Trophic ecology of massive shrimp aggregations at a Mid-Atlantic Ridge hydrothermal vent site

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Abstract

The source of nutrition of shrimp that form giant aggregations at Mid-Atlantic Ridge hydrothermal vents was explored by a combination of different molecular techniques. These animals have been hypothesized either to graze on the free-living, surface microbial community or to feed off epibiotic bacteria growing on their exoskeleton. Stable isotope compositions of potential food sources, consumer tissues, and gut contents suggest that the shrimp derive most of their carbon and nitrogen from the epibionts. However, probing of nucleic acids extracted from shrimp guts points to the existence of a gut microflora that is isotopically similar but genetically distinct from the epibionts. Carbon dioxide fixation experiments were carried out to compare the magnitude of potential primary productivity of the different nutritional sources of the shrimp. Relatively low and comparable rates were observed for both the free-living community and the epibionts but activity associated with gut preparations was surprisingly high. These high CO_2 fixation rates in the gut may be indicative of a nutritional relationship between the shrimp and their gut microflora. Overall, the data indicate a nutritional symbiosis of the shrimp with their epibionts and possibly also with a separate gut microflora.

Hydrothermal vents are well known for unusual fauna engaged in mutualistic, nutritional symbioses with chemoautotrophic bacteria (Cavanaugh 1994; Fisher 1990). These associations are seen as adaptive strategies to exploit efficiently the abundant yet ephemeral local energy sources, primarily geothermally reduced sulfur and methane (Cavanaugh 1994). Indeed, at the well-studied vents in the Eastern Pacific, giant, endosymbiont-bearing tube worms, clams, and mussels account for up to 75% of the biomass (Tunnicliffe 1991) with growth rates that are among the highest known (Lutz et al. 1994). More recently, however, the emerging generalization that large biomass at hydrothermal vents requires nutritional symbioses was challenged by the exploration of vents along the Mid-Atlantic Ridge (MAR). At least two of these sites (Snake-Pit, 23°N, and TAG, 26°N) are dominated by decapod shrimp of the family Bresiliidae that can reach population densities as impressive as the Pacific animals (Van Dover 1995). These shrimp carry epibiotic bacteria on some anterior body parts, but no endosymbionts could be detected in these animals. Their feeding behavior and potential nutritional sources have remained controversial (Van Dover et al. 1988; Segonzac et al. 1993; Gebruk et al. 1993).

Like most of their Pacific counterparts, the Snake-Pit and TAG communities at the MAR receive virtually no input of photosynthetically fixed carbon and are sustained by bacterial chemoautotrophic primary production (Pond et al. 1997; Wirsen et al. 1993). This process utilizes energy released by the oxidation of reduced chemicals, such as sulfur compounds and methane, for the fixation of C_1 compounds and is closely linked to the narrow zones where the reduced vent fluid mixes with the surrounding oxygenated seawater. This leads to a patchwork of areas of potential bacterial productivity that are generally closely matched by the animal distribution (Tunnicliffe 1991). The shrimp reach their highest densities on solid sulfide chimneys around warm, sulfide-rich water emissions that they appear to seek out actively (Van Dover et al. 1988; Gebruk et al. 1993). There, they can form layers several individuals thick, sometimes completely concealing the substrate surface providing a picture reminiscent of a beehive (Van Dover 1995). Five species have been described from these aggregations; however, most studies have concentrated on *Rimicaris exoculata*, which comprises the majority of individuals (Van Dover 1995).

While there is now consensus that the shrimp subsist on a bacterial diet of local origin (Pond et al. 1997), two very different nutritional sources are still under consideration: (1) the free-living bacterial community growing on the massive polymetal sulfide precipitates, or (2) epibiotic bacteria associated with the shrimp body. Grazing on the sulfide surfaces was originally proposed on the basis of an interpretation of the feeding apparatus of *R. exoculata* in combination with the fact that virtually all individuals recovered had guts entirely filled with sulfide particles (Van Dover et al. 1988). However, ingestion of epibionts as a nutritional supplement was deemed likely (Van Dover et al. 1988) and has since been postulated to be the main mechanism of food acquisition (Gebruk et al. 1993; Segonzac et al. 1993). Because the epibionts form dense mats on some of the shrimps' extremities and on the inner surface of the carapace (Van Dover 1995), they may indeed constitute a rich source of food. Genetic analysis of these epibiotic bacteria showed that the

population consists entirely of a single phylotype (defined as organisms with identical 16S ribosomal RNA [rRNA] genes) (Polz and Cavanaugh 1995). This is reminiscent of at least one nutritional ectosymbiosis (Polz et al. 1994) and indicates a highly specific relationship. Surprisingly, the epibiotic phylotype was not restricted to the shrimp body and was also found to contribute over 60% of the detectable rRNA in the sulfide surface-associated microbial community at the Snake-Pit vent (Polz and Cavanaugh 1995). Such dominance is highly unusual and leads to a situation where the shrimp are likely to rely heavily on a single bacterial phylotype as food, independent of whether it is grazed off their body or off the sulfide surfaces.

This study examines whether the hydrothermal vent shrimp engage in a nutritional ectosymbiosis or whether they derive their food by grazing on sulfide surfaces and the epibionts are merely commensals. A combination of three molecular biomarker and tracer techniques was used to address the nutritional biology of *R. exoculata,* the dominant species, and of *Rimicaris* n. sp. (Martin et al. 1997), a smaller and less abundant species at the Snake-Pit vents. The relative importance of potential trophic links was estimated by comparative analyses of the ratios of the stable isotopes of carbon and nitrogen. Consumers generally have carbon isotope compositions similar to their foods and show a consistent increase in the heavier nitrogen isotope relative to the trophic level below them (Minagawa and Wada 1984). The representation of the epibiotic phylotype in shrimp guts was investigated by quantitative nucleic acid hybridization using species- and domain-specific oligonucleotide probes. The potential primary productivity of different food sources was measured by carbon dioxide incorporation into biomass, methane utilization, and assays for ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the CO_2 fixation enzyme of the Calvin–Benson cycle.

Materials and methods

Specimens and samples—All specimens, including shrimp and solid sulfide samples were collected in June 1993 during DSV *Alvin* dives 2,610–2,623 at the Snake-Pit hydrothermal vent site (23°N) on the MAR. Animals were dissected within 1 h upon arrival on board ship. Parts were either stored frozen for nucleic acid extraction and stable isotope analysis or used immediately for carbon dioxide fixation and methane utilization experiments. Surfaces of solid sulfide blocks and black smoker chimneys were scraped and stored frozen for stable isotope analysis. Standard nucleic acids used in quantitative hybridization experiments were extracted from *Escherichia coli* INVαF' (Invitrogen) and *Methanobacterium thermoautotrophicum* (provided by Lee Krumholz, Massachusetts Institute of Technology).

Stable isotope analysis—Individuals of *R. exoculata* and *Rimicaris* n. sp. collected from the Snake-Pit hydrothermal site were frozen individually and stored at −70°C. Carapace lengths were measured to estimate body size. Abdominal muscle was removed for analyses and washed in dilute HCl to remove contaminating carbonates, rinsed with distilled water, dried at 60°C, and ground to a powder. Cardiac stom-

achs were removed from three lots of 20 shrimp each. Stomach contents were gently washed and pooled in three separate dishes and dried. These dishes were then placed overnight in a desiccator containing concentrated HCl fumes to remove contaminating carbonates and finally redried. Corresponding abdominal muscles from each set of 20 shrimp were pooled for analysis and treated as described above. Concentrated epibiont biomass from *R. exoculata* was collected by gently scraping the thick bacterial pads located on the inner surface of the carapace, the exoskeleton that creates the brachial chamber. These scrapings were wiped on a precombusted filter without predetermined pattern. The filter was cut into three pieces, dried, treated with concentrated HCl fumes, redried, and shredded. Sulfide scrapings were taken from the outermost surfaces of samples, dried, treated with concentrated HCl fumes, redried, and ground to a powder for analyses.

Isotope ratios were determined following the methods of Minagawa et al. (1984) on a mass spectrometer (Finnigan) at the MBL Ecosystems Center (Marine Biological Laboratory, Woods Hole, Massachusetts), with Peedee Belemnite (PDB) carbon and atmospheric N_2 as standards for carbon and nitrogen, respectively. Ratios were calculated according to

$$\delta^{13}C \text{ or } \delta^{15}N \ (\text{‰}) = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \times 10^3,$$

where R is ($^{15}N/^{14}N$) or ($^{13}C/^{12}C$).

Nucleic acid extraction—Nucleic acids were extracted for quantitative slot-blot hybridization from frozen preparations of cardiac stomachs and mid- and hindguts of *R. exoculata* (35 individuals pooled per replicate). To ensure efficient cell lysis, a sodium dodecyl sulfate- and phenol-containing buffer was used in a minibead beater (Biospec Products) as described elsewhere (Polz and Cavanaugh 1995). Standard nucleic acids from organisms classified in the three domains (sensu Woese et al. [1990]) were extracted using the same method: for Eukarya, *R. exoculata* tail tissue; for Archaea, *M. thermoautotrophicum*; and for Bacteria, *E. coli.*

Quantitative slot-blot hybridizations—To determine the relative abundance of the shrimp epibiotic phylotype compared to total Bacteria or Archaea in shrimp gut contents, quantitative slot-blot hybridization was performed using radiolabeled oligonucleotides as probes (Polz and Cavanaugh 1995). Nucleic acids extracted from stomachs and guts were blotted in five replicates on nylon membranes (Zeta-Probe, Bio-Rad) (Polz and Cavanaugh 1995). Dilutions of standard nucleic acids, including bacteria scraped off the carapace and representatives of the three domains Eukarya, Archaea, and Bacteria were blotted in duplicates on the same membranes. Subsequently, these membranes were hybridized to oligonucleotide probes specific for the shrimp-epibiont phylotype (Polz and Cavanaugh 1995), the domains Bacteria (Stahl and Amann 1991), Archaea (Stahl and Amann 1991), and Eukarya (Giovannoni et al. 1988), as well as to a negative control probe that differed in two positions from the epibiont-specific probe (Polz and Cavanaugh 1995). All probes were labeled with polynucleotide kinase to an approximate specific activity of 5×10^8 cpm μg^{-1}. Hybridizations were

done overnight at 40°C (except Eukarya at 30°C) followed by two washes (15 min each) at the hybridization temperature in the hybridization and wash buffer recommended for Zeta-Probe membranes. The specific wash temperatures were based on experimentally determined midpoint dissociation temperatures for all oligonucleotides and were: 42, 50, 60, and 56°C for the Eukarya, Archaea, Bacteria, and epibiont-specific probes, respectively (Polz and Cavanaugh 1995). Probe remaining bound to the membrane after the washes was quantified using a Fujix Bio Imaging Analyzer BAS2000 phosphor imager and BAS2000 Image File Manager 2.1 analysis software. Linear regression analysis on standard nucleic acids from shrimp scrapings and positive controls for the domain-specific probes yielded $r^2 > 0.98$ in all cases. The hybridization signals obtained for the individual samples were converted into amount of nucleic acids using the standard curves.

Carbon dioxide fixation and RubisCO assays—Chemoautotrophic potential of bacteria associated with shrimp body parts and sulfide surfaces was tested by incubation with ^{14}C-labeled bicarbonate and comparison of fixation rates in parallel samples with or without addition of reduced sulfur compounds. Samples included scrapings of solid sulfides and the inner surface of the carapace and preparations of scaphognathites, cardiac stomachs, and midguts. Six replicates were analyzed for each type of sample, except for sulfide surfaces where material was scraped off a single block and two separate blocks were collected during dives 2,616 ($n = 1$) and 2,618 (both $n = 3$), respectively. Scrapings of sulfides and carapace (30 individuals pooled) and scaphognathites (10 individuals pooled) were homogenized on ice in a groundglass tissue grinder in 0.22-μm-filtered seawater and subsampled for determination of protein concentration using the Bradford method (Bio-Rad Protein Assay Kit). Cardiac stomachs and mid- and hindguts were dissected and incubated whole (four per treatment) and ground at the end of the experiment. All samples were brought to slightly above 3 ml and aliquoted into three 7-ml glass scintillation vials. One of these vials served as the unamended control, and the others were spiked with sulfide or thiosulfate to a final concentration of 100 μM or 10 mM, respectively. Incubations were initiated at room temperature by the addition of H^{14}CO$_3^-$ (specific activity 56.4 mCi/mmol, ICN Biomedicals) to an intended final activity of 1.0 μCi ml^{-1}. The exact specific activity of the bicarbonate in each of the samples was determined by scintillation counting of a 10-μl subsample transferred to 100 μl of phenethylamine in 5 ml scintillation cocktail immediately after addition of the isotope. Incubations were stopped after 5 h by acidification with 100 μl of 1 N HCl and the samples were stored at -20°C. Incorporation of ^{14}C into acid-stable product was determined by scintillation counting after the samples were further acidified and bubbled with air to drive off residual CO$_2$. Total incorporation of CO$_2$ into tissue was calculated by assuming a seawater bicarbonate concentration of 2.4 mM.

RubisCO activity was determined on scrapings of epibionts and of sulfide surfaces and homogenates of cardiac stomachs, mid-, and hindguts following previously published protocols (Cavanaugh 1983). Partially purified spinach

RubisCO (Sigma) served as a positive control. Samples were homogenized in 3 volumes assay buffer (100 mM Tris, pH 8.2, 5 mM dithiothreitol, 20 mM MgCl$_2$, and 5 mM NaHCO$_3$) and subsequently either sonicated or passed through a French pressure cell (82.4 MPa). Cell debris was pelleted by centrifugation (16,000 $\times$ g), and 50–100 μl of the supernatant was used in assays. The supernatants were preincubated for 10 min after adding H^{14}CO$_3$ to a concentration of 2.12 mM at which point subsamples were taken for determination of specific activity. The reactions were initiated by addition of ribulose-1,5-bisphosphate (RuBP) to a concentration of 10 mM and terminated by the addition of 200 μl acetic acid. Time courses were run by stopping the reaction in parallel samples in 5-min intervals up to a maximum of 30 min. Assays conducted without the addition of RuBP served as a control for background fixation. Acid-stable incorporation of ^{14}C was determined by liquid scintillation counting.

Methane utilization—Methane utilization potential of bacteria associated with shrimp body parts and sulfide surfaces was tested by incubation with ^{14}C-labeled methane. Types of samples and their preparation were as described above. One-milliliter homogenates were added to 25-ml serum vials containing 5 ml 0.22-μm-filtered seawater. The vials were sealed with a rubber stopper and crimped. The experiment was initiated by injecting ^{14}CH$_4$ (specific activity 6.56 μCi; courtesy of Dr. Lacey Daniels, University of Iowa) through the rubber stopper using a gas-tight syringe to a final concentration of 0.4 μM. In parallel samples, the labeled methane was diluted with unlabeled methane to 50 and 200 μM final concentration, respectively. Controls for nonspecific sticking of methane were spiked with acetylene or killed with formalin. Vials were incubated inverted in a 20°C water bath for 30, 60, and 90 min and reactions stopped by the addition of 100 μl 0.1 M NaOH and stored frozen. The amount of ^{14}C-methane oxidized to CO$_2$ and incorporated into acid-stable compounds was determined by transferring 1-ml aliquots to 125-ml Erlenmeyer flasks containing 5 ml distilled water. The flasks were shaken overnight to drive off unincorporated methane. Subsequently, the solution was acidified and CO$_2$ trapped on filter papers soaked in phenethylamine. Radioactivity trapped on the filters (CO$_2$ from methane oxidation) and remaining in solution (methane incorporated into acid-stable products) was determined by liquid scintillation counting. All values were corrected for background by subtracting counts observed for formalin-killed or acetylene-inhibited controls.

Results

Stable isotope ratios—Carbon and nitrogen isotope compositions were measured to estimate the relative importance of potential food sources of *R. exoculata* and *Rimicaris* n. sp. For individual *R. exoculata*, stable isotope ratios of tail muscle tissue ranged from -14.9 to -10.0‰ for carbon (δ^{13}C) and from $+5.4$ to $+8.2$‰ for nitrogen (δ^{15}N). However, the ratios for both isotopes fell in two nonoverlapping classes (Fig. 1) that were strongly dependent on body size (Fig. 2). In the majority of cases, smaller individuals exhib-
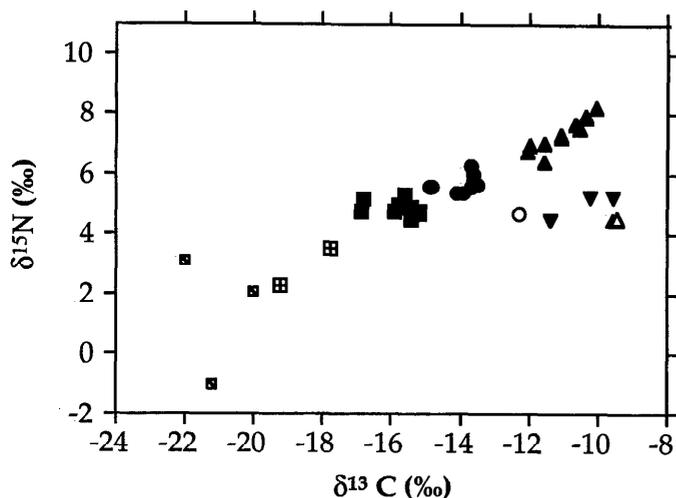
Fig. 1. $\delta^{13}C$ versus $\delta^{15}N$ values for shrimp muscle tissue, gut contents, and their potential food sources. Tail tissue: *R. exoculata*, large (>12 mm carapace length) = ▲ and small (<12 mm carapace length) = ●, *Rimicaris* sp. = ■; epibionts: *R. exoculata*, large = △ and small = ○; gut contents *R. exoculatai* large = ▼; Organic material from sulfide surfaces: massive shrimp covering = ◨ and little or no shrimp covering = ⊞.

ited lower values for both isotopes. Carbon ratios ranged from -12.0 to $-10.0‰$ (average $= -11.0 \pm 0.7‰$) and -14.9 to $-13.5‰$ (average $= -14.0 \pm 0.6‰$) for the classes containing mostly larger and smaller individuals, respectively. Nitrogen isotopes clustered similarly with ranges from $+6.4$ to $+8.2‰$ (average $= +7.3 \pm 0.6‰$) and from $+5.4$ to $+6.3‰$ (average $= +5.7 \pm 0.3‰$). Specimens of *Rimicaris* n. sp. had relatively uniform isotope values that ranged from -16.9 to $-15.2‰$ (average $= -15.8 \pm 0.6‰$) for carbon and $+4.5$ to $+5.3‰$ (average $= +4.9 \pm 0.2‰$) for nitrogen. They were on the average smaller than *R. exoculata* and showed a lesser correlation of size with carbon; size was not correlated with nitrogen isotope ratios (Fig. 2).

Samples from the two possible food sources, the epibiotic bacteria and sulfide-associated organic material, were also analyzed. Pooled epibiotic bacteria collected from 25 specimens of the large shrimp class had isotope ratios of $-9.4‰$ for carbon and $+4.4‰$ for nitrogen (Fig. 1). This amounts to an average difference between shrimp and epibionts of $-1.6 \pm 1.8‰$ for carbon and $+2.9 \pm 0.6‰$ for nitrogen isotope ratios. Epibionts from the small shrimp class ($n = 4$ individuals) showed values of $-12.3‰$ and $+4.7‰$ and differences of $-1.7 \pm 0.5‰$ and $0.9 \pm 1.0‰$ for carbon and nitrogen, respectively. No epibionts were analyzed for *Rimicaris* n. sp.

Isotope values of organic material associated with several sulfide samples had a relatively homogeneous distribution with carbon and nitrogen values centered around $-20‰$ and $+2‰$, respectively (Table 1, Fig. 1). No organic carbon or nitrogen could be detected in some samples (Table 1). Of particular interest are sulfide surfaces that had a high density of shrimp covering with measurable organic material. Their carbon and nitrogen isotope ratios were $7.5‰$ and $4.4‰$ lower than the average large *R. exoculata* and $4.5‰$ and
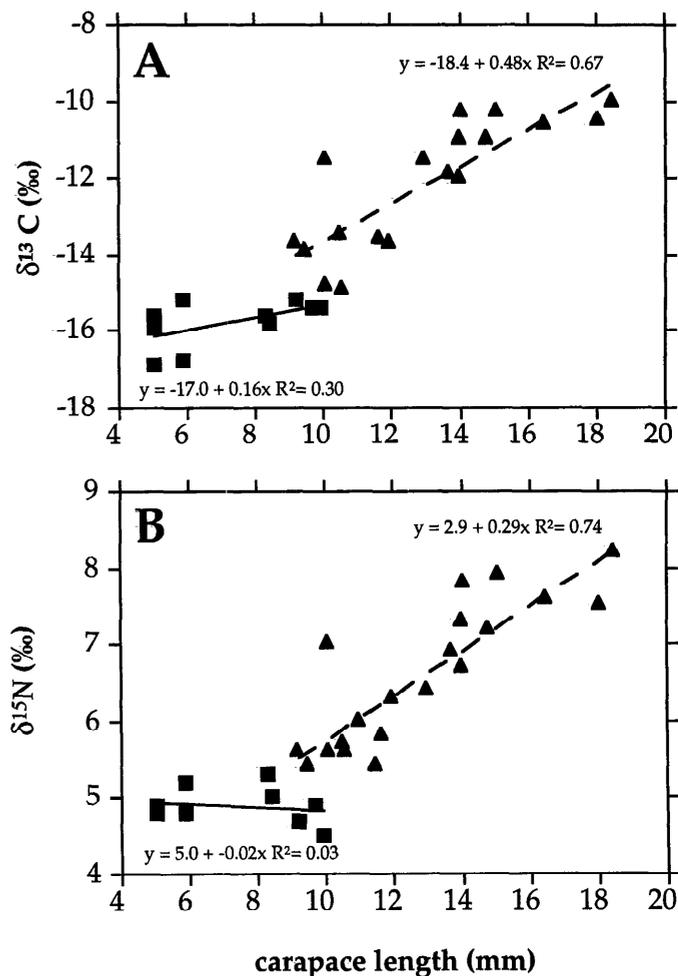

Fig. 2. Carbon ($\delta^{13}C$) (A) and nitrogen ($\delta^{15}N$) (B) stable isotope ratios of shrimp tail tissue versus carapace length as a measure of body size. ▲, *R. exoculata*, tail tissue; ■, *Rimicaris* n. sp., tail tissue.

Table 1. Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) ratios of carbonate-free scrapings of sulfide surfaces.

Sample number	Shrimp covering*	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
95-01	+	-19.2	$+2.3$
95-02	+	-17.8	$+3.5$
94-19†	+	-21.2	ND
94-20†	+	-21.7	ND
94-21†	+	-22.2	ND
95-03	−	-21.2	-1.0
95-04	−	-20.0	$+2.1$
95-05	−	-22.0	$+3.1$
95-06	−	ND‡	ND

* Samples were collected from areas with high (+) and zero to low (−) density of shrimp covering on the sulfide surfaces.
† Subsamples from a single larger piece of sulfide.
‡ ND, below detection limit.

Table 2. Stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) ratios of gut contents of large (>12-mm carapace length) *R. exoculata*.

Dive number	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	Number of individuals*
2,611	−11.3	+4.4	20
2,618	−10.2	+5.2	20
2,620	−9.5	+5.2	17

* Cardiac stomachs, mid-, and hindgut were dissected from individual shrimp and their contents removed and pooled for isotope analysis.

Table 3. Contribution of ectosymbiont-specific nucleic acids to the total microbial community associated with shrimp *R. exoculata* and sulfide surfaces.

Nucleic acids	Replicate slots	ng nucleic acids ± SE		% of total*
		Bacteria	Epibiont-specific	
Shrimp-associated†	5	586.8±59.7	572.9±12.5	97.6
Cardiac stomach‡	5	33.6±2.3	7.2±0.8	21.4
Mid- and hindgut‡	5	76.7±2.1	18.2±0.2	23.7
Sulfide-associated§	3	64.0±1.3	39.2±1.9	61.3

* Ratio of hybridization signal of the epibiont-specific probe to that of the *Bacteria* probe (×100).
† Modified after Polz and Cavanaugh (1995); ng nucleic acid per individual, calculated from pooled nucleic acids from 10 individuals.
‡ Values are in ng nucleic acid per gut, calculated from pooled nucleic acids from 35 guts.
§ Modified after Polz and Cavanaugh (1995); ng nucleic acid per g substrate, calculated from pooled nucleic acids from eight scraped sulfide surfaces.

2.8‰ than the smaller specimens. *Rimicaris* n. sp. isotope values differed by only +2.7‰ and +2.0‰ for carbon and nitrogen, respectively.

Gut contents were extracted and analyzed from three batches of *R. exoculata* specimens (20 specimens each). Two of these had carbon and nitrogen isotope ratios almost identical to the epibionts and the third differed only slightly in its carbon values (Table 2). No gut content data were collected for the smaller *R. exoculata* and *Rimicaris* n. sp.

Quantification of epibionts in shrimp guts—The proportion of the epibiotic bacterial phylotype to total *Bacteria* and *Archaea* was determined by quantitative probing of nucleic acids extracted from pooled shrimp guts (n = 100). Total detectable bacterial nucleic acids amounted to 33.6 and 76.7 ng per individual cardiac stomach and combined mid- and hindgut, respectively (Table 3). No signal was obtained from the probe specific for Archaea. Hybridization with the epibiont-specific probe indicated an average rRNA content of 7.2 ng per cardiac stomach and 18.2 ng per gut. Thus, in both sections of the gut, rRNA from the epibionts represent slightly more than 20% of total rRNA from bacteria contained in the lumen or associated with the wall (Table 3).

Autotrophic potential of implied food sources and shrimp tissues—Carbon dioxide fixation and methane utilization were tested on scrapings of sulfide surfaces and shrimp carapaces and homogenates of shrimp scaphognathites and guts. Methane utilization above killed controls was not detected for any of the samples except for hydrothermal vent mussels containing endosymbiotic bacteria used as a positive control (data not shown).

Rates of CO_2 fixation were comparable for sulfide surfaces, carapaces, and scaphognathites. Most of the sample averages ranged from 3 to 4 nmol C mg protein^{-1} for 5-h incubation periods (Fig. 3A, C). No statistically significant stimulation of fixation was observed in the presence of reduced sulfur compounds. However, variability among the data was large. Homogenates of total shrimp guts showed the highest fixation rates, exceeding some of the other samples by up to two orders of magnitude (Fig. 3B). Preparations of the mid- and hindgut sections incorporated the highest amount of CO_2. Addition of thiosulfate or sulfide did not consistently stimulate CO_2 fixation.

RubisCO activity measurements were carried out to determine whether CO_2 fixation was by autotrophic pathways. Positive results were obtained for pooled epibionts scraped off carapaces (data not shown) that confirmed previous ac-

tivity measurements (Wirsen et al. 1993) and detection of a form II RubisCO by immunoblotting (Cavanaugh and Robinson 1996). However, other samples did not yield enough material to carry out enzyme assays.

Discussion

Energy flow from primary producers to grazers within the "textbook" Pacific vent communities dominated by tubeworms and bivalve mollusks is short-circuited by the endosymbiotic condition of chemoautotrophic bacteria living within host tissues. This characteristic was postulated as a prerequisite for large animal standing stocks at deep-sea vent ecosystems (Tuttle et al. 1983) but was challenged by the discovery of giant shrimp swarms in at least two MAR hydrothermal vent systems. We provide several new lines of evidence that the MAR vent shrimp also depend on symbiotic microorganisms for their nutrition, reaffirming the notion that symbiotic relationships are critical in the autecology of high-biomass species within vent systems.

Our experiments were designed to test the two alternative hypotheses that the shrimp derive their nutrition primarily either from epibiotic bacteria growing on their own body or from organic material associated with sulfide particles that are ingested by the shrimp. A confounding issue was the discovery that over 60% of the rRNA of the microbial community on sulfide surfaces is identical to that of the epibionts (Polz and Cavanaugh 1995), implying that the two potential food resources are primarily a single bacterial phylotype.

Despite the significant presence of the epibiotic phylotype on the sulfide surfaces (Polz and Cavanaugh 1995), stable isotope ratios of the two putative food resources are distinct (Fig. 1, Table 1). This is especially pronounced for $\delta^{13}C$ values (sulfides: −17.2 to −22.2‰; epibionts: −9.4 and −12.3‰) and may be due to the presence of a mixed community of autotrophic bacteria on the sulfide surfaces as opposed to a monoculture of a single phylotype on the shrimp bodies. Free-living and symbiotic sulfur-oxidizing bacteria have been shown to fall into isotopically distinct groups grouped around −30 and −11‰, respectively (Childress and
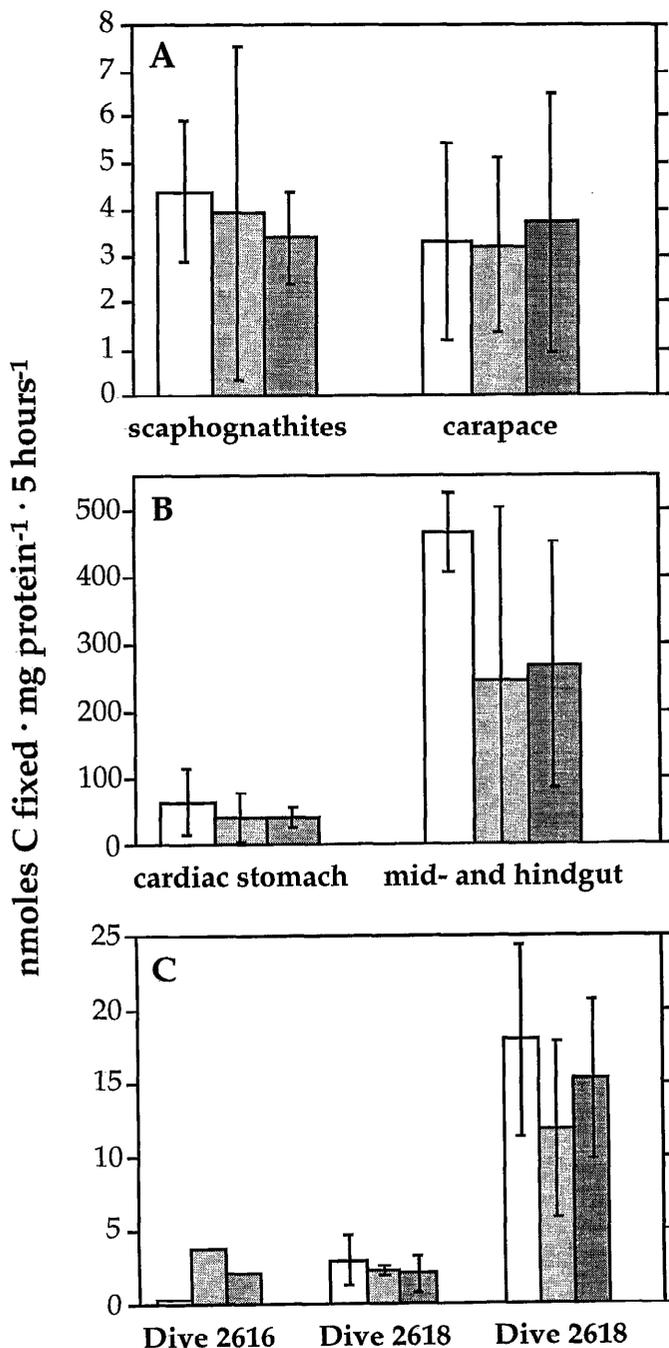
Fig. 3. Carbon fixation activity in pooled samples of epibiont-containing *R. exoculata* body parts (scaphognathites and inner surface of carapace, $n = 3$) (A), of gut homogenates ($n = 6$) (B), and of scrapings of three different sulfide surfaces collected during dives 2,616 ($n = 1$) and 2,618 (both $n = 3$) (C). □, control; □, 10 mM thiosulfate; ▨, 100 μM sulfide.

Fisher 1992). This difference between the two groups correlates well with the expression of either form I ($-30\%o$ group) or form II ($-11\%o$ group) RubisCO for which distinct kinetic isotope fractionations have been observed, e.g., Robinson and Cavanaugh (1995). The shrimp epibionts associate with the $-11\%o$ group and have been shown to express form

II RubisCO (Cavanaugh and Robinson 1996). However, stable carbon isotope values for the organic material on the sulfide surfaces ranged from -17.2 to $-22.2\%o$ (Fig. 1, Table 1), falling between the two groups. This may reflect the presence of a mixed microbial community on the sulfide surfaces in which some bacteria express form I and others form II RubisCO and is consistent with the observation that in sulfide surfaces from the same vents over half of the rRNA originated from the shrimp epibionts (Polz and Cavanaugh 1995) and that other bacteria, including a *Thiomicrospira* phylotype, could be detected (Muyzer et al. 1995).

The difference in isotopic composition between the shrimp epibionts and the sulfide surface organic material allows a comparison of bulk shrimp tissue with the putative nutritional sources to determine which is more likely to be important in the diet. Isotope values of muscle tissue of both size classes of *R. exoculata* were on the average much closer to their respective epibionts than to the sulfides (Fig. 1). However, some size dependency appears to exist with larger shrimp deriving a greater proportion of their diet from epibionts (Fig. 1). Mass balance calculations indicate that the large specimens of *R. exoculata* obtain over 80% of their carbon from grazing on their epibionts if the average carbon isotope values of shrimp tissue ($-11.0\%o$), sulfide surfaces with extensive shrimp covering ($-18.5\%o$), and epibionts ($-9.4\%o$) are taken into account. This estimate does not change significantly if nutritional input is estimated with nitrogen isotopes. For the smaller size class of *R. exoculata*, carbon isotope values suggest up to 30% dependence on sulfide-associated organic material; however, estimates based on nitrogen isotopes are inconclusive and may actually reflect efficiency of food utilization by the animals rather than serve as an indication of trophic level (J. P. Montoya pers. comm.). Isotope ratios of the smaller, newly described species of *Rimicaris* may point to an even greater reliance on grazing on sulfides, but isotope ratios of epibionts are currently not available (Fig. 1). Overall, the data suggest that the shrimp depend primarily on epibionts grazed off their own bodies with input from sulfide organic material varying with body size. Nevertheless, two alternative explanations for the observed correlation of isotope composition and body size are imaginable (Fig. 2). The small shrimp may be exposed to different environmental conditions (e.g., temperature, CO_2 concentration) or substrate limitation may lead to size-dependent depletion due to growth of the epibionts in the enclosed structure of the carapace.

Isotope values of pooled gut contents of large shrimp specimens were almost identical to the epibionts confirming that the sulfides are unlikely to be the major dietary source (Fig. 1, Table 1). This similarity is critical for an interpretation of the feeding habits of the shrimp because of the large proportion of the epibiotic phylotype in the sulfide surface community (Polz and Cavanaugh 1995). Conceivably, the close match in isotope values of shrimp tissues and the epibiotic phylotype is the result of ingestion of total organic material from the sulfide surfaces coupled with preferential digestion of the epibiotic phylotype. However, if organic material other than the epibiotic phylotype remained unaffected by digestion, gut contents would be expected to have a more negative signal than observed, i.e., be more similar to the

sulfide organic material in both carbon and nitrogen isotope compositions.

Potential productivity of the implied food sources also supports feeding off epibionts as the dominant mode of nutrition. Although carbon dioxide fixation rates, measured per unit protein, were comparable for the scrapings of sulfide surfaces (0.5–18.0 nmol C mg protein^{-1} 5 h^{-1}) and shrimp epibionts (3.0–4.5 nmol C mg protein^{-1} 5 h^{-1}) (Fig. 3A, C), total productivity of the sulfide surfaces may actually be very low. Many of the sulfide samples yielded only very little protein, which indicates a small standing stock of microbial cells. Indeed, microbial rRNA per unit area as a mixed measure of growth rate and abundance suggests that bacterial populations are orders of magnitude smaller on the sulfides than on the shrimp body (Polz and Cavanaugh 1995). Therefore, overall productivity of the sulfide microbial community may be low.

Low productivity could be due to an extreme patchiness of the bacterial distribution. Scanning electron microscopy of sulfide surface samples from areas where shrimp were abundant revealed a moderate number of cells in one and virtually no cells in the other (unpubl. obs.). This is consistent with reports ranging from the observation of dense bacterial mats (Wirsen et al. 1993) to almost no surface community (Gebruk et al. 1993; Segonzac et al. 1993). Based on these observations alone, it is questionable whether the massive shrimp populations could be sustained by grazing on the sulfide surfaces. Epibiotic microorganisms growing on the surface of the branchial chamber of the shrimp are thus strongly implicated as the primary source of nutrition for the shrimp.

Although stable isotopes and productivity estimates strongly suggest that the shrimp feed on their epibionts, only slightly more than 20% of the detectable bacterial rRNA in shrimp foreguts (cardiac stomachs), midguts, and hindguts originated from the epibiotic phylotype (Table 3). A further surprising result was that carbon dioxide fixation rates measured for foreguts and combined mid- and hindgut preparations were very high and exceeded rates measured for sulfides and epibionts by as much as two orders of magnitude (Fig. 3). Based on our rRNA analysis and carbon fixation data, we suggest that (1) most epibionts are lysed in the foregut where digestive enzymes are secreted in caridean shrimp (Dall and Moriarty 1983), and (2) there are additional, highly active bacterial populations in the shrimp guts that could serve as a previously unsuspected source of nutrition.

The gut environment may indeed be favorable for autotrophic production by microorganisms via oxidation of polymetal sulfides ingested by the shrimp. This would finally provide a satisfactory explanation for the initial observation by Van Dover et al. (1988) that all shrimp guts examined contained high concentrations of sulfide mineral particles. While carbon fixation in shrimp guts (93.2 nmol C mg protein^{-1} h^{-1}) is several thousandfold lower than in symbiont-containing tissues of the tubeworm *R. pachyptila* and the clam *C. magnifica* (Childress and Fisher 1992), it is higher than in the symbiotic mussel *Bathymodiolus thermophilus* (12.4 nmol C mg protein^{-1} h^{-1}) (Nelson et al. 1995). Furthermore, heterotrophic carbon dioxide fixation generally proceeds at much lower rates (Hammen and Lum 1964;

Clough and Lopez 1993). For example, values ranged from 0.0015 to 0.3 nmol C fixed mg protein^{-1} h^{-1} for the deposit-feeding polychaete *Heteromastus filiformis* that is exposed to high sulfide concentrations in its environment. Although carbon dioxide fixation was not stimulated by reduced sulfur compunds, we suggest that some large part of the fixation in the shrimp guts is carried out by a gut microflora which is genetically distinct from the epibiotic phylotype but has carbon (e.g., a form II RubisCO) and nitrogen fractionation mechanisms similar to the epibionts. Confirmation of the presence of these bacteria and whether they serve as a significant additional food source requires further study.

In conclusion, our results strongly suggest that the shrimp are involved in a nutritional symbiosis with their epibionts. They derive most of their diet from specific bacteria associated with their body that enables them to gain a large degree of independence from environmental food sources. This may be highly beneficial in an extremely patchy environment dominated by steep and unpredictable gradients that determine primary productivity. For similar reasons, the bacteria would be likely to benefit from the association. As in other symbioses (Cavanaugh 1994; Ott et al. 1991), the shrimp act analogous to a chemostat, providing a stable, nutrient-rich environment for their symbionts. The epibionts may benefit from a continuous and simultaneous access to reduced sulfur compounds and oxygen through the shrimps' movement in the gradients of the mixing zone of vent and ocean water. On the other hand, the gut bacteria may oxidize polymetal sulfides ingested by the shrimp in the stable environment of the gut. Thus the MAR shrimp show characteristics similar to many Pacific vents animals that are nourished by endosymbiotic bacteria. In general, this suggests that large animal biomass at hydrothermal vents is indeed linked to nutritional symbioses. It will be important to test new and currently poorly characterized vent fauna to explore further the postulated dependence on symbiosis by high biomass species.

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