Ultrastructure and stable carbon isotope composition of the hydrothermal vent mussels *Bathymodiolus brevior* and *B.* sp. affinis *brevior* from the North Fiji Basin, western Pacific

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ABSTRACT: In hydrothermal vents of the western Pacific several mytilid bivalve species of the genus *Bathymodiolus* attain a dominating ecological role. The ultrastructure of the symbiosis in the vent mytilid *B. brevior* as well as in an undescribed species *B.* sp. affinis *brevior* from a deep-sea hydrothermal vent site in the North Fiji Basin of the southwestern Pacific was studied. The abundance of symbionts was strikingly different in the 2 species, with *B. brevior* gills housing low numbers of bacteria that occurred singly in membrane-bound vacuoles, while *B.* sp. aff. *brevior* gills contained numerous symbionts with up to 12 bacteria per vacuole. Bacterial vacuoles at the outermost region of the bacteriocytes occasionally (*B. brevior*) or regularly (*B.* sp. aff. *brevior*) showed an open connection to the exterior, ambient seawater. In both species only a single symbiont morphotype without intracytoplasmatic membranes occurred, suggesting that these mytilids harbor only chemoautotrophic, sulfur-oxidizing bacteria. Stable carbon isotope ratios provided further information on the trophic interactions between the endosymbionts and their hosts. The $\delta^{13}C$ values of *B. brevior* gill tissues ranged from −30.8 to −35.8‰ and were significantly more negative than in non-symbiotic tissues, indicating that the symbionts may not account for the sole source of dietary carbon in these associations.

KEY WORDS: Symbiosis · Mytilid · Chemoautotrophic bacteria

INTRODUCTION

The mytilid bivalve *Bathymodiolus thermophilus* Kenk & Wilson (1985) was first discovered in 1977 at deep-sea hydrothermal vents in the Galapagos Rift Zone of the eastern Pacific (Corliss & Ballard 1977, Corliss et al. 1979). Since then many new *Bathymodiolus* species from cold-water methane/sulfide seeps and hydrothermal vents in the Atlantic and Pacific oceans have been described (Cosel & Métivier 1994, Hashimoto & Okutani 1994, Craddock et al. 1995). The genus *Bathymodiolus* is characterized by a considerable flexibility in its sources of nutrition. All members of the genus contain intracellular symbiotic bacteria in their large, fleshy gills (reviewed in Fisher 1990, Nelson & Fisher 1995). In some species these symbionts are thioautotrophic (i.e. sulfur-oxidizing, CO_2-fixing), in some methanotrophic (i.e. methane-utilizing) and in others both thioautotrophic and methanotrophic bacteria can co-occur (Fisher et al. 1993, Distel et al. 1995). In addition to their ability to gain nutrition from their symbionts, all *Bathymodiolus* species have retained a gut and at least 2 species are able to filter feed and assimilate organic material from the ingested food (Page et al. 1990, 1991, Le Pennec et al. 1995).

While *Bathymodiolus* species from the eastern Pacific have been studied intensively (see reviews cited above), little is known of the biology of symbiotic mytilids from the western Pacific. Beyond general descriptions of hydrothermal vent communities in the southwestern Pacific (Desbruyères et al. 1994) and some stable isotope data on unidentified mytilid species of the Mariana Trough (Van Dover & Fry 1989) and Okinawa Trough (Kim & Sakai 1991), only a few stud-

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ies have examined the symbioses of western Pacific *Bathymodiolus* species in more detail (Pranal et al. 1995, 1997). Until recently, investigations were hampered by a lack of information on the taxonomy of hydrothermal vent mytilids from the western Pacific. Two *Bathymodiolus* species from hydrothermal vents in the Lau and North Fiji Basin in the southwestern Pacific have now been identified, *B. brevior* and *B. elongatus* (von Cosel & Métivier 1994), which can co-occur at some sites. In this paper we describe the ultrastructure of the symbiosis in the vent mytilid *B. brevior* as well as in an undescribed species *Bathymodiolus* sp. affinis *brevior* (R. von Cosel pers. comm.) from a hydrothermal vent site in the North Fiji Basin. Stable carbon isotope ratios of *B. brevior* provide further information on the trophic interactions between the endosymbionts and their hosts.

MATERIALS AND METHODS

Specimen collection. The mytilid bivalve *Bathymodiolus brevior* was collected in the North Fiji Back Arc Basin, southwest Pacific, from the LHOS hydrothermal vent site (16° 59.65' S, 173° 54.73' E) during the 'Sonne 99' cruise in January 1995 (Auzende et al. 1995, Halbach et al. 1995). Based on photographic surveys with a video-controlled deep-towed sled, the LHOS site is a composite vent field of 200 × 200 m with 5 more or less active vents and at least 4 wide patches of diffuse venting along an open fissure. A TV-controlled grab (GTV) lowered from the ship to 2000 m water depth was used for specimen collection. The specimens described in this study were retrieved from 4 grabs (GTV Stns 91, 92, 98, and 115) that were all taken within approx. 50 to 100 m of each other. The number and diversity of hydrothermal vent species recovered in these grabs was low at Stns 91 (2 *B. brevior*, 1 *B.* sp. aff. *brevior*) and 92 (1 *B.* sp. aff. *brevior* and 3 *Thyasira* sp.), intermediate at Stn 98 (38 *B. brevior*) and highest at Stn 115 [40 *B. brevior*, at least 8 *Ifremeria nautilei* (a gastropod with symbiotic bacteria in its gills, Windoffer & Giere 1997), and 1 unidentified pogonophore] (Michael Türkay, Forschungsinstitut Senkenberg, Frankfurt, Germany, pers. comm.).

Species identification was provided by Rudo von Cosel (Muséum National d'Histoire Naturelle, Paris, France). *Bathymodiolus brevior* has been well described (von Cosel & Métivier 1994) while the characterization of *B.* sp. aff. *brevior* is preliminary and based on only 1 juvenile and 1 adult specimen (von Cosel pers. comm.). This possibly new species is characterized by a somewhat narrower shell than *B. brevior* (but much shorter and stouter than in *B. elongatus*). The ligament does not end as abruptly as in *B. brevior* and *B.*

elongatus. Voucher specimens have been deposited in the Mollusc Department of the Muséum National d'Histoire Naturelle.

Microscopy. For microscopic examinations on *Bathymodiolus* sp. aff. *brevior*, 1 juvenile from Stn 91 (with a shell length of 30 mm) and 1 adult from Stn 92 (with a shell length of 87 mm) were available. For *B. brevior*, 4 adults from Stn 98 (with shell lengths from 64 to 97 mm) were available. The gill filaments were dissected and fixed in 4 % paraformaldehyde and 3 % glutaraldehyde in 0.1 M cacodylate buffer with 15 % sucrose (pH 7.4). The tissues were rinsed in the same buffer, post-fixed in 1 % osmium tetroxide, and stored in the same buffer for up to several months before dehydration in acetone and embedment in SPURR (Spurr 1969). For light microscopy, semithin sections were stained with toluidine blue. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss EM 109-S2.

A quantitative analysis of bacterial abundance was not possible because of the limited number of specimens available for study. Instead, bacterial abundances in *Bathymodiolus brevior* and *B.* sp. aff. *brevior* were compared by counting the number of bacteria per host cell on representative electron micrographs of the mid-filament region of the gill.

Stable carbon isotope analysis. Only *Bathymodiolus brevior* specimens were available for carbon isotope analysis. Three adults from Stn 98 (with shell lengths from 84 to 89 mm) and 3 adults from Stn 115 (with shell lengths from 93 to 108 mm) were examined. At Stn 115 each mytilid carried 1 individual of the commensal polychaete *Branchypolynoe pettibonae* Miura & Hashimoto (1991) in its pallial cavity. The mytilids were frozen at –20°C immediately after collection, thawed in the laboratory and dissected into 3 fractions: gills, foot tissue, and the rest of the soft tissue. The symbiotic polychaetes (n = 3) were pooled and treated as 1 tissue. The tissues were overlaid with 0.1 N HCl for 24 h, rinsed in aqua dest. overnight, and dried at 60°C. Samples were combusted to carbon dioxide at 1050°C in a stream of oxygen using a Heraeus CHN analyzer attached to a Finnigan MAT Delta mass spectrometer with a reproducibility of less than 0.15‰. Results are expressed as parts per thousand in relation to the Pee Dee Belemnite (PDB) standard.

RESULTS

Gill structure

The general morphology of the *Bathymodiolus brevior* gill corresponded to previous descriptions of gills from other members of this genus (Fiala-Médioni et al.

1986, Fisher et al. 1987, 1993). The symbionts occurred in the gills which were composed of numerous filaments, with abundant ciliation at their frontal apex. The filaments were inter-connected by at least 7 or 8 rows of ciliary tufts on their lateral faces. The lateral faces of the filaments were composed of bacteriocytes, which contained the intracellular symbionts and sym-

biont-free intercalary cells interspersed irregularly between the bacteriocytes. In comparison to other mytilid symbioses, *B. brevior* bacteriocytes housed much lower abundances of symbionts, with 10 to 60 bacteria per host cell visible in cross-sections of the mid-filament region (Fig. 1A). The symbionts were found in the apical or surface region of the bacterio-
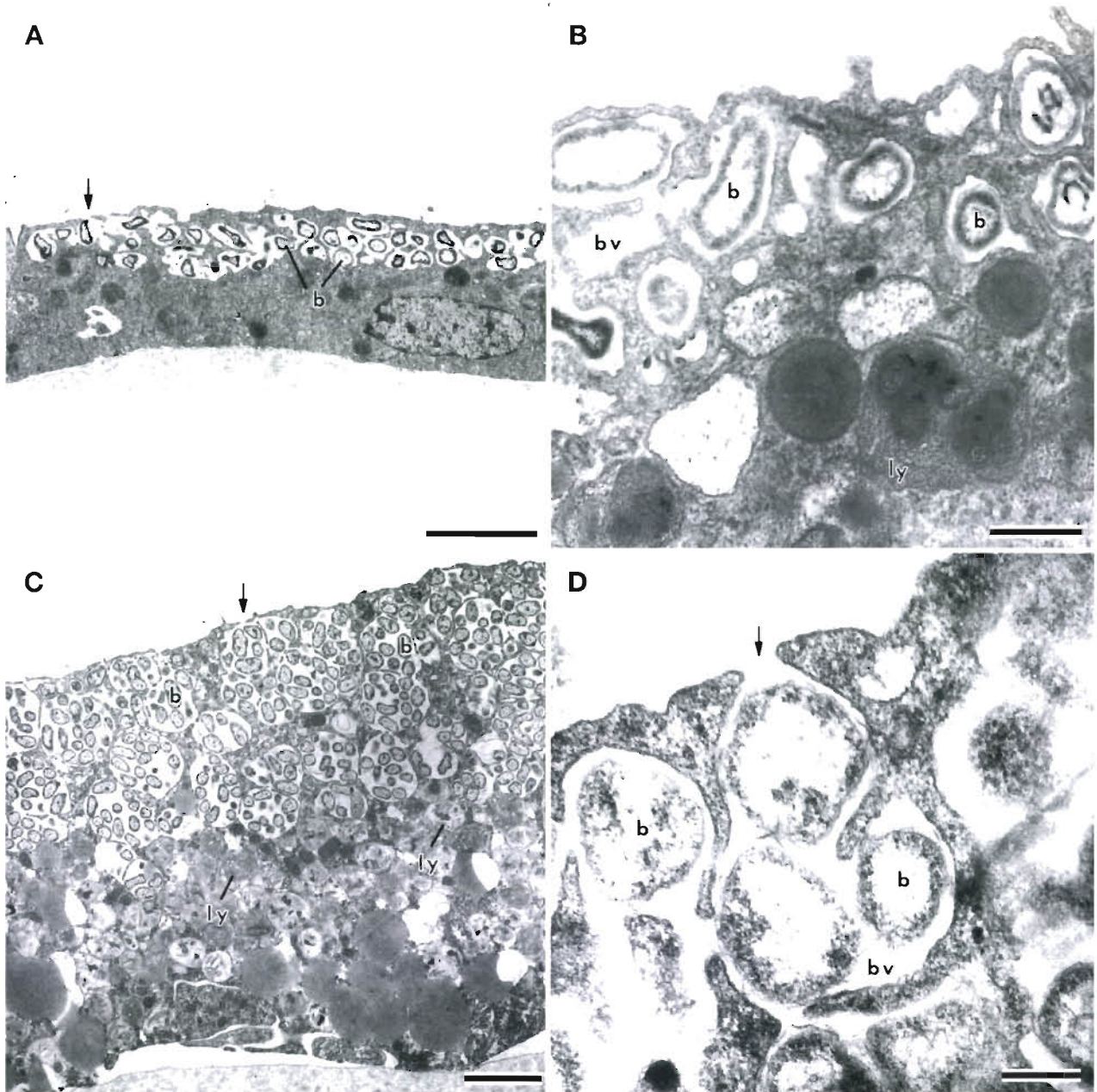

Fig. 1. (A, B) *Bathymodiolus brevior* and (C, D) *B* sp affinis *brevior* Gill tissues from the mid-filament region with symbiotic bacteria. *B. brevior* bacteriocytes are narrow with low symbiont abundances and bacteria are housed singly in membrane-bound vacuoles. *B.* sp. aff. *brevior* bacteriocytes are considerably broader with much higher numbers of symbionts, and vacuoles that contain up to 12 bacteria. Open connections of peripheral bacterial vacuoles to the exterior occur regularly (arrows in A, C, and D), and the host membrane is intact at these openings (D) b· bacteria, bv· bacterial vacuole. ly lysosome Scale bars (A) 2 μm, (B) 0.5 μm, (C) 2 μm, (D) 0 2 μm

cytes in vacuoles that were often interconnected, with usually only 1 symbiont per vacuole (Fig. 1B). Occasionally, symbiont vacuoles at the outermost region of the bacteriocytes showed an open connection to the exterior (Fig. 1A). The symbionts were small cocci or short rods ranging from 0.25 to 0.75 μm in diameter, with a cell wall typical of Gram-negative bacteria, and did not contain membranes or other distinctive structures in their cytoplasm. Towards the basal part of the bacteriocytes electron-dense, membrane-bound lysosomal structures were found that could contain numerous internal membranes (Fig. 1A, B).

While the gross morphology of the *Bathymodiolus brevior* and *B.* sp. aff. *brevior* gill was similar, the abundance of symbionts was dramatically different. The bacteriocytes in *B.* sp. aff. *brevior* harbored many more bacteria than *B. brevior*, with 100 to 350 bacteria per host cell visible in cross-sections of the mid-filament region (Fig. 1C). Correspondingly, the bacteriocytes were much broader than those of *B. brevior* and their basal regions were more densely packed with lysosomal structures (Fig. 1C). In further contrast to *B. brevior*, each vacuole contained more than 1 symbiont, with as many as 12 bacteria occurring in a single vacuole. Open connections of bacterial vacuoles to the exterior occurred more often than in *B. brevior* (Fig. 1C, D). As in *B. brevior*, the host membrane at these openings always appeared intact (Fig. 1D). The morphology and size of the symbionts was similar in *B.* sp. aff. *brevior* and *B. brevior*. Ultrastructural differences between the juvenile and adult *B.* sp. aff. *brevior* specimens were not observed.

Stable carbon isotope ratios

Stable carbon isotope ratios were determined separately for the gills, foot, and other soft body tissues (rest) from a total of 6 *Bathymodiolus brevior* individuals from Stns 98 and 115 (Table 1). At Stn 98 gill tissues ranged from −30.8 to −32.1‰, at Stn 115 from −35.2 to −35.8‰. Gill tissues were consistently more negative

Table 1. *Bathymodiolus brevior.* Stable carbon isotope values of mussel tissues ($\delta^{13}C$, in ‰). Specimens were dissected into 3 parts: gills, foot tissue, and the rest of the soft body

	Gills	Foot	Rest
Stn 98			
B. brevior 1	−31.1	−30.6	−31.0
B. brevior 2	−30.8	−25.7	−30.7
B. brevior 3	−32.1	−30.8	−31.5
Stn 115			
B. brevior 1	−35.8	−32.4	−33.5
B. brevior 2	−35.2	−32.0	−33.8
B. brevior 3	−35.7	−32.2	−33.9

(i.e. lighter or enriched in ^{12}C) than foot and other soft body tissues at both stations. The average difference between gills and the 2 other tissues was significant at Stn 115 (p < 0.05, Wilcoxon, Mann and Whitney *U*-test), with the means of the $\delta^{13}C$ values from the 3 individuals differing by 1.8‰ between gills and rest, and 2.8‰ between gills and foot.

The *Bathymodiolus brevior* tissues from Stn 115 were significantly more negative than those from Stn 98 (p < 0.05, Wilcoxon, Mann and Whitney *U*-test), with average differences between the 2 stations ranging from 4.2‰ in gill tissues to 3.2‰ in foot tissues and 2.7‰ in other soft body tissues (rest). Only mytilids from Stn 115 carried the commensal polychaete scale worm *Branchypolynoe pettibonae*. The $\delta^{13}C$ value of 3 pooled polychaetes was, at −33.2‰, in the same range as *B. brevior* tissues from Stn 115, indicating that the worms and their hosts derive their nutrition from the same trophic sources.

DISCUSSION

As in all other *Bathymodiolus* symbioses (reviewed in Fisher 1990, Nelson & Fisher 1995), the symbionts found in the gills of *B. brevior* and *B.* sp. aff. *brevior* were intracellular within membrane-bound vacuoles and had a cell envelope resembling that of Gram-negative bacteria. The ultrastructure of the symbionts indicates that they are prokaryotic cells, as in all other hydrothermal vent animals with intracellular symbionts (Cavanaugh 1994).

In other *Bathymodiolus* symbioses the prokaryotic symbionts have been identified as thioautotrophic or methanotrophic based on morphological, biochemical, enzymatic, and molecular data, with each species harboring either one or both trophic types (Cavanaugh et al. 1992, Fisher et al. 1993, Distel et al. 1995, Pranal et al. 1997, Pond et al. 1998). In *B. brevior*, the stable carbon isotope values of −25.7 to −35.8‰ are in the range of values reported for both thioautotrophic and methanotrophic *Bathymodiolus* symbioses and thus do not provide unambiguous information on the trophic nature of the symbionts (Nelson & Fisher 1995). The morphological data is more informative and suggests that both *B. brevior* and *B.* sp. aff. *brevior* harbor only thioautotrophic symbionts because (1) only a single symbiont morphotype was observed and (2) this morphotype did not contain intracytoplasmic membranes which are characteristic for all methanotrophic symbionts (Cavanaugh 1992). Further indications for the presence of sulfur-oxidizing symbionts are high concentrations of the amino acid thiotaurine and (n-7) monounsaturated phospholipid fatty acids in the gills of *B. brevior* (Pranal et al. 1995, 1997).

In comparison to other *Bathymodiolus* species, the abundance of endosymbiotic bacteria in *B. brevior* was remarkably low, with most of the bacteriocytes in the mid-filament region showing only very few symbionts per cross-section. [Compare, however, with Pranal et al. (1997) who, based on fatty acid data, suggest symbiont biomass values in *B. brevior* of the same order of magnitude as those estimated for *B. thermophilus*.] Low symbiont abundances were observed in all *B. brevior* specimens examined in this study. Unfortunately, these specimens were all collected at the same site (Stn 98) so that the influence of different microhabitats and nutrient conditions on intraspecific symbiont morphology could not be investigated in this study. Surprisingly, a detailed study of the relationship between a host's microenvironment and its symbiont ultrastructure and distribution has, to our knowledge, not been published. In the case of *B. brevior*, the low abundances of symbionts appear to be characteristic for this association, as similarly low numbers of symbionts were observed in *B. brevior* specimens collected several years earlier from a different hydrothermal vent site in the North Fiji Basin (see Fig. 2 in Pranal et al. 1995). This conclusion is further supported by the fact that *B.* sp. aff. *brevior* individuals collected within the same hydrothermal vent area during this study harbored high abundances of symbionts. Clearly, bacterial abundance varies in a species-specific manner in *Bathymodiolus* hosts, with the number of symbionts per vacuole ranging from one to hundreds (Fisher & Kelly 1992).

In both *Bathymodiolus brevior* and *B.* sp. aff. *brevior*, vacuoles at the apical ends of the bacteriocytes were occasionally open to the exterior ambient seawater. At these sites, the host cell membrane was always clearly intact, indicating that these openings were not caused by fixation artefacts. While not described in other *Bathymodiolus* symbioses, similar openings have been observed in 2 symbiotic gastropod species from western Pacific hydrothermal vent sites, *Alviniconcha hessleri* and *Ifremeria nautilei* (Endow & Ohta 1989, Windoffer & Giere 1997), and it has been suggested that this arrangement mirrors a possible evolutionary pathway from extra- to intracellular symbioses (Windoffer & Giere 1997). In *A. hessleri*, staining experiments with ruthenium red showed that the external medium only reaches the bacteria at the periphery while the symbionts in the basal part of the bacteriocytes are truly enclosed (Endow & Ohta 1989). Unfortunately, in this study fresh material needed for these staining experiments was not available.

The stable carbon isotope ratio ($\delta^{13}C$) of an animal's tissues reflects its source of organic carbon and can therefore be used to examine trophic relationships. Stable carbon isotope ratios in *Bathymodiolus brevior*

ranged from −25.7 to −35.8‰ and were similar to ratios determined in undescribed symbiotic mytilid species from the western Pacific (−26.1 to −34.8‰; Van Dover & Fry 1989, Kim & Sakai 1991). The *B. brevior* values are consistent with the hypothesis of a nutrition source for these mytilids that is not surface-derived photosynthetic carbon (−12 to −22‰; Van Dover & Fry 1989). The $\delta^{13}C$ values of *B. brevior* resemble those found in free-living thioautotrophic bacteria and in other bivalve groups which harbor thioautotrophic symbionts (reviewed in Fisher 1995, Nelson & Fisher 1995). In *Bathymodiolus* symbioses with methanotrophic symbionts, much more negative ratios ranging from −37 to −78‰ have been reported (Fisher 1995, Nelson & Fisher 1995). However, interpretation of stable carbon isotope ratios is complicated by the fact that, in some *Bathymodiolus* species with both thioautotrophic and methanotrophic symbionts, $\delta^{13}C$ values are in the same range as in mytilids with only thioautotrophic symbionts (Cavanaugh et al. 1992, Pond et al. 1998). Thus, while amino and fatty acid (Pranal et al. 1995, 1997) as well as morphological data (see above) indicate that *B. brevior* harbors only thioautotrophic symbionts, its stable carbon isotope composition does not provide unequivocal support for this conclusion.

Bathymodiolus brevior specimens from Stn 115 had consistently more negative $\delta^{13}C$ values than mytilids from Stn 98. These differences could be due to a more negative $\delta^{13}C$ composition of dissolved inorganic carbon (DIC) at Stn 115. Stable carbon isotope composition of DIC can vary by as much as 3‰ between different vent microhabitats (Childress & Fisher 1992). However, the more likely explanation for the observed differences of 2.7 to 4.2‰ in *B. brevior* $\delta^{13}C$ values between Stns 98 and 115 is a higher input of chemosynthetic carbon at Stn 115. This assumption is further supported by the higher number and diversity of hydrothermal vent species collected at Stn 115 than Stn 98 (see 'Materials and methods').

As in all other *Bathymodiolus* species, *B. brevior* has retained its digestive apparatus and it is likely that it has at least a rudimentary ability to filter feed and ingest food, as observed in other symbiotic mytilids (Page et al. 1990, 1991, Le Pennec et al. 1995). This assumption is supported by the difference in $\delta^{13}C$ values of symbiotic gill and non-symbiotic foot tissues in *B. brevior* (rest tissues are not considered here, as they might have been contaminated with minor bits of gill tissue). In *B. brevior*, non-symbiotic foot tissues were as much as 5.1‰ and on average 2.8‰ more positive than symbiotic gill tissues. If the only source of dietary carbon were the symbionts then foot tissues should have, if anything, slightly more negative $\delta^{13}C$ values than gill tissues, as isotopic fractionation of carbon between an animal and its food source is minimal, with

at most a 1‰ more negative $\delta^{13}C$ in animal tissues relative to diet $\delta^{13}C$ occurring (Rau 1985). An isotopic fractionation of 1‰ would increase the average difference between *B. brevior* gill and foot tissues to 3.8‰, lending further support to the assumption that the symbionts do not account for the sole source of dietary carbon in these associations. Indeed, the low abundances of bacteria in these associations make it hard to imagine that the symbionts could provide their hosts with sufficient nutrition. The relationship between symbiont abundance and stable carbon isotope composition has, to our knowledge, not yet been described. Further studies on the intra- and interspecific variation in symbiont distribution and trophic relationships within the genus *Bathymodiolus* are clearly necessary in order to better understand the ecology of these important members of hydrothermal vent macrofauna.

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