Evidence for the Role of Endosymbionts in Regional-Scale Habitat Partitioning by Hydrothermal Vent Symbioses

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Classification: Biological Sciences (Ecology)

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Keywords: chemosynthetic symbiosis, *Alviniconcha*, hydrothermal vent, Lau Basin, niche partitioning

ABSTRACT

Deep-sea hydrothermal vents are populated by dense communities of animals that form symbiotic associations with chemoautotrophic bacteria. To date, our understanding of which factors govern the distribution of host/symbiont associations (or holobionts) in nature is limited, though host physiology is often invoked. In general, the role that symbionts play in habitat utilization by vent holobionts has not been thoroughly addressed. Here we present evidence for symbiont-influenced, regional-scale niche partitioning among symbiotic gastropods (genus *Alviniconcha*) in the Lau Basin. We extensively surveyed *Alviniconcha* holobionts from four vent fields using quantitative molecular approaches, coupled to characterization of high-temperature and diffuse vent fluid composition using gastight samplers and *in situ* electrochemical analyses respectively. Phylogenetic analyses exposed cryptic host and symbiont diversity, revealing three distinct host types and three different symbiont phylotypes (one ε-proteobacteria and two γ-proteobacteria) that formed specific associations with one another. Strikingly, we observed that holobionts with ε-proteobacterial symbionts were dominant at the northern fields while those holobionts with γ-proteobacterial symbionts were dominant in the southern fields. This pattern of distribution corresponds to differences in the vent geochemistry that result from deep subsurface geological and geothermal processes. We posit that the symbionts, likely through differences in chemoautotrophic metabolism, influence niche utilization among these holobionts. The data presented here represent the first evidence linking symbiont type to habitat partitioning among the chemosynthetic symbioses at hydrothermal vents, and illustrate the coupling between subsurface geothermal processes and niche availability.

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INTRODUCTION

Niche partitioning, the process wherein coexisting organisms occupy distinct niches, is thought to be essential in structuring many biological communities (1-3). Classic studies of ecological niche partitioning have focused on how the intrinsic traits of organisms allow them to occupy or utilize distinct habitats or resources (4, 5). However, species can also access novel niche space via symbiotic associations with other organisms. In these cases, the niche of the host is expanded through the addition of the symbiont's physiological capabilities. With increasing awareness of the prevalence of microbe-animal associations, the effect of the symbiont(s) on niche utilization may prove to be key to understanding the coexistence of organisms in many biological communities. This is likely to be especially important in ecosystems structured by coexisting symbiotic associations, such as hydrothermal vents. Therefore, we looked for habitat utilization patterns reflective of symbiont-influenced niche partitioning among a group of closely-related, snail-bacterial symbioses in the Eastern Lau Spreading Center (ELSC) hydrothermal vent system.

Hydrothermal vents are extremely productive environments wherein primary production occurs via chemolithoautotrophy, the generation of energy for carbon fixation from the oxidation of vent-derived reduced inorganic chemicals (6). The dense communities of macrofauna that populate these habitats are typically dominated by

invertebrates that form symbiotic associations with chemolithoautotrophic bacteria (7). In these associations, the endosymbionts oxidize reduced vent-derived compounds –usually hydrogen sulfide- and fix inorganic carbon, which is shared with their host for biosynthesis and growth (8-12). Symbiotic associations between chemosynthetic bacteria and invertebrates have been described for multiple invertebrate taxa from three phyla (13), and these associations often coexist within given vent fields, systems of vent fields (regions), and biogeographic provinces (14).

It is well established that hydrothermal fluid can exhibit marked spatial and temporal differences in temperature, pH, and chemical composition, the result of numerous sub-surface geological, chemical, physical, and biological factors (15-18). This heterogeneity across both space and time provides myriad physicochemical niches and ample ecological opportunity to support a diversity of chemosynthetic symbioses via niche specialization. Previous studies have examined successional changes within a community of chemosynthetic symbioses in relation to temporal changes in vent fluid chemistry (19, 20), the distribution of the symbioses in relation to physicochemical conditions within a vent field (21-27), and the distribution of chemosynthetic symbioses among different vent fields (28, 29). Host tolerance, growth rates and physiological capacities are often invoked when explaining the observed distribution. Given the reliance of chemosynthetic symbioses on vent-derived chemicals for symbiont function (30), variations in symbiont physiological activity have the potential to result in distinct habitat utilization patterns by holobionts. However, no study has yet comprehensively interrogated both host and symbiont to ascertain whether there is evidence for symbiont-influenced niche partitioning at vents.

Despite a convergence of general function among chemosynthetic symbioses, in which the endosymbionts provide primary nutrition for the host, chemoautotrophic symbiont lineages have evolved multiple times from distinct lineages of free-living Proteobacteria (13, 31), and the genetic distance within and among symbiont lineages is sufficient to posit that physiological differences exist among them. Indeed, ongoing studies of chemosynthetic symbioses continue to reveal diverse modes of energy metabolism, such as hydrogen and carbon monoxide oxidation (32, 33). Given the obligate nature of these associations, the ecological implications of differences in symbiont physiological capacity are quite significant as they may enable niche partitioning that results in previously inexplicable or unrecognized distribution patterns. If there are physiological differences among the symbionts of given groups (genera or species) of hosts, symbiont physiological activity would have the potential to constrain host habitat utilization via differences in chemolithotrophic metabolism.

Provannid gastropods of the genus *Alviniconcha* provide a unique opportunity to study symbiont-driven host niche partitioning. *Alviniconcha* are widely distributed at vents in western Pacific (Manus Basin, Marianas Trough, North Fiji Basin, Lau Basin), as well as in the Indian Ocean at vents along the Central Indian Ridge. In addition to the described species of *Alviniconcha*, previous studies have found additional host "types", which are sufficiently divergent that they may represent undescribed species (34-36). These species and host types have been observed to host either intracellular γ- or ε-proteobacterial symbionts in the gill (36-40). Studies of the distribution of these species and types among vent fields examined a modest number of specimens per site (e.g. 2 individuals from each sampling site), with little or no contextual habitat information. As

such, it is impractical to infer from these data the relationship between host type, symbiont type, and habitat utilization.

In order to look for patterns indicative of symbiont-influenced habitat partitioning, we collected 288 *Alviniconcha* individuals from the walls of hydrothermal chimneys and diffuse flow habitats (where hydrothermal fluid is emitted from cracks in the seafloor) (Fig.1, Table S1). *Alviniconcha* were sampled from four vent fields spanning a regional geological gradient, where the two northernmost fields (Tow Cam and Kilo Moana) are dominated by basaltic lava, while the two southernmost vents (ABE and Tu'i Malila) are dominated by andesitic lava (41-45). Co-registered measurements of the physicochemical habitat within the animal collections, as well as characterization of vent end-member fluids from within each field, provide contextual geochemical information for these samples. Both host and symbionts were subject to phylogenetic analyses, and symbiont population compositions from all individuals were quantified via quantitative PCR. Select samples were also analyzed for stable carbon isotopic content. Collectively, these data reveal striking patterns of both host and symbiont (holobiont) distribution along an approximately 300 kilometer length of the ELSC. The observed patterns in holobiont distribution correlate to differences in vent fluid composition along the ELSC, implicating *Alviniconcha* symbionts in governing the distribution of their hosts among vent fields. These data provide the first evidence that symbiont complement might influence niche partitioning within a closely related group of animals, and in this case, as a consequence of differences in geochemical composition along the entire spreading center, yield regional-scale patterns of holobiont distribution.

RESULTS

Phylogenetic analysis of the host mitochondrial CO1 gene: We successfully amplified mitochondrial CO1 from 274 host individuals and recovered a total of 56 haplotypes (Table S2). These haplotypes were distributed among three major clades with high (> 0.95) posterior support, corresponding to three host types from the southwestern Pacific, and are called Type 1 (HT-I), Type 2 (HT-II), and Type Lau (which we renamed here HT-III) (Fig.2). Only HT-III has been previously described from the Lau Basin (38). Our results corroborate the *Alviniconcha* phylogeny as published in Suzuki et al. 2006 (38), in which one major clade includes HT-I, HT-III and *A. hessleri* (from the Mariana trench) and the second major clade includes HT-II and *A.* aff. *hessleri* (from the Indian Ocean). For HT-I and HT-II, reference sequences AB235211 and AB235212 were each identical to the most common experimental haplotype in their respective clade; AB235215, representing HT-III, was identical to a relatively rare haplotype in our dataset, but had only one nucleotide difference from the most common HT-III haplotype. The three host types found on the ELSC were divergent from those observed in the northwestern Pacific (Mariana Trench) and the Indian Ocean.

Some structure was apparent within the major host types in our sample; within HT-III, a clade including 11 of the 22 HT-III haplotypes was supported with a posterior probability approaching 1.0. While structure was also apparent in other host types, none was resolved with a posterior probability exceeding 0.9.

Phylogenetic analyses of symbiont 16S rRNA genes: Based on 16S rRNA gene sequences, three symbiont phylotypes were found to be associated with ELSC *Alviniconcha*, two of which had not been previously observed in this region. Longer sequences were generated from clones of each phylotype for phylogenetic analysis (Fig.3) and revealed that the three phylotypes are closely related to the previously published sequences for the γ- and ε-proteobacterial endosymbionts from *Alviniconcha* in this and other hydrothermal systems in the southwestern Pacific (Manus and North Fiji basins) (36-38). One of the γ-proteobacterial symbiont phylotypes (γ-Lau) was most closely related to the previously published symbiont sequence from *Alviniconcha* in the Lau Basin (98% sequence identity) (38). The second γ-proteobacterial symbiont phylotype (γ-1) and a ε-proteobacterial symbiont phylotype were most closely related (96-97% and 97% sequence identity, respectively) to *Alviniconcha* symbionts previously observed in the North Fiji and Manus basins (38).

Proportion of symbiont phylotypes within *Alviniconcha*: Quantification via qPCR revealed that all *Alviniconcha* individuals analyzed were dominated (>67% of total detected 16S rRNA genes) by either γ- or ε-proteobacterial endosymbionts. The dominant phylotype on average represented 99.5 ± 2.2% S.D. of the total symbiont gene counts within all individuals (Fig.4). We never observed individual snails with approximately equal representation of γ- and ε-proteobacteria, although we did observe individuals with roughly equal representation of the two γ-proteobacterial phylotypes. Accordingly, we refer to *Alviniconcha* individuals as primarily hosting either γ- or ε-proteobacterial endosymbionts.

Relationships among symbiont phylotypes and host types: Our qPCR analysis also revealed specificity among the three host types and three symbiont phylotypes. One-way ANOSIM comparing the symbiont composition among the different host types demonstrated that each host type associated with significantly different symbiont populations (Global $R=0.789$, $p<0.001$; Fig. 4). HT-II were exclusively dominated by ε-proteobacteria, with ε-proteobacteria always representing >99% of the detected symbiont genes. Accordingly, we found no significant differences in the symbiont population among HT-II from three different vent fields (one-way ANOSIM Global $R=0.312$, $p=0.07$). HT-III, conversely, were exclusively dominated by γ-proteobacteria, either γ-1 or γ-Lau. A small number of HT-III individuals (n=7, later called "γ-Both") had relatively equal proportions of both γ-proteobacterial phylotypes. HT-III was found at the two southernmost vent fields (ABE and Tu'i Malila); however, due to the presence of just one HT-III individual at ABE, we were unable to statistically test the effect of geography on symbiont population composition in this host type. Finally, HT-I was dominated by either γ-1 or ε-proteobacteria, though HT-I was most commonly dominated by γ-1 not the ε-proteobacteria (n=93 vs. 6 individuals respectively). In this host type, the associated symbiont population displayed different patterns of symbiont fidelity according to geography. HT-I was found at all four vent fields; however, the dominant symbiont phylotype changed from north to south. Five of twelve HT-I individuals in the northern vent fields were dominated by ε-proteobacteria, compared to only 1 of 87 HT-I individuals in the southern vent fields. This was confirmed via one-way ANOSIM comparing the symbiont population of HT-I by location, which demonstrated that there

were significant differences among HT-I individuals from the different vent fields (Global R=0.385, $p<0.001$).

Geographic patterns in the abundance of *Alviniconcha* host types: The distribution and abundance of each host type varied geographically from north to south (Fig. 5). HT-I was found at all four vent fields, HT-II was found at three vent fields but not Tu'i Malila, and HT-III was found at the two southernmost vent fields ABE and Tu'i Malila. With respect to their relative abundance, *Alviniconcha* populations at the northern vent fields were mainly HT-II, while populations at the southern vent fields were mainly HT-I and HT-III. The relative abundances of host types in the northern two vent fields (Kilo Moana and Tow Cam) versus southern two vent fields (ABE and Tu'i Malila) were significantly different (Global R=0.34, p=0.03).

Geographic abundance of symbiont phylotypes: The abundance of symbiont phylotypes associated with *Alviniconcha* changed along the spreading center (Fig. 5). Individuals dominated by symbiont γ-1 were present at all four vent fields. Individuals dominated by ε-proteobacteria were present three vent fields but not Tu'i Malila. Individuals dominated by γ-Lau were only observed at Tu'i Malila. The dominant symbiont phylotypes in *Alviniconcha* from the two northern vent fields (Kilo Moana and Tow Cam) were significantly different from the southern two vent fields (ABE and Tu'i Malila) (One-Way ANOSIM, Global R=0.409, p=0.024). Specifically, the majority of *Alviniconcha* at the northern vent fields (Kilo Moana and Tow Cam) were dominated by ε-proteobacteria, while the majority of *Alviniconcha* at the southern vent fields (ABE and Tu'i Malila) were dominated by one of the two γ-proteobacterial phylotypes.

Chemistry and temperature at *Alviniconcha* habitats: Chemical and thermal measurements were taken upon the cleared substratum after *Alviniconcha* collections were completed (Table S1, Fig. 6). Free sulfide concentrations in the vent fluids of the northern-most *Alviniconcha* habitats were significantly greater than those of the southern-most habitats (Mann-Whitney U, p=0.038). Though we happened to sample more chimney wall habitats in the north, this does not explain the significant difference in sulfide concentrations between northern and southern fields. Indeed, when grouped by habitat type regardless of region, diffuse flows and chimney wall habitats measured here did not have significantly different sulfide concentrations (Mann-Whitney U, p=0.126, Table S1; Fig.6). This is true for diffuse flows and chimneys within the same region as well (Mann-Whitney U, p=0.182 and p=0.102, north and south respectively). We also did not detect any significant differences in the oxygen concentrations or temperature of the vent fluids among the sample collection sites in the northern and southern vent fields (p=0.180 and p=0.118 respectively).

End-member vent fluid chemistry: End-member aqueous concentrations of hydrogen sulfide (H_2S) and hydrogen (H_2) reveal along-axis geochemical variations from north to south (Fig. 7). End-member aqueous H_2 concentrations varied from 220 to 498 μM in the northernmost vents (at Kilo Moana) and decreased to the south to concentrations that varied from 35 to 135 μM in the southernmost (at Tu'i Malila) vent fluids, nearly an order-of-magnitude difference in concentration. End-member dissolved H_2S

concentrations exhibit a similar trend from north to south, although the ~2-fold change in concentration of 4.9 to 2.8 mM from north to south respectively, is substantially less than was observed for aqueous H_2. In contrast to H_2 and H_2S, end-member CH_4 concentrations in 2009 occupied a very narrow range of 33 to 44 μM and showed no along-axis trends (Fig. 7). End-member aqueous DIC concentrations were highest in the Tu'i Malila vent fluid, reaching a value of 15 mM, and lowest in ABE vent fluids where concentrations varied from 5.4 to 7.0 mM, with fluids from the other vent fields containing intermediate concentrations of DIC (Fig. 7). End-member CH_4 and DIC concentrations did not change markedly from 2005 to 2009.

Stable carbon isotopic composition according to dominant symbiont phylotype: Across the ELSC, the average $\delta^{13}C$ value for gill tissue from *Alviniconcha* dominated by ε-proteobacteria (−11.6 ± 0.4‰ S.D.) was much less depleted than the average value of *Alviniconcha* dominated by γ-proteobacteria (−27.6 ± 2.3‰ S.D.) (Table S5). A one-way ANOVA of Tu'i Malila γ-proteobacteria hosting individuals grouped by dominant symbiont phylotype γ-1, n=23; γ-Lau, n=21; γ-Both, n=8), irrespective of host type, showed that there were significant differences among the groups ($p<0.001$). Tukey's multiple pairwise comparisons showed that individuals dominated by γ-Lau were not significantly different from γ-Both individuals ($p=0.834$), while individuals dominated by either γ-Lau or γ-Both were significantly less depleted than individuals dominated by γ-1 ($p=0.001$, $p=0.004$, respectively). We were unable to compare the possible effects of host type on the stable carbon isotopic composition with this sub-set of individuals, since we did not have enough individuals of different host types with the same dominant symbiont phylotype for statistical analysis.

DISCUSSION

These analyses — which were based on an extensive sampling effort in four different vent fields along the length of the ELSC — uncover previously cryptic, regional-scale patterns in the distribution of *Alviniconcha* holobionts. Our results suggest that regional-scale gradients in geochemistry, which are the surficial expression of sub-surface tectonic processes and water-rock interactions respectively, influence niche availability –and thus partitioning- among hydrothermal vent symbioses. Specifically, we observed striking patterns in the distribution of *Alviniconcha* host types, wherein *Alviniconcha* associated with ε-proteobacteria were substantially greater in abundance at the northern-most, basaltic vent fields (Kilo Moana and Tow Cam). Conversely, *Alviniconcha* associated with γ-proteobacteria were found in greater abundance at the andesitic southern vent fields (ABE and Tu'i Malila) (42, 43). We observed further basin-wide geographic trends in *Alviniconcha* individuals hosting different γ-proteobacterial symbionts, including the absence of individuals dominated by the γ-Lau phylotype from all but the Tu'i Malila vent fields. Together, with geochemical data from high-temperature and diffuse vent fluids from these vent fields, our results indicate that niche partitioning within a genus of chemosynthetic symbioses at deep sea hydrothermal vents is linked to subsurface geological/geochemical processes. These data suggest that interactions between symbionts and the physicochemical habitat, rather than host physiology alone, can govern the distribution of hydrothermal vent symbioses across a biogeographical province.

Symbiont and Host Diversity and Association: Cryptic diversity revealed here reshapes our understanding of the biogeography of this genus. Prior to this study, only HT-III (previously called host type Lau) and one symbiont phylotype (γ-Lau) had been documented in the Lau Basin (38). Our phylogenetic surveys uncovered two additional host types (HT-I and HT-II) and two additional symbiont phylotypes (γ-1 and ε-proteobacterial) within the ELSC. Collectively, these data establish the ELSC as the geographic area with the highest documented diversity for this genus, with the greatest number of host types and symbiont phylotypes compared to any other region. It is possible that *Alviniconcha* hosts and symbionts are comparably diverse at other western Pacific and Indian Ocean vent systems, although this remains to be determined (36-38, 40). Regardless, the data herein have revealed unforeseen holobiont diversity within the genus *Alviniconcha* and emphasize the value of interrogating both host and symbiont identity — at an appropriate sampling scale — to capture cryptic phylogenetic diversity.

The observed patterns of association among the host and symbiont phylotypes were most surprising. 16S rRNA gene qPCR of all sampled individuals revealed that *Alviniconcha* host types exhibited varying degrees of specificity for their symbionts. *Alviniconcha* HT-II solely associated with ε-proteobacteria. HT-III hosted mixed populations of the two γ-proteobacterial phylotypes (γ-Lau and γ-1). Notably, HT-I associated with both γ- or ε-proteobacterial endosymbionts, sometimes within the same individual (though it was always dominated by one). This phenomenon of a single snail simultaneously hosting two symbionts from distinct bacterial classes has not been previously observed. While some species of *Bathymodiolus* hydrothermal vent mussels are known to associate with two endosymbiotic γ-proteobacterial phylotypes (46-48), the ability of an *Alviniconcha* individual to host endosymbionts from two distinct bacterial classes is unprecedented among chemosynthetic symbioses. These symbionts are thought to be environmentally acquired (49), and the observed patterns of symbiont distribution among host types suggests an interplay between host specificity and environmental determinants. This may play a profound role in structuring the distribution of *Alviniconcha* host types across available niche space.

Holobiont distribution and basin-wide geochemical gradients: Further investigation revealed that the holobionts exhibited a structured pattern of distribution across the four vent fields. While *Alviniconcha* HT-I and the symbiont γ-1 were represented at all four vent fields, individuals dominated by the symbiont γ-Lau were observed at only one vent field (Tu'i Malila), and only one HT-III individual was found outside of Tu'i Malila. Structured distributions of marine fauna often result from geographical isolation or other barriers to dispersal (50, 51). However, the representation of host HT-I and symbiont phylotype γ-1 among all the vents studied here, combined with our recovery of host haplotypes identical to previously-collected individuals from thousands of kilometers away, suggests that the existence of such barriers is unlikely. *Alviniconcha* are thought to produce far-dispersing planktotrophic larvae (52), and studies of deepwater circulation in the ELSC have revealed continuity among the sites (53). Thus, the potential for geographic isolation due to limitations on larval dispersal or deepwater circulation along the ELSC seems low.

Geological and geochemical gradients along the spreading center better explain the observed holobiont distributions. The ELSC comprises a series of vent fields in the

Lau back-arc basin created by the subduction of the Pacific plate under the Indo-Australian plate. As the ELSC proceeds from north to south, it approaches the volcanic arc, resulting in an increased influence of the subducting Pacific plate on the crustal rocks (54-56). Consequently, there is a change in crustal rock type, with vent fields in the north being dominated by basalt and vent fields in the south being dominated by basaltic-andesite and andesitic lavas (42, 43). The increasing influence of the subducting slab is reflected in the changing geochemical composition of vent fluids north to south along the spreading center, including sizeable differences in dissolved volatile concentrations (28, 44, 45). Our analyses of high-temperature vent effluents from among the sampling sites revealed variations in gross geochemical composition along the ELSC that appears to be stable over time (44, 45). Both H_2 and H_2S concentrations decrease from north to south, with H_2 showing about an order of magnitude difference in concentration in end-member fluids from Kilo Moana in the north (~500 μM) to Tu'i Malila in the south (~43 μM). As there is often a correspondence between the geochemical composition of a diffuse flow and nearby high-temperature flow (57-59), the elevated H_2 and H_2S concentrations in the high temperature fluids at the northern vent sites likely correspond to higher concentrations of these chemical species in the cooler vent fluids bathing the *Alviniconcha* at these fields. Indeed, *in situ* voltammetry of vent fluids from among the collections corroborated the above geochemical trend and established that sulfide concentrations were higher among the *Alviniconcha* aggregations in the northern vent fields, though temperature and oxygen concentrations were not significantly different among the collection sites.

Niche Partitioning: If there are functional differences among *Alviniconcha* symbionts, then each host type's specificity for a particular symbiont would influence its capacity to exploit different physicochemical niches. Given the aforementioned distribution of phylotypes and the seeming lack of barriers to dispersal, we posit that the observed patterns of distribution of *Alviniconcha* across the ELSC relates to the gradients in vent fluid geochemistry (Fig.7). Holobionts with ε-proteobacterial symbionts dominated in fields with higher H_2 and H_2S concentrations, and conversely holobionts with γ -proteobacterial symbionts were in greater abundance at fields with lower H_2 and H_2S. This is consistent with studies of free-living ε- and γ-proteobacteria in sulfidic environments, which found that ε-proteobacteria dominate over γ-proteobacteria in habitats with higher sulfide (60-62). Both H_2 and sulfur oxidation are known to be common metabolisms among the close relatives (i.e. *Sulfurimonas* spp.) of the ε-proteobacterial symbionts (60, 63-65) and we hypothesize that one or both of these is supporting autotrophy in this phylotype. Previous studies of *Alviniconcha* symbiont metabolism have focused on sulfide oxidation *in vivo* and *in vitro* (39, 66), but did not identify the symbionts, so it is unclear which phylotypes are engaged in this metabolism. We observed that holobionts with ε-proteobacteria did not have visible sulfur granules in their gills, which is a known intermediate in some sulfur oxidation pathways. In contrast, holobionts with γ-proteobacteria had elemental sulfur in their gills, suggesting different modes of sulfur metabolism. This too is consistent with studies of sulfur oxidation by ε- and γ-proteobacteria, which are known to employ different pathways (as reviewed in (60)). We recognize that other factors, yet to be determined, could be influencing the north to south partitioning of ε-and γ-proteobacterial symbionts, as well as the

distribution of holobionts with γ-Lau and γ-1, along the ELSC. Further work identifying the specific reductants and pathways utilized by the three symbiont phylotypes is needed to better understand the connection between symbiont physiology and the observed habitat partitioning.

We also observed evidence for niche partitioning at a local (vent field) scale. Most collections were dominated by holobionts associating with one particular symbiont type (e.g. HT-I and II both hosting ε-proteobacterial symbionts in collection TC-2; Fig 5). This patchiness does not strictly correspond to habitat type (chimney wall vs. diffuse flows), because collections from both habitat types in the north were dominated by ε-proteobacterial symbionts and, conversely, by γ-proteobacterial symbionts in the south. There are anomalous collections from Kilo Moana and ABE, which deviate from the overarching patterns of distribution in this study, that may be reflective of local patchiness in geochemistry. Indeed, if habitat conditions are driving these patterns, we would expect local variation in chemistry to result in patchy holobiont distribution even within a vent field. Unfortunately, we did not collect environmental data at these specific sites, so we cannot determine whether these collections were associated with different geochemistry. While higher resolution sampling of *Alviniconcha* with associated fine-scale chemical measurements is necessary to understand the extent of intra-field habitat partitioning by these symbioses, the existing data suggest interactions between the symbionts and the environment.

Previous studies have hypothesized that differences in the oxygen tolerance of the carbon fixation pathways employed by the γ- and ε-proteobacterial symbionts could influence habitat utilization by the different *Alviniconcha* symbioses (38, 61). Indeed, our measurements of carbon stable isotopic composition are consistent with the use of different carbon fixation pathways by the γ- and ε-proteobacterial symbionts (Table S5). However, the oxygen concentration in the habitats occupied by individuals with the γ- and ε-proteobacterial symbionts was not significantly different. Moreover, it is unlikely that environmental oxygen concentrations are experienced by the symbionts because host oxygen-binding proteins, such as the gill hemoglobin of *Alviniconcha* (67), have a high affinity for oxygen and will govern its partial pressure within the host's tissues. With respect to differences in host physiology influencing the observed distribution patterns, little is known about differences in thermal tolerance or chemotolerance among *Alviniconcha* host types (66). Sulfide tolerance has been suggested to affect animal distribution at vents (23, 27, 68) and is significantly different among collections dominated by the different *Alviniconcha* holobionts at the ELSC. However, the highest sulfide levels detected among the snails in our collections are well below the tolerance limits reported from shipboard experiments on *Alviniconcha*, and thus host tolerance for sulfide is unlikely to be responsible for the patterns we report (66). Additionally, temperature and oxygen concentrations — two key factors often invoked in governing the distribution of animals at vents (23) — were not significantly different among our collection sites. Though both host and symbiont physiology undoubtedly influence the overall niche of these holobionts, we suggest that host physiology is unlikely to be playing a major role in the habitat partitioning observed here.

Conclusions: For vent holobionts, access to vent-derived chemical resources (reduced compounds for chemoautotrophy) requires physical proximity to the emitted vent fluid, as evidenced by the strong association of chemosynthetic symbioses with vent fluid

emissions (e.g., (28)). Competition among these holobionts for chemical resources takes the form of competition for the limited space near vent flows. Within a chemically heterogeneous vent system such as the ELSC, with spatial variability in the composition of vent fluid, resource partitioning among symbioses appears to occur via the differential distribution of the symbioses across the range of geochemical milieus. Here, for the first time, we observed this process occurring both within a genus and at a regional scale, with differential distribution of holobionts among distinct vent fields that are tens of kilometers apart.

In many ecosystems, niche partitioning has been shown to facilitate the coexistence of ecologically similar taxa (as reviewed in (3)), which has generally been considered in the context of the intrinsic differences in organisms, not in differences in their symbionts. Despite growing knowledge of the ubiquity of symbioses in the natural world, evidence for their effects on niche partitioning among similar hosts is surprisingly rare. In a few animal-microbial symbioses, namely coral-algal and aphid-bacterial associations, studies have correlated microbial symbiont genetic and physiological diversity to niche partitioning by the symbioses. In these cases, specificity in partnering among physiologically distinct endosymbiont phylotypes and genetically distinct hosts has been found to correspond to the distribution of corals in different light and temperature regimes on reefs (69-74) or aphids on different plant types (75-77). Prior to this study, research on the relationship between symbiont identity and environmental geochemistry at hydrothermal vents examined how differences in symbiont phylotype and abundance varied within a single species of mussel as a function of habitat (47, 78-80). It is now apparent that the process of symbiont-influenced niche partitioning among genetically distinct hosts is likely playing a role in structuring vent ecosystems, and is driven by subsurface geological and geochemical interactions. The influence of symbiont metabolism on host niche utilization is fundamental to our understanding of hydrothermal vent symbioses and vent ecosystems. With increasing awareness of the prevalence of microbe-animal interactions in our biosphere, the process of symbiont driven niche partitioning is likely to be elemental in other biological systems as well.

METHODS

***Alviniconcha* specimens:** 288 *Alviniconcha* specimens were collected from four vent fields in the ELSC using the ROV *JASON II* during expedition TM-235 in 2009 on board the RV *Thomas G. Thompson* (Fig.1, Table S1). Sites were haphazardly chosen, and live specimens were collected using modified "mussel pots" (81, 82) or large scoop nets, then returned to the ship in insulated containers. On board ship, live specimens were kept in chilled (4°C) seawater until dissection. Symbiont-containing gill tissues were dissected shipboard and frozen immediately at −80°C. The frozen tissue remained at −80°C until it was subsampled for DNA extraction and carbon isotope analysis.

Free sulfide, oxygen and temperature determination via *in situ* voltammetry: *In situ* voltammetry and a temperature probe were used to determine free sulfide and oxygen concentrations, as well as fluid temperatures, associated with a subset of the *Alviniconcha* collections (Table S1) (83, 84). Measurements were made in the same manner for both the diffuse flows and chimney walls. Briefly, animals were collected, then between 1 and 12 scan sets were performed with the tip of the probe directly on the cleared substrate.

Each scan set was comprised of seven to twelve discrete measurements (scans), which were then averaged. At the diffuse flow sites, measurements were made on the cleared substratum after the animal collections. At the chimney wall sites, the probe was positioned directly along the side of the structure after the animal collections, perpendicular to chimney wall, so that the tip was touching -or was within a cm of touching- the chimney wall (based on the laser scale from the *ROV Jason*). In all cases, shimmering water was often visible, and temperatures were never higher than 60°C. The instrument's quantitative limits of detection for free sulfide and oxygen are 0.2 μM and 15 μM respectively. For statistical analyses, values below the quantitative limits of detection were treated as in Podowski et al. 2010 (28).

End-member vent fluid sampling and analyses: Hydrothermal fluids were recovered from high temperature orifices (temperatures ranged from 268–320°C) using the ROV *Jason II* and isobaric gas-tight fluid samplers (85) during expedition TM-236 in June-July 2009 on the RV *Thomas G. Thompson.* Samples were analyzed for dissolved methane (CH_4), hydrogen sulfide (H_2S) and dissolved inorganic carbon (DIC). Dissolved CH_4, DIC and hydrogen (H_2) were also measured in vent fluids at the ELSC in April-May 2005 during expedition TUIM05MV on the RV *Melville*, at the same vent fields sampled during this study (see Mottl et al. 2011 (44) for 2005 sample information). All fluid samples were processed via gas chromatography or gravimetry as in Mottl et al. 2011 (44). See SI Methods for details of end-member calculations.

DNA extraction: Approximately 25 mg of gill tissue was sub-sampled while frozen for DNA extraction. Each subsample was placed into one well of a 96-well plate containing a proprietary lysis buffer from the AutoGenprep 965/960 Tissue DNA Extraction kit (AutoGen,Inc.) and DNA was extracted with the AutoGenprep 965 automatic extraction system. Prior to downstream analysis, all DNA extracts were diluted 1:100 in molecular-grade sterile water to minimize the effect of any co-extracted inhibitors on downstream molecular analysis.

Phylogenetic analysis of the host mitochondrial CO1 gene: DNA extracts from all *Alviniconcha* individuals were used as template to amplify the cytochrome C oxidase subunit 1 (CO1) mitochondrial gene, and the resulting amplicons were cleaned, trimmed and aligned, then used to produce a Bayesian inference phylogeny using the SRD06 model of nucleotide evolution (86), which partitions protein coding sequence into first + second and third codon positions, estimating parameters for each. Details of these analyses can be found in the Supp. Methods. Host CO1 gene sequences were deposited in GenBank, and accession numbers are presented in Table S2.

Phylogenetic analysis of symbiont 16S rRNA genes: Universal bacterial primers were used to amplify symbiont 16S rRNA genes from the DNA extracts of 30 individuals from ABE and Tu'i Malila. A clone library was constructed from the pooled amplicons of individuals from each vent field and sequence diversity was assessed via partial sequencing of clones (see SI Methods for Genbank accession numbers). The clones were found to represent three phylotypes with >96% identity to previously sequenced *Alviniconcha* symbionts. Bidirectional sequencing of clones representative for each

symbiont phylotype yielded longer sequences (accession numbers JN377487, JN377488, JN377489), which were cleaned, trimmed and aligned with other 16S rRNA gene sequences from both free-living and symbiotic Proteobacteria, then used to produce a Bayesian inference phylogeny with BEAST (87) implementing the GTR+I+G model of substitution. Details of these analyses can be found in the SI Methods.

Symbiont quantitative PCR assay development: SYBR Green quantitative PCR (qPCR) primers (Table S3) were designed for the three symbiont phylotypes using the aforementioned 16S rRNA gene alignment. Each phylotype assay was designed to target *Alviniconcha* symbiont 16S rRNA gene sequences from this study and others to capture intra-phylotype sequence diversity. See SI Methods for details of qPCR assay design and optimization.

Assessing symbiont composition via qPCR: To confirm that our subsamples yielded symbiont populations typical of the entire gill, we took 3 subsamples each from the whole gills of six individuals (at either end and the middle of each gill), extracted DNA as described above and found that the proportion of symbiont phylotypes varied by <1% among subsamples (Table S4). We accordingly estimated the proportion of each symbiont phylotype in the original *Alviniconcha* gill DNA extracts by applying all three qPCR assays to 2 µl of each sample (in duplicate), which were compared against duplicate standard curves and no-template controls, then averaged to determine copy number. Reactions in which the C_t was greater than the C_t for the lowest standard (10 copies) were documented as zero copies. Additionally, all quantities were adjusted for amplification inhibition (see SI Methods). Symbiont population within an individual were assessed by assuming each 16S rRNA gene to represent a single symbiont genome (see SI Methods for discussion of this assumption).

Analysis of carbon isotopic composition: Approximately 300 mg gill tissue was subsampled while frozen for carbon isotopic analysis. Samples were lyophilized for 24 hours, then acidified with 0.1 N HCl to remove any inorganic carbon contamination. The samples were subsequently dried for 24-48 hours at 50-60°C, homogenized to a fine powder and sealed within tin capsules. The carbon isotopic composition was determined by combustion in an elemental analyzer (Eurovector, Inc.) and separating the evolved CO_2 by gas chromatography prior to introduction to a Micromass Isoprime isotope ratio mass spectrometer (IRMS) for determination of $^{13}C/^{12}C$ ratios. Measurements are reported in δ-notation relative to the Peedee belemnite (PDB) in parts per thousand deviations (‰). Typical precision of analyses was ± 0.2‰ for $\delta\,^{13}C$. Egg albumin was used as a daily reference standard.

Statistical Analyses: Comparisons of the symbiont composition between *Alviniconcha* individuals at different vent fields and among the four host types was assessed via analysis of similarity (ANOSIM) using Bray-Curtis dissimilarity (88) (see SI Methods for details of ANOSIM). In these analyses, the symbiont composition for each individual represented an independent community profile. Additionally, the collections were also compared by classifying each individual based on its dominant symbiont phylotype (γ-1, γ-Lau, ε) or "γ-Both" (for the few individuals that hosted relatively equal proportions of

the two γ-proteobacterial symbionts). In these analyses, Bray-Curtis dissimilarity from standardized collection profiles was used.

One-way ANOVAs with post-hoc pairwise comparisons (Tukey's) were performed (SPSS Statistics v19) in order to compare the average carbon stable isotope values among individuals from the same vent field (Tu'i Malila) with different dominant γ-proteobacterial symbiont phylotypes.

To compare the temperature and environmental sulfide and oxygen concentrations from among the collections at all sites as measured via cyclic voltammetry, a non-parametric test (Mann-Whitney U, SPSS Statistics v19) was used. The statistical comparisons were conducted between the northern and southern vent fields, representing the habitats occupied by ε- and γ-proteobacteria-dominated *Alviniconcha*, respectively (see Table 1 for information on measured collection sites).

Acknowledgements: This material is based upon work supported by the National Science Foundation (OCE-0732369 to PRG, OCE-0732333 to CRF, OCE-1038124 and OCE-0241796 to JSS and OCE-0732439 to GWL, to RAB and JGS under GRF No. DGE-1144152). JSS also received support from the Woods Hole Oceanographic Institution. BF was supported by a Lavoisier research fellowship from the French Ministry of Foreign and European Affairs. We would like to thank the crews of the RV *Thomas G. Thompson*, RV *Melville* and the ROV *JASON II*. We would like to thank E. Podowski and S. Hourdez for extensive assistance in lab and on board ship, T. Yu and T. Galyean for preparing samples for stable isotopic analysis, and K. Fontenaz for her help with phylogenetic analysis. We thank C. Cavanaugh, A. Knoll, C. Marx and A. Jones for their helpful comments and discussion.

Author contributions: RAB, PRG and JGS designed the research. CRF and GWL directed the *in situ* collections and measurements. RAB, BF and JGS performed the shipboard sampling, dissections and molecular analyses. RWL performed the stable isotope analysis. JSS, SPS, GWL and AG performed the chemistry collections and research. RAB, JGS and BF analyzed the data. PRG, CRF and ELB assisted with analytical design and data interpretation. RAB and PRG wrote the manuscript with input from all co-authors.

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865 **Figure 1: (A)** Map of Eastern Lau Spreading Center (ELSC) depicting the four vent
866 fields sampled herein. Inset map shows location of ELSC in the South Pacific **(B)** A
867 typical assemblage of *Alviniconcha* (Al) and other vent animals in the Lau Basin
868 (Courtesy of James Childress). **(C)** An individual *Alviniconcha* snail.
869
870 **Figure 2:** Bayesian inference phylogeny of the *Alviniconcha* host mitochondrial CO1
871 haplotypes from this and previous studies, as well as sequences from the sister genus
872 *Ifremeria*, with boxes showing the three *Alviniconcha* host types found here. The
873 haplotype ID number is shown at the tip of each branch, with the gray bars representing
874 the total number of individuals recovered for each haplotype. Accession numbers for
875 haplotypes found in this study can be found in Table S3. Posterior probabilities are
876 indicated above nodes if >0.7.
877
878 **Figure 3:** Bayesian inference phylogenies of 16S rRNA sequences showing the three
879 *Alviniconcha* symbiont phylotypes found at the ELSC. All *Alviniconcha* symbionts, from
880 this study and others, are shown in bold. Gray highlight indicates the representative
881 sequences from this study. Boxes show the *Alviniconcha* symbiont phylotypes defined
882 here and in other studies. Posterior probabilities are indicated above nodes if >0.7. (A) γ-
883 proteobacterial phylogeny, with β-proteobacteria as the outgroup. (B) ε-proteobacterial
884 phylogeny, with δ-proteobacteria as the outgroup.
885
886 **Figure 4:** Ternary plots of the symbiont composition of each *Alviniconcha* host type,
887 with each point showing the symbiont composition of a single individual. The vertices of
888 the triangle represent 100% of each symbiont phylotype and the tick marks on the axes
889 represent decreasing intervals of 10%. The symbiont phylotypes are indicated by γ-1 (γ-
890 proteobacteria type 1), γ-Lau (γ-proteobacteria type Lau) and ε (ε-proteobacteria). Vent
891 fields are indicated by ● (Kilo Moana), □ (Tow Cam), ✕ (ABE), ▽ (Tu'i Malila).

892 **Figure 5:** The distribution of *Alviniconcha* host types and dominant symbiont type across
893 the ELSC, with each individual colored by dominant symbiont phylotype (>67% of the
894 total detected 16S rRNA genes) and shaped by host type. The four vent fields are
895 separated by solid lines and distinct collections from within each vent field are divided by
896 dashed lines, with the Collection ID indicated (see Table S1). Symbiont phylotypes are
897 indicated by colors: green, γ-proteobacteria type 1 (γ-1); yellow, γ-proteobacteria type
898 Lau (γ-Lau); blue, ε-proteobacteria (ε). Host types are indicated by shapes: ○ Host type 1
899 (HT-I); □ Host type II (HT-II); △ Host type III (HT-III); ◇ Host type undetermined.
900 The individuals that had relatively equal proportions of two of the symbiont phylotypes
901 are split into two colors.

902 **Figure 6**: Cyclic voltammetry measurements made on the cleared substratum after
903 *Alviniconcha* collections, showing **(A)** temperature, **(B)** free sulfide concentration

(sulfide) and **(C)** oxygen concentration at northern collections versus the southern collections. North (N) includes the vent fields Kilo Moana (KM) and Tow Cam (TC); South (S) includes ABE and Tu'i Malila (TM). Symbols with horizontal lines = samples from diffuse vent flows; symbols without lines = chimney wall habitats. Median values for each region are indicated by a dashed horizontal line.

Figure 7: The end-member fluid concentrations of **(A)** hydrogen (H_2), **(B)** hydrogen sulfide (H_2S), **(C)** methane (CH_4) and **(D)** dissolved inorganic carbon (DIC) at the four vent fields along the ELSC from which *Alviniconcha* were collected. Symbols indicate year of sampling: × (2005); ● (2009). DIC and H_2S data from 2005 were previously published in Mottl et al. 2011 (44).