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Diet of *Haplognathia ruberrima* (Gnathostomulida) in a Caribbean marine mangrove

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Abstract

Haplognathia ruberrima is a cosmopolitan gnathostomulid species found in sulfur bacterial mats in mangroves in Guadeloupe (French West Indies). *Haplognathia ruberrima* presents a $\delta^{13}C$ value lower than all measured meiofaunal grazers and lower than the available measured food sources of this environment. This low $\delta^{13}C$ value can not be due to specific ingestion of ^{13}C-depleted methanogenic bacteria because abundances of those bacteria are reduced in surficial and deep sediments as revealed by $\delta^{13}C$ of bacterial fatty acid. According to scanning electron microscope observations, no bacterial ectosymbionts were observed at the surface of the gnathostomulids, and transmission electron microscope views revealed the absence of bacterial endosymbionts. Energy-dispersive X-ray spectroscopy analysis detected low levels of sulfur (0.32%±0.8) in biological tissues of *H. ruberrima*, confirming the absence of thioautotrophic bacterial symbionts in these animals. Consequently, the low $\delta^{13}C$ value of *H. ruberrima* can not be due to the presence of sulfur-oxidizing symbionts but more probably to the selective and exclusive consumption of free-living, sulfur-oxidizing bacteria.

Introduction

Gnathostomulida are a group of non-segmented marine meiobenthic acoelomates. Distributed worldwide, they are known predominantly from the North Atlantic, Mediterranean, South Pacific and the Caribbean (Sterrer 1998). They move by gliding through the small interstices of sand grains by means of long cilia (Tyler & Hooge 2001). Usually located in sediments with high organic matter content (Sterrer 1971), their maximum abundances are reported at the discontinuity layer between aerobic surface and anaerobic subsurface sediment (Riedl 1969). They are common in sulfur environments (Fenchel & Riedl 1970; Powell *et al.* 1983) and their association with polychaete burrows has been linked to the activity of sulfur bacteria (Reise 1981).

This worm group was first described as aberrant flatworms (Ax 1956) and then raised to phylum rank (Riedl 1969). In the 10 years following the first description, a limited number of species was described but the taxonomic diversity greatly increased from the mid-1960s to the late 1970s and in the late 1990s about 100 species of this phylum are known (Sterrer 1998). The phylogenetic position of Gnathostomulida has been highly debated over time from both morphological and molecular perspectives (Sterrer *et al.* 1985; Littlewood *et al.* 1998), although the monophyly of the group has never been questioned (Giribet *et al.* 2000). Archaic characteristics of Gnathostomulida make them potentially useful for understanding early protostome evolution.

Gnathostomulida are vermiform animals with hermaphroditic reproduction. The phylum presents two

main characteristics: a monolayered epithelium with monociliated cells (Riedl 1969; Lammert 1991) and a bilaterally symmetric muscular pharynx usually containing complex cuticular mouth-parts. The mouth is ventral and the alimentary canal leads through the muscular pharynx to the blind gut (Knauss 1979). Jaws and the basal plate are particularly useful in gnathostomulid taxonomy as these structures preserve well and do not change in size or shape with body growth or sexual maturity. For instance, they provided new ultrastructural support for a homology between gnathostomulid and rotifera (Rieger & Tyler 1995).

Only scant data on feeding in Gnathostomulida are available; however, scraping off microorganisms from sediment grains by means of the cuticular mouth-parts is the most probable way of feeding (Sterrer 1971). Distinct prey items in the gut are uncommon but some bacteria, cyanobacteria and fungal hyphae have been observed (Riedl 1969; Kristensen & Nørrevang 1977, 1978). The spatial distribution of Gnathostomulida in sediment has been linked to areas with highest microbial production, suggesting an important trophic role of bacteria (Powell & Bright 1981; Reise 1981). By contrast, the sulfur environment is known to support a significant number of often mouthless meiofauna taxa, such as Nematoda (Ott *et al.* 2004), Turbellaria-Catenulida (Leisch *et al.* 2011) and Oligochaeta (Giere 1996).

Haplognathia ruberrima (Sterrer, 1966) is a bright red, relatively large (>3 mm), slender and cosmopolitan gnathostomulid worm species that has been reported from the North Atlantic and Caribbean, South Pacific and Mediterranean (Sterrer 1998). It has also been found in sulfidic mangrove sediment in Guadeloupe in the French West Indies (FWI).

The aim of the present study was to explain the carbon and nitrogen isotopic composition of *H. ruberrima* (Sterrer, 1966) in Guadeloupe mangrove. First, available food items (bacteria, diatom, mangrove leaf and particulate organic matter) were considered to see whether or not they explained isotopic composition of this species. Secondly, microscopic observations [transmission electron microscope (TEM), scanning electron microscope (SEM), energy-dispersive X-ray spectroscopy (EDXs) analysis used with envrionmental SEM (ESEM)] were used to ensure that the isotopic signature was not linked to the presence of bacterial associates.

Material and Methods

Study area

Manche-à-eau is a small tropical lagoon connected to the marine channel, Rivière Salée, separating the two main islands of Guadeloupe in the FWI (Fig. 1). In this lagoon, tides are semidiurnal, with a mean tidal amplitude of 30 cm (tide gauge of Pointe-à-Pitre, REFMAR^{TM}), and the seawater has an annual average temperature of 28 °C and salinity of 35‰.

It is bordered by mangrove forest dominated by *Rhizophora mangle*. Shallow sediment (>1 m water depth) located between roots of mangrove trees has high sulfide concentrations and anaerobic conditions characteristic of a sulfide system (Fenchel & Riedl 1970). In some areas, this shaded and muddy sediment is covered by a patchy white mat of bacteria (*Beggiatoa* spp.) associated with interstitial organisms such as gnathostomulids, nematodes and turbellarians. These colonies of bacteria are organized in large filaments (20−60 μm diameter) visible with the naked eye.

Samples were taken by snorkeling. Samples were collected from the surficial (1 mm) layer of sediment containing the bacterial mat using a 50-ml syringe. Back in the laboratory, these sediment samples were allowed to settle for a few minutes in a large Petri glass dish in order to permit the formation of the bacterial mat of *Beggiatoa* spp. on the surface of the sediment as observed in the field. Diatoms, filamentous sulfur-oxidizing bacteria and *Haplognathia ruberrima* were individually picked up live

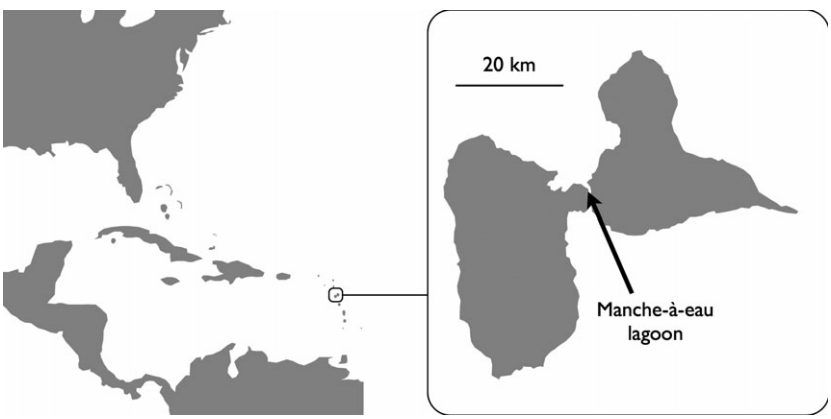


Fig. 1. Location of the Manche-à-eau lagoon in Guadeloupe (French West Indies).

using glass pipettes. Neither sieves nor narcotics were used.

Molecular analysis

DNA was extracted from *Haplognathia ruberrima* using a DNeasy Blood and tissue kit (Qiagen) according to the manufacturer's instructions. 18S rDNA was amplified using the primers 1F and 5R as previously described (Giribet *et al.* 1996). Touchdown PCR conditions were performed as follows: initial denaturation at 94 °C for 5 min, followed by 10 cycles at 94 °C for 1 min, 65 °C to 52 °C for 1 min and 72 °C for 1 min and by 25 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, then a final elongation step at 72 °C for 7 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced by Genoscreen (http://www.genoscreen.com).

The 18S rDNA gene sequences obtained (875 bp) were compared with the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) database using BLAST (Altschul *et al.* 1990). The best hits were included in the phylogenetic analyses. The phylogenetic analyses were performed using Geneious pro version 5.4.4 software (Biomatters; http://www.geneious.com). Sequences were aligned using multiple sequence comparison by log-expectation (Muscle) and alignments were checked manually. The phylogenetic tree was constructed from the multiple-aligned data using the neighbor-joining (NJ) method with Tamurai−Nei as genetic distance model. Node robustness was assessed by performing 1000 bootstrap replicates, and only bootstrap values above 50% are indicated at the nodes of the tree. *Glycera dibranchiata* was used as an outgroup. The sequence obtained in this study was deposited in the GenBank database under accession number KF438168.

Stable isotopes

For stable isotope analyses, freshly collected biological material was randomly selected, cleaned of debris, rinsed and then dried. A minimum of 60 specimens of *Haplognathia ruberrima* was pooled for each sample (n = 3). Microphytobenthos and bacteria *Beggiatoa* spp. were collected individually under a dissecting microscope and a minimum dried biomass of 250 µg was combined for each sample (n = 3). A similar protocol was used for the mangrove leaf samples (n = 3). Surficial sediment (1 cm) was collected with a syringe and analysed to determine the isotopic composition of sediment particulate organic matter (POM; n = 3). For stable isotope measurements of nitrogen (N), sediment was untreated whereas for carbon (C), sediment was previously acid treated using 1 mol · L^{-1} HCl in order to remove inorganic carbonates.

Samples were analysed at the Isotope Facility at the University of California, Davis, using an elemental-analyser isotope ratio mass spectrometer. Measured values were reported relative to the standards atmospheric N_2 and Vienna PeeDee Belemnite carbon. Stable isotope values are reported in δ notation: $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 10^3$ where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively.

The surficial (1 cm) layer of sediment was collected with a syringe and deeper sediment located 15−25 cm below the surface was sampled using a sediment corer (diameter = 4 cm). This sediment was freeze-dried and phospholipid-derived fatty acids (PLFAs) were extracted and their isotopic compositions determined (n = 3) using a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c-IRMS) as in Boschker *et al.* (1999). Concentrations and $\delta^{13}C$ of PLFA specific to bacteria (i14:0, i15:0, a15:0, 18:1ω7c) and algae (20:5ω3) were used to estimate their weighted-average $\delta^{13}C$ composition.

Ultrastructural analyses

The samples collected were observed and photographed with a stereomicroscope before preparation for SEM and TEM observations in order to detect possible symbioses. Samples for SEM observations were fixed for 2 h at 4 °C in 2% glutaraldehyde solution in cacodylate buffer (900 milliosmoles, pH 7.2). They were then dehydrated in graded concentrations of acetone, critical point-dried in CO_2 and sputter-coated with gold before observation with a Hitachi S 2500 at 20 kV. For TEM observations, four individuals were pre-fixed for 1 h at 4 °C in 2.5% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer adjusted to 900 mOsm. After a brief rinse in the same buffer, they were fixed for 45 min at room temperature (RT) in 1% osmium tetroxide in the same buffer with the final osmolarity adjusted to 1000 mOsm, then rinsed in distilled water and post-fixed with 2% aqueous uranyl acetate for one more hour at RT before dehydration using graded concentrations of acetone, embedding in epon-araldite resin and observation in a Leo 912 TEM at 120 Kv. Five grids (from the head to the tail) containing four to five ultra-thin sections were observed per individual; the corresponding semi-thin sections were also examined for overview analyses. The TEM micrographs presented in this study are representative of all individuals examined.

Energy-dispersive X-ray spectroscopy analysis

In order to detect elemental compounds (such as sulfur or iron) from individuals, freshly fixed samples (in 2%

glutaraldehyde solution in sea water) were observed using an environmental scanning electronic microscope (FEI Quanta 250) operating from 1 to 20 kV under an environmental pressure of 7 Torrs at 5 °C. EDX spectra were obtained using a M-max 500 mm^2 Oxford detector.

Two individuals were divided into two parts under a dissecting microscope; one part was used for SEM analysis and the second for TEM analysis as described above in order to combine the two analyses from the same individual.

Results and Discussion

Molecular and morphological descriptions

Studied organisms were morphologically identified as *Haplognathia ruberrima* (Sterrer 1966). A phylogenetic tree based on partial 18S rDNA sequences (875 bp) showed that this metazoan forms a distinct clade with the closest relative *Haplognathia rosea* with 97.6% pairwise identity and *H. ruberrima* (Bermuda) with 98.4% pairwise identity (Fig. 2). The sister-group relationship between *H. ruberrima* (Bermuda) and *H. ruberrima* (Guadeloupe FWI) was supported by a robust branch of the phylogenetic tree (98% bootstrap support from 1000 replicates), which suggests that these populations are genetically different. Moreover, they inhabit different types of sediments (sand *versus* mud, respectively), supporting the hypothesis that they could form genetically distinct *Haplognathia* 'types'. They would form a complex of cryptic species as commonly observed in the marine environment (Knowlton 1993, 2000; Barroso *et al.* 2010), and as suggested by the wide range of features (such as jaw size and shape) throughout the global distribution of this morpho-species (Sterrer 1998). As a result, the feeding ecology of *H. ruberrima* described in the present study may be specific to this population and therefore should be generalized to other populations of this species with caution.

Methodological considerations

The direct observation of foraging behavior is a traditional method for assessing the diets of organisms. Sediment is an obscure habitat; moreover, the small size of meiofaunal benthic grazers makes direct observation particularly difficult. Such visual observation requires experimental conditions that are oversimplified compared with real natural complexity. Experimental environments are usually free of sediment to allow observation, and the number of food items available is often limited. These biases are potentially important for the determination of

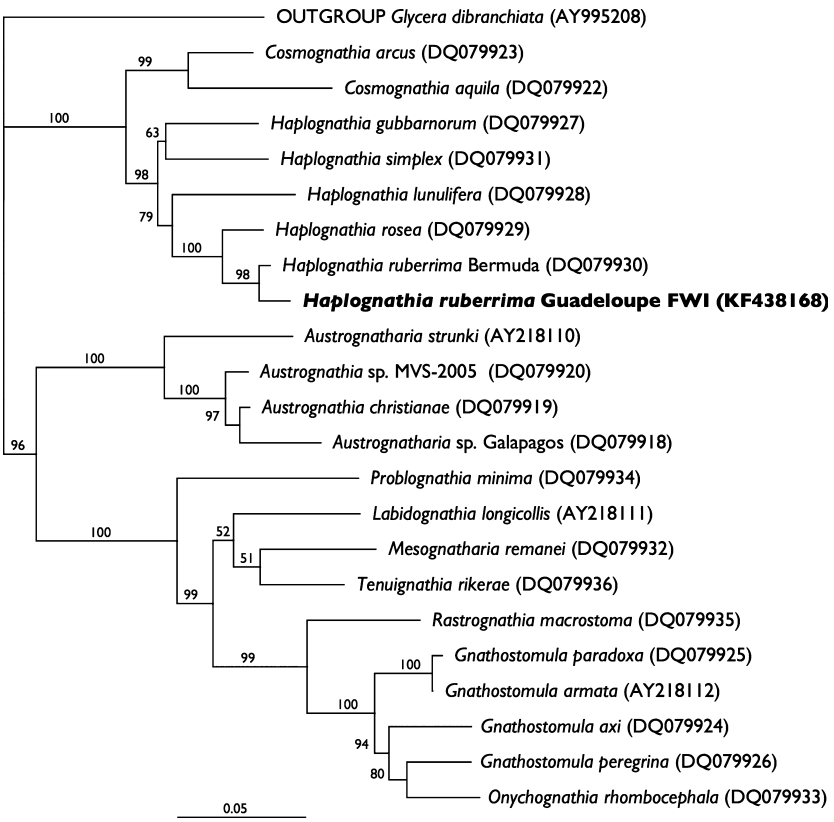


Fig. 2. Neighbor-joining tree displaying the phylogenetic relationships between *Haplognathia ruberrima* Guadeloupe, French West Indies (in bold), and other Gnathostomulida based on the analysis of 18S rDNA gene sequences of 875 nucleotides. *Glycera dibranchiata* was used as the outgroup. Only bootstrap values higher than 50% are shown at each node. The scale bar corresponds to 0.05 changes per nucleotide.

diet composition, as many meiofaunal organisms are opportunistic feeders that change their feeding behavior according to the accessibility of food (Moens & Vincx 1997). Another classic method is the observation of the gut content of a grazer in order to determine the composition of recently ingested food. Gnathostomulida gut-content contained bacteria, cyanobacteria and fungal hyphae (Sterrer 1971), but this method does not seem adequate because a majority of observed items are unrecognizable (Riedl 1969; Sterrer 1971). Food disintegration may occur due to the action of sharp teeth during the ingestion process (Sterrer & Farris 1975). Gut content method presents another potential bias as the retention times and digestibility of food items differ (Afik & Karasov 1995); moreover, ingested material is not necessarily assimilated.

The use of multiple natural abundance of stable isotopes has become an important tool in investigating trophic interactions as it allows the reconstruction of diets and evaluation of the relative importance of each food item to the consumer (Fry 2006). Carbon and nitrogen isotope ratios of an organism resemble its ingested food, with slight enrichment of heavier isotopes (^{13}C, ^{15}N) as the lighter isotopes (^{12}C, ^{14}N) are preferentially used in metabolism (DeNiro & Epstein 1981). Compared with traditional methods, stable isotope analysis presents certain advantages as it provides information on assimilated food and on the long-term diet of organisms.

Membrane lipids such as PLFAs can be used as biomarkers as their compositions can be specific to different groups of microorganisms (*e.g.* bacteria and algae) and because they are representative of living material due to their rapid degradation after the death of the organism (Boschker & Middelburg 2002). Stable isotope ratios of those specific PLFAs can be analysed using GC-c-IRMS. This method was used to determine the isotopic compositions of PLFAs specific to potential food items in surficial (1 cm) and deeper (15–25 cm) sediment.

Stable isotope approach

Diatoms manually extracted from the sediments had a $\delta^{13}C$ value ($-23.5 \pm 0.1‰$) similar to values observed in PLFAs specific to algae ($-21.5 \pm 6.2‰$) and slightly more depleted than in other mangrove studies (-22.1 to $-17.3‰$ in Lee 2000; Bouillon *et al.* 2002; Bouillon *et al.* 2004) . Mineralization of mangrove detritus generates inorganic carbon depleted in ^{13}C (Hemminga *et al.* 1994; France 1998). In the studied mangrove, benthic microalgae would be expected to have a low $\delta^{13}C$ as the amount of organic matter is particularly elevated in areas influenced by marine water (Lallier-Vergès *et al.* 1998). Bulk sediment had a $\delta^{13}C$ value ($-24.3 \pm 0.3‰$) similar to values found in other environments (Lee 2000; Bouillon *et al.* 2002, 2004) and that was less depleted than in mangrove leaves ($-30.3 \pm 0.5‰$). Such a discrepancy is commonly observed in mangrove (Rodelli *et al.* 1984; Stoner & Zimmerman 1988; Hemminga *et al.* 1994; Dittel *et al.* 1997; Bouillon *et al.* 2002) and was previously observed locally in Guadeloupe (Lallier-Vergès *et al.* 1998). Imported suspended POM was not considered as an available food source here because the studied mangrove is stagnant due to low incoming tidal velocities (Lallier-Vergès *et al.* 1998). To our knowledge, *Beggiatoa* spp. have never been studied in mangrove before but their carbon isotopic composition ($\delta^{13}C$: $-31.7 \pm 0.9‰$) is similar to values reported for bacteria in environments associated with gas hydrate (Zhang *et al.* 2005) and hydrocarbon vents (Aharon *et al.* 1992; Larkin *et al.* 1994). *Beggiatoa* spp. are sulfur-oxidizing chemoautotrophs with a typically high degree of isotope fractionation relative to the carbon source of ambient seawater (Larkin *et al.* 1994). In surface sediment, *Beggiatoa* spp. had the lowest $\delta^{13}C$ value of all food sources taken into account (Fig. 3).

Methanotrophic bacteria usually have $\delta^{13}C$ fluctuating between -50 and $-110‰$ (Peterson & Fry 1987; Boschker

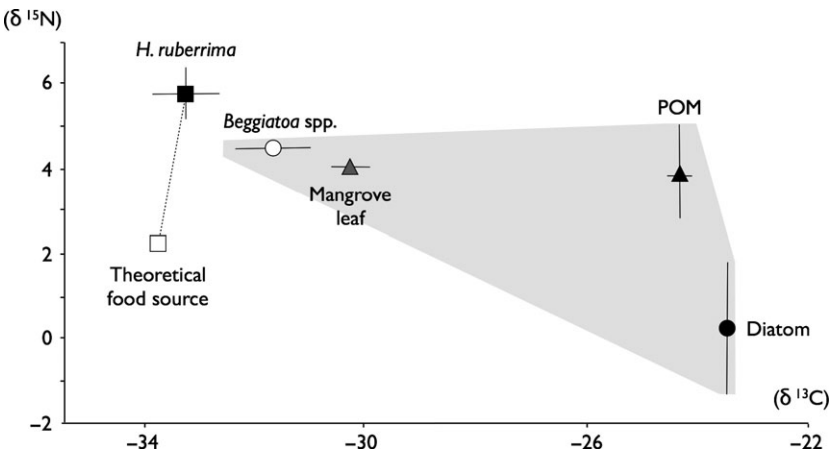


Fig. 3. Isotopic composition of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) of *Haplognathia ruberrima* and its potential food sources: *Beggiatoa* spp., mangrove leaf, particulate organic matter (POM) and benthic diatoms (mean±SD; n = 3). The signature of the available food is represented by a grey polygon. The open box represents the theoretical isotopic composition of the food sources of *H. ruberrima* taking into account trophic enrichments of 0.5 for $\delta^{13}C$ (Fry 2006) and 3.4 for $\delta^{15}N$ (Minagawa & Wada 1984).

& Middelburg 2002) due to high ^{13}C depletion of methane (Milkov 2005) and low carbon fractionation (Alperin *et al.* 1988). Bacteria located under mats of *Beggiatoa* are potential producers of methane (Fenchel & Bernard 1995). Methanogenesis can be important in anthropogenically impacted mangrove (Giani *et al.* 1996) but is generally thought to be limited in pristine mangrove (Alongi *et al.* 2000, 2001). In deeper layers of sediment, fatty acids specific to bacteria were more depleted in ^{13}C, reaching values of $-34.5 \pm 1.3‰$ mainly due to i15:0 and a15:0 (Table 1), which are PLFAs not found in methanotrophs (Murase *et al.* 2011). This limited bacterial depletion suggests a reduced abundance of methanogenic bacteria in deep layers of sediment (15–20 cm). Methanotrophy is also limited in more oxygenated superficial sediment, as this type of metabolism is specific to anaerobic environments. In living environment of *Haplognathia ruberrima*, bacteria dwelling in deeper layers of sediment were the only food sources with isotopic compositions more depleted in ^{13}C than *Beggiatoa* spp.

The studied grazer *H. ruberrima* ($-33.3 \pm 0.9‰$) was more depleted in ^{13}C than all the food sources available in surficial sediment. The classical application for stable isotope analysis in ecology is to study the food resources used by an organism based on the well-established principle 'you are what you eat'. A fractionation of $0.5‰$ between a consumer and his food $\delta^{13}C$ (Fry 2006) implies that food of *H. ruberrima* presents a $\delta^{13}C$ of $-33.8‰$ (Fig. 3). Therefore, most of the food items studied here (diatoms, POM and fungi) do not constitute a dominant part of the diet of *H. ruberrima*. Fungal hyphae are one of the rare distinct structures previously observed in gnathostomulid guts (Riedl 1969; Kristensen & Nørrevang 1977, 1978). The present study suggests that the diet of *H. ruberrima* consists principally of bacteria: *Beggiatoa* spp. from the surficial sediment and/or bacteria from the deeper layers. In order to show a depleted $\delta^{13}C$ value, *H. ruberrima* must be highly selective and exclusive in its ingestion of bacteria. *Haplognathia ruberrima* had the lowest $\delta^{13}C$ value of all of the meiofaunal grazers studied in the area: nematode, copepod, turbellarian, rotiferan (Pascal *et al.* 2014) and consequently appears to be the only local grazer with this specialized diet. Gnathostomulid abundances are higher at the discontinuity layer between the aerobic surface and the anaerobic sediment and the redox potential discontinuity layer is a site of high bacterial activity involving sulfide oxidation and sulfate reduction (Riedl 1969; Reise 1981). The spatial distribution of gnathostomulids associated with the highest microbial production could be explained by the use of bacteria as food resource (Powell & Bright 1981; Reise 1981; Powell *et al.* 1983). Methods used in the present study confirm this trophic role.

Table 1. $\delta^{13}C$ values of phospholipid-derived fatty acids (PLFAs) specific to bacteria in surficial (1 cm) and deep (15–25 cm) sediment (mean ± SD).

Organism	PLFA	Surficial sediment	Deep sediment
n (replicates)		25	3
Bacteria	i14:0	−29.6 ± 1.8	n/a
	i15:0	−27.8 ± 1.3	−34.5 ± 1.3
	a15:0	−26.2 ± 1.0	−32.5 ± 3.0
	18:1ω7c	−29.9 ± 1.7	−30.2 ± 1.1
Algae	20:5ω3	−21.5 ± 6.2	n/a

n/a, not available.

Sulfur-oxidizing chemoautotrophic bacteria discriminate heavy carbon isotopes to a greater degree than other bacteria (Ruby *et al.* 1987). In symbiotic relationships, the host uses carbon from its symbionts either through symbiotic metabolic pathways, such as the Calvin–Benson cycle that transfers carbonated metabolites to the host cells, or directly from the lyosomal digestion of the symbionts by the host (Dubilier *et al.* 2008). Marine invertebrates known to possess sulfur-oxidizing symbionts that use sulfides to fix CO_2 usually have low $\delta^{13}C$ values of between -39.8 and $-23.4‰$ (Berg & Alato 1984; Spiro *et al.* 1986; Southward *et al.* 1994; Dubilier *et al.* 2008). As a result, one explanation of the low $\delta^{13}C$ observed in *H. ruberrima* could be the existence of chemoautotrophic symbiotic bacteria.

In order to validate this hypothesis, individuals were analysed at the ultrastructural level. No bacterial ectosymbionts could be observed on the surface of the gnathostomulids analysed in the SEM images. Usually, ectosymbionts form a coat around the body of the colonized host, as described in several marine invertebrates inhabiting sulfur environments (Laurent *et al.* 2009; Maurin *et al.* 2010; Muller *et al.* 2010). Previous investigations conducted in marine mangrove environments have shown the presence of a specific meiofauna (sessile or vagile) living in association with sulfur-oxidizing bacterial symbionts and covering their entire body, such as the ciliate *Zoothamnium niveum* (Laurent *et al.* 2009; Maurin *et al.* 2010), vorticellid (Laurent *et al.* 2009) and the nematode *Eubostrichus dianae* (Himmel *et al.* 2009). These thioautotrophic bacteria oxidize the sulfides emitted from the anoxic sediment, resulting in the detoxification of this environment for its invertebrate host species. Such symbiotic organisms can thus colonize an environment that remains toxic (due to the high levels of sulfides) for most of the metazoans present. In our case, no bacterial symbionts were detected either by SEM (Fig. 4) or by Fluorescent in situ hybridization using eubacterial universal probes (data not shown). The long filaments

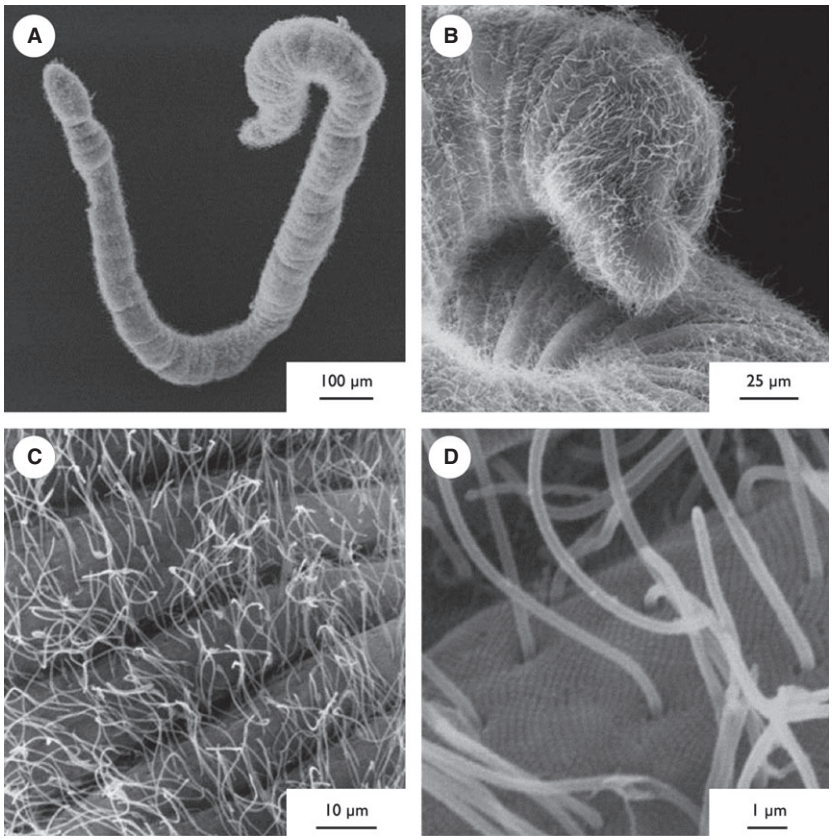


Fig. 4. Scanning electron microscope analysis of *Haplognathia ruberrima* individuals freshly collected from marine mangrove. (A): In low magnification, the entire body surface of the individuals analysed presented this general aspect. (B and C): The general aspect is due to the presence of numerous long filaments individually attached to the body surface of the animal. (D): Higher magnification reveals these filaments to be long cilia that are likely responsible for the gliding movement observed in *H. ruberrima* individuals.

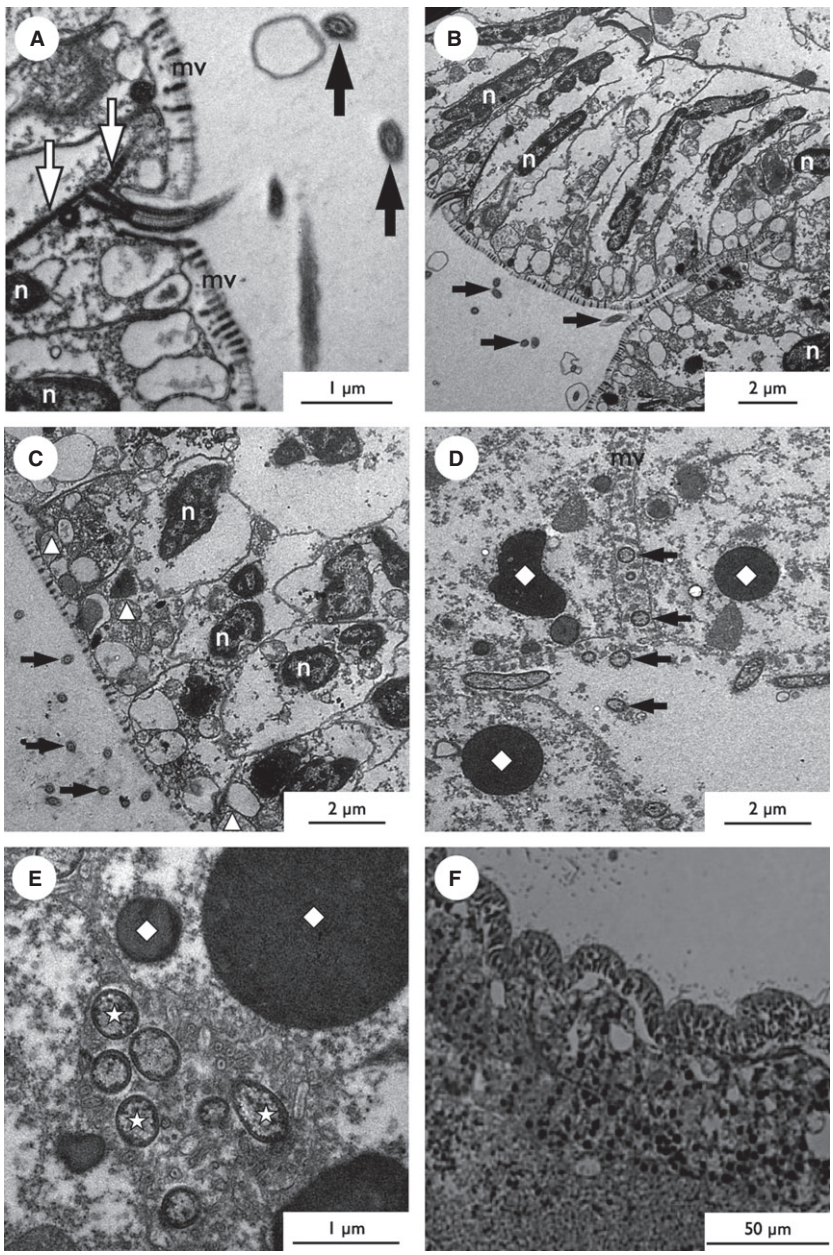
observed covering the whole organism are in fact eukaryotic cilia as shown by thin sections observed with TEM (Fig. 5). These cilia permit the gliding movements through the sediment of this organism, as already described (Tyler & Hooge 2001). Between these long cilia, microvilli from the cell membrane were apparent, demonstrating the lack of a cuticle in these organisms.

Some marine invertebrates inhabiting the sulfur sediment of marine mangroves have also been described as harboring intra-cellular sulfur-oxidizing bacteria, such as the lucinid bivalve *Lucina pectinata* (Frenkiel *et al.* 1996), which lives in the present study area but deeply burrowed in the sediment (*c.* 50 cm depth). According to the TEM views, there were no bacterial endosymbionts in the gna-

Fig. 5. Ultrastructural analyses of *Haplognathia ruberrima* individuals freshly collected from marine mangrove (A–E: Transmission electron micrographs; F: Light micrograph). (A): Thin section across the long cilia that cover the entire body surface of *H. ruberrima* individuals. Few transverse sections of cilia (black arrows) are observed in this micrograph while the basal body is deeply anchored into the cytoplasm (white arrows) by an anterior and a posterior rootlet. The axonemal cytoskeleton appears to be typical of eukaryotic cilia, with two central microtubule singlets in addition to the outer doublets. (B): Low magnification image of the periphery of the epidermis characterized by long, thin and apparently empty cells. These cells harbor thin nuclei, which are mostly located in the center of the cell. The apical pole of such cells is characterized by numerous heterogeneous inclusions in contact with the cytoplasmic membrane. The arrows indicate transverse sections of cilia that cover the body of the animal. (C): Higher magnification image focusing on the heterogeneous inclusions (white triangles) located at the apical pole of the cells constituting the body wall of *H. ruberrima* individuals. These envacuolated inclusions are not intra-cellular bacteria due to the lack of a double membrane typical of Gram-negative bacteria. Moreover, they do not look like lysosomal residual bodies. Their function remains unknown. The arrows indicate transverse sections of cilia that cover the body of the animal. (D): Only a few bacteria (arrows) were observed outside the eukaryotic tissue in the lumen of the digestive tract or between microvilli of two adjacent cells from the intestinal and/or stomach epithelium. The differences in the shapes and sizes of the bacteria observed are probably due to the section plan. Diamonds indicate pigment granules inside the cells as observed from the semi-thin section (see F). (E): The bacteria (asterisks) encountered in the lumen of the digestive tract probably correspond to environmental bacteria ingested by the animal. They do not have sulfur granules, which are usually observed in the periplasm of sulfur-oxidizing bacteria. Diamonds indicate pigment granules located inside the cells of the digestive epithelium. (F): Semi-thin transverse section of an entire individual showing the body wall covered by cilia. Pigment granules located inside the epidermis are smaller than those located inside cells of the digestive tract. mv, microvilli; n, nucleus.

thostomulids analysed in this study. The rare bacteria observed were located in the lumen of the digestive tract (probably ingested as food by the animal) and do not have the characteristics of sulfur-oxidizing bacterial symbionts, lacking periplasmic sulfur granules for instance (Vetter 1985; Lechaire *et al.* 2008; Maurin *et al.* 2010). A lack of identifiable *Beggiatoa* spp. in the gut of *H. ruberrima* does not necessarily imply an absence of consumption as these chain-like bacteria could have been destroyed during ingestion by the action of sharp teeth (Sterrer & Farris 1975). Present study reveals that individuals of *H. ruberrima* isolated from the marine mangrove environment in Guadeloupe do not live in association with sulfur-oxidizing bacteria either as ectosymbionts or as endosymbionts.

Energy-dispersive X-ray spectroscopy analysis performed at a low voltage (5 kV) did not detect the presence of iron in the cells of gnathostomulid individuals (Fig. 6). In our study, the EDXs analysis was performed using an environmental SEM, which allowed the observation of fully hydrated biological samples, and so elemental sulfur was not dissolved during the prepara-



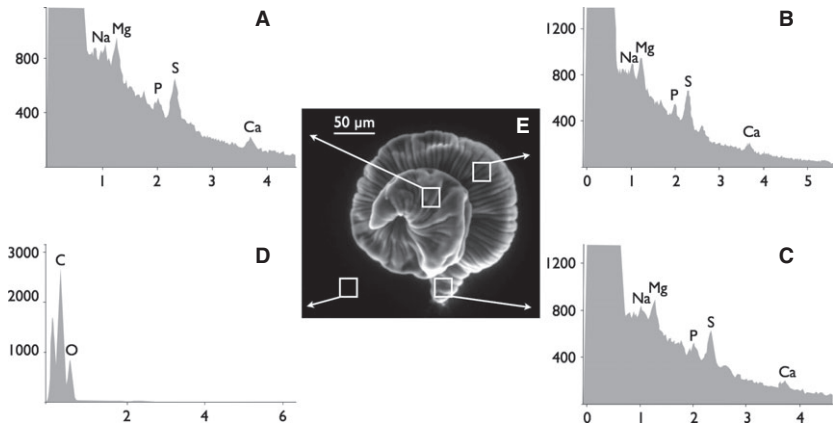


Fig. 6. Energy-dispersive X-ray spectroscopy (EDXs) analysis of a freshly collected individual of *Haplognathia ruberrima* from marine mangrove using an environmental scanning electron microscope (A–D: EDXs spectrum, E: ESEM view of the individual analysed). Elemental sodium, magnesium, phosphorous, sulfur and calcium were detected from various locations within the *H. ruberrima* individual (A–C) while only carbon and oxygen were detected from the specimen holder [used as negative control (D)]. Thus, this gnathostomulid individual does not contain either iron or sulfur in high concentrations, rejecting the possibility of circulating iron-rich hemoglobin or the presence of sulfur-oxidizing bacterial endosymbionts.

tion process of the samples. Sulfur content appeared to be very low at the surface of the tissue analysed at low voltage (from 1 to 5 kV). When the same area was analysed at higher voltage (20 to 25 kV), with deeper penetration and spreading of the electron beam into the specimen, no more sulfur content measured was not higher even if metals from the specimen holder were detected, *i.e.* iron, nickel and chromium. These data suggest that the electron beam has completely crossed the body of the individual, without a high concentration of elemental sulfur detected. Moreover, we used as a positive control the marine colonial ciliate *Zoothamnium niveum*, known to harbor sulfur-oxidizing bacterial symbionts. The spectrum obtained for this species under similar conditions of voltage and pressure showed a large peak of sulfur (with more than 8000 counts detected) compared with the small peak obtained with *H. ruberrima* individuals (around 800 counts detected). EDXs analysis of *H. ruberrima* revealed a low level of sulfur ($0.32 \pm 0.8\%$) throughout the animal, which probably corresponded either to sulfur associated with sulfur-rich proteins or to thioautotrophic free-living bacteria consumed as food by the gnathostomulids. Usually, in thioautotrophic bacteria living either as free-living bacteria in sulfur environments or as symbionts in association with marine invertebrates, sulfur levels are higher (up to 12% of the atomic weight, O. Gros, personal observations). Thus, the lack of detection of high sulfur levels by EDXs analysis from freshly collected individuals of *H. ruberrima* confirmed the absence of thioautotrophic bacterial symbionts in these animals. Moreover, individuals that were divided into two parts to allow a dual analysis (TEM and EDXs) from the same individual confirmed this observation by the absence of bacterial detection with TEM and the limited levels of sulfur detected through the body.

The low $\delta^{13}C$ value measured for *H. ruberrima* can therefore not be due to the presence of sulfur-oxidizing symbionts but more probably results from the consumption of free-living sulfur-oxidizing bacteria.

Conclusions

The SEM and TEM observations performed here both confirmed the lack of ectosymbiotic and endosymbiotic bacteria in *Haplognathia ruberrima*. Furthermore, the EDXs analysis showed that sulfur, which is specific to this type of symbiosis, had limited abundance. The low $\delta^{13}C$ value of *H. ruberrima* can consequently be explained entirely by diet. Those results indicate that *H. ruberrima* would be highly selective during the feeding process and would ingest principally chemoautotrophic sulfur-oxidizing bacteria. The specific diet of anoxic bacteria suggests that *H. ruberrima* originated in, and/or survived an ocean depleted in oxygen, and thus constitutes a primitive acoelomate.

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