tissue. **b** Longitudinal and transverse sections of twisted rods (*r*) below sponge cuticle. **c–d** Detail of mid- and distal regions of rod, showing cytoplasmic membrane (*m*), bacterial wall (*w*), and marked differences between nucleoplasm (*np*) and riboplasm (*rp*)

commenced once the disease had peaked in Granada populations (i.e., in October), we cannot reliably compare temperature effects between 2008 and 2009 for this study site. Likewise, we lack disease data for the Chafarinas population in 2008 and cannot make an appropriate between-site assessment of potential temperature effects.

In addition to temperature, some studies have suggested that lateral gene transfer from terrestrial bacteria (used in agricultural control of animal disease) to marine bacteria could have given rise to new marine strains suited to attack animal cells (e.g., Cervino et al. 2006; Webster 2007). Although it has recently been demonstrated that *Bacillus thuringiensis* is unable by itself to infect corals and sponges, and that ectopic laboratory application of its endotoxin has no effect on these organisms (Negri et al. 2009), the possibility of a transference of genes responsible for the "virulence" to bacteria that can infect marine invertebrates remains a plausible hypothesis. Unlike the Moroccan coast close to the Chafarinas Islands, the coast of Granada has extensive plantations of tropical fruit trees, grown using modern techniques that include routine biological control based on *Bacillus thuringiensis*. However, in the absence of a genomic assessment of "virulence factors", it is impossible to decide whether a greater use of biological control on the Granada coast could be related to a much higher incidence of the disease in this area relative to the Chafarinas Islands. It is also noteworthy that additional observations suggest that the incidence of mortality may vary greatly between local populations, irrespective of agricultural development. For instance, on 3 October, 2009, an additional random transect at a site on the Malaga coast (only 5 km from the monitoring sites on the Granada coast) had a higher disease level, with 72 dead sponges (59%) out of 122 inspected. In contrast, mortality never exceeded

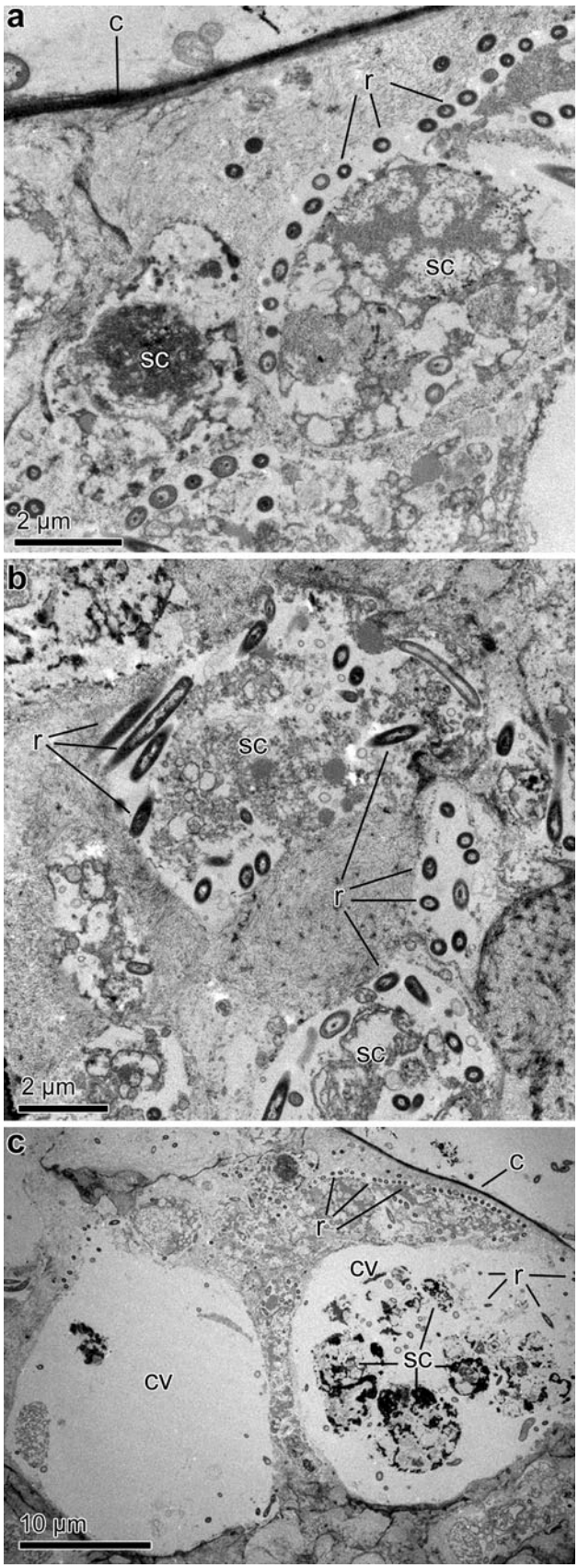


**Fig. 8** Bacterial invasion of sponge peripheral tissue. **a** Abundant rods (transverse-sectioned) surrounding sponge cells (*sc*) immediately below sponge cuticle (*c*). **b** More advanced stage of sponge cell (*sc*) destruction in presence of putative pathogenic rods (*r*). **c** Large cavities (*cv*) formed in peripheral mesohyl after sponge cell lysis (*sc*) in presence of rods (*r*)

30% in Granada populations. Likewise, during October–November 2009, there were "informal reports" by diving colleagues that many individuals of the "*I. variabilis-I. fasciculata*" complex" were dying at several sites in the northwestern Mediterranean (e.g., Columbretes Islands, Murcia Coast, Balearic Islands, Corsica Island, etc.). Some of these reports also suggest higher levels of mortality than those detected in the present study. Overall, these data might also suggest that the disease originated in the northwestern Mediterranean and has only recently reached more southern sponge populations.

One of the most interesting aspects of the present study was that many sponges stopped the microbial infection and eventually recovered. The ultrastructural study revealed that the sponges secreted successive collagen barriers at the diseased area and abandoned decaying body parts external to the barrier. Likewise, we noticed that abundant amoebocytes (migrating phagocytic cells) acted as immune cells, engulfing and digesting the pathogen. These amoebocytes, which are totipotent cells, probably facilitate cellular regeneration in the necrotic areas. The abundant glycogen granules and mitochondria in their cytoplasm indicated high metabolic activity. The fact that these ameobocytes were still abundant in sponges that were recovering from the disease also supports their participation in regeneration processes. One non-corneous sponge (*Tethya lyncurium*) has been described to stop a bacterial invasion using the same mechanism as *Ircinia* spp., i.e., sealing off a diseased area with collagen barriers, abandoning decaying body parts, and accumulating totipotent phagocytic cells at the infected zones (Connes 1967). A variation on this defensive strategy has been reported in a disease of the sponge *Geodia papyracea*, where the infection was caused by an internal "symbiotic" cyanobacterium that proliferated beyond host control (Rützler 1988). When the symbiont became virulent, many sponge amoebocytes migrated to the areas where bacteria were proliferating and these were sealed off by thick collagen envelopes. Therefore, collagen barriers and phagocytic-totipotent cells appear to be common elements in the most primitive immune system of animals.

It is also worth noting that the pustule disease appears to be restricted to the "*I. variabilis-I. fasciculata*" species pair. The sponge *Ircinia oros,* another common Mediterranean member of the genus that often shares habitat with *I. variabilis* and *I. fasciculata*, never showed signs of

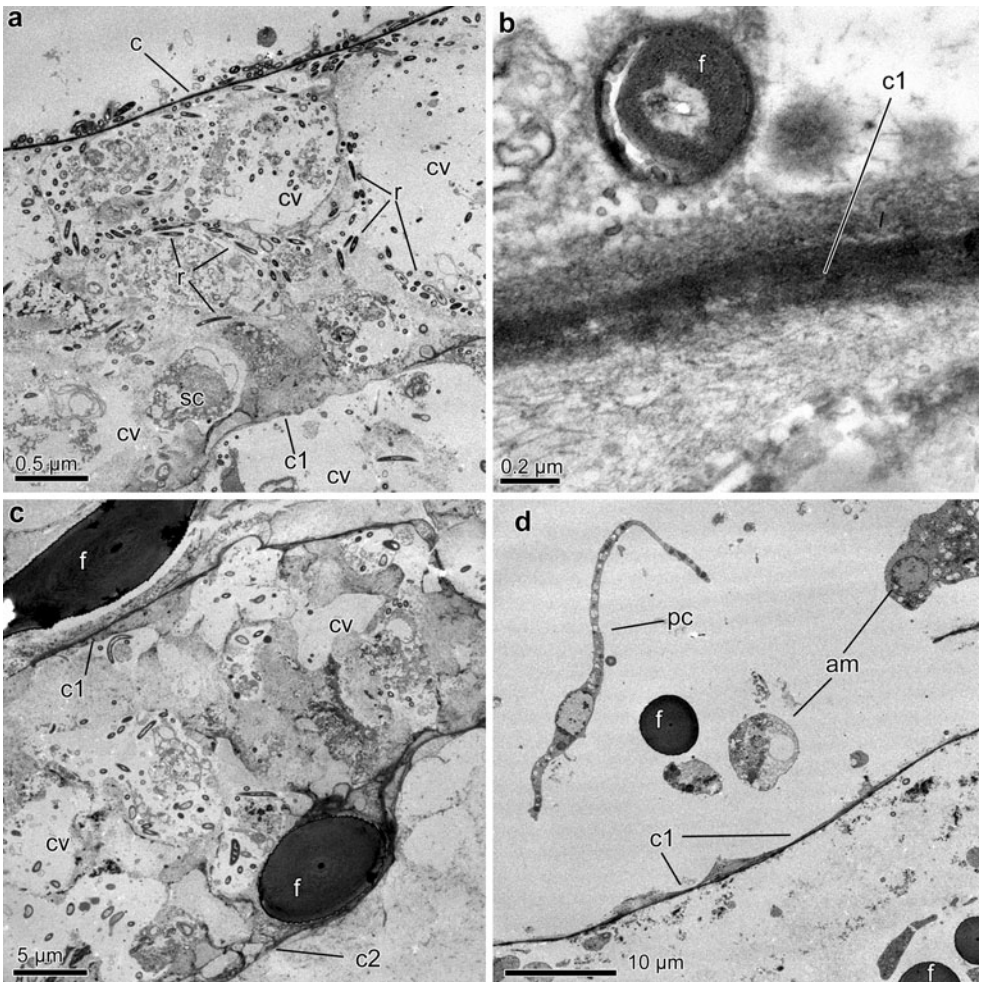


**Fig. 9** *Ircinia* spp. defensive collagen barriers against bacterial progression. **a** First collagen barrier (*c1*), secreted below external cuticle (*c*). Mesohyl shows many cavities (*cv*) resulting from destruction of sponge cells (*sc*) by rods (*r*). **b** Detail of first collagen barrier (*c1*) showing progressive compaction of collagen fibrils on both sides. Note spongin filament (*f*) at the side. **c** General view of a mesohyl portion comprised of a first (*c1*) and a second (*c2*) collagen barrier. Note that rods have penetrated deeper into sponge and produced large cavities (*cv*). **d** General view of highly disorganized ectosome after bacterial attack, showing detached epithelial cell (*pc* = pinacocyte), several subepithelial amoebocytes (*am*), spongin filament (*f*), and collagen barrier (*c1*)

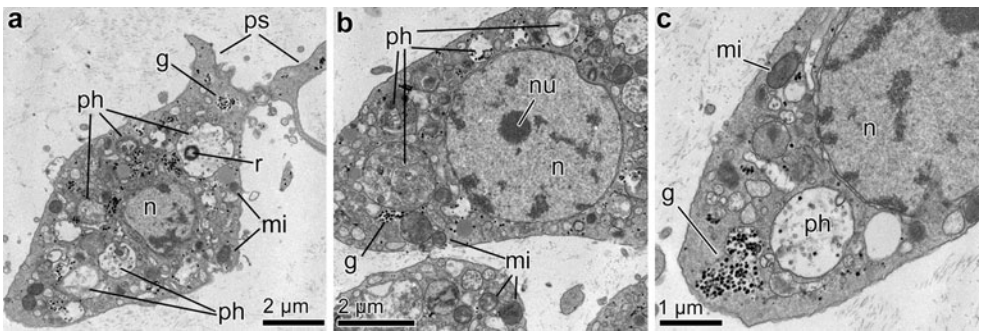


**Fig. 10** Defensive *Ircinia* spp. cells. **a–c** Details of amoebocytes, showing some pseudopodia (*ps*), a nucleolate (*nu*) nucleus (*n*), abundant glycogen granules (*gl*), small abundant mitochondria (*mi*), many phagosomes (*ph*) at various digestion stages, including obvious traces of engulfed rods (*r*)

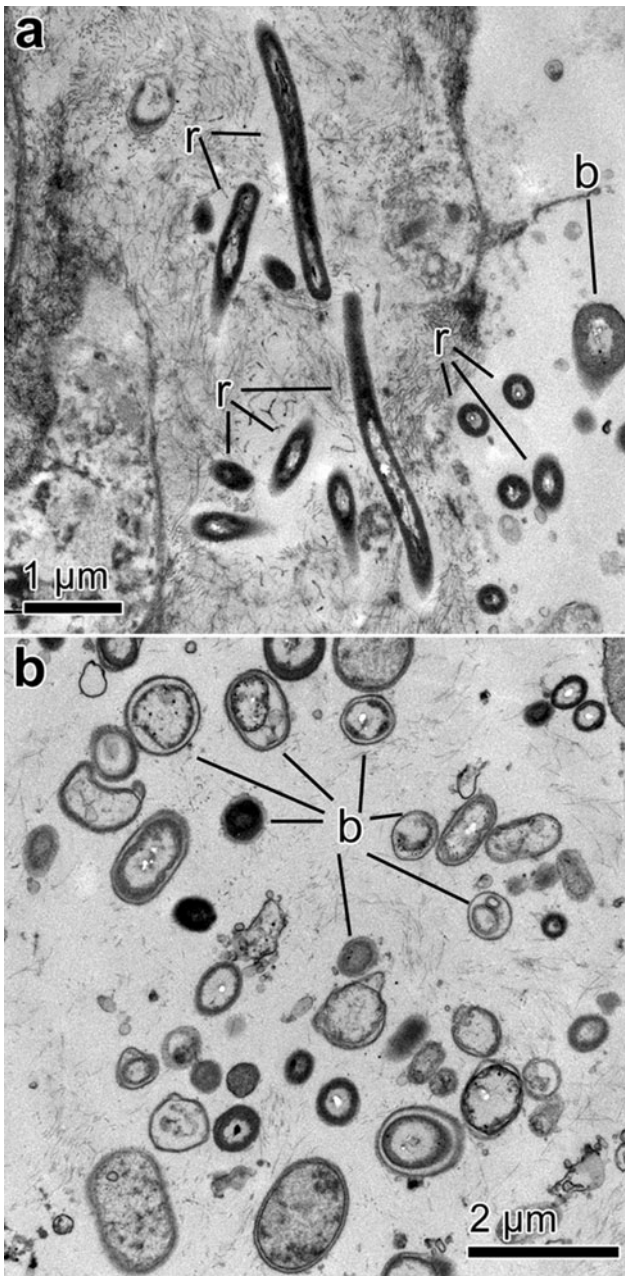


**Fig. 11** Microbial diversity in healthy and diseased *Ircinia* spp. tissue. **a** Peripheral mesohyl of seriously diseased sponge, in which twisted rods (*r*) became the dominant microbe, with near absence of other bacteria (*b*) that regularly occur in healthy sponges. **b** Regular abundance and diversity of bacteria in peripheral mesohyl of healthy sponges

pustule disease. Members of the sister genus *Sarcotragus*, such as *S. spinulosum*, were only occasionally affected by a disease with very different symptoms, not including production of pustules (Online Resource: Supplementary Figure 4). The fact that *I. variabilis* and *I. fasciculata* showed similar susceptibility to the infective agent and identical symptoms (pustule progression) supports the idea that they may not be different species but ecomorphs of a single species.

Infectious diseases are foreseen to increase among marine animals, accelerating the loss of biodiversity and causing major community shifts in coastal ecosystems (Cervino et al. 2006; Bally and Garrabou 2007; Webster 2007). To establish control strategies for future conservation, we need to advance our current understanding of both etiological processes and factors that determine the susceptibility of animal populations, particularly in invertebrates of no commercial value but with relevant ecological roles.

**Acknowledgments** The authors thank J. Zapata, J. Díaz, I. Busturia, and C. González for help with field work, A. García for help with TEM sample processing, R. Bernardello for help in processing HDF temperature data, and A. de la Linde for help with maps. This study benefited from a BFU2008-00227/BMC grant by the Spanish Ministry of Science and Innovation.

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