Population and Feeding Characteristics of Hydrothermal Vent Gastropods Along Environmental Gradients with a Focus on a Bacterial Symbiosis Hosted by *Lepetodrilus fucensis* (Vetigastropoda)

By

Amanda Elizabeth Bates
B.Sc., Simon Fraser University, 1998

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Dr. V. Tunnicliffe (Department of Biology, School of Earth and Ocean Sciences)

Supervisor

Dr. L. Page (Department of Biology)

Departmental Member

Dr. R. Roy (Department of Biology)

Departmental Member

Dr. K. Gillis (School of Earth and Ocean Sciences)

Outside Member

Dr. K. Juniper (Center for research in Geochemistry and Geodynamics, Université du Québec à Montréal)

External Examiner

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Abstract

Three gastropods occupy a range of habitats along gradients in hydrothermal flux at Juan de Fuca Ridge vents. I examined how these species co-exist and identified mechanisms driving their abundances. First, I measured temperatures and spatial patterns in adult densities of the three species at three distances from vents to test if thermal regime relates to their habitat selection. *Lepetodrilus fucensis* and *Depressigyra globulus* were most dense in-vent (0-25 cm) at variable temperatures (10±5°C): 2100 and 240 ind. dm^{-2} (respectively). *Provanna variabilis* was most abundant far-vent (51-75 cm: 60 ind. dm^{-2}) at stable temperatures (3±0.5°C). Thermal conditions are key in their habitat selection; behavioural experiments showed that these gastropods select fluid temperatures

<18°C. *L. fucensis* and *D. globulus* preferred 5-15°C, while *P. variabilis* preferred 4-12°C.

The next studies sought to explain how *Lepetodrilus fucensis* reaches order of magnitude higher densities in comparison to other gastropods. First, I quantified *L. fucensis* recruitment and sex ratio patterns to identify innovative life history traits. I meaured size structure and density at in- and far-vent locations. Early postlarval juveniles occupied far-vent at remarkable densities (2419 ind. dm^{-2}). To test for sex ratio biases, I sexed animals from different habitats and sizes. Populations nearest vents hosted the largest females (>6.0 mm), while peripheral habitats were male-biased. A transplant experiment showed that female survivorship and gonad fullness were significantly lower than males in far-vent locations. Sex ratio biases are driven by two mechanisms: females maximize their reproductive output by selecting optimal habitats and suffer relatively higher mortality in low flux.

Next, I hypothesized that the *Lepetodrilus fucensis* gill symbiosis is a key adaptation. I used multiple approaches to determine if the prevalence of the association and relationship to the limpet's condition support this hypothesis. FISH probes specific to the 16S rRNA molecule of a gamma-Proteobacteria hybridized where bacteria were present. Direct sequencing using symbiont-specific primers gave a single unambiguous sequence, indicating high specificity. Light and TEM micrographs of gill tissue from a range of species also showed that the symbiosis is ubiquitous. In addition, the gills of in-vent animals had high surface area, dense symbiont populations and healthy tissues, while far-vent animals showed the reverse trend, suggesting that the symbiosis benefits *L. fucensis*. Carbon fixation by gill tissues was stimulated by inorganic sulfide and related

to the abundance of bacteria on the gill. These data indicate a persistent and specific symbiosis that is dependent on access to sulphide.

I further examined feeding by *Lepetodrilus fucensis* to determine if the bacteria contribute to their host's nutrition. The morphology of feeding structures were compared among *Lepetodrilus* species. *L. fucensis* exhibited specialized features: the gill is enlarged, the lamellae are free of the mantle, do not narrow and are stabilized by ciliary junctions. The radula and stomach of *L. fucensis* are also reduced. Shipboard observations confirmed suspension feeding by *L. fucensis*. In addition, the symbiont may be ingested because its phylotype was well-represented in food material on the gill. The limpet's morphological specializations are consistent with dependence on suspension feeding and/or symbiont farming; however, *L. fucensis* also grazes, a mechanism likely important in peripheral locations.

Lepetodrilus fucensis populations are partitioned by size and sex along environmental gradients near vents. Peripheral populations are dominated by recruits and adults tend to be male; grazing is likely their primary feeding mode. Larger animals form stacks in venting fluids and are female-biased. These populations access suspended particles for food and sulphide, which generates dense symbiont populations for ingestion. Multiple feeding modes sustain high *L. fucensis* densities in a space-limited environment and may be an innovative strategy that drives its remarkable abundances.

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I have been lucky to work with excellent hydrothermal vent biologists. Colleen Cavanaugh adopted me as a student for four months and, in spite of having almost no lab experience, gave me a bench space and pointed me in the direction of the PCR machine. It has been rewarding to work with someone who has such an unbounded love for invertebrate-bacterial symbioses and who provided a different perspective on the scientific process. Chuck Fisher also treated me like one of his students during a cruise to the East Pacific Rise and, with Janet Voight, gave me a seat on ALVIN to visit the bottom of the ocean to see newly formed basalt and the EPR fauna with my own eyes.

My research cruises were a highlight during my degree (yes, the memories of nausea are starting to fade). I am privileged to have worked with the ROPOS crew. In addition to their piloting skill and technical expertise, they continually tainted the control room with humor. Keith Shepherd, in particular, was supportive of my experiments in spite of technical challenges. My work was prioritized during several research cruises by principal investigators and, as a result, I was able to gain datasets that were pivotal to my

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Dedication

For my parents, who introduced me to the ocean.

CHAPTER 1

INTRODUCTION

Scientists were astounded to discover teeming communities of giant tubeworms and clams in anoxic hydrothermal fluids laden with toxic metals on the Galapagos Rift in 1977 (Corliss & Ballard 1977, Corliss et al. 1979). Further explorations have documented vent communities at seafloor spreading centers in all oceans at hydrothermally active sites on mid-ocean ridge spreading centers (Von Damn 1995). These communities are of special interest because they are sustained by geothermally driven chemosynthetic bacteria, a novel source of primary production. Many of the dominant fauna, such as vestimentiferan tubeworms (Cavanaugh et al. 1981, Felbeck et al. 1981), are gutless and gain nutrition from symbiotic bacteria within their tissues, while others may ingest bacteria cultivated on their body surfaces (Alayse-Danet et al. 1987). Primary consumers graze microbes from surfaces (e.g., scaleworms), remove free-living microbes suspended in vent effluent (e.g., serpulid polychaetes) (Hessler & Smithey 1983), and/or consume detrital material (e.g., Levesque et al. 2005).

Biological research over the last three decades has focused on describing a diverse set of physiological mechanisms employed by animals to withstand exposure to the toxic elements present in vent fluids (Bischoff & Seyfried 1978, Childress & Fisher 1992). Studies have also characterized trophic relationships between invertebrates and bacteria (Hessler & Smithey 1983, Cavanaugh 1994), the reproductive biology of many common species (Van Dover 2000) and provided insights into the evolution and biogeography of vent fauna (Tunnicliffe 1991, Van Dover et al. 2002). In addition, classic ecological

studies show that vent fluid chemistry controls species patterns and community structure (reviewed in Tunnicliffe 1991).

Hydrothermal vent ecology is developing along a similar path to coastal marine ecology and is transitioning from research dominated by descriptive work to experimental studies that test ecological hypotheses (Underwood et al. 2000, Van Dover & Lutz 2004). Initial research was primarily observational in nature because experimental studies are challenging to execute in deep-sea habitats (e.g., Hessler et al. 1985, Johnson & Tunnicliffe 1988). However, experiments at vents are currently on the rise due to advances in submersible technology that have led to more efficient use of bottom time and ease of equipment deployment (Van Dover & Lutz 2004). Such research is yielding exciting findings; for example, Micheli et al. (2002) deployed exclusion cages and discovered that predation by deep-sea zoarcid fish on mobile gastropods facilitates increased settlement by sessile invertebrates. The continued application of *in situ* experiments will likely aid in tackling ecological questions that remain unanswered in vent systems.

Hydrothermal activity and faunal patterns

Hydrothermal venting occurs globally along mid-ocean ridges where oceanic plates diverge and geological processes create new ocean crust. Ridges are segmented by transform faults, ridge offsets and other axial discontinuities and vent sites on these segments are comprised of vent fields, or clusters of vents. Vent-dependent communities may therefore be isolated on segments by tens to hundreds of kilometers (Seyfried & Mottl 1995). Topographic features of mid-ocean ridge systems, such as discontinuities in the ridge axis, can alter oceanographic circulation patterns and limit larval dispersal

among discrete vent communities, thus impacting species distributions and biogeographic patterns (e.g., Craddock et al. 1995, Van Dover et al. 2002). Although different segments tend to host similar species assemblages, the depth of each segment, its volcanic and tectonic history, and the intensity of venting can vary. Consequently, biological communities on different segments experience unique spatial and temporal patterns in hydrothermal activity that influence community longevity and extent.

The availability of hydrothermal fluid flow in space and time structures biological communities. Although the visually stunning black smoker chimneys that billow super-heated hydrothermal fluids (>350°C) from focused conduits are an icon of hydrothermal habitats, most megafauna are found in areas of diffuse venting where fluids are typically diluted by ambient seawater and cooled to temperatures below ~60°C (Figure 1.1). Vent fauna cluster around these low temperature fluids emerging from cracks in basalt and through porous sulphide structures as single or diffuse outflows to form a patchwork community that mirrors the complex pattern of low temperature venting. Over time, the persistence of a biological community depends on the longevity of a vent source; fast spreading ridges tend to host ephemeral vents that senesce within tens of years, while slow spreading ridges host vents that persist for thousands of years (MacDonald 1982, Campbell et al. 1988, Lalou et al. 1995).

Vent fluid chemistry also varies in space and time and influences community structure and successional patterns (Tunnicliffe et al. 1997, Luther et al. 2001, Marcus 2003). Emergent hydrothermal fluids are a mixture of chemically transformed seawater, volatile-rich vapor and metal-rich brine, and the character of emergent fluids is dependent

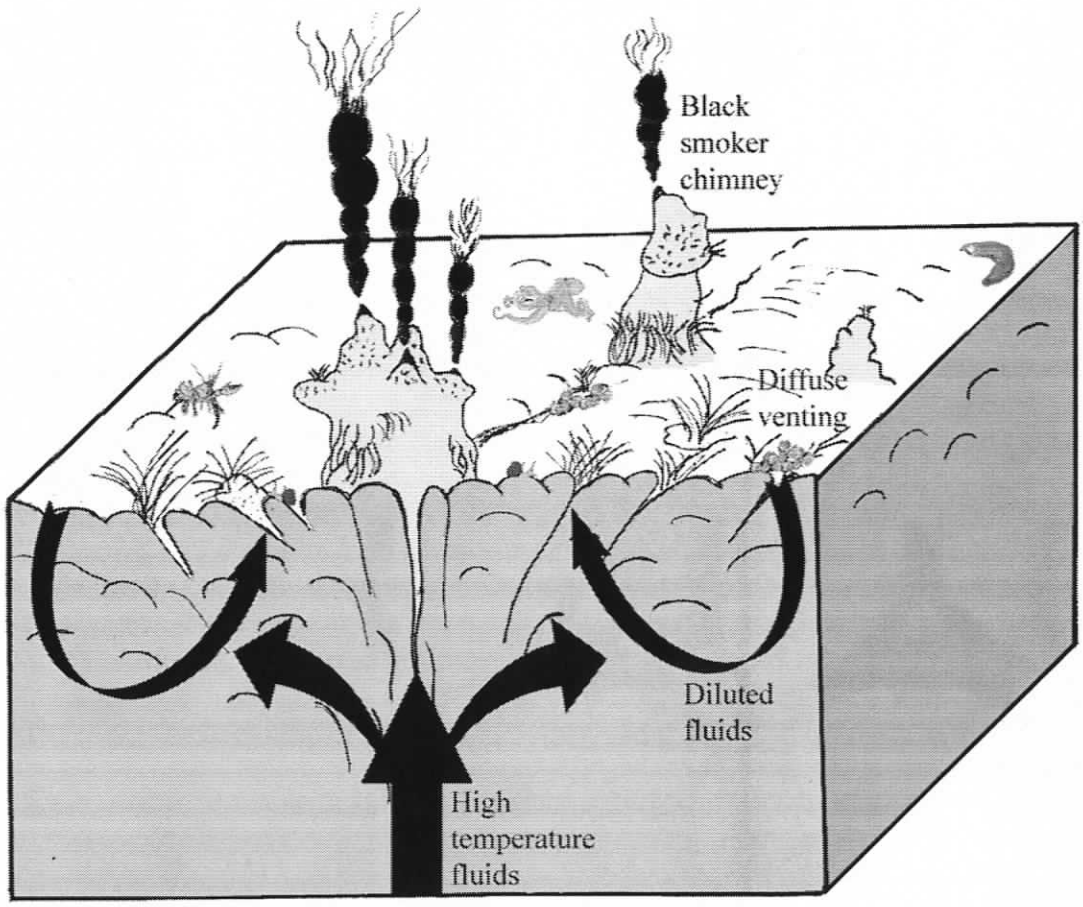

Figure 1.1
Schematic of a vent field showing focused flow (high temperature fluids) through black smoker chimneys and diffuse flow (low temperature diluted fluids) through cracks in basalt and near the bases of sulphide structures. Adapted from Tunnicliffe (1991).

on the relative contribution of each of these different components (reviewed in Kelley et al. 2002). As a result, vent fluid chemistry can differ between ridge segments and vent fields (e.g., Butterfield et al. 1994). Spatially, fluid chemistry can also differ among vents within a vent field due to differences in fluid transit time through the crust, chemical alteration of rock and subsurface interactions with microbial communities (Butterfield et al. 1997). In addition, fluid chemistry changes predictably over time following volcanic or tectonic events (e.g., chlorine, sulfide and temperature are initially high and then decline) (Butterfield et al. 1997).

Geological setting of study sites

My study sites were in the northeast Pacific Ocean where three intermediate rate spreading centers (~6 mm yr^{-1}) lie off the west coast of North America at the junctions with the Pacific plate: the Gorda, the Juan de Fuca and the Explorer Ridges (Crane et al. 1985) (Figure 1.2). The Juan de Fuca Ridge is comprised of seven principal segments (Baker & Hammond 1992). Studies in this thesis were conducted at hydrothermally active vent sites on two of these segments: Axial Volcano and Endeavour. In addition, Explorer Ridge was sampled where active venting is known on the southern-most segment (Tunnicliffe et al. 1986).

Axial: Axial Volcano is a one of the most seismically active sites on the Juan de Fuca Ridge and summits at 1500 m (45°58'N 130°03'W') in an elongate three-sided caldera (3 x 8 km) that lies between the two rift zones. Three major hydrothermal fields support similar biological communities near the caldera fault and along the rift zones (Marcus 2003). The ASHES and CASM vent fields host high temperature venting

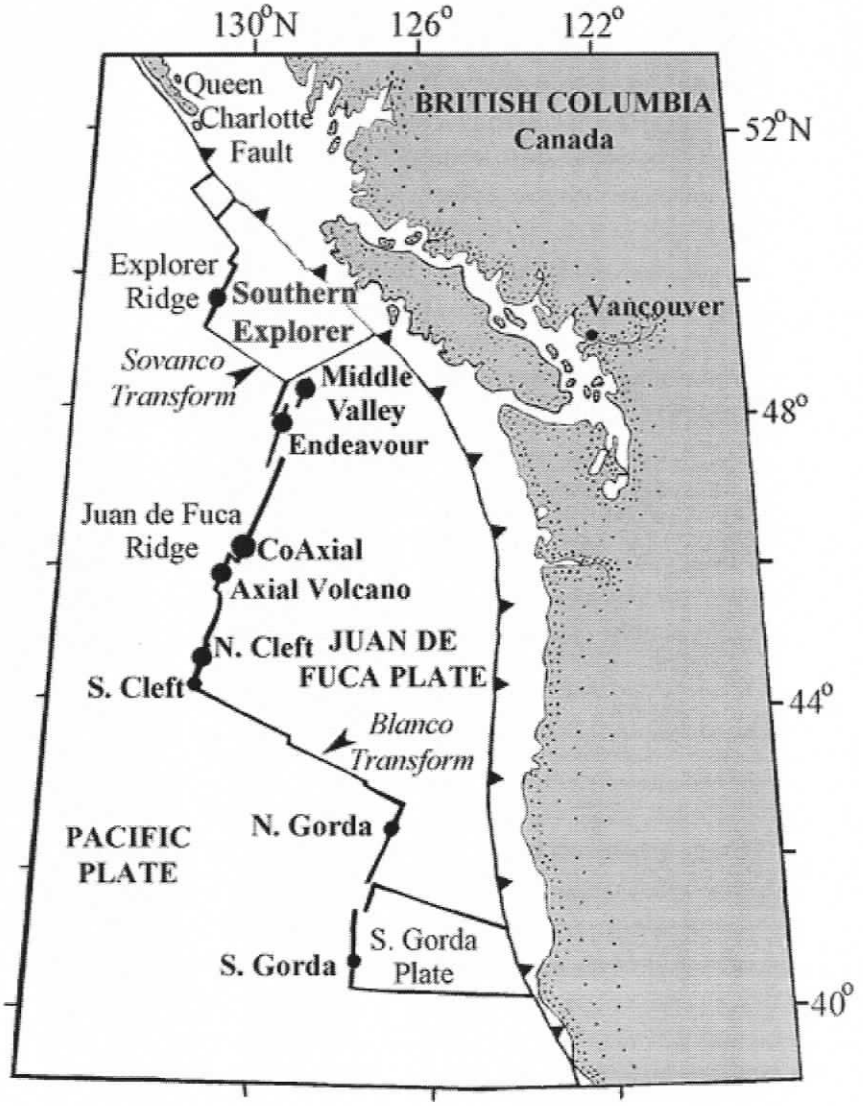

Figure 1.2
The Juan de Fuca Ridge in the northeast Pacific. Dots on the ridge represent segments with active vent sites. Samples in this thesis were from Axial Volcano and the Endeavour and Southern Explorer Segments. Adapted from Tunnicliffe et al. (1997).

through sulphide chimney complexes, while diffuse flows emerge through cracks in basalt on the 1998 Lava Flow vent field (Chase et al. 1985, Embley et al. 1990, Embley et al. 1999).

Endeavour: The Endeavour Segment vent site lies 220 km north of Axial at 47°57'N 129°06'W. Hydrothermal venting occurs primarily within a rift valley (1 by 10 km) on deep faults away from the main spreading area at depths from 2300 m in the south to 2170 m in the north. Four major vent fields are separated by ~2 km along the axial valley (Robigou et al. 1993). Vent fields are characterized by complex areas of diffuse lava vents and sulphide structures that host high temperature venting (>350°C).

Southern Explorer: The Explorer Ridge is relatively unsampled and lies at the northern end of the chain of spreading centers at 49°46'N 130°16'W, depth ~1700 m. Many areas of extinct sulphide chimneys occur within the rift valley. The Explorer Ridge vent site sustains long-lived hydrothermal activity; currently, most active venting occurs at four vent fields characterized by complex massive sulphide structures on a topographic high located outside the primary rift valley (Embley 2002). Megafauna are relatively sparse, although the dominant species are shared with the Juan de Fuca Ridge (Tunnicliffe et al. 1986).

Research focus

Hydrothermal vents are ideal systems to study the interaction of organisms with their environment at small-scales. Steep gradients in parameters such as fluid flux, chemical concentrations and biological productivity are maintained at centimeter scales around vent outflows and thus, species patterns are relatively easy to detect (Sarrazin et

al. 1999). However, after three decades of study, the biology and responses of many vent species to environmental factors are still poorly understood and, as a result, interpretation of community patterns is difficult (Marcus 2003). Whole organism studies focusing on the basic characters of dominant species are therefore important.

In the northeast Pacific, vent sites on the Juan de Fuca Ridge are among the best-studied (Figure 1.2). The most abundant species are typical of eastern Pacific fauna, e.g., vestimentiferan tubeworms and polychaetes; however, gastropods are also remarkably abundant and form dense colonies around diffuse flows (Tsurumi & Tunnicliffe 2003). *Lepetodrilus fucensis* McLean 1988 (Vetigastropoda, Lepetodrilidae) reaches densities an order of magnitude higher than other gastropods and forms prominent stacks in active hydrothermal fluid flow where it probably suspension feeds and grazes (Figure 1.3). *L. fucensis* also hosts epibiotic bacteria on its gill that appear to be phagocytosed by the epithelial membrane and digested in lysozomes (de Burgh & Singla 1984). Two abundant grazing snails co-occur with *L. fucensis*, *Depressigyra globulus* Warén and Bouchet 1989 (Neomphalida, Peltospiridae) and *Provanna variabilis* Warén and Bouchet 1986 (Caenogastropoda, Provannidae) (Marcus 2003, Tsurumi & Tunnicliffe 2003) (Figure 1.3B). Although fluid parameters are probably key in controlling the distribution of these three gastropods (Sarrazin & Juniper 1999, Marcus 2003), the mechanisms driving their habitat selection have not been investigated. It is also unknown what characteristics allow these species to co-exist at high abundances.

The overall objective of my thesis was to determine if these three species partition habitats near vents and to identify mechanisms driving their abundance patterns. I focused on *Lepetodrilus fucensis*, because it achieves densities (up to 100 000 individuals

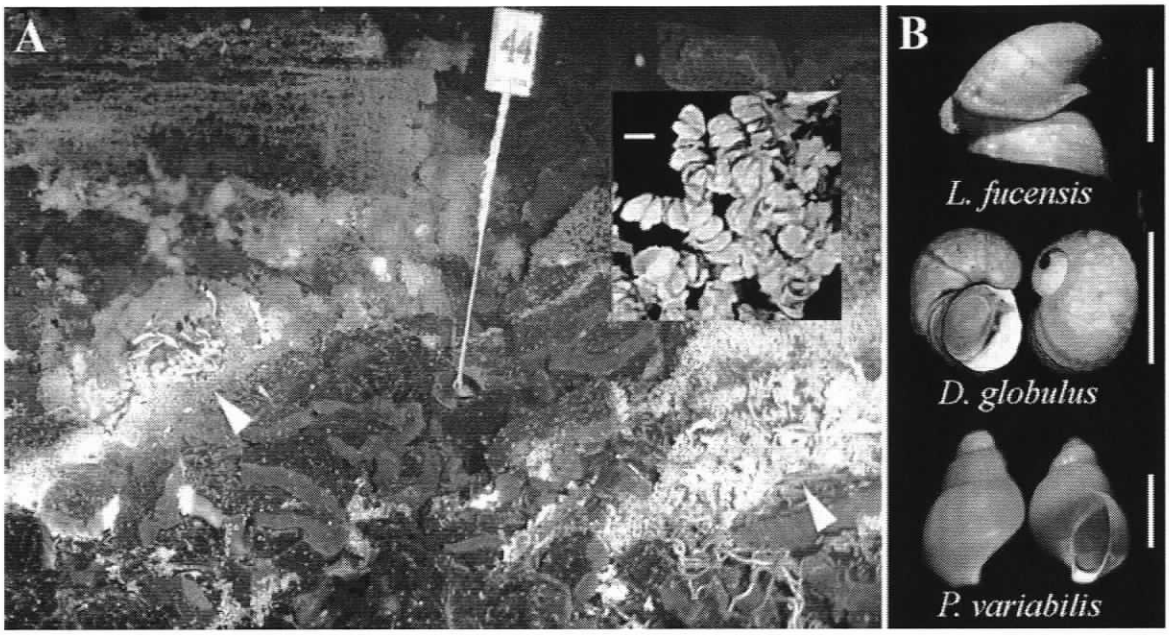

Figure 1.3
Gastropods from hydrothermal vents on the Juan de Fuca and Explorer Ridges. (A) Pillow basalts on a young lava flow near Mrk N44, Axial Volcano. Low temperature vent fluid emerges from meter-long cracks (arrows). Fauna clustered around venting fluids is dominated by the dense stacks of *Lepetodrilus fucensis* pictured in inset (scale bar is 1 cm) that reach densities of 100 000 m^{-2}. (B) The three most common gastropod species are *L. fucensis*, *Depressigyra globulus* and *Provanna variabilis* (scale bars are 5 mm).

m^{-2}: Sarrazin & Juniper 1999) one order of magnitude greater than *Depressigyra globulus* and *Provanna variabilis*. First, I determined the distribution patterns and processes controlling where these three species of gastropods occur and predicted that their habitat selection would relate to thermal regime. I then tried to identify innovative life history traits or feeding mechanisms of *L. fucensis* that might explain its high densities by comparing its population characteristics and feeding morphology to those of other gastropods. Last, I used molecular tools and microscopy to test if the *L. fucensis* association with epibiotic gill bacteria is beneficial to the limpet, thus suggesting a key adaptation.

Sampling strategy

I made an effort to sample vents from different substrata types, vent fields, segments and ridges so that any documented patterns could be widely generalized. In addition, I used multiple approaches. Observational studies included faunal collections, video imagery and high resolution pictures. Small-scale samples (<10 cm^{-2}) were made by suctioning animals into contained jars, while whole community collections (>25 cm^{-2}) included grab samples of tubeworm bushes and sulphide structures for recovery in closed boxes. *In situ* and shipboard experimental manipulations were also conducted.

Fluid heat provides a good relative measure of hydrothermal fluid flux, especially within a vent, because temperature is correlated to most vent fluid parameters, such as oxygen and sulphide (Johnson et al. 1986, Butterfield et al. 2004). It was therefore possible to sample along a gradient in environmental conditions with increasing distance from a single vent outflow based on fluid heat. Point temperature measures of different vent outflows also accompanied animal collections where possible to provide an

approximation of relative hydrothermal fluid flux. The collections in this thesis targeted vents where emergent fluids were less than 20°C.

In order to understand the response of different biological characteristics of *Lepetodrilus fucensis* populations to different habitat conditions and maximize the scientific gain from small-scale samples along a gradient in hydrothermal fluid flux and a transplant manipulation, these collections were subsampled to address several hypotheses. As a result, Chapter 3, 5 and 6 describe the same small-scale samples and transplant experiment.

Data chapter overview

(1) Chapter 2: *Role of thermal conditions in habitat selection by hydrothermal vent gastropods*. I was interested in what factors might drive the abundance patterns of *Lepetodrilus fucensis*, *Depressigyra globulus* and *Provanna variabilis*, and hypothesized that fluid heat is key in their habitat selection. I designed novel observational and experimental approaches to test if the small-scale abundance patterns of each gastropod related to variation in thermal conditions and their behavioural responses to temperature.

(2) Chapter 3: *Size and sex based habitat partitioning in hydrothermal vent gastropods.* My aim was to isolate factors that might provide insight into the remarkable abundances of *Lepetodrilus fucensis* by identifying its recruitment habitat and densities in comparison to *Depressigyra globulus*. I also examined its sex ratio patterns among and within *L. fucensis* populations to determine if the sexes show differential habitat selection. I further tested if males and females experience differential mortality in low flux environments by moving a large population of animals to the periphery of a vent for one year.

(3) Chapter 4: *A hot vent gastropod hosts a novel gill episymbiosis with gamma-Proteobacteria.* The nature of the symbiosis between *Lepetodrilus fucensis* and its gill bacteria was, prior to this thesis, little studied. The phylogenetic relationship of the symbionts to other bacteria and whether the bacterial symbionts were represented by one or multiple phylotypes were unknown. I used molecular tools to determine the phylogenetic identity of *L. fucensis* gill bacteria in relation to other chemoautotrophic symbionts and determine the specificity of the association from animals at different vent sites.

(4) Chapter 5: *Influence of habitat on the persistence and morphology of a symbiosis between a gastropod and gamma-Proteobacteria.* My aim was to test if the symbiosis benefits the host by characterizing its persistence and morphology, and testing if the condition of limpets relates to symbiont abundance. First, I determined the prevalence of the symbiosis over a range of animal sizes from vents representing a broad geographic range. I also examined host gill morphology, bacterial abundance and size, and host condition in high and low fluid flux to isolate biological characteristics of the symbiosis that are influenced by fluid regime. Last, I tested if the bacteria are thioautotrophic to explain the high bacterial abundance in vent flow.

(5) Chapter 6: *Feeding strategy, morphological specialization and the presence of bacterial episymbionts in lepetodrilid gastropods from hydrothermal vents.* My objective was to determine if the feeding strategy of *Lepetodrilus fucensis* is innovative and can explain its high densities. To do so, I compared the morphology of feeding structures among *Lepetodrilus* species with and without symbionts for evidence of differential reliance on grazing, suspension feeding or the symbiosis for nutrition. The feeding

behaviours of live *L. fucensis* in shipboard pressure vessels were also observed. Last, I characterized the food material present on the gill using microscope techniques.

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CHAPTER 2

Role of thermal conditions in habitat selection by hydrothermal vent gastropods

Chapter 2 is a research article in Marine Ecology Progress Series:

Bates AE, Tunnicliffe V, Lee RW (2005) Role of thermal conditions in habitat selection by hydrothermal vent gastropods. Marine Ecology Progress Series 305: 1-15.

Dr. Tunnicliffe (Thesis supervisor: University of Victoria) executed the time-lapse imaging study at Easter Island, provided the resources for this work and assisted with the writing of this article. Dr. Lee (University of Western Washington) designed and built the pressure vessels used in shipboard experiments.

In addition to work presented in Chapter 2, I completed a series of pilot experiments in experimental chambers that examined the responses of gastropods to temperature and sulphide gradients at surface pressures. The objectives, methods, results and discussion of this work appear in Appendix 2. 1.

Abstract

Habitat selection by three Juan de Fuca Ridge gastropod species relates to their thermal environment. Both collection and images along transects document the small-scale abundance patterns of each species with respect to temperature and distance from vent flows. *Lepetodrilus fucensis* and *Depressigyra globulus* were most abundant at distances of 0 to 25 cm in warm fluids with high temperature variability over several time scales (10±5°C). Both species were also abundant at 26 to 50 cm where temperatures were lower with less variability (4±1°C). *Provanna variabilis* was most abundant from

51 to 75 cm where temperatures were stable (3±0.5°C). All species were absent where maximum fluid temperatures reached 18°C and their substratum coverage was related to temperature. When presented with a choice in vent fluids from 10 to 2°C, *L. fucensis* and *D. globulus* moved to temperatures above 5°C, while *P. variabilis* showed no preference. In species-specific temperature preference experiments, *L. fucensis* and *D. globulus* accumulated between 5 and 13°C, while *P. variabilis* occupied areas with significantly lower temperatures from 4 to 11°C. These experimental temperature preferences correspond with their thermal environments. Upper temperature limits are moderate; extreme abiotic variability in higher temperature fluids may constrain these three species. We conclude thermal conditions are a primary determinant of habitat selection, thereby driving gastropod abundance patterns. However, other factors likely contribute. Space competition nearest vent flows may result in the displacement of individuals of these species to low quality habitats.

INTRODUCTION

The high variability of abiotic conditions in many extreme environments poses challenges to explaining species abundance patterns. Steep gradients of increasing harshness in physical and chemical conditions characterize hydrothermal vents where focused warm fluids rich in reduced compounds emerge from cracks in basalt or porous sulphide structures and mix with surrounding ambient seawater. This mixing generates rapid fluctuations in chemical variables at any fixed position (Johnson et al. 1988) that organisms must tolerate. Isolating how a specific variable such as temperature, hydrogen sulphide or oxygen concentration influences faunal patterns is difficult with observational

studies, as vent fluid characters are highly correlated. Furthermore, the fragmented nature of vent habitats presents another layer of complexity. Most vents are not isolated, but exist as part of a disordered field of venting sources that vary in intensity and size. In this patchy habitat, organisms persist in a complex mosaic of temporally and spatially shifting vent fluid concentration.

Many vent studies focus on key fluid characters that likely affect processes such as habitat selection and recruitment by relating abiotic factors to species patterns in the field. Because deep-sea access by researchers is time limited, variables that can be measured expeditiously and accurately constitute the majority of the data. Studies of venting vigour (Sarrazin et al. 1999), temperature (Hessler & Smithey 1983), hydrogen sulphide and iron concentration (Sarrazin et al. 1999, Luther et al. 2001) suggest that abiotic factors relate to spatial patterns in species abundance. Observations of distinct species zones on sulphide structures (Tunnicliffe & Juniper 1990) and around basalt-hosted vents (Fustec et al. 1987) support the hypothesis that fauna select specific conditions at larval and/or adult stages. Larval studies show that patterns in species richness and abundance on settlement plates are related to temperature, suggesting recruitment processes are influenced by abiotic factors (Mullineaux et al. 2000).

Behavioural responses to habitat variables are largely unexplored for hydrothermal vent fauna (Van Dover & Lutz 2004). In comparison, decades of observational and experimental work from intertidal habitats demonstrate that animals exhibit flexible behavioural responses to various environmental cues. Complex species interactions act in concert with these responses to determine abundance patterns (Underwood et al. 2004). While we have yet to build such complex models in

hydrothermal habitats, preliminary work can identify the importance of specific abiotic factors to species abundance patterns. Multiple approaches have successfully linked faunal patterns on intertidal shores to species-specific selection of habitats based on abiotic factors (Chapman 2000).

Thermal conditions are primary determinants of habitat selection by marine species (Heath et al. 1993, Williams & Morritt 1995). Shorelines are a model system for research examining links between heat exposure and habitat selection. The upper distribution limits of organisms typically relate to their temperature tolerance. Avoidance by mobile species of specific temperatures occurs in gradient experiments in lab and field settings and preferred temperatures typically match environmental exposure (e.g., Pulgar et al. 2005). Heat shock can also influence the outcome of species interactions, thus altering abundance patterns (Tomenack 2002). Understanding how habitat selection relates to environment variability, organismal body temperature and physiological stress is emerging as an important research goal (see Tomenack & Helmuth 2002). Because hydrothermal fluid temperatures are highly variable, studies aiming to link thermal conditions to habitat selection by vent fauna can contribute to this goal (Chevaldonné et al. 1991).

A remarkably high abundance of gastropods carpet the substrata around warm fluid sources at vents on the Juan de Fuca Ridge. The dominant gastropod is *Lepetodrilus fucensis* McLean 1988, a non-coiling species found at densities of 10^5 individuals m^{-2} (Sarrazin et al. 1997, Tsurumi & Tunnicliffe 2003). Also common are two snails, *Depressigyra globulus* Warén and Bouchet 1989, and *Provanna variabilis* Warén and Bouchet 1986. Abundance patterns of these species in diffuse flows may

relate to fluid parameters such as sulphide-to-heat ratios (Marcus 2003). Gastropod density on a sulphide structure is correlated with decimeter scale changes in key physical and chemical variables (Sarrazin et al. 1997, Sarrazin & Juniper 1999, Sarrazin et al. 1999). Zonation of these three species is apparent in video imagery; *L. fucensis* and *D. globulus* occur near focused vent fluids, while *P. variabilis* is visually distinct on the substratum surrounding vents. This hypothesis that *P. variabilis* may occupy a different habitat is supported by a measured increase in the relative abundance of *P. variabilis* with distance from a vent source (Marcus & Tunnicliffe 2002).

Research conducted by submersibles can limit the scope of ecological studies by restricting the number of sampling and experimental replicates possible. Precision in fine scale studies depends on manipulator dexterity. Two lessons from observational and experimental studies in the intertidal are relevant. First, it is critical to replicate samples and experiments at different sites to generalize patterns (Chapman 2000). Second, to relate species patterns to fluid variables, sampling of each must be performed at the same scale (Chapman 2000). With these lessons in mind, the goal of this research was to use novel *in situ* techniques and experiments to test if the small-scale abundance patterns of vent gastropods relate to (1) variation in thermal conditions and (2) their behavioural responses to temperature. To achieve this goal, we used complementary observational and experimental approaches.

The first objective was to describe small-scale spatial patterns in the abundance of *Lepetodrilus fucensis*, *Depressigyra globulus* and *Provanna variabilis* with respect to thermal conditions and distance from focused vent flows using *in situ* transect and survey approaches. We hypothesize that these three species use fluid temperature to select

habitat. The second objective was to test this hypothesis by determining if species-specific behavioural responses to temperature correspond with spatial patterns in their abundance. We employed image analysis of a fixed area over time and a manipulative experiment to examine whether *in situ* behavioural responses relate to thermal regime. To ascertain if temperature is a behavioural cue, we designed controlled experiments shipboard to test whether the three species prefer specific temperatures.

METHODS AND MATERIALS

Sites and sampling. Hydrothermal vent fields were visited during summer months (May–September). Sites on the Juan de Fuca Ridge (1994–95 and 2001–03) were Axial Volcano (45°56'N, 130°00'W; depth 1570 m) and Endeavour Segment (47°57'N, 129°06'W; depth 2220 m). On Explorer Ridge (2002), the study site was Southern Explorer Segment (49°45'N, 129°42'W; depth 1850 m) (Figure 2.1). *In situ* manipulations and observations were conducted with the Canadian remotely operated vehicle, ROPOS. All studies were at outflows diffusing through basalt or low sulphide mounds. A summary of the studies included in this paper are presented in Table 2.1.

Spatial Patterns

Four observational studies relate spatial patterns of gastropod abundance, heat and distance from focused vent fluid flow (Table 2.1).

Within-vent abundance patterns. Spatial patterns of gastropod abundance were studied within 75 cm from fluid flows at five Axial Volcano vents, two Endeavour Segment vents and three Southern Explorer Segment vents in 2001 and 2002. Vents

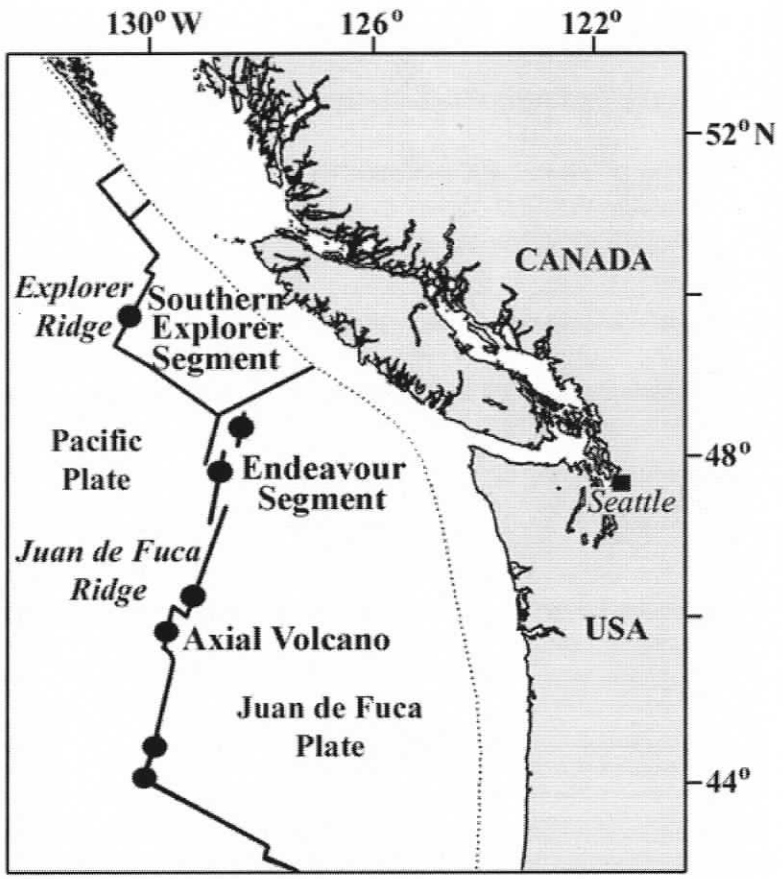

Figure 2.1
Locations of Axial Volcano, Endeavour Segment (Juan de Fuca Ridge) and Southern Explorer Segment (Explorer Ridge), northeast Pacific Ocean. Black circles are active vent sites. Redrawn from Tunnicliffe et al. (1997)

Table 2.1
Summary of studies undertaken in this work. Studies A(1) and A(2) related spatial patterns in gastropod abundance and heat to distance from focused vent flows. Studies A(3) and A(4) related spatial patterns in substratum occupation by gastropods to heat. Studies B(1) to B(3) tested whether *in situ* and *in vitro* behavioural responses relate to vent fluid parameters. Study type was observational (obs) or manipulative (man). Vent sites were Axial Volcano (Ax), Endeavour Segment (En) and Southern Explorer Ridge (Ex).

	Study approach	Type	Measurement	Parameters	Sites
A. Spatial patterns					
1	*in situ* transect: 75 cm n = 10	obs	gastropod relative abundance and density	- distance from flow - point temperatures	Ax, En, Ex
2	*in situ* transect: 65 cm n = 2	obs	multi-day temperature time-series	- distance from flow	Ax, En
3	*in situ* multi-vent survey n = 46	obs	gastropod presence/absence	- point temperature	Ax, En, Ex
4	*in situ* transect: 65 cm n = 2	obs	% gastropod cover	- temperature regime	Ax, En
B. Behavioural responses					
1	fixed-area monitoring n = 1	obs	7-month time-series of % gastropod cover in images	- temperature regime	En
2	*in situ* choice n = 1	man	end position in track-way after 77 h	- distance from flow - temperature range	Ax
3	*in vitro* choice n = >2	man	end position in gradient after 4&12 h	- temperature range	Ax

were selected based on the following criteria: (1) focused shimmering fluids were present; (2) tubeworms, known to change fluid flow patterns, were either small or absent; and (3) a single outflow was isolated by at least 3 m from other visible hydrothermal sources. We grouped samples into three distance categories from the focused fluid flow: in-vent, 0 to 25 cm; near-vent, 26 to 50 cm; and far-vent, 51 to 75 cm (categorized by ROPOS pilots using laser spots at 10 cm apart). In-vent samples were typically immersed in shimmering water (n = 10). Near-vent samples were at the edge of shimmering (n = 7) and, at far-vent locations, no shimmering was evident (n = 10). Point measurements of maximum temperature were recorded for ~5 min for most sampled sites with a rugged transition joint probe (Adam's module thermistor, ±1°C). Appendix 2.2 summarizes the location of each vent, flux characteristics, temperature and substratum type.

Gastropods were collected from approximately 100 cm^2 of substratum in each distance category using a suction sampler; removal efficiency was near 100% as determined by close-up video (Appendix 2.2 provides the exact area sampled for each location). Samples were preserved in 7% formalin. All gastropods with greater than 1 mm shell length were identified and relative abundances were calculated. More than 100 gastropods occurred in all samples.

High quality video imagery with zoom included laser spots at 10 cm apart. A digital processing program (Image-Pro Plus© 4.5) was used to quantify sample area when a distinct cleared patch was visible. It was possible to estimate density for five in-vent, three near-vent and five far-vent samples at Axial Volcano. Differences in species

densities between in- and far-vent were tested with a Monte Carlo Randomization (Manly 1991) routine; sample size was too low to include near-vent samples in this analysis.

Temperature variability. Temperatures were measured every 30 min along a transect running away from vent flow using digital temperature data loggers (DS1921L-F50 Thermochron iButton©, ±0.5°C) at Endeavour (15 d) and Axial Volcano (3 d). Deployment duration depended on the submersible dive schedule. We attached five loggers to the experimental apparatus described later (see 'Methods and materials' section 'Location preference') for the two deployments. For each logger, distance to the nearest fluid source was estimated from high resolution digital images. At the Endeavour location (distances 0, 10, 28 and 30 cm from flow), two fluid sources were visible in imagery, while a single fluid source was present at Axial (5, 20, 35, 50, and 65 cm). Because temperature loggers were embedded in epoxy resin for deep-sea use, a lag in temperature response was present. Response time was in the range of 0.25 to 1.25 °C per min for temperature shifts of 1 to 25°C.

Substratum occupation in relation to temperature. To determine the range of temperatures at which gastropods occur, we measured the maximum temperature over a 1 to 5 min period in venting fluids between 5 and 25°C at Axial Volcano (n = 40), Endeavour (n = 2) and Southern Explorer (n = 4). During these temperature measurements, we collected video imagery of the substratum surrounding the custom-made platinum resistance temperature probe (Q396 Pt wirewound sensor, ±0.5°C; Logan Enterprises, West Liberty Ohio; courtesy of D.A. Butterfield). Presence or absence of any of the three gastropod species within a 1 cm radius of the probe was recorded.

Abundance in relation to temperature. Small-scale spatial changes in substratum use by the three gastropods were examined relative to temperature measurements using high resolution digital still images (Sony DSC 707F) around each of the 5 temperature loggers deployed in 2 transects (see 'Temperature variability' section). The percentage of substratum covered by gastropods in a 10 cm^2 area surrounding each temperature logger was measured (Image-Pro Plus©4.5); animals down to 1 mm shell length were easily resolved. Mean temperature and one standard deviation were calculated for each record.

Behavioural responses

Studies were conducted over lengths of time that allowed individual gastropods to respond to fluid conditions (Table 2.1). *In situ* experiments tested for behavioural responses to changes in fluid heat and/or chemical variables. We assumed that measured temperature changes represented shifts in relative concentrations of vent fluids. Shipboard experiments identified behavioural responses to temperature.

Temporal changes in gastropod abundance related to temperature. A time-lapse camera (Photosea model 1200L) focused on a tubeworm bush at Easter Island (47°56.9'N, 129°05.9'W), Endeavour. An image was taken every 31 h as described in Martell et al. (2002). One sector of the image included a temperature probe (YSI 44000 deep-sea thermistor, ±0.5°C; courtesy of I.R. MacDonald) that recorded every 10 min; data were averaged for the 31 h surrounding each image. To compare temporal changes in gastropod abundance to temperature, the percent of substratum occupied by the three gastropod species in the same 225 cm^2 area (represented by Figures 2.2A & B) around the temperature probe was measured in 170 high resolution colour

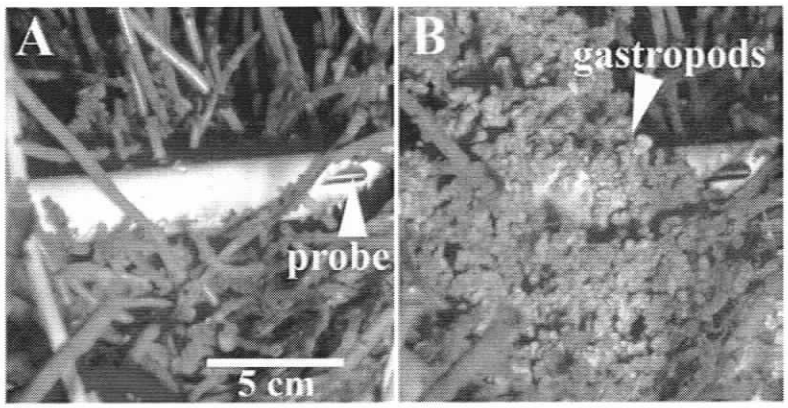

Figure 2.2
Gastropod movement near a thermistor probe. Images are the same 225 cm^2 area of larger colour high resolution images captured every 31 h by a time lapse camera (n = 170). The substratum occupation by *Lepetodrilus fucensis* and *Depressigyra globulus* was quantified as the percent substratum covered by both species for each image. Measured distance is up to 15 cm from the thermistor probe. (A) The probe measured fluid temperature at one point (arrow) every 10 min. Minimum gastropod presence (1.5% substratum cover) occurred on October 8, 1994. (B) A gastropod mass, dominated by *L. fucensis* (arrow), reached maximum substratum cover (60%) on February 21, 1995.

images taken from July 17, 1994 to February 21, 1995.

Location preference. To determine if specific distances from a focused flow are preferred by each of the three species, we placed individuals in contained flow-through tracks with one end positioned in venting fluid. The apparatus resembled a racetrack with four lanes, each being 75 cm long by 1.0 cm wide by 1.5 cm high; it was constructed with lexan sides and covered top and bottom with 2 mm plastic mesh (Figure 2.3). The tracks were divided by gates into 5 regions at incremental distances from focused vent fluid: 0–15, 16–30, 31–45, 46–60, 61–75 cm (Figure 2.3). The gates were left open during the deployment, allowing unrestricted movement within the track, until recovery when a bar was released causing the gates to close. Thus, numbers of animals in each region of the track could be determined accurately upon recovery of the apparatus. Temperatures were measured every 30 min at 15 cm intervals from the vent flow by attaching digital temperature data loggers (see 'Temperature variability') to the outside of the experimental apparatus (Figure 2.3).

Mrk 33 Vent (Embley et al. 1999: 45°56.0'N, 129°58.9'W) on Axial Volcano was selected because fluid emerges from a discrete crack in basalt (verified with temperature measures prior to the deployment of the experiment). Animals within 50 cm of the crack were collected by low power suction and brought immediately to the surface. Three species-specific tracks contained 100 individuals of *Lepetodrilus fucensis*, *Depressigyra globulus* and *Provanna variabilis*; in the fourth track we pooled 100 individuals of each species. Animals were stored in 4°C sea water for 6 h until their return to the seafloor. During descent, all animals were jostled to one end of the apparatus. We positioned this end away from Mrk 33 vent in 3°C fluid. We returned after an 85 h acclimation period

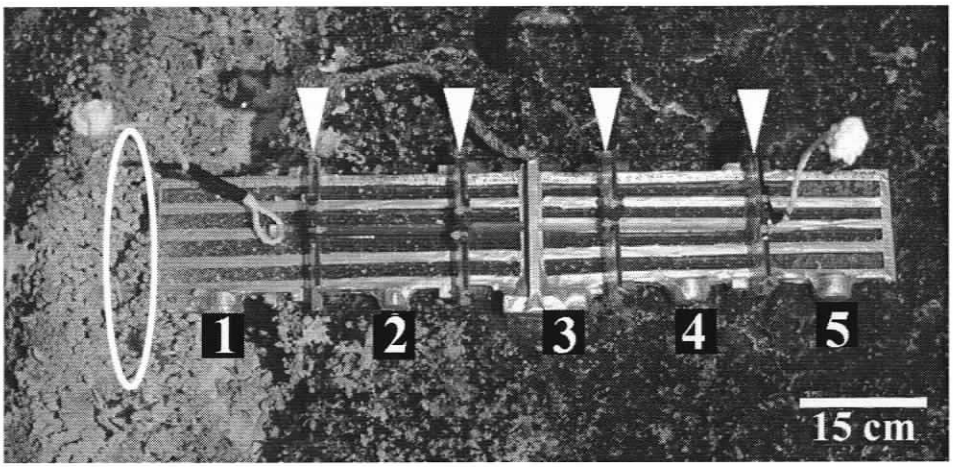

Figure 2.3
Experimental apparatus deployed at Mrk 33 vent (Axial Volcano) to determine if the three gastropod species move to specific distances from a focused flow (see 'Location preference'). White arrows indicate the positions of spring-close gates. The white oval marks the vent flow. Numbers one to five show the position of digital temperature data loggers (see 'Temperature variability') attached to the outside of the apparatus.

(determined by the research dive schedule) and turned the apparatus 180°. The animals were left to disperse for 77 h, after which we closed the gates and recovered the apparatus. Due to weather constraints, we were unable to replicate this experiment.

***In vitro* temperature response.** To determine if these gastropod species respond to gradients in temperature, we established shipboard experimental chambers with spatial gradients (in 2003). A pressurized experimental vessel with a horizontal temperature gradient was used (pilot studies identified geotaxis in vertical chambers as a confounding factor: see Appendix 2.1). Animals were placed in a chamber made from an anodized aluminum block with free access along a groove 20 cm long by 1 cm wide by 1.5 cm high. A polycarbonate window allowed viewing of the animals under pressure. Filtered seawater was pumped into the chamber inlet with a Beckman 110B high pressure liquid chromatography pump at a rate of 1 ml min^{-1}. Pressure in the chamber was maintained at 13 789 kPa (2000 psi) by a Whitey backpressure regulator on the chamber outflow tubing. A temperature gradient was created by placing the chamber in a waterbath cooled with ice at one end and heated with a submersible heating element at the opposite end. Every 30 min, we measured the temperature gradient with a digital thermometer probe (VWR, ±0.2°C) placed in holes spaced at intervals of 1 to 2.5 cm along the aluminum block. We extrapolated temperature for intermediate positions based on a linear relationship between temperature and distance. Block temperature occasionally fluctuated by a maximum of 1°C due to melting and replacement of ice. Preliminary trials at surface pressure showed that fluid temperature in the chamber responded to a 5°C change in the aluminum block in less than 5 min.

Gastropod collections were made at Axial Volcano (1580 m) because a notable decrease in moribundity at surface pressure occurred compared to Endeavour (2220 m). Each trial consisted of 30 individuals of each species (animals ranged between 3 and 7 mm shell length); animals in different trials originated from different vents. Immediately after collection, animals were acclimated at pressure for 4 h at 4°C. We recorded the position of each individual in control and temperature gradient treatments after 4 (six trials) and 12 h (two trials). In controls temperature was maintained at 8°C (this temperature was selected because *L. fucensis* did not move at 4°C after 4 h, but was mobile at 8°C). Two temperature treatments were run: (1) 4 h in a steep (4–30°C) gradient, and (2) 12 h in a shallow (4–18°C) gradient. Prior to the start of the gradient experiments, animals were positioned in one end of the chamber. This end was subsequently heated to create the gradient over 30 min with a maximum temperature increase of 1°C min^{-1}. All individuals in pressurized experiments remained active.

The species-specific distributions between trials where animals were collected from different vents were similar ($p > 0.3$, Kruskal-Wallis H test, $\chi^2 < 2$). Thus, the data sets from each trial for each species were pooled and potential differences due to site of origin were ignored to examine differences between treatments and species. Standard box plot diagrams represent these pooled distributions. SPSS 11.0 was used to test for significant differences in the distributions between species using a Kruskal-Wallis H test for independent samples. A *post-hoc* Mann-Whitney U Test for two independent samples identified significantly different data sets.

RESULTS

Spatial patterns

Within-vent abundance patterns. Although most vents do not fit the sample location requirements we imposed, isolated focused flow occasionally did create 'islands' on the substratum for use as model sites (e.g., Figure 1.3A). The three gastropod species were present at all sampling locations (Figure 1.3B). However, the relative abundance of each species changed with distance from vent (Figure 2.4). *Lepetodrilus fucensis* was dominant in-vent (0–25 cm), with a relative abundance of 75%; it decreased to 60% near-vent (26–50 cm) and to 40% far-vent (51–75 cm). The relative abundance of *Depressigyra globulus* ranged between 21 and 36% in all distance categories. *Provanna variabilis* was relatively more abundant far-vent (40%) than in- and near-vent (1%). The numbers of individuals counted for each species (>1 mm shell length) in the three distance categories are reported in Appendix 2.3.

The species density in each distance category provides information on habitat use (Figure 2.5). *Lepetodrilus fucensis* was most abundant (mean of 2100 ind. dm^{-2}) in-vent and decreased an order of magnitude through near-vent (400 ind. dm^{-2}) to far-vent (90 ind. dm^{-2}). *Depressigyra globulus* had similar densities in- and near-vent (means of 240 and 160 ind. dm^{-2}, respectively) and decreased to 60 ind. dm^{-2} at far-vent locations. *L. fucensis* and *D. globulus* reached maximum densities of 2900 and 950 ind. dm^{-2} (respectively) in-vent where their mean densities were significantly greater than far-vent ($p < 0.001$, Monte Carlo Randomization). *Provanna variabilis* displayed the reverse trend. Its mean density far-vent (60 ind. dm^{-2}) was significantly greater than in-vent (10

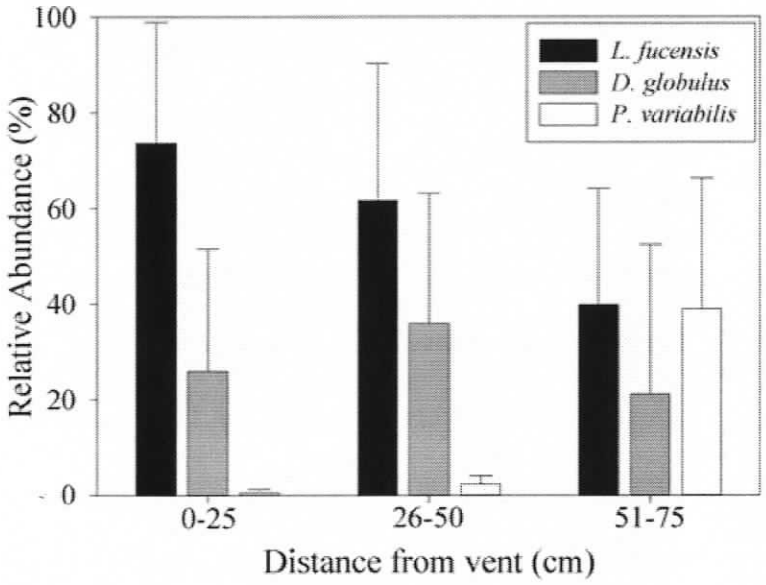

Figure 2.4
Lepetodrilus fucensis, *Depressigyra globulus* and *Provanna variabilis*. Relative abundance (mean ±1 SD) with distance from focused vent flows for collections in-vent at 0 to 25 cm (n = 10, 5–15°C), near-vent at 26 to 50 cm (n = 7, 3–5°C) and far-vent at 51 to 75 cm (n = 10, <3°C). *L. fucensis* is the dominant species. The relative abundances of *L. fucensis* and *D. globulus* are greatest in- and near-vent, while *P. variabilis* is relatively abundant far-vent

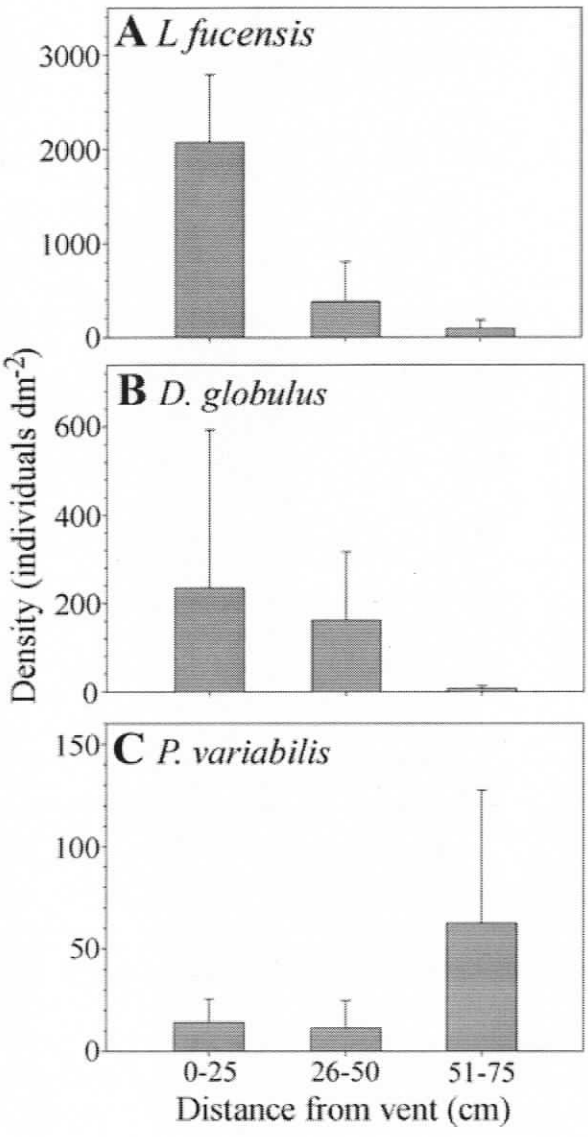

Figure 2.5
Lepetodrilus fucensis, *Depressigyra globulus* and *Provanna variabilis*. Density (mean ±1 SD) with distance from focused flows for collections in-vent at 0 to 25 cm (n = 5, 5–15°C), near-vent at 26 to 50 cm (n = 3, 3–5°C) and far-vent at 51 to 75 cm (n = 5, <3°C). Y-axes differ by an order of magnitude. *L. fucensis* and *D. globulus* densities are each significantly greater ($p < 0.001$; Monte Carlo Randomization Test) in- versus far-vent. *P. variabilis* displays the reverse pattern ($p < 0.05$)

ind. dm^{-2}) ($p < 0.05$, Monte Carlo Randomization). The maximum density of *P. variabilis* was 200 ind. dm^{-2} in a far-vent sample.

Temperature variability. Point temperatures of fluids where animals were collected from in-, near- and far-vent locations ranged from 5 to 15°C, 3 to 5°C and <3°C, respectively. We also recorded temperatures every 30 min with temperature loggers for several days along two 75 cm transects from focused flow. In-vent temperatures were highly variable: mean ±1 SD, minimum, and maximum at the Endeavour location for 3 in-vent sites were (0 cm from flow = 12.3±2.0, 7.5, 15.0°C), (0 cm = 7.3±1.5, 5.0, 10.5 °C) and (10 cm = 6.0±1.8, 3.0, 10.0°C). Two in-vent sites at Axial were monitored: (5 cm from flow = 8.1±2.3, 3.5, 12.5°C) and (20 cm = 5.3±1.6, 3.0, 8.5°C). In comparison, near- and far-vent locations were relatively stable (Figure 2.6). For example, loggers placed 35 (near) and 65 cm (far) from flow at Axial returned the following mean ±1 SD, minimum, and maximum (respectively): (3.6±0.4, 3.0, 5.0°C) and (2.6±0.2, 2.5, 3.0°C). The temporal temperature records also indicate high variability at in-vent locations at various time scales (Figure 2.6). In-vent temperature changes of 3 (Endeavour) and 5°C (Axial) in 30 min occurred. In-vent animals were also subject to longer term variability than near- or far-vent locations. Diurnal variations and a 5°C warming trend were evident in the Endeavour in-vent record (Figure 2.6A). A 5°C daily change in the running average temperature at Axial was also recorded (Figure 2.6B).

Substratum occupation in relation to temperature. Point temperature measurements coupled with images from numerous vents documented the range of fluid conditions in which the three gastropod species occur (Figure 2.7). *Lepetodrilus fucensis*

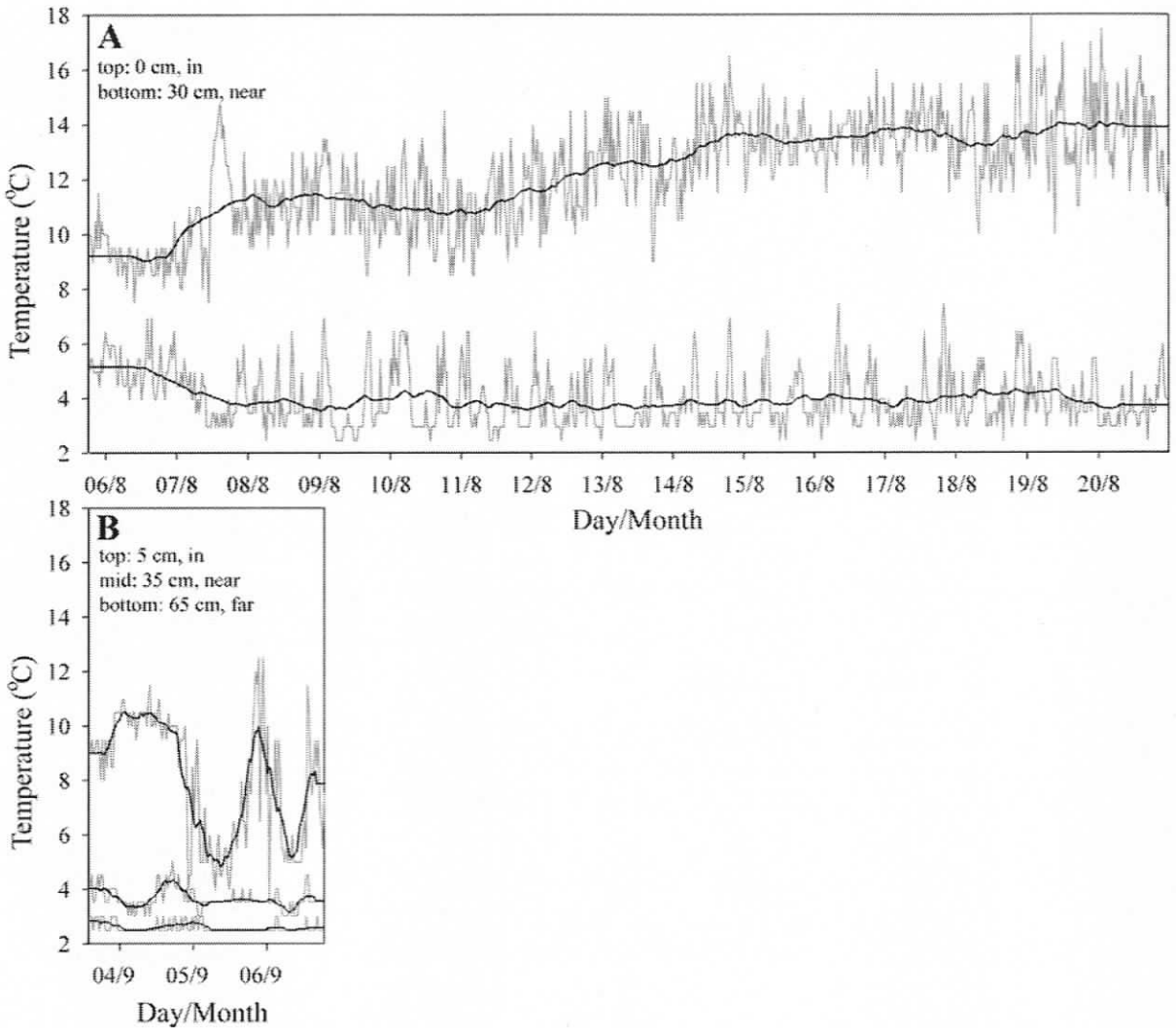

Figure 2.6
Temperature at increasing distances from focused vent flows measured every 30 min (gray); running averages are black lines. Absolute temperatures and variability decrease with distance from flow. Both records illustrate the short time scales on which large shifts in temperature can occur. (A) Endeavour: temperature records are 0 cm (in-vent) and 30 cm (near-vent) from focused flow. There is a 5°C change over 15 d in the running average at 0 cm from vent flow and a weak diurnal tidal signature. (B) Axial Volcano: temperature records are from 5 cm (in-vent), 35 cm (near-vent) and 65 cm (far-vent) from focused flow. The running average varies daily by 5°C at 5 cm

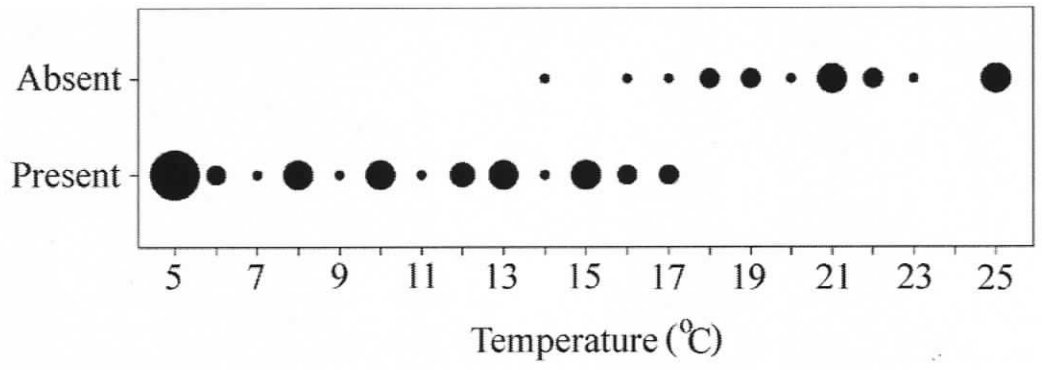

Figure 2.7
Observations of gastropod presence relative to point temperature measurements over a 5 to 25°C range. Circle size represents number of sites (largest = 5, smallest = 1) with gastropods present or absent from the substratum within a 1 cm radius of the temperature probe. Sites from Axial Volcano (n = 40), Endeavour (n = 2) and Southern Explorer (n = 4) were surveyed. Gastropods were visible at all locations below 14°C (n = 21) and at eight locations in 14 to 17°C fluids. Gastropods were absent at all locations (n = 14) in fluid temperatures of 18°C or higher and from another three locations with maximum fluid temperatures between 14 and 17°C

and *Depressigyra globulus* were visible at all 21 locations below 14°C and at eight locations in 14 to 17°C fluids. The three species were absent from all 14 locations surveyed where maximum fluid temperatures reached 18°C or higher. These gastropods were also absent from another three locations with maximum fluid temperatures between 14 and 17°C. *Provanna variabilis* was only rarely distinguishable among the other two species in video images. Therefore, the upper temperature limit for *P. variabilis* may be less than the other two species.

Abundance in relation to temperature. Loggers recorded mean temperatures between 3 and 12°C at Endeavour and Axial (Figure 2.8). *Lepetodrilus fucensis* was dominant in the images, *Depressigyra globulus* was always visible and *Provanna variabilis* was sporadically visible. Because we could not determine the percent substratum cover by species from images, our values are a combined estimate for all three species. Substratum coverage in the 10 cm^2 areas surrounding the loggers increased at higher temperatures. *L. fucensis* was visible in images on 100% of the available substratum and formed stacks when temperatures ranged between 8.1±2.3 and 12.3±2.0°C; coverage dropped below 10% in temperatures below 4.0±0.9°C.

Behavioural responses

Temporal changes in gastropod abundance versus temperature. Early images from the time-lapse camera series showed a mass of gastropods in the area immediately surrounding the thermistor (Figure 2.2). The gastropod mass disappeared then reappeared in the same area over 220 d. These temporal changes in the percent substratum cover by the gastropods correspond to changes in the temperature record (Figure 2.9A). However, the temperature range recorded in this study (2–5°C)

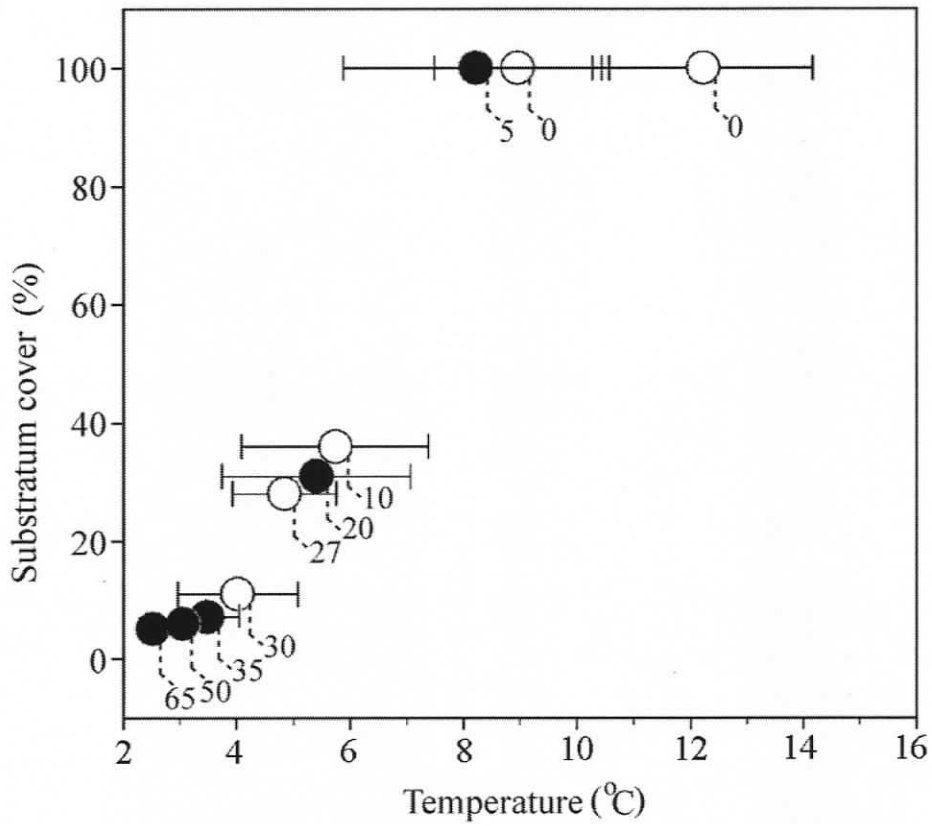

Figure 2.8
Relationship between gastropod substratum cover (%) and temperature (mean ±1 SD) along 2 transects at Endeavour (white circles) and Axial Volcano (black circles). Numbers indicate distance (cm) from focused flow. Substratum cover was quantified from high resolution digital images in a 10 cm^2 square area adjacent to each temperature logger. Mean temperatures were calculated from 30 min recordings over 15 d at Endeavour and 3 d at Axial. Substratum cover is 100% at temperatures above 8°C and approaches zero when fluid temperatures are near ambient (2°C). See Figure 2.6 for examples of complete temperature records

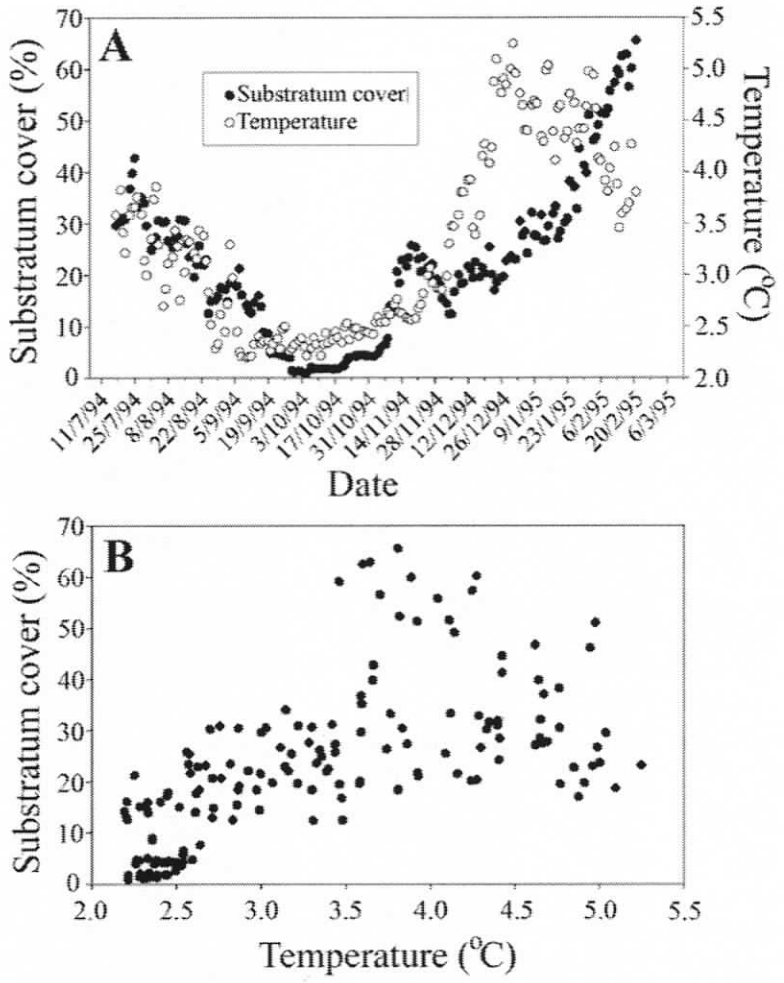

Figure 2.9
Changes in gastropod abundances in a tubeworm aggregation over 7 mos seen in time-lapse camera images at Endeavour. Image interval was 31 h (n = 170). See Figure 2.2 for further details. (A) Gastropod substratum cover and mean fluid temperatures show similar trends with time. (B) Gastropod substratum cover and mean temperature are significantly correlated ($p < 0.01$, Spearman's rho = 0.73, df = 168). Substratum cover approaches zero when fluid temperatures are near ambient (2°C)

represents the low-end of habitat temperatures for these gastropods. Although the thermistor probably does not record the actual temperature within the gastropod mass, percent cover and temperature are significantly correlated ($p < 0.01$, Spearman's Rank Correlation = 0.71, df = 168) (Figure 2.9B). Substratum cover ranged from 0 to 20% when fluid temperatures were below 2.5°C (Figures 2A, 9B). However, the maximum values for gastropod substratum cover (66%) (Figures 2B, 9B) do not correspond with maximum temperatures (5°C).

Location preference. *Lepetodrilus fucensis* was less mobile than the other two species in the trackway. After the acclimation period (85 h), a clump of *L. fucensis* was visible in their starting position at one end of the track in 3°C fluids while the other two species were more dispersed. Consequently, when the trackway was turned, many *L. fucensis* started the experiment within 10 cm from the vent (see Methods). When we returned after the experimental duration (77 h), camera images revealed that these *L. fucensis* had moved. Nearly all *L. fucensis* and *Depressigyra globulus* were found within 30 cm of flow (Figure 2.10). Video showed that this area was bathed in vent fluid with temperature values of 8.1±2.3°C at 5 cm and 5.3±1.6°C at 20 cm over 3 d (Figure 2.10 inset). *Provanna variabilis* occurred in similar numbers in all distance categories of the track in fluids with temperatures ranging from 8.1±2.3 to 2.6±0.2°C (Figure 2.10 inset). The lane with all three species combined returned comparable results to the single species lanes (Figure 2.10).

***In vitro* temperature response.** Temperature had a significant influence on the distributions of all three gastropod species in both 4 and 12 h trials ($p < 0.001$, Mann

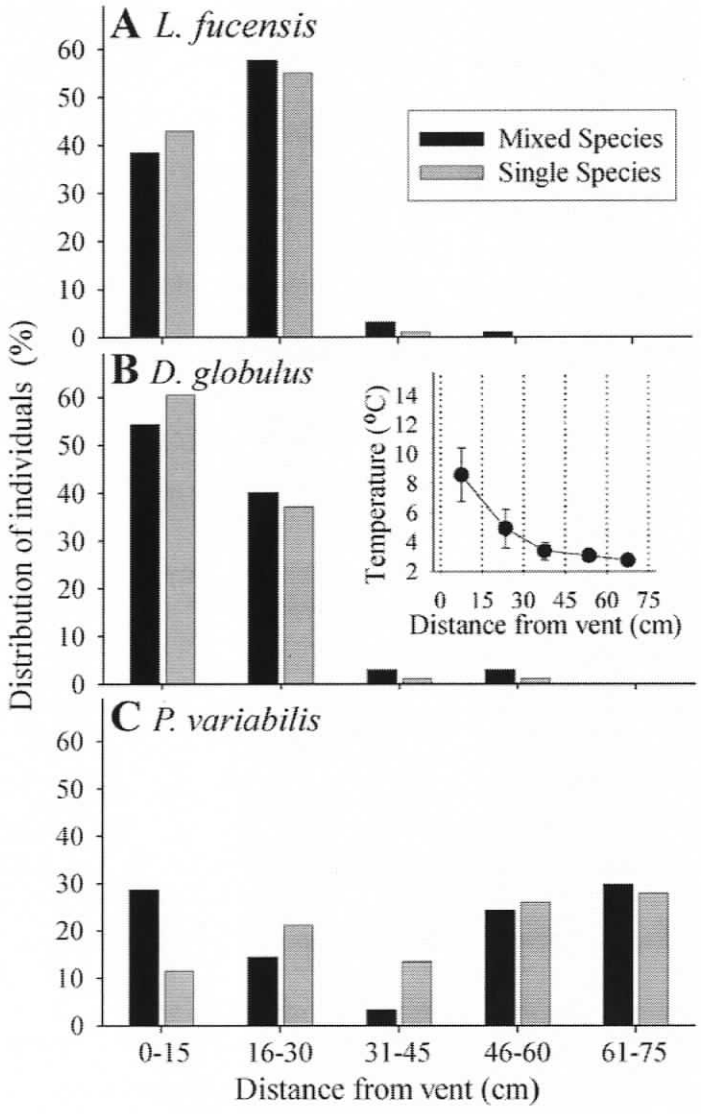

Figure 2.10
Lepetodrilus fucensis, *Depressigyra globulus* and *Provanna variabilis*. Final positions after 77 h along a 75 cm trackway oriented with one end in vent flow to create a gradient in vent fluid concentration (see Figure 2.3). A single species lane contained 100 individuals from one species (gray); the mixed species lane contained 100 individuals from each species (black). The inset shows the relationship between temperature (mean ±1 SD) and distance from focused vent fluids (see Figure 2.6B for examples of complete temperature records). (A) *L. fucensis* cluster within 30 cm of the vent where mean temperatures are 5 to 8°C. (B) *D. globulus* cluster within 30 cm of the vent where mean temperatures are 5 to 8°C. (C) *P. variabilis* are uniformly distributed with distance from vent at 3-8°C

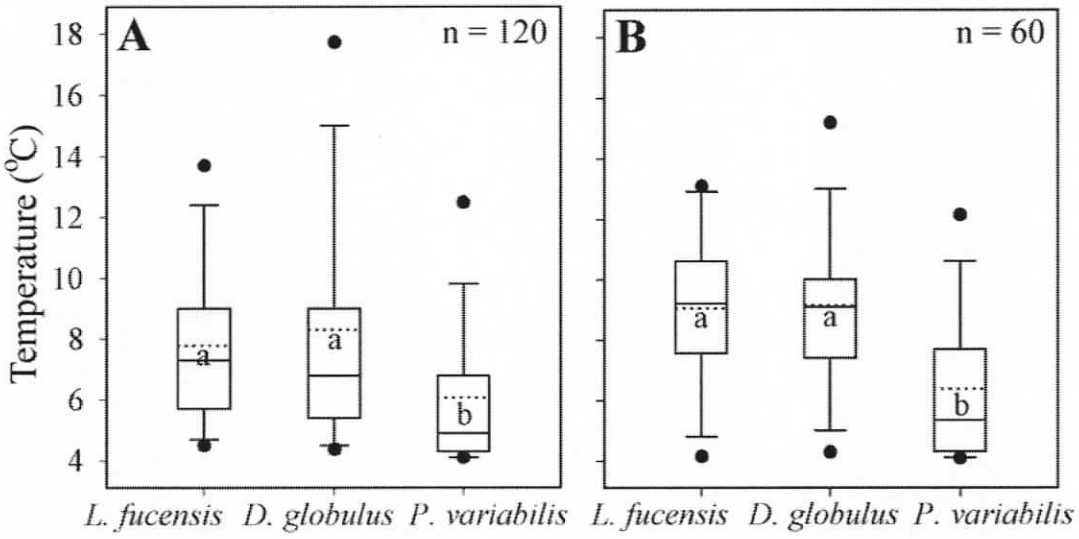

Figure 2.11
Lepetodrilus fucensis, *Depressigyra globulus* and *Provanna variabilis*. Behavioural responses to temperature gradients in pressurized (13 789 kPa) horizontal chambers; positions were recorded after 4 (6 trials) and 12 h (2 trials). Box plots present 5th, 25th, 50th, 75th, 95th percentiles; the dotted line is mean. Means with dissimilar letters differ significantly from each other ($p < 0.01$; Mann-Whitney U Test). Significant differences in responses exist both among species and compared to controls. N = individuals per species. (A) Distributions in a 4 to 30°C temperature gradient after 4 h; species-specific distributions in A are similar to B. (B) Distributions in a 4 to 18°C temperature gradient after 12 h. 90% of *L. fucensis* and *D. globulus* occupy 5 to 13°C; 90% *P. variabilis* occupy significantly cooler temperatures from 4 to 10°C

evenly dispersed. *Lepetodrilus fucensis* and *Depressigyra globulus* in the 4 h (4–30°C, steep gradient) and the 12 h (4–18°C, shallow gradient) temperature treatments occupied similar temperature ranges (Figure 2.11). In the 4 h trial with a steep temperature gradient, 90% of *L. fucensis* individuals were between 5 and 13°C, while *D. globulus* were between 5 and 15°C. The distributions of both species were skewed toward lower temperatures. In comparison, 90% of individuals for both species were between 5 and 13°C in the 12 h trial and were symmetrical around the mean. For *Provanna variabilis*, the distributions were similar for 4 and 12 h trials; 90% of *P. variabilis* individuals occurred between 4 and 11°C and the distribution was skewed toward lower temperatures (50% of animals between 4 and 5°C) (Figure 2.11). Individuals of *P. variabilis* were distributed to significantly ($p < 0.001$, Mann-Whitney U Test, $Z < -5.5$) lower temperatures than *L. fucensis* and *D. globulus* in both the 4 and 12 h trials. We observed that *D. globulus* and *P. variabilis* dispersed rapidly at 4°C, while *L. fucensis* did not. However, all three species moved in chambers at 8°C.

DISCUSSION

Spatial patterns

Three gastropod species at Juan de Fuca vents showed small-scale patterns in abundance. *Lepetodrilus fucensis* displayed the most marked gradient with distance from focused vent flows, and had higher densities than *Depressigyra globulus* and *Provanna variabilis* in all in-vent samples. The stacking behaviour of *L. fucensis* (Figure 1.3A) probably allows increased access to suspended food particulates when population densities are high. *D. globulus,* also most abundant in-vent, may exploit interstitial

spaces by virtue of its small size. *P. variabilis* was relatively less abundant than the other two species and its density increased from in- to far-vent. Its occupation of far-vent habitats might explain results from studies that report a unique carbon isotope signature for *P. variabilis* (Levesque 2003).

The thermal regime changed over spatial scales that correspond with observed gastropod density gradients. In-vent animals experienced higher mean temperatures and variability at both short and long timescales while animals distant from flows are in relatively stable, low temperature fluids. Diurnal variation in our in-vent temperature record is likely tidally driven. Tivey et al. (2002) show that fluid mixing is affected by tidal and bottom currents over hours to days. We also observed a 5°C warming trend over 15 d at an Endeavour in-vent location. The cause is unknown but other studies record spikes in fluid heat after earthquake swarms; fluids at Endeavour vents increased 4 to 11°C, and ±5°C temperature oscillations were present for 8 to 12 d (Johnson et al. 2000).

Change in thermal regime indicates differences in environmental conditions. Butterfield et al. (2004) show that temperature in a Juan de Fuca vent field is a good proxy for chemical mixing especially within one vent. Currently the assessment of the physico-chemical status of habitats both at and beyond the fluid exit point is challenged by lack of sufficient data. Therefore, extrapolation to other fluid parameters should be done cautiously. Future studies aiming to experimentally determine the influence of additional environmental parameters (e.g., hydrogen sulphide concentration) in habitat selection by these species are important.

Substratum occupation and coverage by the three gastropod species also relates to heat. All species were absent above 17°C and presence was unpredictable between 14 and 17°C, suggesting an *in situ* thermal limit for these species within these temperatures. This uncertainty range likely reflects the difficulty in estimating a thermal limit by recording point temperature measurements in highly variable conditions. Further, the percent cover by *Lepetodrilus fucensis* and *Depressigyra globulus* at decimeter scales corresponded to temperature differences; above mean temperatures of 8°C, gastropods occupy all available substrata and *L. fucensis* begins stacking. These observations are consistent with the hypothesis that heat and/or vent fluid chemistry is important to habitat selection by these three species.

Responses to vent fluids

Lepetodrilus fucensis and *Depressigyra globulus* actively seek specific conditions. When placed in cold temperatures, they selected warmer vent flows and a mass of gastropods grew in size as temperature increased. Substrata bathed in vent fluids probably offer the greatest food availability for animals that graze and suspension feed, as these fluids deliver subsurface bacteria, particulates and reduced metals for further surface bacterial growth (Jannasch 1995, Giere et al. 2003). It is also likely that the association between *L. fucensis* and a chemoautotrophic symbiont (de Burgh & Singla 1984, Fox et al. 2002) requires access to dissolved sulphide and oxygen (Chapter 5). Although maximum substratum occupation by these species did not co-occur with maximum temperatures in our time-lapse images, the dense stacks of limpets visible below the thermistor may have re-directed flow around the probe during the time of maximum coverage, thus reducing measured temperature.

Lepetodrilus fucensis and *Depressigyra globulus* selected locations in focused vent fluids when inter-specific interactions and other habitat cues were eliminated experimentally. This result suggests that the relative scarcity of *L. fucensis* and *D. globulus* in far-vent habitats is because these species prefer habitats in vent flows. In contrast, *Provanna variabilis* was evenly distributed with distance from focused vent fluids. The process giving rise to relatively higher densities of this species far-vent is probably not a behavioural preference for specific fluid parameters.

Although manipulation of experimental animals can influence behavioural responses (e.g., Chapman 1999, Chapman 2000), the results from the location preference experiment are consistent with the time-series observations of animals in their natural environment. Shock due to change in pressure may also have influenced behaviour. Although *Depressigyra globulus* and *Provanna variabilis* were mobile when returned to the seafloor, *Lepetodrilus fucensis* was less active. However, the acclimation period was conducted at 3°C and shipboard observations suggest difference in mobility is more likely due to cold temperatures than to pressure shock.

Responses to temperature

In the shipboard gradient experiments, the temperature ranges chosen by animals collected from different vents were similar. Because access inside experimental chambers was unimpeded, we infer that these temperature zones represent their preferences. Animals in gradient treatments were exposed to temperatures at which they are absent *in situ* (up to 30°C) and individuals moved away from the high heat. Active selection of specific temperatures indicates preferred ranges (Voss et al. 2001). Also, the

range of temperatures occupied by each species reflects results from the location preference experiment.

Temperature is probably a cue by which *Lepetodrilus fucensis* and *Depressigyra globulus* locate preferred vent flows. They selected temperatures (5–13°C) typical of in-vent habitats where they are found at their highest densities. In comparison, *Provanna variabilis* preferred significantly cooler temperatures. Although *P. variabilis* is most abundant out of vent flows from 3 to 4°C, it did not avoid warmer fluids. Thus, the relatively high far-vent densities of *P. variabilis* are probably not driven by a behavioural response to temperature.

Each species selected a wide range in temperatures (*Lepetodrilus fucensis* and *Depressigyra globulus*: 5–13°C; *Provanna variabilis*: 4–11°C). These ranges likely reflect the extreme thermal variability of vent flows in space and time. Point measures of different vents ranged between 4 and 17°C. Fluctuations up to 5°C in 30 min were evident in-vent; the magnitude of these fluctuations was underestimated due to a delay in logger response. As *D. globulus* has a higher temperature tolerance than *L. fucensis* (Lee 2003) and moves more quickly, it may be able to better tolerate short-term exposures to higher fluid temperatures than *L. fucensis*. The greater tolerance and mobility of *D. globulus* may explain why several individuals ventured for short time periods (<5 min) into fluids up to 20°C in our gradient experiments, while *L. fucensis* avoided these temperatures.

Our experiments also indicate that these three species have moderate upper thermal limits, around 13°C for *Lepetodrilus fucensis* and *Depressigyra globulus* and 11°C for *Provanna variabilis*. These temperatures are lower than *in situ* thermal limits

that ranged from 14 to 17°C; this difference is likely a consequence of the heat stability in the shipboard experiments relative to *in situ* conditions. These species can survive only short exposure to temperatures over 30°C (Lee 2003). Most individuals became inactive in our study after 72 h at 17°C. In the fluctuating vent habitat, gastropods may occupy positions that leave a margin of safety from heat exposure that exceeds their physiological tolerances. A next step is to examine the response of these species to fluctuating temperatures.

Temperature responses may minimize exposure to a combination of environmental stresses, as is found for shoreline gastropods (Underwood 1979, McMahon 1990). Fluids up to 15°C contain hydrogen sulphide concentrations well below 500 μM (e.g., Johnson et al. 1986, Butterfield et al. 2004, Marcus 2003); however, toxicity and duration of anoxia increases with temperature (Johnson et al. 1986, Johnson et al. 1988). Extreme fluctuations in these variables may also act synergistically to impose limits on these three gastropods, which may not be able to adapt to the variability in higher temperature flows. Physiological flexibility in extreme environments is likely key to surviving high variability (Bergquist et al. 2004, Peck 2004).

Alternatively, these gastropods may occupy habitats in moderate temperature fluids as a result of interactions with other species. If so, their preferred temperatures could simply reflect adaptation to the thermal regime presented by these habitats. For example, Marcus (2003) report that, over a range of sulphide-to-heat ratios, the combined relative abundances of *Paralvinella* polychaete worms (*P. palmiformis*, *P. pandorae* and *P. sulfincola*) is inversely proportional to the relative abundance of *Lepetodrilus fucensis*. These worms aggregate at the fluid exit point. Perhaps *Paralvinella*, known to possess

novel sulphide detoxification pathways (Martineu et al. 1997), better tolerates periodic exposure to higher temperature fluids with high sulphide levels. The worms may also be able to physically displace the gastropods.

Space competition

Temperatures above 15°C may trigger avoidance of vent flows that present physiologically limiting conditions. Differences between habitat occupation and temperature preferences by these three gastropods suggest further studies need to investigate the role of other abiotic and biotic factors in habitat selection. For example, *Lepetodrilus fucensis* and *Depressigyra globulus* are abundant in near-vent habitats despite a preference for in-vent temperatures. These peripheral individuals may be displaced to lower quality habitats by competition for food or space. The high densities of *L. fucensis* and *D. globulus* measured in this study suggest density-dependent competition may force the use of a variety of habitats besides those preferred (Rosenzweig & Abramsky 1985). *Provanna variabilis* is also probably excluded from in-vent habitats by the other gastropods.

Responses to other factors

Temperature likely interacts with other factors such as flow vigour (Sarrazin et al. 1997) in habitat selection. A pilot on-ship experiment introduced 60 individuals from each of the three gastropod species to flows of 4.4 cm s^{-1} (surface pressure, 6°C). Directional orientation was not observed (A. Bates, unpubl. data). However, in flowing water, *Lepetodrilus fucensis* lifted its shell (similar to 'mushrooming' by littorines) and *Depressigyra globulus* and *Provanna variabilis* initiated random crawling patterns. Thus, an encounter with flowing fluid probably initiates movement and positioning. In

addition, all three species are negatively geotaxic, *Depressigyra globulus* notably so. Geotaxic behaviour may serve to re-orient individuals that fall off chimney structures and tubeworm bushes, as in the case of many intertidal gastropods that use geotaxism to maintain positions on the shore (Underwood 1979).

Hydrogen sulphide concentration is a principal factor correlated with faunal distributions (Sarrazin et al. 1999). Marcus (2003) reports a correlation between the relative abundance of *Lepetodrilus fucensis* and sulphide-to-heat ratio. Further, a shrimp species from Mid-Atlantic Ridge vents exhibits a chemosensory response to sulphide that may serve in orientating individuals to vent flows (Renninger et al. 1995). Gastropods may use multiple environmental cues in habitat selection.

Broader implications

Several studies examine time-series temperature measurements from probes positioned among vent animals in diffuse flows (Table 2.2). Maximum temperatures in these flows do not exceed 22°C, unlike the extreme temperatures reported for alvinellid polychaetes (Chevaldonné et al. 1992, Chevaldonné 2000, Di Meo-Savoie et al. 2004). Although fluid temperatures in shrimp swarms range up to 40°C, inducible stress proteins in the blind shrimp from the Mid-Atlantic Ridge also indicate an optimal thermal habitat for this species below 25°C (Ravaux et al. 2003). Point temperature measurements may be misleading.

Although temperatures are moderate in diffuse flows, variability is short-term, extreme, and unpredictable. Multiple degree temperature ranges (4–20°C) were present in all time-series records and changes up to ±15°C occur at short and long time scales (Table 2.2). At scales of seconds to minutes, turbulent mixing of emerging fluids with

Table 2.2
Summary data from studies at Pacific Ocean vent sites reporting time-series of temperature at fixed positions in vent fluids where fauna are abundant. The measurement interval (Meas int) and duration (Meas dur) of each temperature series vary between studies; studies are ordered by decreasing interval. Times are at year (yr), month (mo), day (d), hour (h), minute (min) and second (s) scales. Maximum (max) temperatures are less than 23°C. Temperature range, the spread between maximum and minimum values, varies from 4 to 20°C. Temperature change occurs at long and short scales. In some vent flows the overall temperature change (Overall Δ Temp), the difference between start and end temperature, reached 15°C. At shorter time scales within each record temperature changes of ± several degrees are also evident (Short Δ Temp). In all studies, temperature variability is high and unpredictable. The vent fauna in these studies experience moderate but extreme thermal variability.

Study Location	Meas Int	Meas Dur	Temp Range (°C)	Overall Δ Temp (°C)	Short Δ Temp (°C)	Source
Juan de Fuca	31 h	7.2 mo	3-20	15	7 (mo)	Martell et al. (2002)
Juan de Fuca	30 h	1 yr	2-22	12	10 (mo)	Urcuyo (2000)
			3-16	10	6 (mo)	
			4-16	8	4 (mo)	
			3-12	6	4 (mo)	
Juan de Fuca	30 min	15 d	8-17	5	5 (h)	This study (Figure 2.6)
	30 min	3 d	4-12	1	5 (h)	
Guaymas Basin	5 min	7 d	3-9	5	2 (h)	Chevaldonné (1996)
	5 min	3 d	6-12	4	5 (h)	
Galapagos	15 min	3 d	2-15	5	10 (h)	Johnson et al. (1988)
			2-10	3	5 (h)	
Juan de Fuca	5 min	30 h	3-7	1	3 (h)	Tunnicliffe et al. (1985)
East Pacific Rise	3.8 min	47 h	2-8	1	2 (h)	Chevaldonné et al. (1991)
			2-12	1	5 (h)	
			3-16	0	8 (h)	
Lau Back-Arc Basin	3.8 min	27 h	2-4	1	1 (h)	
			3-13	2	10 (h)	
			4-18	12	8 (h)	
North Fiji Basin	3.8 min	27 h	5-12	2	5 (h)	
			4-17	5	10 (h)	
			8-22	12	8 (h)	
Galapagos	32 s	18 d	2-7	0	5 (h)	Johnson et al. (1994)
			4-20	10	15 (h)	
East Pacific Rise	5.2 s	5 min	3-7		3 (min)	Chevaldonné et al. (1991)
			4-10		5 (min)	
			4-12		7 (min)	
Galapagos	0.5 s	2 min	4-8		1 (s)	Johnson et al. (1988)

ambient seawater creates temperature fluctuations up to ±7°C. At hour to month scales ±10°C fluctuations are common and tidal spectra are present in most records (i.e., Tunnicliffe et al. 1985, Chevaldonné 1991, 1996, Urcuyo 2000). At longer scales, seismic activity can initiate unpredictable temperature and vent flow changes through fluid injection or re-routing (Johnson et al. 2000), cooling trends are also evident over the lifetime of a hydrothermal vent (Butterfield et al. 2003).

Thus, hydrothermal habitats offer a natural laboratory where fluctuations in fluid heat and associated characters are large, rapid and unpredictable. This study complements findings from other extreme environments where animal responses to highly variable thermal conditions have been examined; temperature responses play a large role in habitat selection. However, the variability presented by vent fluids may be unique. For example, although intertidal habitats oscillate up to 25°C diurnally (e.g., Helmuth & Hofmann 2001), vent fluid temperatures fluctuate by several degrees at much shorter time scales. Further studies are needed to understand how these extreme fluctuations relate to body temperature variation, physiological stress and species patterns (Tomenack & Helmuth 2002, Fitzhenry et al. 2004).

It is possible to manipulate animals from difficult environments to interpret responses to habitat variables. The congruence of field and lab approaches demonstrates that shipboard and deep-sea experiments can yield robust insights. The gastropods manipulated in behavioural studies were responsive both shipboard and when returned to the seafloor where species-specific responses in shipboard experiments were replicable. These findings encourage the continued use of manipulative experiments to examine the

role of abiotic and biotic factors as controls of small-scale patterns in vent species abundance.

Summary

A combination of in vivo and *in vitro* studies of hot vent gastropods demonstrated the importance of temperature in habitat selection and, ultimately, the small-scale distribution patterns of three species. Animals responded to temperature gradients in the absence of other vent fluid characters and selected temperatures typical of their habitats. All three species exhibited moderate upper thermal preferences (~15°C) that corresponded with their *in situ* temperature limits, suggesting that warm flows may pose physiological constraints. Temperature is likely an important habitat cue for two species whose preferred thermal ranges approximated the fluid temperatures where each was most abundant. Our study indicates that abiotic variability is a key factor controlling the distributions of these species. Additionally, interspecies interactions probably modify species patterns. Relating animal responses to environmental variability using *in situ* and shipboard approaches will be important in further studies aiming to understand how habitat selection relates to vent faunal distributions.

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Appendix 2.1

Gastropod response to *in vitro* gradients in temperature and hydrogen sulphide conducted in non-pressurized experimental vessels

INTRODUCTION

The following study describes pilot experiments with live animals conducted in non-pressurized experimental vessels during research cruises to Axial Volcano in 2001 and 2002. The aim was to determine if gastropods respond to gradients in temperature and hydrogen sulphide concentration to provide a focus for further investigations. Animals responded to temperature and the results from trials conducted in non-pressurized vessels corroborate the datasets obtained using a similar experimental design, but with pressurized experimental vessels (as described in Chapter 2: '*In vitro* temperature response'). However, animals did not respond to a sulphide gradient or pulses of high sulphide, and as consequence, I focused on quantifying responses to temperature in the pressurized experiments.

Controlled experiments at ambient pressure were executed shipboard to test if *Lepetodrilus fucensis*, *Depressigyra globulus* and *Provanna variabilis* exhibit differential responses to gradients in fluid temperature and/or hydrogen sulphide concentration.

METHODS AND MATERIALS

Shipboard experiments. To determine if the three gastropod species respond to gradients in temperature or hydrogen sulphide concentration, I established shipboard experimental chambers with spatial gradients. Collections were made at Axial Volcano (1500 m) because a notable decrease in moribundity occurred compared to Endeavour

(2250 m). I selected animals between 3 and 7 mm, put them in the chambers and observed their positions after a set interval of time. In each trial, animals originated from a different vent.

Temperature. In 2001 and 2002, temperature gradients were established in non-pressurized vertical glass cylinders. Temperature was measured hourly at 1 cm intervals with a YSI 402 Telethermometer (±0.2°C). Thirty active animals were placed in the bottom (4°C) of species-specific chambers. Individual positions were recorded as height (cm) from the chamber bottom after 4 hr. Animals seen foot-up on the chamber bottom were not included; hence, the number of individuals varied between trials and treatments.

In 2001, 100 ml graduated cylinders (2.7 cm inner diameter, 21 cm tall) were adapted as flow-through chambers. Filtered seawater (0.2 μm) was circulated through the chambers using a peristaltic pump at a constant rate of 2 ml min^{-1}. Temperature was maintained by sealing experimental chambers in 4°C water jackets; controls had full jackets. Temperature gradients from 4 to 21°C were created by jacketing the bottom half of the chamber. Less than 10 individuals moved in some trials; morbundity was near 50%. I pooled data from 11 trials with *Lepetodrilus fucensis* and 7 trials with *Depressigyra globulus*. The total number of *L. fucensis* and *D. globulus* (respectively) in control treatments were n = 139 and n = 112. In temperature gradient treatments numbers were n = 145 and n = 104.

In 2002, chamber diameter was increased (4 cm inner diameter, 21 cm height) to reduce the degree of curvature as *Lepetodrilus fucensis* frequently detached in the 2001 chambers; morbundity decreased to 20%. To maintain temperature, I immersed racks of sealed chambers in a 4°C waterbath either fully (controls) or partially to create a 4 to

21°C gradient. Total numbers of individuals from eight trials with *L. fucensis* and six trials with *Depressigyra globulus* are (respectively) control treatments, n = 234, n = 174 and temperature gradient treatments, n = 242, n = 189.

Sulphide. In 2001 and 2002, chambers were set up as described for temperature above and the same controls used. Sulphide response was not examined in 2003 as neither preference (2001) nor avoidance (2002) was observed. In 2001, the stable sulphide gradient was created bottom (0 μM) to top (150 μM) by adding 40 Mm sodium sulphide solution (pH = 8. 5) to the chamber top at 3 ml hr^{-1} with a syringe pump. Hydrogen sulphide concentrations were assessed by the methylene blue method using a spectrophotometer at 670 nm (Cline 1969). Combined numbers of individuals for all trial numbers were 131 for *Lepetodrilus fucensis* and 121 for *Depressigyra globulus*. In 2002, the gradient was reversed by injecting a 1 ml of sodium sulphide at the chamber bottom to create a pulse of high hydrogen sulphide concentration. Initial hydrogen sulphide concentrations were 1000 μM 1 cm from the bottom, 100 μM 2 cm from the bottom and no detectable hydrogen sulphide for the rest of the chamber. I conducted two trials with a combined total of 54, 53 and 48 individuals for *L. fucensis*, *D. globulus* and *P. variabilis*, respectively.

Statistics. I compared the distributions within species between vent-specific trials and found no significant differences in all years (Kruskal-Wallis H test for independent samples). Thus data were pooled and potential differences due to site of origin were ignored to examine differences between treatments and species. Standard box plot diagrams represent these pooled distributions; outliers are 5^{th} and 95^{th} percentiles. SPSS 11.0 was used to test for significant differences in the distributions

between treatments using a Kruskal-Wallis H test for independent samples. A *post-hoc* Mann-Whitney U Test for two independent samples identified significantly different data sets.

RESULTS

Temperature. Shipboard manipulations in vertical chambers in 2001 and 2002 indicate that these species respond to temperature gradients; the distribution of each species in controls was significantly different than in the temperature gradients ($p < 0.001$ for each species). However, comparisons of control distributions between species in 2001 and 2002 indicate that *Depressigyra globulus* climbed higher in the chambers than the other two species. Its distribution in controls and in gradient treatments was significantly different than *Lepetodrilus fucensis* (2001 and 2002) and *Provanna variabilis* (2002) (Figure 2.12). Thus, I can not compare the temperatures occupied by *D. globulus* in the gradient treatments to the other two species. *L. fucensis* also frequently detached from the chamber walls in 2001 but not in 2002 in the larger chambers (see control distributions for 2001 and 2002, Figure 2.12). Although control distributions were similar for *P. variabilis* and *L. fucensis* in 2002 experiments (Figure 2.12B), *P. variabilis* was found at significantly cooler temperatures in gradient treatments ($p < 0.001$) (Figure 2.12D).

Sulphide. The distributions of all three gastropod species in the sulphide gradient (2001) and sulphide pulse (2002) treatments did not differ significantly from controls.

DISCUSSION

The three gastropod species clearly responded to heat in the vertical non-

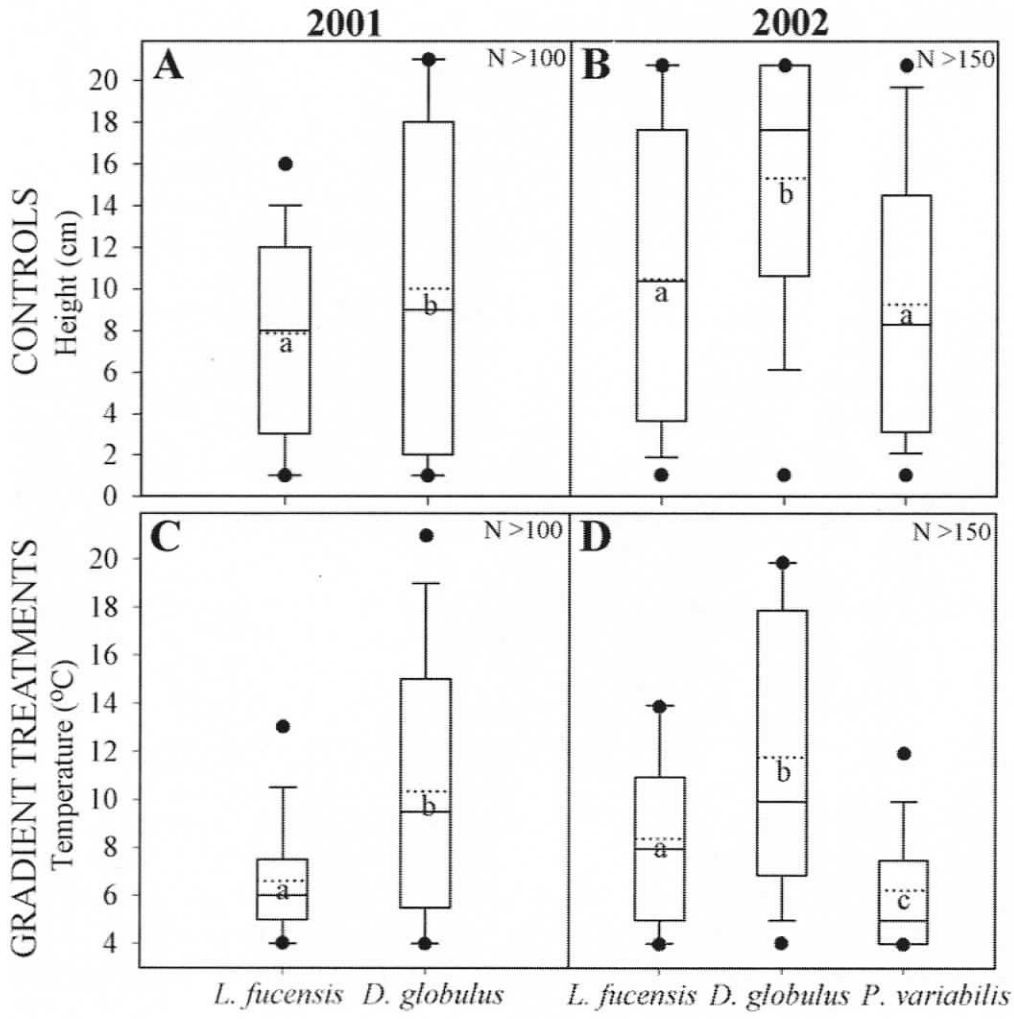

Figure 2.12

Lepetodrilus fucensis, *Depressigyra globulus* and *Provanna variabilis*. Behavioural responses to temperature gradients in non-pressurized vertical chambers; positions were recorded after four hours. N = total # of individuals for each species from pooled data. Box plots present 5th, 25th, 50th, 75th, 95th percentiles; the dotted line is the mean. Means with dissimilar letters differ significantly from each other ($p < 0.01$; Mann-Whitney U Test for two independent samples). Significant differences in responses exist both among species and compared to controls.

(A) 2001 control distributions at 4°C: no temperature gradient.

(B) 2001 distributions in a 4 to 21°C temperature gradient.

(C) 2002 control distributions at 4°C: no temperature gradient.

(D) 2002 distributions in a 4 to 21°C temperature gradient.

pressurized chambers. This finding was corroborated by similar experiments in pressurized vessels with a horizontal orientation and the significance of species-specific responses are discussed in Chapter 2: Discussion 'Responses to temperature'. However, the differences among species in their climbing behaviour and high moribundity suggest that although studies at surface pressure may be useful for indicating key parameters, behavioural experiments are best conducted under pressure. In addition, good controls are key to interpreting species responses. For example, the curvature of the cylinder influenced the crawling ability of *L. fucensis*, but did not impede *D. globulus*.

The control treatments returned significant differences between *Depressigyra globulus* and the other two gastropods: *D. globulus* was highly positively geotaxic and accumulated in the top of the chambers in the controls, while the other two species were less geotaxic. In the gradient treatments where the fluid temperatures increased vertically, the geotaxic behaviour of *D. globlulus* probably shifted its distribution to include higher temperatures. In comparison, *Lepetodrilus fucensis* and *Provanna variabilis* had similar distributions in the control chambers, but their behaviours differed in the gradient treatments. *L. fucensis* ranged from 4 to 14°C, while *P. variabilis* aggregated at lower temperatures and ranged from 4 to 10°C, indicating that *L. fucensis* prefers higher temperatures than *P. variabilis*. Experiments under pressure returned similar results for these two species and, as suggested in the discussion of Chapter 2, the differences in their thermal preferences likely relates to their habitat selection. *L. fucensis* prefers warm flows in active venting, while *P. variabilis* occupies a range of temperatures, and is probably adapted to the lower temperatures where it is most abundant.

Sulphide may provide important cues in habitat selection by vent species. For example, a shrimp species from Mid-Atlantic Ridge vents exhibits a chemosensory response to sulphide that may serve in orientating individuals to vent flows (Renninger et al. 1995). However, no preference for specific sulphide concentrations or avoidance of high (1 Mm) sulphide by the three gastropod species was observed. These species may not be limited by or may not prefer any specific value within the range of experimental micromolar concentrations and/or over the time intervals I reported in this study. Alternatively, the animals may have required a directional cue in order to orient along a sulphide gradient (e.g., Williams et al. 1983, Rittschof & Brown 1986), while our experimental design presented stable conditions. The response of these species to sulphide in combination with another environmental cue, such as fluid flow or temperature may be more important. While gastropod behavioural responses to in-vitro sulphide gradients were not detected it is impressive that individuals retained mobility in fluids with high sulphide concentrations. The ability of these three vent gastropods to survive high sulphide deserves investigation.

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Appendix 2.2

In- (0-25 cm from a vent source), near- (26-50 cm) and far-vent (51-75 cm) locations were sampled from vents at Axial Volcano, Endeavour Segment and Southern Explorer Segment in 2001 and 2002. These samples were used to address multiple hypotheses. Sample is the ROPOS dive log number. Vent features include substratum area sampled and temperature where indicated. Substratum type was basalt or sulphide.

Vent site Vent field Vent	Lat Long depth (m)	Year	Sample	Distance category (cm)	Area (cm^2)	Temp (°C)	Substratum
Axial South Caldera Caspar	45°55.1'N 129°59.6'W 1538	2001	630-10	0-25	83	13.5	Sulphide
Axial ASHES Gollum	45°56.0'N 130°00.8'W 1547	2001	624-07	0-25	85	11	Basalt
Axial ASHES Hell	45°55.9'N 130°00.9'W 1550	2002	662-08	0-25	-	-	Sulphide
Axial 98 Lava Flow Mrk 33	45°55.9'N 129°58.9'W 1524	2002	661-12	0-25	-	12	Basalt
Axial CASM Shepherd	45°59.3'N 130°1.6'W 1580	2001	628-04	0-25	93	9	Basalt
Endeavour Hi-Rise S&M 'site 1'	47°56.9'N 129°05.9'W 2202	2001	592-03	0-25	120	8	Sulphide
Endeavour Hi-Rise S&M 'site 2'	47°56.9'N 129°05.9'W 2201	2001	592-05	0-25	85	5	Sulphide
Explorer Mystic Area Einstein	49°45. 5'N 130°15.2'W 1798	2002	665-12	0-25	-	5	Sulphide
Explorer Merlin Mound Limpet Land	49°45.6'N 130°15.5'W 1778	2002	670-07	0-25	-	13	Sulphide
Explorer Merlin Mound Tubeworm Chimney	49°45. 5'N 130°15.4'W 1781	2002	666-04	0-25	-	-	Sulphide
Axial South Caldera Caspar	45°55.1'N 129°59.6'W 1538	2001	630-09	51-75	112	3.5	Sulphide
Axial ASHES Gollum	45°56.0'N 130°00.8'W 1547	2001	624-08	51-75	205	3	Basalt
Axial ASHES Hell	45°55.9'N 130°00.9'W 1550	2002	662-09	51-75	-	-	Sulphide

Axial 98 Lava Flow Mrk 33	45°55.9'N 129°58.9'W 1524	2002	661-03	51-75	-	3	Basalt
Axial CASM Shepherd	45°59.3'N 130°1.6'W 1580	2001	628-03	51-75	193	4	Basalt
Endeavour Hi-Rise S&M 'site 1'	47°56.9'N 129°05.9'W 2202	2001	592-04	51-75	140	3	Sulphide
Endeavour Hi-Rise S&M 'site 2'	47°56.9'N 129°05.9'W 2201	2001	592-06	51-75	105	3	Sulphide
Explorer Mystic Area Einstein	49°45. 5'N 130°15.2'W 1798	2002	665-11	51-75	-	3.5	Sulphide
Explorer Merlin Mound Limpet Land	49°45.6'N 130°15.5'W 1778	2002	670-08	51-75	-	3	Sulphide
Explorer Merlin Mound Tubeworm Chimney	49°45. 5'N 130°15.4'W 1781	2002	666-05	51-75	-	-	Sulphide
Axial 98 Lava Flow Mrk 33	45°55.9'N 129°58.9'W 1524	2001	623-06	26-50	155	4.5	Basalt
Axial 98 Lava Flow Mrk N3	45°56.6'N 129°59.1'W 1529	2001	622-40	26-50	260	4	Basalt
Axial 98 Lava Flow Nascent	45°56.2'N 129°58.9'W 1520	2001	625-07	26-50	273	5	Basalt
Endeavour Hi-Rise S&M 'site 3'	47°56.9'N 129°05.9'W 2201	2001	590-10	26-50	-	4.5	Sulphide
Endeavour Hi-Rise S&M 'site 4'	47°56.9'N 129°05.9'W 2201	2001	590-09	26-50	-	4.5	Sulphide
Explorer North Lucky Find	49°45.7'N 130°15.3'W 1781	2002	669-25	26-50	-	4	Sulphide
Explorer Northeast Zooarium	49°45.7'N 130°15.3'W 1791	2002	670-05	26-50	-	-	Sulphide

Appendix 2.3

Numbers of *Lepetodrilus fucensis*, *Depressigyra globulus* and *Provanna variabilis* greater than (bold) and less than 1 mm in- (0-25 cm), near- (26-50 cm) and far-vent (51-75 cm) at Axial Volcano, Endeavour Segment and Southern Explorer Segment. Sample information is provided in Appendix 2.1. nc = not counted.

Vent site **Vent field** **Vent**	**Sample**	**Distance category (cm)**	***# L. fucensis***	***# D. globulus***	***# P. variabilis***
Axial			23	0	0
South Caldera					
Caspar	630-10	0-25	**988**	**8**	**0**
Axial			9	4	0
ASHES					
Gollum	624-07	0-25	**1057**	**31**	**25**
Axial			0	0	0
ASHES					
Hell	662-08	0-25	**1269**	**128**	**1**
Axial			11	24	0
98 Lava Flow					
Mrk 33	661-12	0-25	**835**	**717**	**0**
Axial			111	48	11
CASM					
Shepherd	628-04	0-25	**2657**	**47**	**11**
Endeavour			114	0	0
Hi-Rise					
S&M 'site 1'	592-03	0-25	**3192**	**1138**	**5**
Endeavour			123	8	0
Hi-Rise					
S&M 'site 2'	592-05	0-25	**102**	**21**	**18**
Explorer			12	3	0
Mystic Area					
Einstein	665-12	0-25	**51**	**277**	**7**
Explorer			0	0	0
Merlin Mound					
Limpet Land	670-07	0-25	**1614**	**1852**	**0**
Explorer			0	0	0
Merlin Mound					
Tubeworm Chimney	666-04	0-25	**24**	**192**	**53**
Axial			3520	43	0
South Caldera					
Caspar	630-09	51-75	**83**	**2**	**27**
Axial			387	129	0
ASHES					
Gollum	624-08	51-75	**13**	**0**	**73**
Axial			3120	43	0
ASHES					
Hell	662-09	51-75	**98**	**12**	**141**

Axial 98 Lava Flow Mrk 33	661-03	51-75	1227 **19**	623 **38**	6 **120**
Axial CASM Shepherd	628-03	51-75	3007 **489**	399 **2**	1 **370**
Endeavour Hi-Rise S&M 'site 1'	592-04	51-75	7834 **56**	267 **8**	2 **61**
Endeavour Hi-Rise S&M 'site 2'	592-06	51-75	3101 **102**	18 **21**	0 **18**
Explorer Mystic Area Einstein	665-11	51-75	59 **51**	867 **277**	0 **7**
Explorer Merlin Mound Limpet Land	670-08	51-75	24 **24**	283 **192**	1 **53**
Explorer Merlin Mound Tubeworm Chimney	669-06	51-75	27 **64**	46 **8**	0 **32**
Axial 98 Lava Flow Mrk 33	623-06	26-50	nc **189**	nc **591**	nc **47**
Axial 98 Lava Flow Mrk N3	622-40	26-50	nc **121**	nc **148**	nc **6**
Axial 98 Lava Flow Nascent	625-07	26-50	nc **2662**	nc **126**	nc **4**
Endeavour Hi-Rise S&M 'site 3'	590-10	26-50	nc **2265**	nc **398**	nc **68**
Endeavour Hi-Rise S&M 'site 4'	590-09	26-50	nc **389**	nc **39**	nc **5**
Explorer North Lucky Find	669-25	26-50	nc **293**	nc **892**	nc **32**
Explorer Northeast Zooarium	670-05	26-50	nc **3247**	nc **1369**	nc **5**

CHAPTER 3

Size and sex based habitat partitioning by hydrothermal vent gastropods.

Abstract

I investigated the population characteristics of the northeast Pacific Ocean hydrothermal vent limpet, *Lepetodrilus fucensis* McLean, to isolate potential life history traits that might drive its high abundances. To determine if the limpet's recruitment success is exceptional, I compared its size structure and recruit densities to another common gastropod, *Depressigyra globulus* Warén and Bouchet 1989, in two distance categories from a vent fluid source: 0 to 25 cm (in-vent) and 51 to 75 cm (far-vent). Juveniles (<1 mm) of both species were abundant far-vent, but were rare in-vent where adults were most abundant. The density (individuals m^{-2}) of juvenile *L. fucensis* was one order of magnitude greater than *D. globulus* in far-vent locations: 2419 and 170 (respectively), and 76 and 11 in-vent. Peripheral locations may be important refuges for gastropod recruitment. Next, I hypothesized that *Lepetodrilus fucensis* might employ a specialized reproductive strategy, such as protandry. I quantified sex ratio patterns among *L. fucensis* populations from different hydrothermal fluxes. High flux locations were female biased, while low flux locations were male biased. I then measured the within population sex ratio of different size classes at in- and far-vent locations and from five different regions on a flange. In-vent locations hosted high numbers of females >6.0 mm and were consequently female biased in comparison to far-vent. Large females also dominated regions on a chimney flange that corresponded with the highest animal densities. These findings suggest that females are better competitors than males for optimal habitats, thus driving female-biases at in-vent locations. Next, *L. fucensis* were

moved away from venting for one year to test for differential survivorship between the sexes. 2% of the original female population survived compared to 27% of males. In addition, the gonads of post-transplant females were empty while males contained some seminal fluid, indicating females suffer a greater cost of reproduction. These results support that sex based habitat partitioning by *L. fucensis* populations optimizes female reproductive output, but because its recruitment patterns are similar to *D. globulus*, do not indicate remarkable life history traits.

INTRODUCTION

Spatial gradients in environmental conditions are important in structuring animal populations and can lead to differences in intraspecific survival and growth (e.g., Werner & Gilliam 1984, Mercurio et al. 1985, McMillan et al. 1995). Hydrothermal vents present the opportunity to examine relationships between habitat and population characteristics because steep environmental gradients in physical (e.g., substratum type and turbulence) and chemical (e.g., hydrogen sulfide and oxygen concentration) parameters occur at centimeter spatial scales (Johnson et al. 1986, Sarrazin et al. 1999, Luther et al. 2001). Consequently, animals that occupy a range of habitats near venting fluids are exposed to strikingly different environments. However, the role that spatial variability in environmental parameters plays in determining the population structure of hydrothermal vent fauna is potentially important (Bates et al. 2005), yet poorly understood, largely because experimental investigations of deep-sea animals are constrained by the difficulty of *in situ* studies.

Lepetodrilus fucensis McLean 1988 (Vetigastropoda, Lepetodrilidae) is a remarkably abundant limpet (densities >250 000 m^{-2}) around warm fluid sources at vents on the Juan de Fuca Ridge (Sarrazin et al. 1999, Tsurumi & Tunnicliffe 2001, Bates et al. 2005) and occupies a range of habitats from post-eruption to senescing stages (Tsurumi & Tunnicliffe 2003, Marcus 2003). Factors that drive the high abundances of *L. fucensis* are currently unknown, but may relate to its life history traits, such as its recruitment patterns or reproductive strategy. For example, adult and juvenile gastropods in shoreline habitats commonly partition habitats along environmental gradients to maximize offspring survival: juveniles select microhabitats where predation is low and their food acquisition is high (Vermeij 1972, Werner & Gilliam 1984, Gosselin & Chia 1995, Gosselin 1997). Female gastropods can also achieve greater relative fecundity with increases in size in comparison to males and species exploit this size-fecundity relationship in various ways (e.g., Grahame 1973). For example, females can grow faster than males in the same habitats (e.g., Burke 1978, Baghurst & Mitchell 2002), resulting in larger females in populations with equal numbers of males and females. Several gastropods also maximize their fecundity by changing sex: for example, protandry is a strategy where juveniles mature as males and change sex at larger sizes, leading to size separation between males and females (Coe 1936, Collin 1995).

Lepetodrilus fucensis is a dioecious species with a non-feeding planktonic larval stage (Lutz et al. 1986); protoconch-stage recruits settle out of the water column in high numbers in venting habitats (Metaxas 2003). Recruitment may be higher in the periphery, either as a result of differential settlement or habitat-specific survivorship, based on the observations that *L. fucensis* exhibits marked size decreases with increasing

distance from vent flows (Marcus & Tunnicliffe 2002). However, *L. fucensis* recruit densities along environmental gradients in hydrothermal flux have not been quantified. Comparisons of its densities to similar recruit-stages of other gastropods may provide insights into how *L. fucensis* achieves such high adult abundances, for example, juvenile abundances several orders of magnitude higher than other species may indicate relatively higher recruitment success. It is also possible that *L. fucensis* possesses a unique reproductive strategy because large animals are often female (McLean 1988). Hence, the primary sex ratio of *L. fucensis* and relationships between sex, size and habitat may be important.

The aim of this investigation was to determine if recruitment and sex ratio patterns of *Lepetodrilus fucensis* populations indicate specialized life history traits. First, I quantified the size structure and juvenile density of *L. fucensis* with increasing distance from a vent fluid source. I compared these results to a co-occurring gastropod, *Depressigyra globulus* Warén and Bouchet 1989, to determine if recruitment patterns differ between these two species. Next, I hypothesized that *L. fucensis* might optimize female reproductive output using a specialized reproductive strategy, such as protandry, or that females might simply select the best habitats for growth. To address these hypotheses, I quantified *L. fucensis* population sex ratios in habitats with different levels of hydrothermal flux. I then determined if sex ratio varies with size classes and between decimeter-spaced locations. Last, I quantified the survivorship of females and males in a population transplanted to the periphery of a vent to test if differential mortality between males and females might contribute to sex ratio variation.

METHODS AND MATERIALS

In-situ collections

All *in situ* collections were made with remote operated (ROPOS) and manned (ALVIN and PISCES) vehicles at vent sites on the Juan de Fuca Ridge: Axial Volcano (45°56'N 130°00'W; depth 1570 m) and Endeavour Segment (47°57'N,129°06'W; depth 2220 m), and Explorer Ridge: Southern Explorer Segment (49°45'N 129°42'W; depth 1850 m). Collections were fixed in 7% seawater formalin immediately following sample recovery and transferred to 70% EtOH in the lab. Point measurements of maximum temperature were recorded for most sampled sites with a rugged transition joint probe (Adam's module thermistor, ±1°C).

The population size structure and densities of juvenile and adult *Lepetodrilus fucensis* and *Depressigyra globulus* were quantified from in- and far-vent samples described in 'Small-scale gradient' (below). Investigations of sex ratio patterns of *L. fucensis* were based on comparing (1) the inter-population sex ratio among ('Population collections') large collections that varied in exposure to hydrothermal fluid flux and (2) the within-population sex ratios of samples that were separated by centimeter distances ('Small-scale gradient' and 'Flange'). Small-scale gradient samples are described first because they were used to characterize size, density and sex structure.

Small-scale gradient. Sampling (using suction) targeted two locations at different distances from isolated vent outflows: 'in-vent' was 0-25 cm from the vent source and 'far-vent' was 51-75 cm from flow (see Bates et al. 2005). In-vent locations were typically immersed in shimmering water (maximum temperatures ranged from 5 to 15°C), while at far-vent locations, no shimmering was evident (<4°C). In addition, near-

vent locations (25-50 cm) were at the margin of visible flow (n = 5) (4-5°C) and were not paired with in- and far-vent samples.

Size: The sizes of *Lepetodrilus fucensis* and *Depressigyra globulus* were measured for six pairs of in- and far-vent locations at Axial Volcano (4 vents: Gollum, Shepherd, Mrk 33, and Caspar), Endeavour (2 vents at S&M: labeled 'site 1' and 'site 2') and Southern Explorer Ridge (2 vents: Tubeworm Chimney and Einstein). Sample descriptions for these locations are in Appendix 2.2. The four vents at the base of sulphide structures were not included because larval delivery may be influenced by fluid flow around physical structures. The shell lengths (Figure 3.1) of up to 500 individuals were measured using a digital processing program (Image-Pro Plus© 4.5) to construct size frequency distributions (<50 *D. globulus* were found at two far-vent locations, thus size frequencies are not shown for these vents). A Mann-Whitney U Test identified significant differences between in- and far-vent size frequencies where the separation between juveniles and adults was not complete.

Juvenile density: Here, juveniles refer to early post larval stages up to 1 mm shell length (this shell length was arbitrarily assigned). Juvenile density was measured at five in- and far-vent locations described in Appendix 2.2 (Axial Volcano: Caspar, Gollum and Shepherd; Endeavour: S&M 'sites 1 & 2'). Sample area from high quality video imagery was quantified using a digital processing program (Image-Pro Plus© 4.5) with laser spots at 10 cm apart. Protoconch identifications were verified using light and scanning electron microscopy (juveniles were dehydrated in 100% EtOH, critical point dried, sputter-coated in gold and viewed with a Hitachi S-3500N microscope). Animals >1 mm shell length were removed from these samples and their densities are reported in Bates et al. (2005).

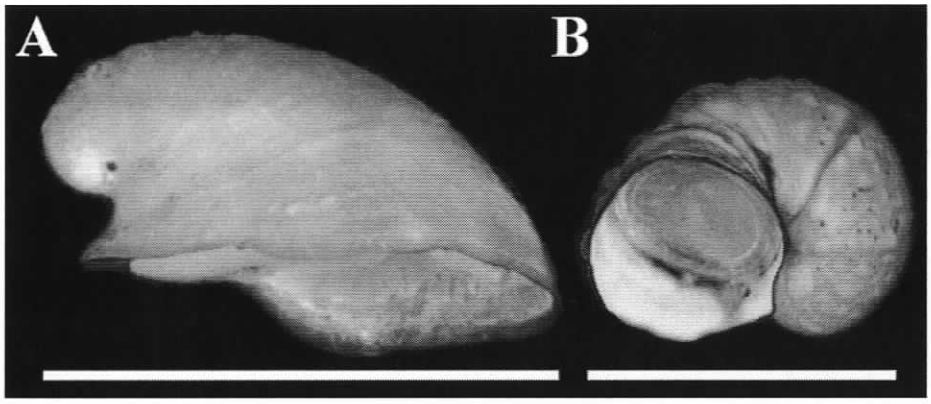


Figure 3.1
Shell lengths were measured using a light microscope on (A) *Lepetodrilus fucensis*: the distance from the apex to the posterior edge of the shell (indicated by a white bar), and (B) *Depressigyra globulus*: the longest shell dimension (indicated by a white bar).

Sex ratio: Lepetodrilus fucensis lacks an operculum, thus the sex of large numbers of individuals can be easily determined by the presence (male) or absence (female) of a conspicuous penis (Figure 3.2). Sex ratio is reported as proportion male. The sex ratios of *L. fucensis* (sample size ranged from 10 to 50 individuals) at ten vents with paired in- and far-vent samples were compared to determine if sex ratio varies at small spatial scales; a paired t-test identified significant differences between the two distance categories (see Appendix 2.2 for sample information). The size-frequency distribution of females and males at both in- and far-vent locations (combined from the ten vents) tested for size differentiation between the sexes. Next, twenty individuals from four size classes (every 1.5 mm from 4.5 mm to 9 mm; measured using an ocular micrometer) were sexed from five different locations in the following distance categories: in-vent (Appendix 2.2: Gollum, Nascent, S&M 'site 1', Shepherd; Appendix 3.1: Salut Vent), near-vent (Appendix 2.2: Lucky Find, S&M site 3 & 4, Village, Zooarium) and far-vent (Appendix 2.2: Hell, Lucky Find, Shepherd, Tubeworm Chimney; Appendix 3.1: Salut Vent), to determine (1) if sex ratio is related to distance from a vent source and size class. A two-way ANOVA tested whether there is a significant interaction between distance category and size; one-way ANOVAs, followed by Tukey's HSD post hoc tests, identified significantly different locations within each size class.

Population collections. To determine the population sex ratio of *Lepetodrilus fucensis* at sites with different levels of hydrothermal flux, *L. fucensis* specimens in each collection) were examined from preserved collections (V. Tunnicliffe, Department of Biology, University of Victoria) sampled at vent sites on the Juan de Fuca and Explorer Ridges (1984 to 2003) (see Appendix 3.1 for collection information). 'High flux'

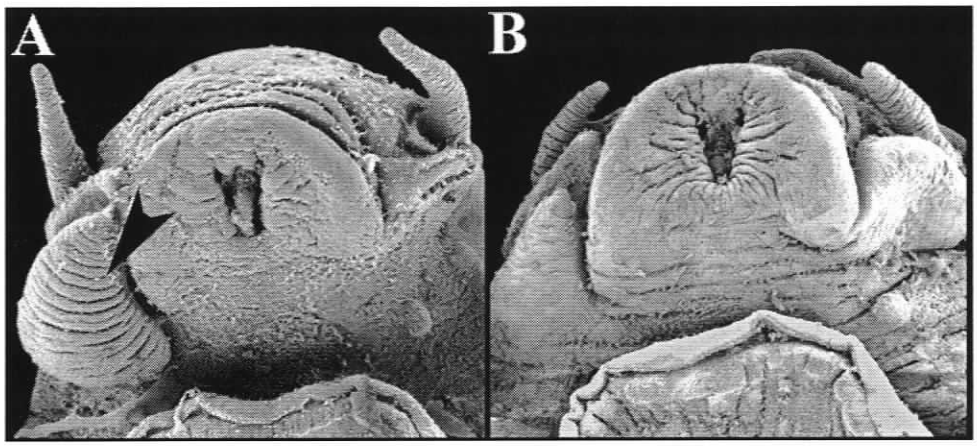


Figure 3.2
Scanning electron micrograph of *Lepetodrilus fucensis* depicting the ventral surface of the anterior region for a male (A) and female (B). Males were identified by the presence of a penis (black arrow).

samples (n = 7) were from sulphide chimneys in active fluid flow; 'mixed flux' (n = 7) collections were tubeworm bushes where venting intensity decreased from a focused source in the middle of the bush to no visible venting at the edge of the bush; 'low flux' were at locations from 0.5 to 3 m from vents (n = 4), or at senescent vents (n = 3). One hundred randomly selected *L. fucensis* were sexed from each population. Typically, sulphide substrata were sampled by suction, while entire tubeworm bushes were grabbed with the manipulator arm and transported to the surface in a closable box. A one-way ANOVA, followed by Tukey's HSD post hoc tests, identified flux categories with significantly different sex ratios.

Flange. The sex and shell length of individuals on five different regions of the top and bottom surfaces of a flange (Crypto Vent, Main Field, Endeavour: 47°56.9'N, 129°05.9'W) were measured to determine if sex-ratio varies at small spatial scales and in five size classes: 4.5, 6.0, 7.5, 9.0 and 10.5. The microhabitats on a chimney flange (69 x 43 x ~10 cm) overhanging a 206°C temperature pool were characterized by *in situ* temperature measures and observations of fluid flow. The flange was detached, wrapped in a blanket to immobilize the fauna, and transported to the surface (Tsurumi et al. 2003). Colour images of animal fauna on the top surface were taken during sampling and both surfaces were photographed upon recovery. To record the spatial orientation of *Lepetodrilus fucensis*, a grid was overlaid on the top and bottom surfaces and individual animals were removed from each grid square (2.5 x 2.5 cm). The overall sex ratio of the flange population was estimated from 100 *L. fucensis* that were jostled from the flange during collection. Cumulative binomial probability tests estimated the probability of sampling male- or female-biased sex ratios in a population where the sex ratio is 0.5.

Transplant manipulation

To test whether the two sexes of *Lepetodrilus fucensis* exhibit differential mortality in response to reduced fluid flow, animals were transplanted away from flow. Specimens (~600) from a focused fluid source at Hell Vent (Axial Volcano: 45°55.9'N, 130°00.8'W) were collected by suction and without recovery to the surface, were deposited in a powder-coated stainless steel framed cage (30 x 25 x 25 cm, 2 mm polypropylene mesh) and repositioned with ROPOS to a location where no shimmering was visible, 50 cm away from fluid flow (3°C fluid temperature). In-vent (sub-sample of transplant population) and far-vent limpets (sampled from the substratum near the transplant cage) were also collected (see 'Small-scale gradient'). The transplant cage was recovered after 12 months. The sex ratio of the transplanted animals was determined and compared to co-collected specimens from fluid flow and specimens that were near the transplant cage at the start of the experiment. Cumulative binomial probabilities were calculated to test whether the transplant animals showed a significantly different sex ratio than their population of origin.

Gonad comparisons

The gonads of *Lepetodrilus fucensis* were examined from different locations using light microscopy. *L. fucensis* transplanted to a far-vent location at Hell Vent, Axial Volcano (surviving limpets: 9 females, 48 males), were compared to animals that were co-collected (but immediately fixed) from in-vent (25 females, 25 males). The gonads of specimens from active (Axial Volcano: Nascent and Hairdo) and senescent (Axial: Old Worms and Co-Axial: HDV) tubeworm bushes (25 females and 25 males in each habitat) were also examined (see Appendix 3.1 for collection information). Animals ranged

between 4 and 12 mm shell length. A qualitative scoring system was based on the following criteria: if eggs or seminal fluid (white shimmering evident) were visible in the entire gonad (testes occur in the posterior gonad) animals were scored as a '2'. If eggs or seminal fluid were absent from the posterior region of the gonad, and the testes were reduced (males), but present on the ventral surface of the gonad, animals were scored as '1'. Animals with no eggs or seminal fluid visible under light microscopy were assigned a '0'. Each specimen was examined for the co-occurrence of a penis, seminal fluid and eggs to identify hermaphrodites. A log-linear analysis (three-way contingency table) tested for significant differences in the frequency of individuals in each gonad score between the sexes and sites.

RESULTS

Size structure: Lepetodrilus fucensis (Figure 3.3; 6 vents) and *Depressigyra globulus* (Figure 3.4; 4 vents) exhibited size-based spatial partitioning. Mean *L. fucensis* shell lengths at in-vent locations ranged from 3.2 to 7.6 mm; at least 95% of in-vent populations were greater than 1 mm shell length and protoconchs were absent. In comparison, far-vent limpets were mostly small: mean shell lengths were ~0.5 mm (Figure 3.5A & B). The density of protoconchs at five far-vent locations varied from 5 to 477 ind. dm^{-2}; the mean (± 1 SD) was 209 ± 232 ind. dm^{-2}. There also appears to be a relationship between maximum vent temperature and the mean shell length of in-vent *L. fucensis*: the largest limpets occurred in vent flows where maximum temperatures were greater than 10°C (Figure 3.3D, E & F). *D. globulus* was also larger in-vent, where mean shell length ranged from 1.8 to 3.9 mm, compared to far-vent, 0.5 to 2.0 mm (Mann-Whitney U, $p < 0.001$, $Z < -10$). Unlike *L. fucensis*, the proportion of *D. globulus*

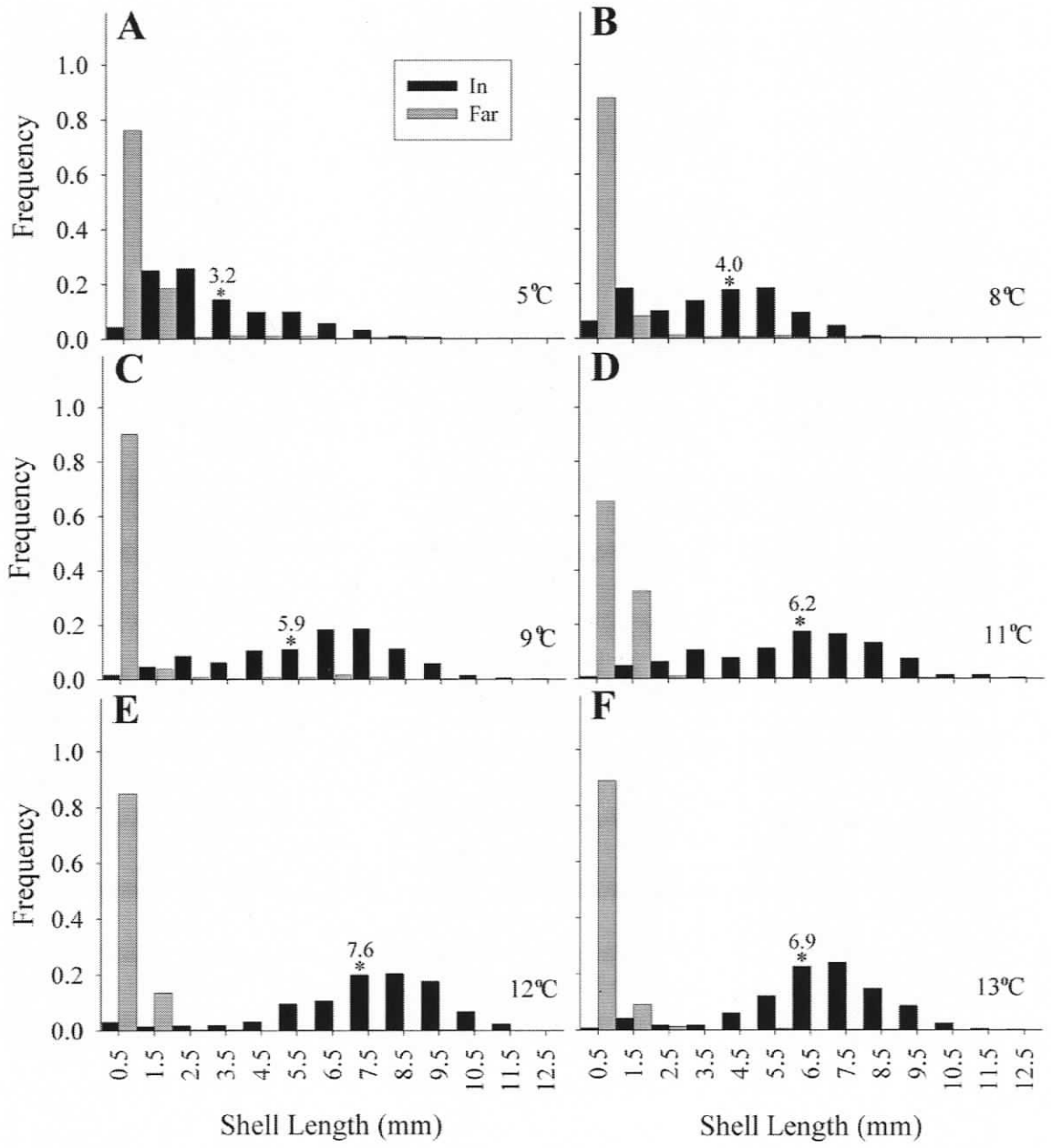


Figure 3.3
Size frequency of *Lepetodrilus fucensis* collected in- (black) and far-vent (grey) at six locations. Sizes classes are 1.0 mm bins. Two sulphide-hosted vents at Endeavour Segment are shown (A) S&M 'site 1' and (B) S&M 'site 2'. Four basalt-hosted vents were sampled at Axial: (C) Shepherd, (D) Gollum, (E) Mrk 33 and (F) Caspar. The maximum in-vent fluid temperature over a 5 min interval is indicated. Higher temperature flows tended to have larger animals. Stars indicate the size class that includes the mean. Mean size for in-vent samples are shown above the star. The mean size of all far-vent locations occurred in the 0 to 1 mm size class. There is a significant difference between in- and far-vent size frequency distributions at all vents (Mann-Whitney U, $p < 0.001$, $Z < -100$).

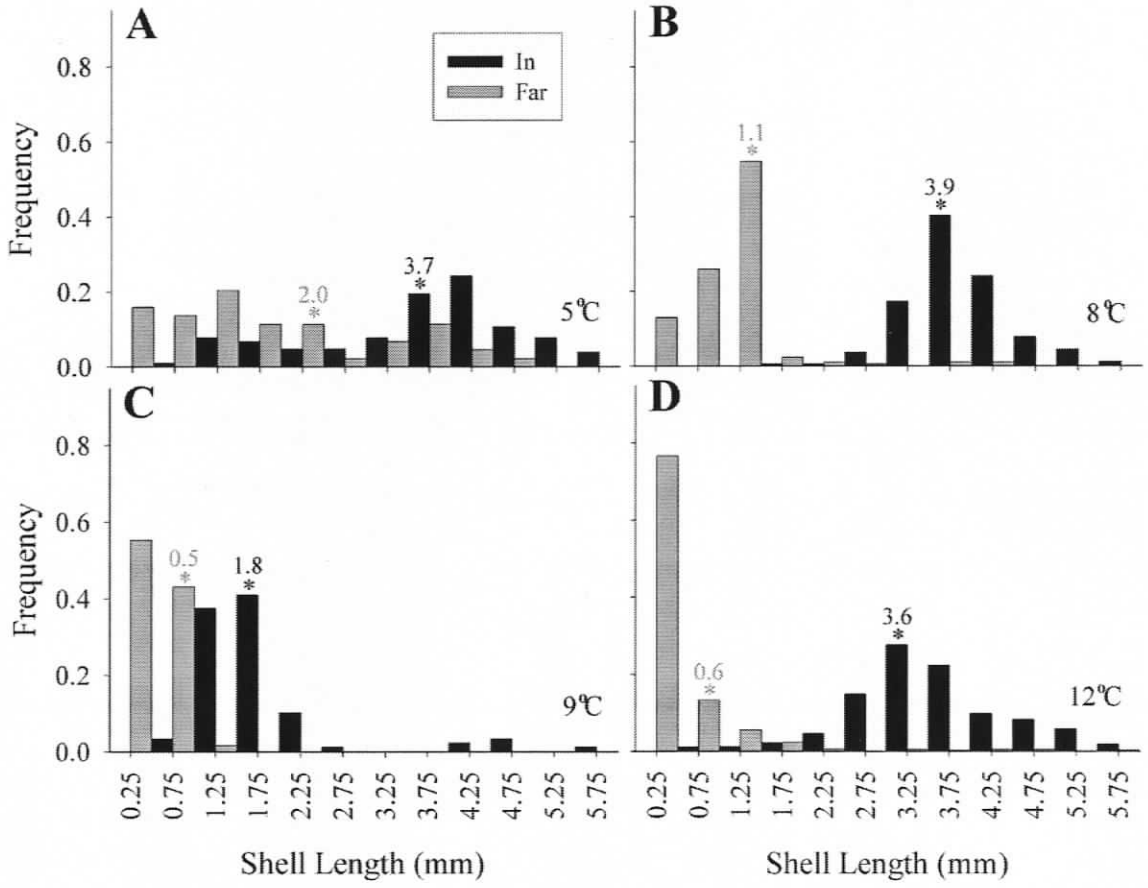


Figure 3.4
Size frequency distribution of *Depressigyra globulus* collected in- (black) and far-vent (grey) at four locations. Two sulphide-hosted vents at Endeavour Segment are shown (A) S&M site 1 and (B) S&M site 2. Data from two basalt-hosted vents are shown from Axial: (C) Shepherd, (D) Mrk 33. The maximum fluid temperature during sampling is indicated. Higher temperature flows tended to have larger animals. Stars indicate the bin size that includes the mean. Mean size for in- and far-vent samples are shown above the star. There is a significant difference between in- and far-vent size frequency distributions at all vents (Mann-Whitney U, $p < 0.001$, $Z < -10$).

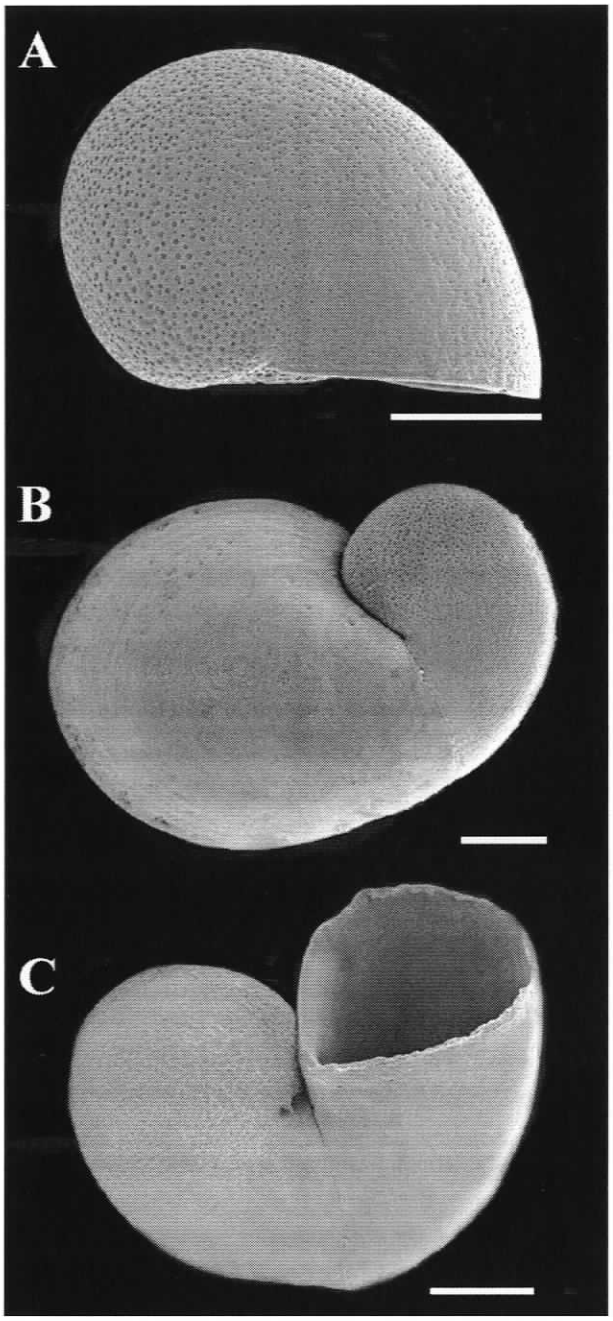


Figure 3.5
Scanning electron micographs of *Lepetodrilus fucensis* protoconchs (A) and juveniles (B), and *Depressigyra globulus* juveniles (C), abundant in peripheral habitats. Scale bar = 30 μm.

in the smallest size classes varied between vents. At two Endeavour vents (Figure 3.4A and B), the mean shell length far-vent was greater than 1.0 mm, while at two Axial vents, (Figure 3.4C and D) the far-vent locations were dominated by *D. globulus* less than 1.0 mm (Figure 3.5C). Furthermore, a relationship between maximum in-vent fluid temperature and *D. globulus* shell length was not apparent; mean shell length was similar in 5°C (Figure 3.4A) and 12°C (Figure 3.4D) vent flows.

Juvenile density: The densities of juvenile *Lepetodrilus fucensis* and *Depressigyra globulus* less than 1 mm in shell length were significantly greater (Monte Carlo Randomization test, $p < 0.001$) in far-vent habitats. Mean juvenile *L. fucensis* density in-vent ($n = 5$) was 56±46 individuals dm^{-2}, two orders of magnitude less than far-vent, 2616±2002 ind. dm^{-2}. The mean in-vent density of juvenile *D. globulus* was 8±10 dm^{-2}, while far-vent habitats hosted 103±88 dm^{-2}. The densities of the two gastropods above 1 mm in shell length show the reverse trend and are reported for the same samples in Bates et al. (2005): mean adult *L. fucensis* density was 2100 ind. dm^{-2} in-vent and 90 ind. dm^{-2} far-vent, and *D. globulus* mean densities in- and far-vent were 240 and 60 ind. dm^{-2} (respectively). Raw data for juveniles and adults at in- and far-vent locations are presented in Appendix 2.3.

Sex ratio: Figure 3.6 shows the relationship between sex ratio and fluid flux. The sex ratios of *Lepetodrilus fucensis* populations collected in high flux (chimney flange collections) were significantly female-biased (mean proportion of males = 0.34±0.07) in comparison to samples from low flux (senescent tubeworm bushes and peripheral locations) (0.64±0.08) (Figure 3.6) (one-way ANOVA, $F = 9.8$, $p < 0.01$; a post-hoc Tukey HSD test identified significant differences between the fluxes). In comparison, the

mean sex ratio from tubeworm bushes where fluid flux was mixed was unbiased (0.49±0.08). Raw data are presented in Appendix 3.1.

The sex ratios of *Lepetodrilus fucensis* collected from in- and far-vent locations at ten vents were significantly different (paired t-test, $p < 0.001$, $t = -8.7$). In-vent locations were more female biased than far-vent (Figure 3.7A). The 600 *L. fucensis* transplanted from in- to far-vent (Hell Vent, Axial) had an initial sex ratio of 0.3 (~420 females and 180 males). However, females suffered higher mortality than males during the year-long transplant to a far-vent location: 9 females (~2% survivorship) and 48 males (~27%) comprised the post-transplant population. The size frequency distribution of all the females examined from the 10 in- and far-vent locations combined was significantly different than males (Mann-Whitney U, $p < 0.01$, $Z = -3.3$) (Figure 3.7B). The difference was driven by the larger size classes; 75% of the animals >12 mm in shell length were female.

Figure 3.8 shows the sex ratios of *Lepetodrilus fucensis* in different size classes for samples collected from in- (0 to 25 cm from fluid flow), near- (26 to 50 cm) and far-vent (51 to 75 cm) locations. There was a significant interaction between location and size (two-way ANOVA: $p < 0.05$, $F_{crit} = 2.3$, df =6). There was no difference in the sex ratio of animals 4.5 mm in shell length among locations; this size class includes the first reproductive animals judging from the occurrence of eggs (at ~4.0 mm) and sperm (at ~3.5 mm) in gonads. As size increased (6.0, 7.5 and 9.0 mm size classes), in-vent animals were significantly female-biased in comparison to far-vent (one-way ANOVA: $F > 8$, $p < 0.01$; post-hoc Tukey HSD tests identified significantly class). The largest size

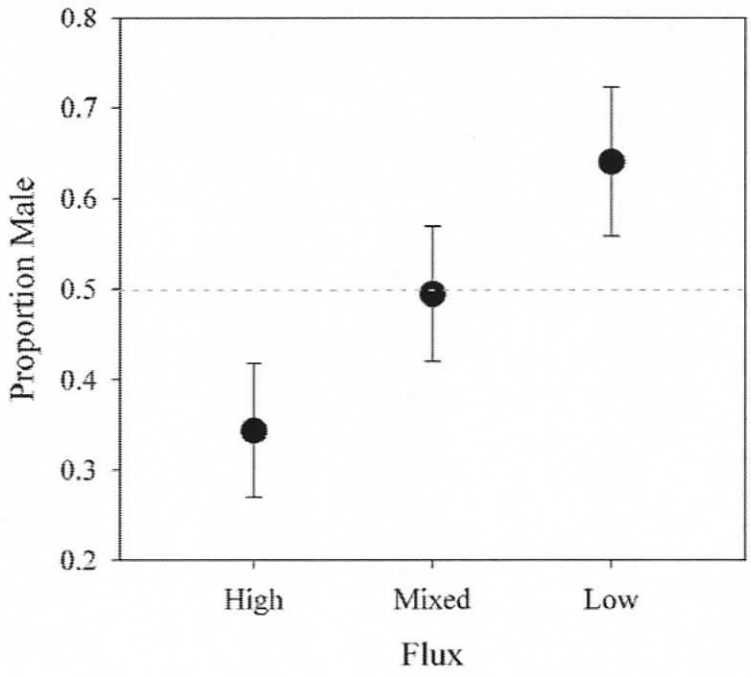
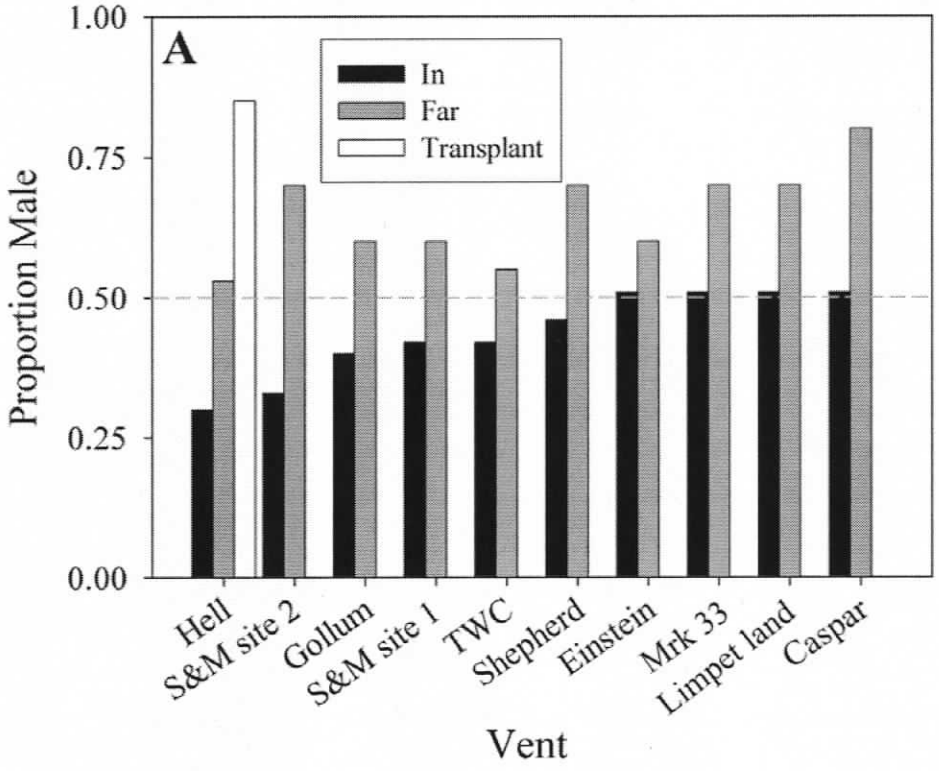


Figure 3.6
Sex ratio (mean ±1 SD) of *Lepetodrilus fucensis* populations from habitats in different types of hydrothermal activity: high (chimney flanges), mixed (tubeworms were fluid flow was visible in a portion of the bush) and low flux (senescent tubeworm bushes and peripheral locations). Seven collections were examined for each flux type; 100 individuals were sexed in each collection. High and low flux populations had significantly different sex ratios (t-test for Equality of Means, $p < 0.001$, $df = 12$): high flux were female-biased, while male-biased populations were in low flux. Mixed flux had relatively even numbers of males and females, as indicated by the dotted line.


A
In
Far
Transplant
Proportion Male
1.00
0.75
0.50
0.25
0.00
Hell
S&M site 2
Gollum
S&M site 1
TWC
Shepherd
Einstein
Mrk 33
Limpet land
Caspar
Vent

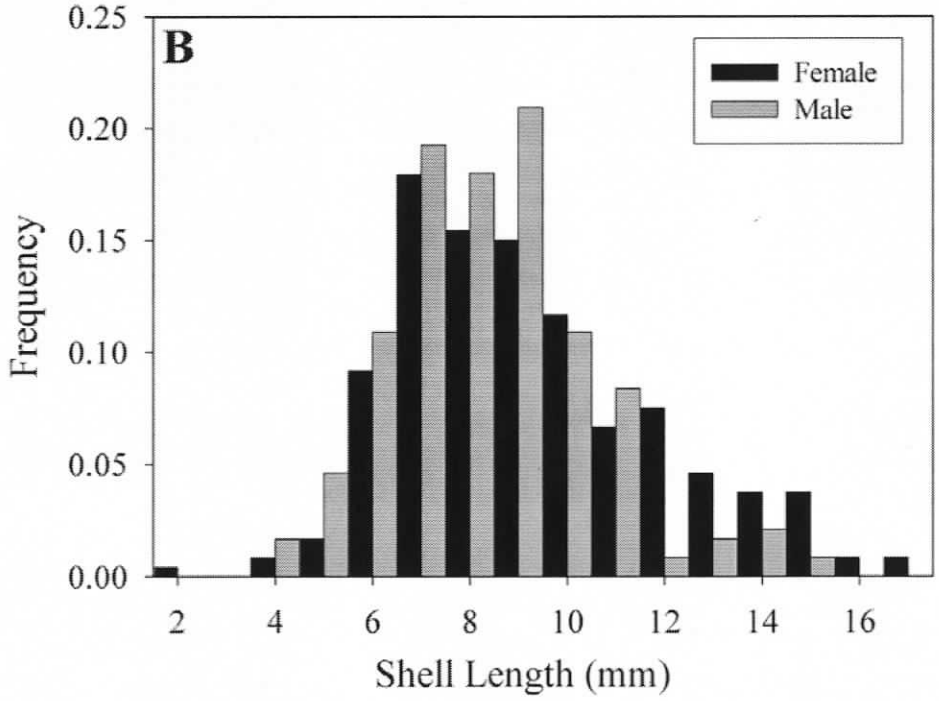

B
Female
Male
Frequency
0.25
0.20
0.15
0.10
0.05
0.00
2
4
6
8
10
12
14
16
Shell Length (mm)

Figure 3.7
(A) Sex ratio (mean ±1 SD) for *Lepetodrilus fucensis* between 4 and 16 mm shell length in- and far-vent at sites on the Juan de Fuca and Explorer Ridges. In-vent samples were female-biased in comparison to far-vent samples. The sex ratio of the transplant treatment, 0.85, is after one year in a far-vent habitat. The sex ratio of the pre-transplant population was 0.25. The sample size at each vent varied: n = 50 for Hell, Tubeworm Chimney (TWC) and Shepherd; n = 10 for all remaining samples. The dotted grey line shows an even sex ratio (0.5 male).
(B) Size frequency distribution of females (n = 221) and males (n = 219) included in (A) (except transplant treatment: Hell Vent). Animals with the largest shell length tended to be female. Females and males had significantly different size frequency distributions (Mann-Whitney U, $p < 0.01$, $Z = -3.3$).

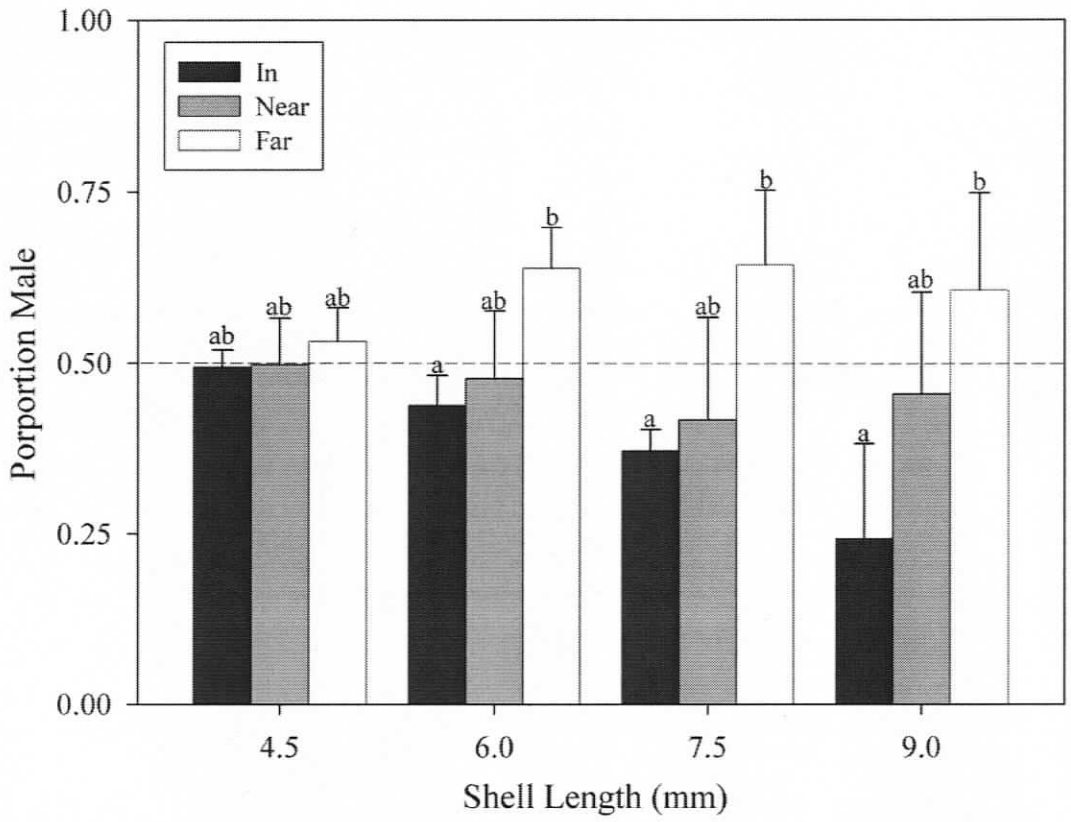


Figure 3.8
Sex ratio of *Lepetodrilus fucensis* in different size classes for collections (n = 5) in-vent (0-25 cm from flow), near-vent (26-50 cm) and far-vent (51-75 cm). 20 individuals were sexed in each size class. Dissimilar letters identify significantly different locations. There is a significant difference in the proportion male between in and far-vent for animals with shell lengths of 6.0, 7.5 and 9.0 mm (t-test for Equality of Means, $p < 0.01$, $df = 8$). The proportion male of animals 4.5 mm in shell length did not vary between in, near and far-vent collections. Near-vent collections had a mean sex ratio of 0.5 for all size classes.

class (9.0 mm) showed the most extreme difference in mean sex ratio: 0.25 in-vent versus 0.65 far-vent. The sex ratios of *L. fucensis* from near-vent locations were near 0.5 and were not significantly different to those limpets from in- or far-vent locations.

Figure 3.9 is a schematic of the flange (Crypto, Endeavour Segment) depicting the spatial location of *Lepetodrilus fucensis* in relation to fluid flow and measured temperatures. Fluids on the top right side of the flange were warmer (maximum of 105°C at the fluid exit points) than the top left side (maximum of 10°C). On the bottom surface, fluids from the high temperature pool flowed through a conduit that exited at three burn holes, and then to the right. The overall population sex ratio of *Lepetodrilus fucensis* from the flange was significantly female biased (proportion male = 0.39), similar to other chimney collections in high flux (Figure 3.6). However, *L. fucensis* separated into five regions on the flange (Figure 3.9) and the size and sex ratio of limpets from these regions varied (Figure 3.10). Regions where *L. fucensis* was relatively low in abundance hosted smaller sized individuals with male biased sex ratios, conversely, locations with higher abundances hosted larger limpets that were primarily female. Specifically, limpets in regions 1 and 3 (top) were mostly in the 4.5 mm size class; larger individuals tended to be male (proportion male >0.6) and animals with shell lengths greater 10 mm were absent (Figure 3.10A). In region 5 (bottom), limpets 6.0 mm in shell length dominated the sample (Figure 3.10B) and animals greater than 4.5 mm were male dominated (proportion male > 0.65) (except in the 9.0 mm size class where only six *L. fucensis* were present). In regions 2 (top) and 4 (bottom), animals ranged up to 12 mm shell length and were evenly distributed across the size classes (Figure 3.10); larger animals tended to be female (proportion male <0.40).

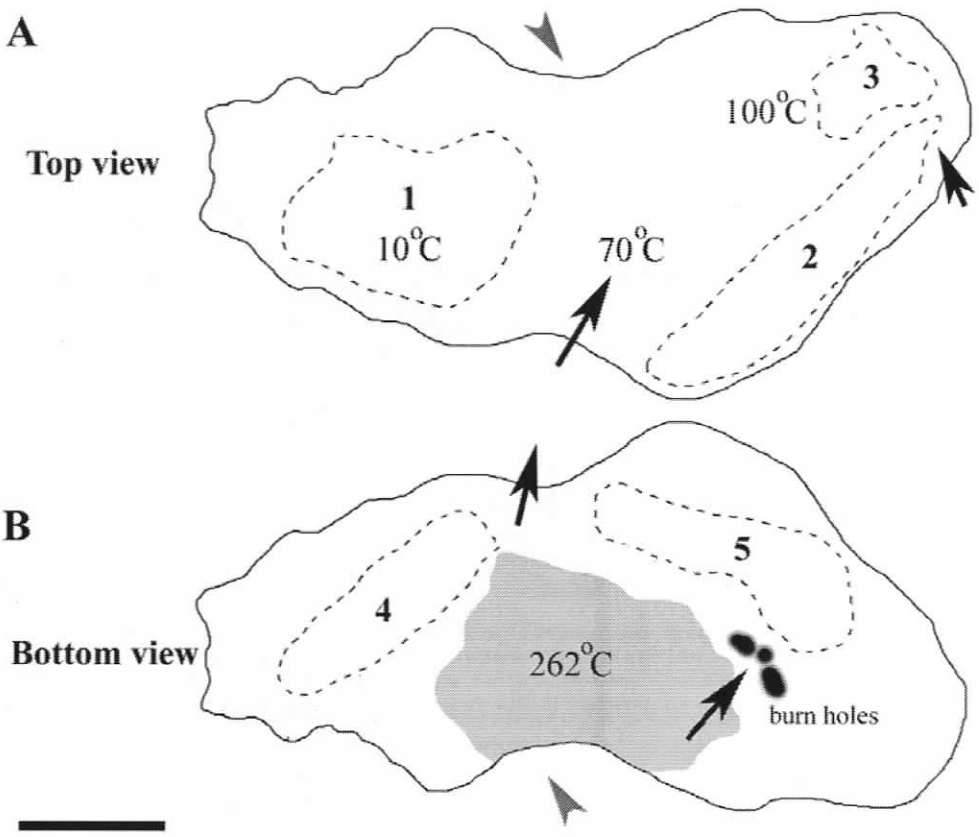


Figure 3.9
Top and bottom view of Crypto flange collected at Endeavour Segment. The dotted lines enclose regions where *Lepetodrilus fucensis* were found. The grey shading indicates the high temperature pool of water trapped on the bottom surface of the flange. Arrows are observed directions of fluid flow on the flange. Near region 3, water was diffusing through the flange and flowed up over the edge from the pool. The right side (70-100°C) was hotter than the left side (10°C). Grey arrows indicate the broken edge. Scale bar = 25 cm.

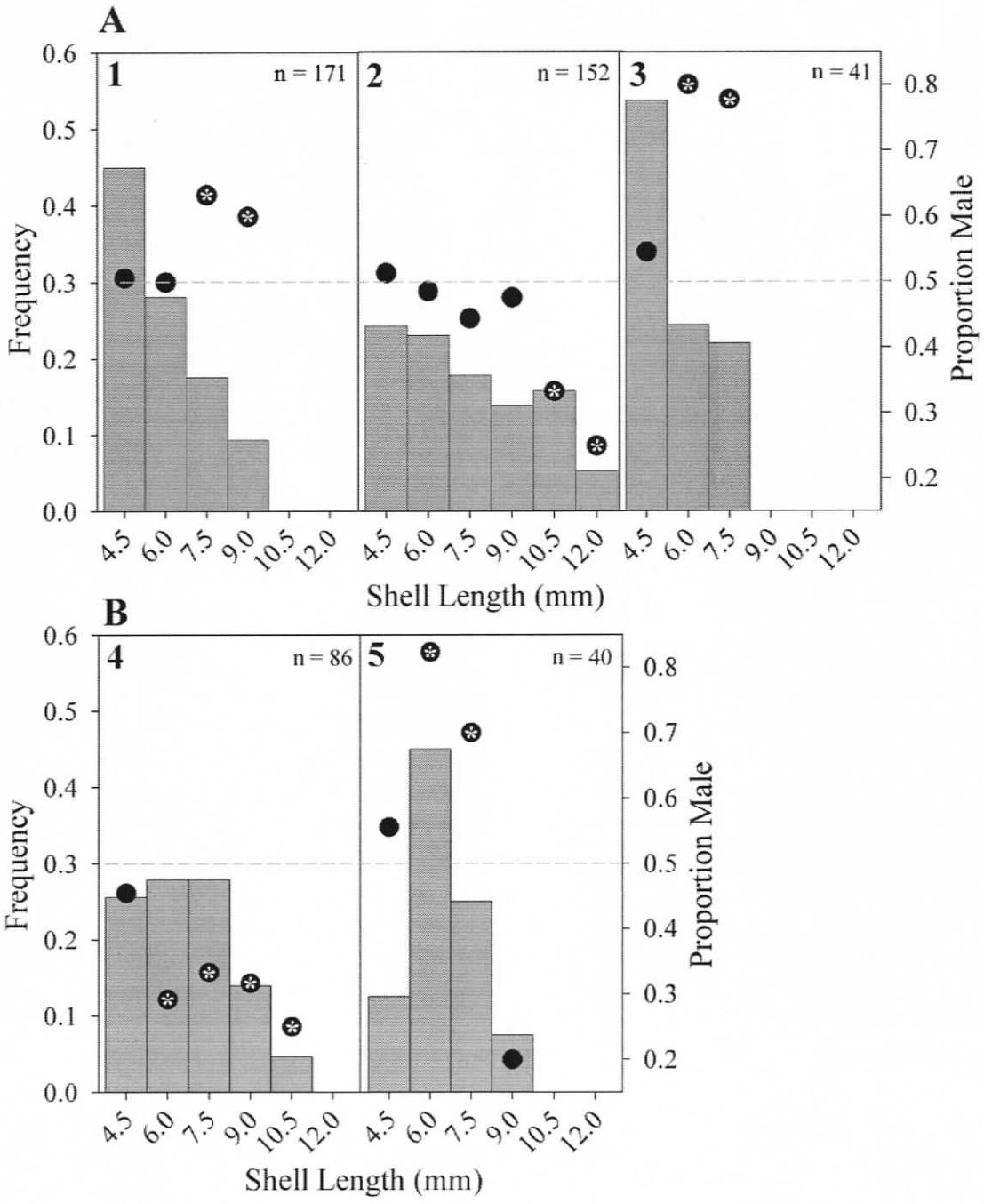


Figure 3.10
Size frequency (left y-axis; grey bars) and sex ratio (right y-axis; black dots) for *Lepetodrilus fucensis* collected in different regions on the top (A) and bottom (B) surface of Crypto flange. White stars indicate sex ratios with a <0.05 probability of occurrence if the true population sex ratio is 0.5. Bold numbers indicate regions represented on Figure 3.9. Regions with animals greater than 10 mm in shell length (Regions 2 and 4) were female-biased in larger size classes. Male-biased sex ratios occurred in the 6 to 9 mm size classes in regions with smaller animals (Regions 1, 3 and 5).

Table 3.1
Percent females and males collected from different flow conditions for three qualitative categories of gonad fullness. A gonad score of '0' indicates gonads without eggs or seminal fluid present under light microscopy. '1' identifies animals with eggs or seminal fluid present on the ventral surface of the gonad, but absent from the posterior gonad (the gonadial duct is posterior). Males with a score of '1' typically showed reduced testes. A score of '2' was assigned to animals with the entire gonad full of eggs or seminal fluid and for males, ripe testes.
(A) Transplant Manipulation: Pre-transplant animals (n = 25 for each sex) were collected in-vent and compared to animals from the same place but after transplantation for a year to a far-vent location (survivorship: females = 8, males = 49).
(B) Tubeworm bushes: Gonads of animals (n = 25 for each sex) from active versus senescing tubeworm bushes were compared.

Gonad Score	Pre-transplant % female	% male	Post-transplant % female	% male
0	0	0	78	17
1	0	4	22	83
2	100	96	0	0

B. Tubeworm bushes

Gonad Score	Active % female	% male	Senescing % female	% male
	Nascent		*Old Worms*	
0	0	0	32	6
1	8	4	68	56
2	92	96	0	38
	Hairdo		*HDV*	
0	0	0	60	0
1	0	0	4	45
2	100	100	0	55

Table 3.1 summarizes gonad fullness scores for females and males in different habitats. Gonad fullness scores were significantly different between males and females in the transplant group and the senescing tubeworm bushes ($G^2 > 33$, $df = 2$, $p < 0.001$). Following transplantation to the periphery of a vent for one year, surviving females had empty gonads (78%), while males retained visible seminal fluid in a large portion of their gonad (83%) (Table 3.1). Likewise, a greater percentage of females from senescing vents had empty gonads (32% at Old Worms and 60% at HDV) in comparison to males from the same samples (6% and 0%), while many males (38% and 55%) had full gonads and ripe testes; no females from this habitat were full of eggs. The difference in gonad scores between the low (transplant and senescing) and high (in-vent and active) habitats ($G^2 > 21$, $df = 3$, $p < 0.001$) was also significant. 90% animals collected from the same in-vent site as the transplant group had full gonads. Likewise, in the active tubeworm bushes, the majority of animals (>98%) had full gonads.

No evidence was found for sex change, i.e., penises were never observed on animals with eggs visible in their gonads, but were present only on animals with visible seminal fluid.

DISCUSSION

Size based habitat partitioning

Lepetodrilus fucensis and *Depressigyra globulus* exhibited size based spatial partitioning along gradients in hydrothermal flux. In preferred habitats nearest vent flows, adults of both species were abundant (Bates et al. 2005), animals less than 1 mm in shell length were scarce and protoconchs were absent; instead, recruits occupied

peripheral locations and probably migrate into active fluid flow at larger sizes. In particular, *L. fucensis* juveniles reached very high densities in peripheral habitats, up to 550 000 individuals m^{-2}, dominated by early post larval stages. A similar size separation is reported by Marcus and Tunnicliffe (2002). Although the proximate cause for size partitioning by *L. fucensis* and *D. globulus* along environmental gradients is not known, studies have attributed similar size based habitat partitioning in intertidal gastropods to factors such as intraspecies competition, differential mortality and diet shifts to increase lifetime survival (e.g., Gosselin 1997, Kiyomoto & Yamaski 1999, Golden et al. 2001).

Here, I discuss two hypotheses for why *Lepetodrilus fucensis* and *Depressigyra globulus* exhibit size separation. One possibility is that protoconch stages settle in equal abundance in all vent habitats, but experience greater mortality in active flow. For example, juvenile gastropods from East Pacific Rise vents are consumed by mobile grazers (Micheli et al. 2002). Likewise, recruits of *L. fucensis* and *D. globulus* may also be preyed upon by larger animals in active vents flows where grazer densities are high, thus maintaining a recruit-biased size distribution in peripheral areas. On shorelines, predation pressure also drives motile juveniles of gastropods to take refuge in different habitats than adults (Werner & Gilliam 1984, Martel & Chia 1991, Gosselin & Chia 1995). Alternatively, protoconchs may settle preferentially in far-vent habitats to avoid hydrothermal fluids and, as they increase in size, may gain greater physiological tolerances to the chemical and temperature fluctuations characteristic of vent flows. Indeed, juveniles of many species tend to exhibit greater susceptibility to environmental extremes than adults (e.g., Davies 1969, Vermeij 1972). Further study is needed to two determine the mechanism driving the size differences in the population structures of these

vent gastropods and to determine how differential use of the habitats near vents relates the lifetime survivorship of *L. fucensis*.

The presence of early post larval stage *Lepetodrilus fucensis* at far-vent locations sampled from different vent fields, both sulphide and basalt-hosted vents, and in two different years (2001) suggests that recruitment occurs primarily in peripheral locations. In addition, *L. fucensis* recruitment may occur year-round; preliminary work by N. Kelly (Dalhousie University) suggests that its reproduction is continuous. However, the size structure of in-vent populations varied between vents and displayed distinct modes, suggesting that abiotic and biotic controls on settlement and recruitment may vary between years and locations. For example, Metaxas (2004) documented inter-annual variability in larval supply at Axial Volcano between 2001 and 2002. Factors such as food availability and mortality may also be vent-dependent, as suggested by Sadosky et al. (2002), who found similar inter-vent variation in the size structure of *L. elevatus* from 13°N on the East Pacific Rise. In comparison to *L. fucensis*, *Depressigyra globulus* in far-vent locations exhibited a more variable size structure and a less strict separation between large and small populations. Factors such as discontinuous reproduction or recruitment, or greater breadth in habitat selection by different sized *D. globulus* may contribute to the variability in its size structure.

Lepetodrilus fucensis may be producing an exceptional number of larvae. *L. fucensis* larvae in passive collectors were almost two orders of magnitude greater than *D. globulus* (Table 3.2). In addition, the densities of the recruit biased *L. fucensis* populations found in peripheral locations are at least an order of magnitude greater than for a congener, *L. elevatus* (East Pacific Rise) (Table 3.2).

Table 3.2
Density and larval supply in *Lepetodrilus fucensis* (Juan de Fuca Ridge), *L. elevatus* (East Pacific Rise) and *Depressigyra globulus* (Juan de Fuca Ridge) as reported in multiple studies. Replicate samples are reported as means (±1 SD); when sample size was one, a single value is reported. Densities are for locations where tubeworms were very small or absent at three temperature ranges: cool = <4°C (shaded grey), warm = 4-19°C, hot = >20°C. Maximum fluid temperatures in tubeworm bush collections were >4°C. Populations in cool habitats were dominated by animals were <1 mm, while warm and hot habitats hosted primarily animals >1 mm. Alphabetical superscripts indicate citations.

	L. fucensis	***L. elevatus***	***D. globulus***
1. Density (# m^{-2})			
cool	261 600±200 [a]	4 115±4 066 *[h]	10 319±8 889 [a]
warm	207 600±71 500 [b]	12 563±5 327 *[h]	23 400±35 900 [b]
hot	37 129 [i]		20 903 [i]
	944 [i]		630 [i]
tubeworm bushes	20 020±26 410 [f]	2 158±1 474 [c]	3 410±5 110 [f]
	108 226 [i]		64 839 [i]
	95 578 [d]		27 730 [d]
2. Larval supply			
passive (# m^{-2} d^{-1})	~50 000 [g]		~100 [g]
nets (# m^{-3})	~10 to 1 000 [g]	<0.03 [e]	~10 to 1 000 [g]

[a] this study, [b] Bates et al (2005), [c] Govenar et al (in press), [d] Govenar et al (2002), [e] Kim SL, Mullineaux LS (1998), [f] Marcus (2003), [g] Metaxas (2004), [h] Mullineaux et al. (1998), [i] Sarrazin and Juniper (1999)

These interspecies comparisons suggest that the recruitment and larval success of *L. fucensis* is remarkable and likely relates to the relatively high abundances of adults in active vent flows.

Sex based habitat partitioning

Lepetodrilus fucensis populations exhibited sex based habitat partitioning. Populations in warm vent flows comprised the largest animals and were increasingly female biased with increasing shell lengths. The smallest reproductive size class (sexual maturation occurs between 3.5 and 4.0 mm based on light microscope observations of gonad tissues) consisted of equal numbers of males and females and each animal exhibited characteristics of one sex only, indicating that *L. fucensis* is not protandrous, and has a primary population sex ratio of 0.5. Instead, the data from the present study suggest that aggregation by larger females in active vent flows is the proximal mechanism driving sex ratio biases. The rationale for this conclusion is discussed below.

In many species, female fecundity increases with size due to greater ability to access resources in optimal habitats and/or increased body volume for egg production (e.g., Wilber 1989, Pardo & Johnson 2005). In contrast, males produce an excess of low-cost sperm, so their reproductive output is often relatively independent of body size and habitat. This can lead to sex ratios that are biased by larger females because they compete more successfully for habitats with rich food resources, thus maximizing reproductive output (e.g., Boaventura et al. 2003, Shine et al. 2003). In *Lepetodrilus fucensis*, males and females were equally represented in populations where a variety of fluid conditions were available, such as tubeworm bushes and at the edge of venting. However, females and males occupied different microhabitats and were separated at

small spatial scales. Females were most prevalent in warm vent flows at locations that are actively preferred (Bates et al. 2005), while males were more common in suboptimal habitats (e.g., vent periphery). A parsimonious explanation for the over-representation of large females in optimal habitats is that females compete successfully for access to food, thus experiencing increased growth.

Suboptimal habitats may also include locations in high temperature fluids where animals are near their tolerance limits. Sarrazin and Juniper (1999) show that limpets are absent or rare from chimney locations where fluid temperatures are >20°C and vent flux is highest (see Table 3.2). This may be because limpets are avoiding higher temperature substrata or the higher sulphide-to-heat ratios of some chimney flows (Bates et al. 2005, Marcus 2003). Indeed, two regions on the flange were bathed in fluids that flowed from the high temperature pool and these regions were sparsely populated with relatively greater proportions of males. It is possible that these males are forced to occupy less preferred locations on the flange to avoid competition with large females in habitats where limpet densities were highest. However, density dependent habitat selection by males and females remains to be tested.

Female *Lepetodrilus fucensis* appear to incur a greater cost of reproduction than males, probably because oocytes require greater energy investment than sperm, as described for intertidal gastropods (Cheung & Lam 1999). This hypothesis is supported by data from the transplant experiment. Female survivorship was far less than males in low flux conditions, yielding a heavily male biased sex-ratio, and the proportion of reproductive males (seminal fluid present) was relatively greater than reproductive females (eggs present) in low flux. Female mortality at the onset of reproduction in

suboptimal habitats may also drive the sharp increase in the sex ratio observed between the 4.5 to the 6.0 mm size class in far-vent habitats. However, these ideas remain to be tested as male reproductive effort may be more costly than earlier studies have indicated (Paukku & Kotiaho 2005).

Conclusions

Although some gastropods with novel reproductive strategies, such as a clonal invasive freshwater mudsnail (*Potamopyrgus antipodarum:* Richards & Shinn 2004), exist in colonies up to 300 000 individuals m^{-2}, the recruitment patterns and sex ratio data presented here suggest *Lepetodrilus fucensis* is a typical dioecious species with a primary population sex ratio of 0.5. However, *L. fucensis* separates habitat based on size and sex, a strategy that probably maximizes the lifetime success of individuals. Animals live for at least one year, indicated by the transplant experiment, and appear to be continuously reproducing (N. Kelley pers. comm.). Eggs are likely fertilized in the mantle cavity, followed by release into the water column as non-feeding planktonic larvae (Lutz et al. 1986, Hodgson et al. 1997) that probably survive for at least weeks based on genetic studies that indicate continuous larval dispersal along the Juan de Fuca and Explorer Ridges (Johnson et al. in press). While larvae settle out of the water column in high abundances across vent fields (Metaxas 2004), this study showed that early post larval stages are sustained in peripheral habitats. Migration into vents from the periphery occurred primarily at sizes >0.5 mm to locations dominated by densely stacked adults (Bates et al. 2005). Habitats in warm vent flows are key to larval production by *L. fucensis*: these locations host high adult densities, the gonads of in-vent animals were full of gametes and large females, presumably with the highest fecundity, were most

abundant. In comparison, low flux populations were sparsely populated by adults and survivorship was low, especially for females, leading to male-dominated populations. The gonads of low flux animals were also rarely full, suggesting that low flux animals do not contribute significantly to the larval pool. However, these peripheral locations were important for recruitment. Understanding factors that drive larval survival at the edges of hydrothermal flow and how reproductive output varies between the sexes in different habitats will be important areas for future studies.

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Appendix 3.1

High, mixed and low flux collections from Axial Volcano, Endeavour Segment and Southern Explorer Segment from 1994 to 2003 used to quantify *Lepetodrilus fucensis* population sex ratios: presented as proportion of males (n = 100). Sample is the preserved collection number (V. Tunnicliffe, University of Victoria). Vent features include a qualitative estimate of visible fluid flux. Substratum type is indicated.

Vent site Vent field Vent	Lat Long Depth (m)	Year	Sample	Flux	Substratum	Proportion Male
Axial ASHES Porkchop flange	45°55.9'N 130°00.8'W 1550	1998	R470-8031	High	Chimney	0.35
Axial ASHES Embley's Inferno	45°59.5'N, 130°03.5'W 1545	1986	P1721-2030	High	Chimney	0.44
Endeavour Main Field Salut	47°56.9'N 129°05.9'W 2202	2003	R710-01	High	Chimney	0.23
Endeavour Main Field Crypto flange	47°56.9'N 129°05.9'W 2194	1988	A2415-2105	High	Chimney	0.33
Endeavour Main Field Dual Smoker	47° 57'N, 129° 05'W 2200	1984	A1451-2010	High	Chimney	0.39
Endeavour Main Field Grotto	45° 51'N, 129° 06'W 2192	1986	A2409-2103	High	Chimney	0.39
Explorer Magic Mountain Lunch Hour	49°46'N 130°18'W 1800	1984	P1494-214	High	Chimney	0.27
Axial South Rift Zone Mrk 113	45°55.4'N 129°59.3'W 1525	2000	R552-8049	Mixed	Tubeworms	0.51
Axial ASHES Hairdo	45°56.01'N 130°00.84'W 1546	2000	R466-2193	Mixed	Tubeworms	0.56
Axial ASHES Gollum	45°56.0'N 130°00.8'W 1547	1998	R471-2175	Mixed	Tubeworms	0.49
Axial 98 Lava Flow Nascent	45°56.2'N 129°58.9'W 1520	2000	R543-8046	Mixed	Tubeworms	0.42
Axial 98 Lava Flow Cloud	45°56.0'N 129°58.9'W 1523	2000	R543-8039	Mixed	Tubeworms	0.51
Axial 98 Lava Flow Snail	45°56.0'N 129°58.9'W 1524	2000	R549-8043	Mixed	Tubeworms	0.42

Explorer Merlin Mound Tubeworm Chimney	49°45. 5'N 130°15.4'W 1781	2002	669-06	Mixed	Tubeworms	0.63
Axial South Rift Zone BagCity	45°55.0'N 129°59.4'W 1523	2000	R479- 8031	Low	Basalt	0.73
Axial 98 Lava Flow Cloud	45°56.0'N 129°58.9'W 1523	2000	R549- 8054	Low	Basalt	0.57
Axial South Rift Zone Mrk 113	45°55.4'N 129°59.3'W 1524	1998	R476- 2158	Low	Basalt	0.53
Axial East Rift Zone Old Worms	45°56.19'N 129 °58.9'W 1520	1998	R478- 2169	Low	Tubeworms	0.61
CoAxial Floc Site HDV	46°18.6'N 129°42.5'W 2254	1996	R365- 2145	Low	Tubeworms	0.68
Endeavour Main Field Easter Island	47°56.9'N 129°05.9'W 2193	1995	R306- 2065	Low	Tubeworms	0.75
Endeavour Main Field Salut	47°56.9'N 129°05.9'W 2202	2003	R710- 01	Low	Chimney	0.61

CHAPTER 4

A hot vent gastropod hosts a novel gill episymbiosis with gamma-Proteobacteria

AE Bates, T Harmer, E DeChaine, CM Cavanaugh

Dr. Harmer (Assistant Professor: Division of Biology, Richard Stockton College of New Jersey) assisted in designing and executing the FISH studies. Dr. DeChaine (Post-doctoral Fellow: Organismic and Evolutionary Biology, Harvard University) completed the phylogenetic analyses. Dr. Cavanaugh (Associate Professor: Organismic and Evolutionary Biology, Harvard University) supervised this work and provided the space and resources for the molecular studies.

Abstract

Lepetodrilus fucensis is a common limpet that forms prominent stacks in diffuse hydrothermal fluids at sites on the Juan de Fuca Ridge complex in the northeast Pacific Ocean. A unique character of this species is dense colonies of filamentous bacteria found partially embedded in a specialized region of the gill lamellae epithelium. We investigated the nature of this association using molecular approaches. Our objectives were to determine (1) the phylogenetic identity of the gill bacteria and (2) whether specimens from different sites hosted bacterial populations comprised of similar sequences. A clone library of 16S rRNA genes amplified by PCR from the gill tissue of limpets collected at different vent sites was dominated by gamma- and epsilon-Proteobacteria sequences (identified by comparative phylogenetic analyses). The gamma-Proteobacterial clones shared >99% sequence similarity, while the epsilon-Proteobacteria sequences divided into four groups based on $\geq$ 97% similarity. To

determine which of the five Proteobacterial clone groups were associated with the bacteria-hosting region on the gill, we designed clone group-specific oligonucleotide probes for fluorescence *in situ* hybridization experiments to target two different sites on the 16S rRNA molecule. Only probes specific to the gamma-Proteobacteria hybridized to the gill epithelium where bacteria were present. Direct sequencing of PCR-amplified products using primers specific to the putative *L. fucensis* symbiont gave a single unambiguous sequence for specimens collected from different vent sites. Our results indicate that *L. fucensis* hosts a specific and persistent bacterial symbiosis with a distinct lineage of gamma-Proteobacteria that is distantly related to known chemoautotrophic symbiont clades.

INTRODUCTION

Examination of the dominant fauna clustered around deep-sea hydrothermal vents revealed novel chemosynthetically-based symbioses between invertebrates and bacteria (Cavanaugh et al. 1981, Felbeck et al. 1981). These highly evolved invertebrate-bacterial associations exploit the reduced chemicals in geothermally altered hydrothermal fluids and oxygen from ambient seawater. Detailed examinations of fauna from various marine habitats where reduced compounds co-occur with oxygenated fluids identified remarkably diverse invertebrate hosted symbioses with bacteria that had been overlooked (Cavanaugh, 1994). Chemosynthetic symbioses are globally represented and have arisen independently in shallow and deep-sea reducing habitats (reviewed in Cavanaugh et al. 2004). The metabolic requirements of symbiotic bacteria tend to reflect host environment; reduced sulphur is probably the most common energy source utilized by

symbiotic bacteria (reviewed in Cavanaugh et al. 2004) and, where methane is abundant, methanotrophic symbionts also occur (Cavanaugh 1993). The widespread incidence of bacterial symbioses hosted by key community animals indicates that the ecological significance of these associations may be underestimated.

Although invertebrates hosting bacterial symbionts often possess unusual morphological features to access chemical substrates from their environment (reviewed in Fisher 1990, Cavanaugh 1994, Cavanaugh et al. 2004), the level of integration between the host and symbiont forms two categories even in divergent taxa. In the most intimate associations, endosymbiotic bacteria are intracellular within vacuoles and invertebrates with endosymbionts tend to derive their nutrition from released bacterial metabolic products or lysosomal degradation of the bacteria (e.g., *Riftia pachyptila*, Bright et al. 2000). In episymbiotic associations, populations of bacteria colonize the interface between invertebrate hosts and their environment (e.g., rimicarid shrimp, Polz & Cavanaugh 1995). Episymbionts can provide a food resource and may also serve in the detoxification of hydrothermal fluids (e.g., Alayse-Danet et al. 1987).

To date, chemosynthetic bacterial symbionts have not been cultured and comparative analysis of the 16S rRNA gene is a primary tool for characterization (Cary et al. 1993). The list of chemosynthetic bacterial symbionts is extensive (McKiness 2004) and continually growing as molecular tools improve (e.g., Blazejak et al. 2005) and new symbioses are discovered (e.g., Goffredi et al. 2004). Typically, symbiont populations in a single host are dominated by one phylotype, identified as a primary symbiont. Bacterial phylogenies based on 16S rRNA sequence data show that the majority of these primary chemosynthetic symbionts fall into three main clades within the gamma-Proteobacteria:

(1) bathymodiolin mussel and vesicomyid clam symbionts, (2) vestimentiferan tubeworm, solemyid, lucinid and thyrasid clam symbionts and (3) nematode and oligochaete worm symbionts (McKiness 2004). Secondary symbionts tend to be less abundant and rigorous sequencing protocols and *in situ* hybridization techniques have revealed stable associations with epsilon-, alpha-, beta- and delta-Proteobacteria (Cavanaugh et al. 2004, Blazejak et al. 2005). Molecular characterization of symbiotic bacterial populations is also a useful indicator of the level of specificity, or fidelity, between host and bacteria. Highly specific symbioses between one or more bacterial type(s) and one invertebrate taxon are common (Dubilier et al. 2001, McMullin et al. 2003, McKiness 2004). In less specific associations, the identity of bacteria in symbiont populations may vary between host individuals (Di Meo-Savoie et al. 2004).

Lepetodrilus fucensis is an abundant gastropod from the Juan de Fuca Ridge complex in the northeast Pacific (Bates et al. 2005, Marcus 2004) that hosts a novel gill episymbiosis: uniform bacterial filaments lie partially embedded in the host's gill epithelium and extend into the fluids circulating around the gill (de Burgh & Singla 1984). Although *L. fucensis* morphology reflects capabilities for grazing and suspension feeding (Fretter 1988), transmission electron micrographs suggest that its gill bacteria are harvested by endocytosis and degraded in lysosomes (de Burgh & Singla 1984). Several lines of evidence indicate that the bacteria rely on sulphide for an energy source. The abundance of bacteria on the gills of animals from vigorous vent flows is significantly higher than in peripheral locations (Chapter 5). Bacterial abundance on the gill is also positively related to the total carbon fixed in the presence of radiolabelled carbon dioxide, inorganic sulphide and oxygen (Chapter 5). Calvin cycle and sulphur-oxidation enzymes,

diagnostic of chemoautotrophy, show high activities in gill tissues (Fox et al. 2002). Furthermore, carbon isotope ratios of *L. fucensis* tissues are consistent with thiotrophic bacterial production and values range from $\delta^{13}C$ -18 to -12 ‰ at Axial Volcano (Levesque 2003).

However, to date, the phylogenetic affiliation of the *Lepetodrilus fucensis* gill bacteria and the specificity between bacteria and host are unknown. We hypothesized that the *L. fucensis* gill symbionts would be represented by a primary epsilon-Proteobacterial phylotype, as found for the majority of known invertebrate-bacterial episymbioses (e.g., Polz et al. 1994, Haddad et al. 1995, Polz & Cavanaugh 1995) and used the ribosomal RNA framework to test this hypothesis. We (1) characterized the phylogenetic placement of *L. fucensis* gill bacteria from multiple specimens and vents; (2) verified the location of the symbiont phylotype within individuals for different vent sites on the Juan de Fuca Ridge complex by means of fluorescence *in situ* hybridization; and (3) obtained direct sequence data using symbiont-specific primers for specimens collected at distant vent sites.

METHODS AND MATERIALS

Specimen Collection

Lepetodrilus fucensis specimens were collected from hydrothermal fields on the Juan de Fuca Ridge (2003) and Explorer Ridge (2002) at Axial Volcano (45°56'N 130°00'W; depth 1570 m), Endeavour Segment (47°57'N, 129°06'W; depth 2220 m) and Southern Explorer Segment (49°45'N 129°42'W; depth 1850 m). All *in situ* collections were made with the Canadian remotely operated vehicle, ROPOS. Animals were

transported to the surface and sub-samples from each collection were immediately preserved for ultrastructural analysis, DNA extraction (frozen at -80°C) and fluorescence *in situ* hybridization (95% EtOH).

Transmission Electron Microscopy

The afferent region of gill lamellae (sensu de Burgh & Singla 1984) were examined using TEM to (1) verify the presence of gill bacteria and (2) produce images of gill tissue in the same orientation as FISH sections. Gill tissues were dissected from 10 specimens of every collection, rinsed in 0.2 μm filtered water and fixed in 2.5% glutaraldehyde buffered in sodium phosphate (pH = 7.4). Samples were stored in glutaraldehyde fixative at 4°C until return to the laboratory. Individual lamella were post-fixed with osmium tetraoxide, dehydrated in a graded ethanol series, transferred to 100% propylene dioxide and embedded in Epon 812 resin (TAAB). Semi-thin transverse sections (500 μm) of lamellae were treated with 1% Methylene Blue, and imaged using a Zeiss Universal compound light microscope. Thin sections (70 nm) were stained with lead citrate and uranyl acetate and examined with a Hitachi H-7000 transmission electron microscope.

PCR, cloning and sequencing of 16S rRNA

To resolve the phylogenetic identity of the *Lepetodrilus fucensis* gill bacteria and determine the specificity of the association, gill tissues were isolated by dissection, ground with mortal and pestle in liquid nitrogen, chemically lysed using GITC, followed by gDNA extraction with phenol-chloroform (Sambrook & Russell 2001). The universal bacteria primers, 27f and 1492r (Weisburg et al. 1991), were used to amplify the 16S rRNA gene from the gill gDNA. PCR thermal cycling was as follows: denatured at 94°C

for 1 min, annealed at 54°C for 1 min, and extended at 72°C for 90 s for a total of either (1) 30 or (2) 18 cycles. The number of PCR cycles was varied to reduce bias in the clone product (see Polz & Cavanaugh 1998). For reactions with 30 cycles, gill tissue samples from four Axial limpets and five Endeavour limpets were used (see Table 4.1 for vent latitude and longitude). For 18 cycles, three specimens from one vent at both Axial and Endeavour were selected. In order to obtain sufficient PCR product from 18 thermal cycles for cloning, the product from 5 replicate reactions were combined for each sample. PCR products were then cloned (TOPO TA cloning kit, Invitrogen Corporation). DNA was extracted from clones (QIAprep Spin Miniprep Kit, Qiagen) and screened using PCR with M13 vector primers. Eight to ten positive clones were selected for sequencing from each specimen; in total, 128 clones were screened (gills from 15 limpets). DNA was labeled using BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems) with M13 vector primers. Screening of the clones was conducted by sequencing (ABI 3100 sequencer) ~700 bp from position 27 on the 16S rRNA gene relative to *Escherichia coli*. Five clone groups were identified as symbiont candidates because of amplification from the gills of at least two limpets from both Axial and Endeavour vent sites. Gamma-Proteobacteria clones (identified by preliminary phylogenetic analyses) were >99% similar and are referred to as Lf-1 gamma. Four epsilon-Proteobacteria clone groups were identified based on ≥ 97% sequence similarity, labeled Lf-1 to 4 epsilon. A representative clone from each group was selected for sequencing (hereafter Lf-1.1 gamma, and Lf-1.1, Lf-2.1, Lf-3.1, Lf-4.1 epsilon) in the reverse direction (total fragment size was ~1400 bp) to use in phylogenetic analyses (see Appendix 4.1 for sequences). Replicate sequencing reactions were completed to resolve ambiguous sites.

Unfortunately, only ~900 bp of the Lf-4.1 epsilon clone were sequenced and it was consequently excluded from phylogenetic analyses. However, probes specific to the Lf-4 epsilon clone group were tested for hybridization with *L. fucensis* gill tissue (see below: 'Fluorescent *in situ* hybridization').

Phylogenetic Analysis

Four clone groups isolated from *Lepetodrilus fucensis* gill tissue (~1400 bp: Lf-1.1 gamma and Lf-1.1, 2.1, and 3.1 epsilon), were imported into the ARB database (Ludwig et al. 2004) for alignment with 118 16S rRNA bacterial sequences, comprised of 1 alpha-, 14 epsilon- and 37 gamma-Proteobacterial symbionts of deep-sea hydrothermal vent and cold-seep invertebrates, shallow water reducing sediments and 68 free-living bacteria. The alignment was manually edited in MacClade 4.0 (Maddison & Maddison 2000) and variable regions of uncertain homology were excluded from the phylogenetic analysis (based on the secondary structure of *Escherichia coli,* positions: 198-214, 456-479, and 838-852).

The phylogenetic relationships among ~1300 bp of the 16S rRNA sequence (with the *Olavius loisae* alpha bacterial symbiont as an outgroup) were inferred using Bayesian analyses implemented with MrBayes v3.0b4 (Huelsenbeck & Ronquist 2001). Support values for the tree topology are posterior probabilities. For the Bayesian analysis, a four-chain (1 cold and 3 heated) Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analysis was employed. Base frequencies were determined empirically and substitution rates and the gamma distribution were estimated (corresponding to GTR+T^{1} model). The first half of 1 500 000 generations were discarded as the "burn-in". Chains were sampled every 100 generations and inferences were based on a total of 7,500 sampled trees. A

consensus tree with posterior probabilities was generated in PAUP 4.10b (Swofford 2003) from the base frequency, substitution rate, gamma distribution and Bayesian trees. The Bayesian analyses were run three separate times to check for convergence in parameters.

Fluorescent *in situ* hybridization (FISH)

Specimen preparation. Specimens were from Axial (Marshmallow Vent: 45°56.02'N, 130°00.82'W) and Endeavour (Salut Vent: 47°56.9'N, 129°05.9'W) vent sites. Whole gills were fixed at the time of collection in 95% EtOH and stored at 4°C. Lamellae were dissected from the anterior, middle and posterior regions of the gill, dehydrated in a graded EtOH series, and embedded in LR White media. Serial sections (750 μm) of five lamellae were cut in transverse orientation. Sections from all three gill regions were included in experiments; three sections were mounted in each well (10 wells total) on chromalum-gelatin coated slides. Two replicate reactions (2 wells) were completed for each experimental trial and for each probe.

Probe design. FISH probes were designed to determine which clone groups (Lf-1 gamma and Lf-1, 2, 3, and 4 epsilon) isolated from *Lepetodrilus fucensis* gill tissue hybridize to the symbiont-hosting region of the gill. Several criteria for probe design were determined *a prior*: (1) only positions on the 16S rRNA molecule promising high signal intensity were selected (Behrens et al. 2003), (2) probe G+C content was required to fall between 40 and 60% (Pernather et al. 2001) and (3) positions on the 16S rRNA were required that were invariable within a clone group but contained at least two base mismatches between any pair of clone groups. To design the probes, the five representative sequences of each clone group (~1400 bp) were aligned to *Escherichia coli*

using ClustalX (Thompson et al. 1997) and visually inspected to ensure that domain structure of 16S rRNA was preserved. Positions 128-147 and 643-662 (relative to *E. coli*) on the 16S rRNA molecule fit the criteria outlined above and Cy3-labeled (Biometra, Germany) 20-mer oligonucleotide probes were designed to target these positions (Appendix 4.1 shows the target sites in relation to *E. coli*). FISH probe sequences (5^1 to 3^1 direction) for each of the five clone groups at positions 128-147 and 643-662 relative to *Escherichia coli* are as follows (probe names are bolded and 128 and 643 indicate the target position): **Lf-1 gamma128**: CCCCA-CTATT-CGGCA-ATTTC, **Lf-1 gamma643**: ACCAT-ACTCT-AGTGA-GCCAG; **Lf-1 epsilon128**: CCATC-TTCAA-GGCAC-ATTAC, **Lf-1 epsilon643**: CCCAT-ATTCT-AGGTA-TTCAG; **Lf-2 epsilon128**: TAGTC-TTCGA-GGCAG-ATTAA; **Lf-2 epsilon643**: CCCAT-ACTCT-AGGTT-ACCAG, **Lf-3 epsilon128**: CAGAC-TAAGA-GGCAC-GTTAC; **Lf-3 epsilon 643**: CCCAT-ACTCT-AGGTA-ACCAG; **Lf-4.epsilon128**: CAAAC-TAAAA-GGTAT-GTTAT, **Lf-4 epsilon643**: TCCAT-ACTCT-AGGAA-AACAG. The universal bacterial probe EuB338 (Amann 1995), labeled with FITC, provided a positive control.

Although it is ideal to design probes that are specific only to the target sequence (Pernather et al. 2001), it was not possible to find a region of rRNA that was both accessible to probes and variable among all five clone groups. Consequently, while the Lf-1 gamma and Lf-4 epsilon probes varied by at least two mismatches to other GenBank sequences (BLAST searches, October 2005), the Lf-1, Lf-2 and Lf-3 epsilon probes matched uncultured environmental epsilon-Proteobacteria clones from hydrothermal vents and reducing sediments in GenBank and are reported in Appendix 4.2. We therefore decided to first test the ten probes listed above for binding in hybridization

experiments to isolate putative symbiont types and then to design specific probes to confirm the symbiont identity as determined by the results from these experiments.

Hybridization experiments. For FISH, gill tissue sections were incubated for 15 min in 0.5 ug/ml Proteinase K per ml hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl [pH 8.4]) at 44 or 46°C, rinsed (10 min in hybridization buffer) and exposed to a 15% formamide hybridization buffer made with 50 ng/ul probe concentration for 2.5 hours (Amann 1995). The slides were then rinsed in washing buffer (0.3 M NaCl, 0.01% SDS, 5 mM EDTA, 20 mM Tris-HCl [pH 8.4]) for 15 min (conducted at 2°C higher than for the hybridization step). Each experimental trial was repeated three times. All solutions were made with DEPC-treated water. VECTASHEILD (Vector Laboratories, Burlingame, California, USA) mounting media was applied to slides, which were subsequently viewed with a Leica DMRB Microscope. Images were acquired with a Digital Camera (RETIGA Exi with RGB/LCD Slider) using Openlab 3.5.1 (Improvision Ltd., Coventry, UK).

Specificity experiments. Lf-1 gamma probes (positions 128-147 and 643-662) bound consistently in the known location of the gill bacteria. These probes did not match any known sequences in GenBank, thus, we proceeded with mismatch probe design (one mismatch: middle G to A transformation) to test for binding specificity: **Lf-1A.128**: 5^1-CCCCA-CTATT-CAGCA-ATTTC-3^1, **Lf-1A.643**: 5^1-ACCAT-ACTCT-AATGA-GCCAG-3^1. Binding by the Lf-1 gamma probes and mismatch probes (Lf-1A.128 and Lf-1A.643) was tested under increasing hybridization stringency at 15, 30 and 45% formamide at 46°C (see 'Hybridization experiments' for protocol).

Direct sequencing with specific primers

Direct sequencing was conducted to determine if the *Lepetodrilus fucensis* symbiont is represented by an unambiguous sequence for gills collected at different vent sites. DNA was extracted from individual gill tissues using the DNeasy Tissue Kit (Qiagen). PCR reactions with a forward primer based on our probe sequence for positions 128-147 (128F.Lfsym: 5[1]-GAAAT-TGCCG-AATAG-TGGGG-3[1]) and 1492r (Weisburg et al. 1991) were conducted. Direct sequencing of the PCR products from Axial (Mrk 33: 45°56.0'N, 129°58.9'W), Endeavour (Clam Bed: 49°57.3'N, 129°03.8'W) and Explorer (Tubeworm Chimney: 49°45.5'N, 130°15.4'W) vent sites (3 specimens per vent site) were completed (BigDye Terminator v3.0 Cycle Sequencing Kit, Applied Biosystems; ABI 3100 sequencer).

RESULTS

Bacterial phylotypes

Co-collected *Lepetodrilus fucensis* from the same vents as specimens used for gDNA extraction had bacterial symbionts based on examination of both light and TEM micrographs. Sequence analyses of a 700 bp region (position 33 to 733 relative to *Escherichia coli*) of the 16S rRNA gene clustered clones into five groups determined by sequence identity. All gamma-Proteobacteria clones shared >99% sequence identity (Lf-1 gamma), while four epsilon-Proteobacteria clone groups were identified (Lf-1, 2, 3 and 4 epsilon) based on ≥97% sequence identity. The relative abundance of clones in each group varied between specimens and vent sites, as well as the number of thermal cycles used for PCR (Table 4.1). For example, the total relative abundance of epsilon clones in

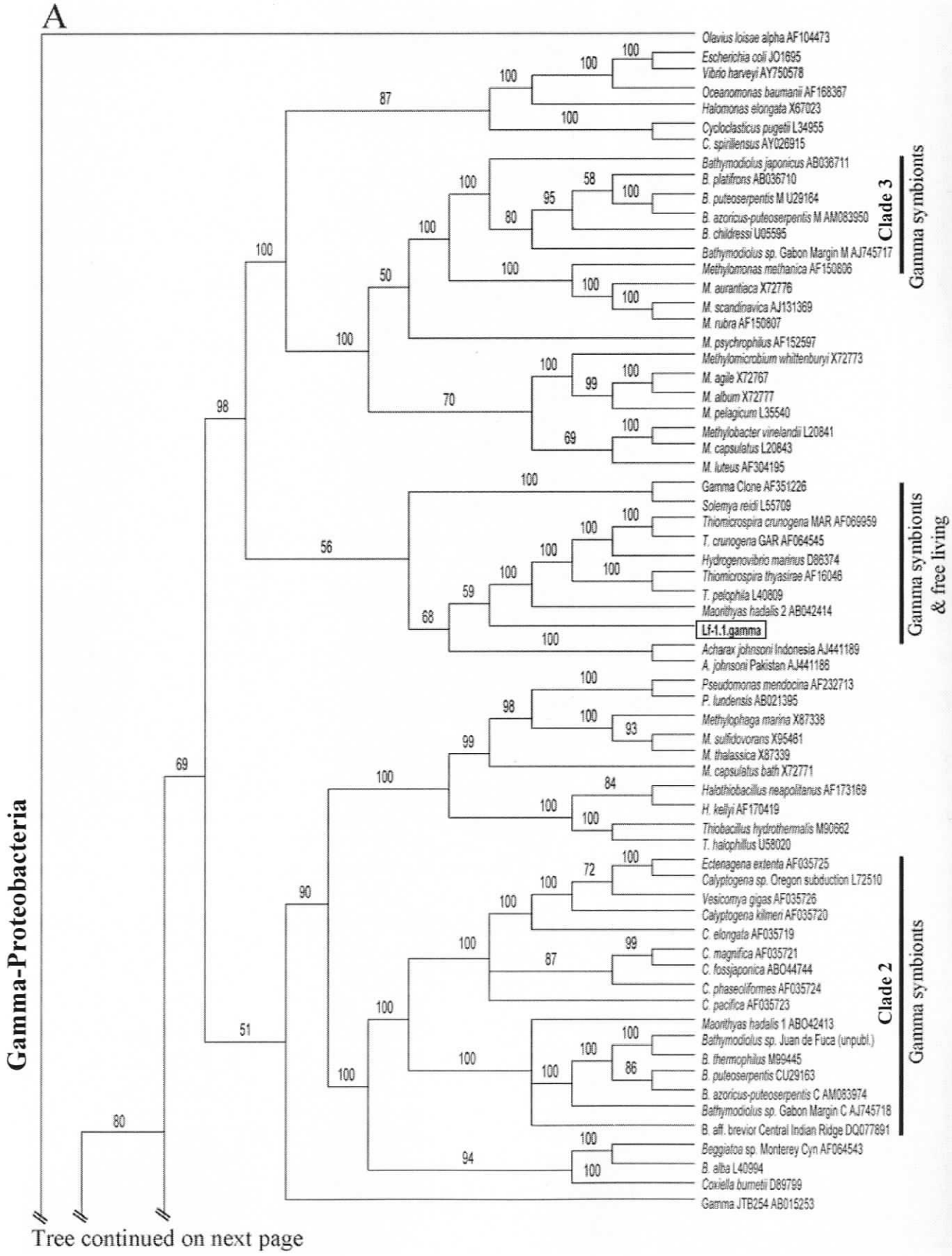
Table 4.1
The number and percentage of 16S rRNA gene clones (700 bp) isolated from *Lepetodrilus fucensis* gill tissue from the Axial (Ax) and Endeavour (End) vent sites in each clone group (Lf-1 gamma; Lf-1 to 4 epsilon) based on ≥ 97% sequence identity. Vents are identified with their published names. #specimens = total number of specimens contributing clones. #clones = total number of clones sequenced. % = (number of clones in each group) / (total number of clone sequenced). #PCR cycles: 30 and 18 PCR thermal cycles were used. One 700 bp clone from each clone group was sequenced in the forward and reverse directions to obtain ~1400 bp sequences for phylogenetic analyses.

#PCR cycles	Site	Vent Lat Long	#specimens: #clones	Lf-1 epsilon	Lf-2 epsilon	Lf-3 epsilon	Lf-4 epsilon	Lf-1 gamma	Other
30	Ax	Mrk 33 45°56.0'N 129°58.9'W	2:16	1 6%	3 19%	7 44%	1 6%	3 19%	1 6%
		Marshmallow 45°56.0'N 130°00.8'W	3:24	1 4%	6 25%	10 42%	2 8%	5 20%	0
	End	Clam Bed 49°57.3'N 129°03.8'W	1:10	4 40%	0	4 40%	1 10%	1 10%	0
		Salut 47°56.9'N 129°05.9'W	2:20	9 45%	2 10%	5 25%	0	3 15%	1 1%
		TOTAL	***8:70***	***15 21%***	***11 16%***	***26 37%***	***4 6%***	***12 17%***	***2 3%***
18	Ax	Mrk 33	2:16	6 38%	1 6%	1 6%	0	7 44%	1 6%
	End	Salut	2:16	4 25%	3 19%	3 19%	1 6%	5 31%	0
		TOTAL	***4:32***	***10 31%***	***4 13%***	***4 13%***	***1 3%***	***12 37%***	***1 3%***

the Lf-1 group for 30 PCR cycles at Axial were 4 and 6%, while at Endeavour, the relative abundances were 40 and 45%. 17% of clones were represented by the Lf-1 gamma group when 30 thermal cycles were employed, in comparison to 37% for 18 cycles.

Phylogenetic analysis

A Bayesian phylogeny included the five representative sequences of the different clone groups isolated from *Lepetodrilus fucensis* gills with free-living and symbiotic Proteobacteria (Figure 4.1). Clones are distinct lineages within the gamma- (Lf-1.1 gamma) and epsilon-Proteobacteria (Lf-1.1, 2.1, and 3.1 epsilon). The Lf-1.1 gamma clone is distantly related to three major chemosynthetic symbiont clades identified by McKiness (2004). The clade including the Lf-1.1 gamma clone is mixed; free-living (e.g., *Thiomicrospira* spp.) and symbiont taxa (e.g., *Solemya reidi* symbiont) are represented. The closest relative to the Lf-1.1 gamma clone is the *Maorithyas hadalis* 2 symbiont, however, the support for this node is low (59% posterior probability), suggesting that the tree topology might change with the addition of new sequences. Epsilon clones Lf-1.1, 2.1, 3.1 are closely related to free-living taxa from sediment and fluid samples at sulphide-reducing habitats with high support (100% posterior probability). The Lf-1.1 epsilon clone is closely related to an isolate from Okinawa Trench sediments and falls out in monophyletic clade (100% posterior probability) with two *Arcobacter* species. Lf-2.1 epsilon and Lf-3.1 epsilon group with isolates from Japan Trench hydrothermal sediments and are included in a clade with two species in the genus *Sulfurimonas* (100% posterior probability). The Lf-4.1 epsilon clone, omitted for the Bayesian analysis due to the shorter sequence length (~900 bp), grouped with the



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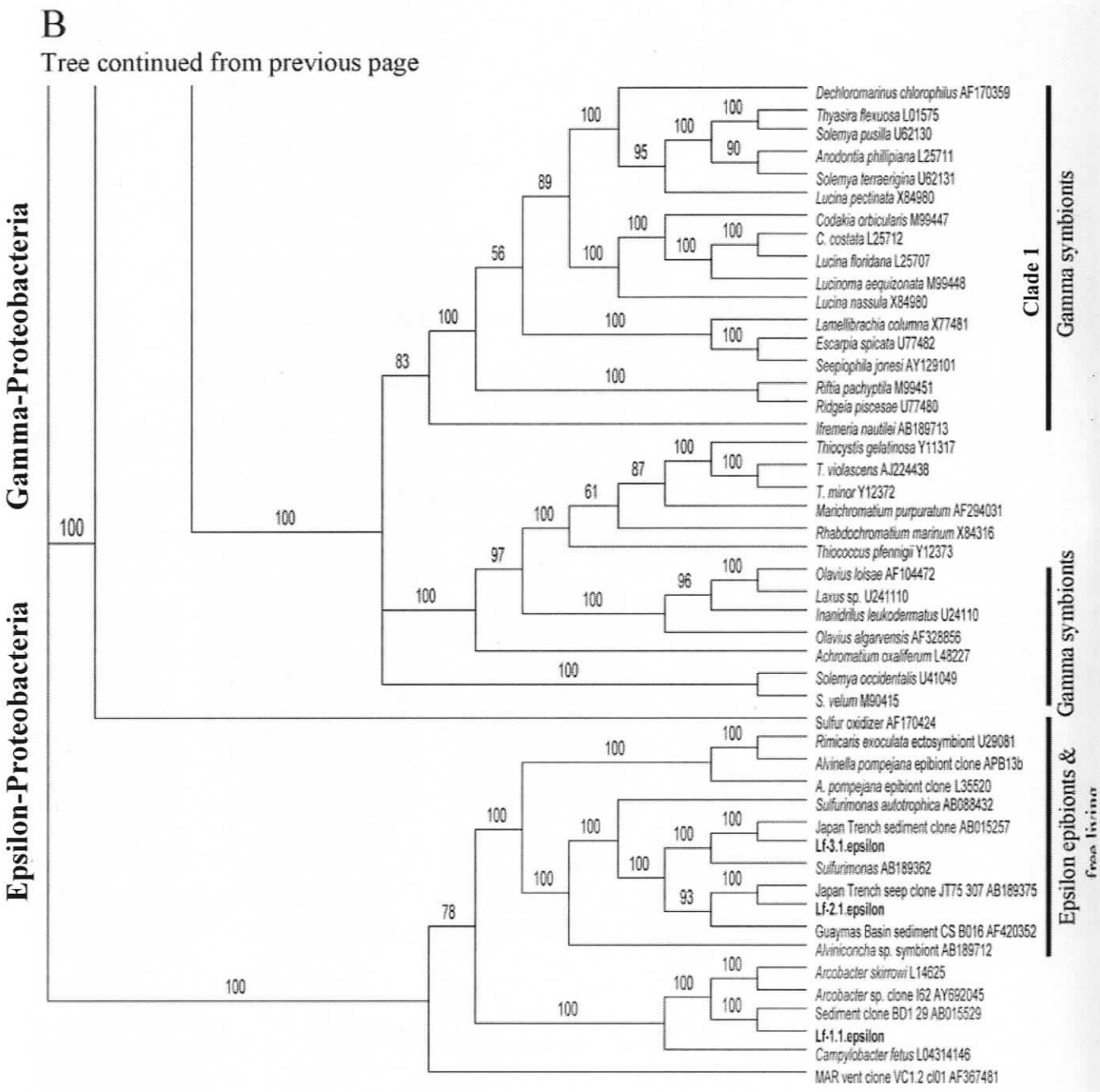

Figure 4.1
(A) and (B) comprise two parts of a Bayesian phylogenetic tree inferred from comparative analysis of ~1400 bp of the 16S rRNA gene sequences among the epibionts reported in this study and representative symbiotic and free-living Proteobacteria taxa. The alpha-Proteobacteria symbiont (*Olavius loisae*) is the outgroup. Posterior probabilities are from Bayesian analysis; parameters were estimated empirically. Symbionts are identified by a black line; the three major gamma-Proteobacteria clades are labeled (McKiness 2004). Phylotypes isolated in the present study are in bold. The *Lepetodrilus fucensis* symbiont: Lf-1.1 gamma is indicated by a box.

Table 4.2
Summary of results from FISH experiments. Initial hybridization experiments tested for positive binding for FISH probes designed to target phylotypes Lf-1 gamma and Lf-1 to 4 epsilon at sites 127 and 643 on the 16S rRNA molecule. Binding was visually scored based on the percentage of the bacterial zone found with probe label on each section: 100% and 0% were observed. Partial binding was defined as fluorescence on less than 5% of the total bacterial zone; partial binding occurred in only one trial (*) for the Lf-1 and Lf-3 epsilon probes. Only probes specific to Lf-1 gamma displayed 100% binding in all replicates and trials. Lf-2 and 4 epsilon were not detected. Stringency of the Lf-1 gamma 128 and 643 probes were tested against a one base pair mismatch probe (identified a Lf-1A). At higher stringency, Lf-1 gamma probes bound while mismatch probes (Lf-1A) did not bind. n = number of trials.

Experiment	**n**	**Temp (°C)**	**% Form-amide**	**Probe**	**100% binding**	**Partial binding**	**0% binding**
1. Initial Hybridization	3	44	15	127	Lf-1 gamma	Lf-1 epsilon*	epsilons: Lf-1,2,3,4
				643	Lf-1 gamma		epsilons: Lf-1,2,3,4
	3	46	15	127	Lf-1 gamma	Lf-3 epsilon*	epsilons: Lf-1,2,3,4
				643	Lf-1 gamma		epsilons: Lf-1,2,3,4
2. Stringency	3	46	15	127	Lf-1 gamma	Lf-1A	
				643	Lf-1 gamma	Lf-1A	
	3	46	30	127	Lf-1 gamma	Lf-1A	
				643	Lf-1 gamma		Lf-1A
	3	46	45	127		Lf-1 gamma	Lf-1A
				643	Lf-1 gamma		Lf-1A

epsilon clones isolated in this study based on maximum parsimony analysis. The placement of Lf-4.1 epsilon was supported by bootstrapping (n = 500 replicates; the data are not shown).

Fluorescent in situ hybridization (FISH)

FISH studies with probes designed to target the Lf-1 gamma and L.f-1 to 4 epsilon clone groups at sites 128 and 643 on the 16S rRNA molecule unambiguously identified the *Lepetodrilus fucensis* gill symbiont. Table 4.2 summarizes the results from the hybridization and stringency experiments. In the hybridization experiments, the Lf-1 gamma probes at positions 128 and 643 exhibited consistent and complete binding over the entire location coincident with the bacteria zone based on comparisons between fluorescent and light micrographs (Figure 4.2). Hybridization of Lf-1 gamma probes was observed on 100% (estimated by visually comparing light and fluorescent micrographs) of the bacteria-hosting region of the gill for: (1) five gill lamellae included in each section, (2) three replicate sections per slide well, (3) two replicate slide wells and (4) three different trials. Probe 128 for the epsilon clones Lf-1 (at 44°C) and Lf-3 (at 46°C) hybridized with less than 5% (and in one case 40%) of the area on gill where bacteria are located. However, hybridization was not observed at all five replicate lamellae or for all three replicate sections when it occurred, and was observed in only one of the three trials. Hybridization of probes designed to target the epsilon clone groups Lf-2 and Lf-4 (at positions 128 and 643), and Lf-1 and 3 (at position 643) were not detected. At high stringency, the Lf-1 gamma probes at both the 128 and 643 positions bound consistently to the bacterial zone on the *Lepetodrilus fucensis* gill, while the mismatch probes (Lf-1A.128 and 643) did not hybridize.

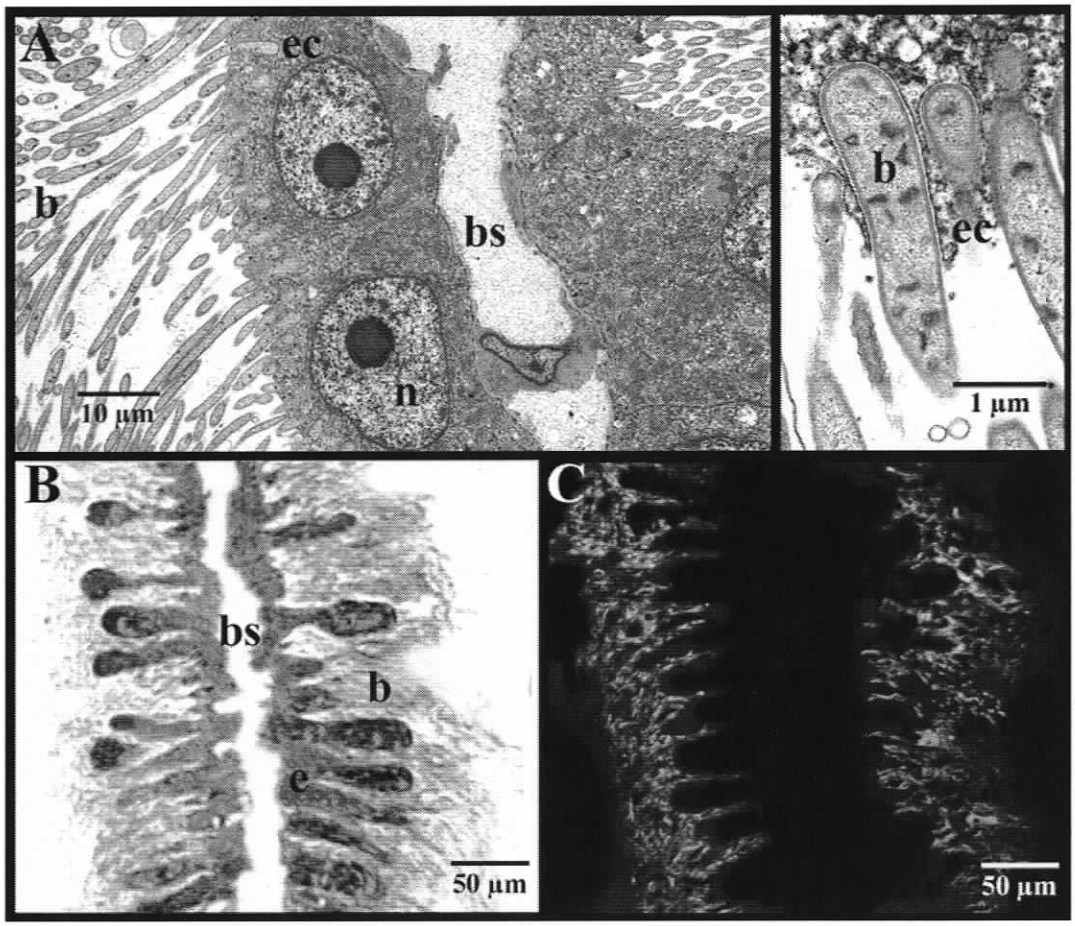

Figure 4.2
Transmission electron and light micrographs of the bacterial zone on the afferent region (sensu de Burgh and Singla 1984) of *Lepetodrilus fucensis* gill lamellae.
(A) TEM micrograph showing a transverse section through the bacterial zone. Bacteria (b) are embedded in the apical surface of gill epithelial cells; the blood space (bs) and cell nucleus (n) are visible. Insert is a high-magnification image of the bacterial double membrane in relation to the host epithelial cell (ec). (B) Light micrograph of the bacterial zone. (C) Fluorescence micrograph showing positive binding (probe 643) in the bacterial zone. Orientation is similar to A and B.

Direct sequencing using specific primers

The *Lepetodrilus fucensis* symbiont is represented by an unambiguous sequence based on comparison of direct 16S rRNA gene sequences obtained using specific primers on gills from specimens collected at distant vent sites. Direct sequences from the gill tissue of specimens (n = 3) collected at Axial Volcano, Endeavour Segment (northern Juan de Fuca Ridge), and Explorer Segment (southern Explorer Ridge) shared >99.5% sequence identity.

DISCUSSION

The *Lepetodrilus fucensis* gill bacteria are dominated by a primary symbiont, a novel lineage in the gamma-Proteobacteria. We show that the *L. fucensis* gill symbiosis is persistent and highly specific for specimens collected over a broad geographic area (~200 km) on the Juan de Fuca Ridge complex. As found for other gastropod and bivalve gill symbioses, *L. fucensis* hosts a highly specific symbiosis, suggesting host-bacterial recognition mechanisms are in place. However, while all molluscs characterized to date host endosymbioses where the bacteria are fully encapsulated in vacuoles (intracellular) and/or are found extracellularly within the microvilli (reviewed in Cavanaugh et al. 2004), the *L. fucensis* symbiont lies in the fluids circulating across the gill. This intermediate morphology may be a useful model to better understand processes that drive the evolution from extra- to intracellular associations.

PCR Bias

PCR bias may limit detection of symbionts in animal tissues and from environmental samples. Although the epsilon-Proteobacteria isolated by the present

study are related to several environmental isolates, the putative *Lepetodrilus fucensis* symbiont has not yet been reported from environmental samples on the Juan de Fuca Ridge (BLAST search, January 1 2006) which may be attributable to PCR bias. In this study, the epsilon-Proteobacteria were relatively rare on the gill based on *in situ* hybridization techniques, but dominated the PCR product (~75%). The relative abundances of clones in each group also depended on the number of thermal cycles employed to isolate genomic bacterial DNA from the host gill tissue (see also Polz & Cavanaugh 1998). *In situ* hybridization techniques are critical in studies aiming to identify symbionts and locate specific bacterial phylotypes in the environment due to PCR bias.

Epsilon-Proteobacteria

Lepetodrilus fucensis collected from different vent fields harboured similar epsilon-Proteobacteria sequences on their gills. These phylotypes may represent secondary symbionts which are more common than previous studies have suggested (e.g., Blazejak et al. 2005). Although two sizes of bacteria have been reported on the *L. fucensis* gill, the smaller morphotype is limited to the middle region of lamella and are not associated with the gill epithelium, while the larger morphotype (presumably the *L. fucensis* symbiont) is found embedded in the gill epithelium (Fox et al. 2002). Although probes specific to the epsilon clone groups Lf-1 and 3 bound in the region where the smaller morphotype is present, these clones are unlikely symbiont candidates because binding occurred on only a few sections and, when evident, was not consistent among replicate lamellae, sections, or experimental trials. A. Warén (pers. comm.) observed rod-shaped bacteria associated with the gill axis in scanning electron micrographs, the

significance of which are unknown, and it may be that these bacteria are represented by one of the clone groups consistently isolated in the present study.

The epsilon-Proteobacteria on *Lepetodrilus fucensis* gills may be environmental isolates and are closely related to free living sulphur-oxidizers (e.g., Lf-2.1 and 3.1 epsilon are related to *Sulfurimonas autotrophica*), suggesting that the gill may serve as a surface for colonization in fluids where sulphide and oxygen are available. For instance, the bacterial epibiont phylotypes most common on the body of a vent endemic polychaete, *Alvinella pompejana*, are also abundant on surrounding substrata, shown by *in situ* hybridization techniques (Cary et al. 1997). The epsilon-Proteobacterial isolates may also represent species found in venting fluids that are concentrated on the gill from the seawater via filtration by the host. For example, both the FISH probes designed to target the epsilon clone group Lf-1 also matched two epsilon clones isolated from a subseafloor habitat following a deep-sea volcanic eruption at Axial Volcano (Huber et al. 2003). Furthermore, environmental bacterial populations tend to share lower sequence identities than symbiotic populations (Peek et al. 1998). The sequence identity among the epsilon-Proteobacterial clones (≥97 %) was less in comparison to the *L. fucensis* symbiont (Lf-1 gamma: >99%), supporting that the epsilon-Proteobacteria are free-living.

Gamma-Proteobacteria

Surprisingly, we discovered that the primary *Lepetodrilus fucensis* symbiont is a gamma- rather than an epsilon-Proteobacteria, as reported for filamentous epibacteria hosted by rimicarid shrimps (Polz & Cavanaugh 1995) and alvinellid polychaetes (e.g., Polz & Cavanaugh 1995). The *L. fucensis* symbiont is a novel lineage in the gamma-Proteobacteria, distantly related to previously characterized chemoautotrophic symbionts,

suggesting that the symbiosis has an independent evolutionary origin within Phylum Mollusca. Furthermore, *L. fucensis* appears to host the same bacterial phylotype over a broad geographic range. Direct sequencing returned a single unambiguous sequence from specimens on the Juan de Fuca and Explorer Ridges and probes designed to target this sequence bound consistently to the bacterial zone on gills from animals collected at different vent sites.

The *Lepetodrilus fucensis* symbiont groups with a mixed bacterial clade that includes free-living sulphur oxidizing genera (e.g., *Thiomicrospira spp.*) and endosymbionts of *Solemya reidi* and *Maortihyas hadalis*. Although phylogenetic position does not necessarily reflect bacterial metabolism, Fox et al. (2002) also suggest the *L. fucensis* symbionts are chemoautotrophic based on high activity levels of Nitrate Reductase, ATP Sulfurylase and RuBisC/O in gill tissues. In addition, the bacteria probably use inorganic sulphide as an energy source because the abundance of bacteria increases in active vent flows and relates to the amount of carbon fixed by gill tissues (Chapter 5). Although BLAST searches (January 1, 2006) indicated that *Cycloclasticus* species share the highest sequence identity (89%) to the *L. fucensis* symbiont, the *L. fucensis* symbiont does not fall with this clade. *L. gordensis* n. sp., a sister species to *L. fucensis* from the Gorda Ridge (Johnson et al. in press), also hosts bacterial episymbionts on its gill (Chapter 6) and identification of these bacteria may provide a close relative of the *L. fucensis* symbiont.

Implications

Mollusc-hosted gill symbioses have multiple independent evolutionary origins and exhibit flexibility in the types of bacterial lineages that establish gill-hosted

symbioses (Distel et al. 1994, Urakawa 2005). The present study highlights an additional gill symbiosis with a novel evolutionary origin and further indicates that the formation of animal-bacterial associations is not lineage dependent. Instead, the host's fluid regime may be one important factor determining what types of bacteria are successful symbionts (Urakawa et al. 2005). Free-living and epibiotic epsilon-Proteobacteria are common in fluids between 30 and 50°C, while gamma-Proteobacteria (both free-living and symbiotic species) tend to occur in fluids with lower temperatures. For example, a Provannid gastropod (*Alvinochonca sp.*) from the western Pacific occupies warm fluids up to 30°C and hosts an epsilon-Proteobaceria endosymbiont (Urakawa et al. 2005), while a related species (*Ifremaria nautili*) selects habitats at lower temperatures and harbours gamma-Proteobacteria. This study provides an additional example of a gamma-Proteobacteria symbiosis that occurs in a moderate thermal regime; *Lepetodrilus fucensis* is present primarily in vent fluids below 15°C (Bates et al. 2005). Further characterization of the thermal and chemical environment of symbiotic bacteria may identify what factors select for certain lineages.

The *Lepetodrilus fucensis* association with gamma-Proteobacteria has a unique morphology: the bacterial symbionts are attached to the gill epithelium at one end but reside primarily in the spaces between the lamellae (de Burgh & Singla 1984). In comparison, provannid gastropods and bivalves typically host bacterial endosymbionts that are fully or partially enclosed within vacuoles of the epithelial membrane. One hypothesis is that the *L. fucensis* symbiosis is an intermediate morphology in transition from an extracellular to an intracellular symbiosis based on its unique morphology. It is also possible that *L. fucensis* symbiosis represents an alternate type of symbiosis that can

be successfully hosted by the gill tissues. However, the association is little studied. For example, determining if the bacteria contribute fixed carbon to their host and characterization of the recognition factors between host and bacteria will provide a basis for comparisons to endosymbiotic associations.

Conclusions

In conclusion, the primary *Lepetodrilus fucensis* gill symbiont is a novel lineage in the gamma-Proteobacteria and is distantly related to previously characterized symbionts. Although the symbionts are epibiotic, the *L. fucensis* gill symbiosis is a stable and highly specific association for specimens collected over a broad geographic area, as reported for the majority of mollusc gill-hosted symbioses to date.

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Appendix 4.1

Gamma- and epsilon-Proteobacteria sequences of the 16S rRNA gene in the 5^1 to 3^1 direction isolated for phylogenetic characterization from position 27 to ~1425 relative to *Escherichia coli* (alignment was conducted in ClustalX). Bolded numbers are base pair counts. Grey shading identifies the FISH probe target sequences at positions 128 and 643.

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                      5          15         25         35         45
LF-1.1 epsilon    AGAGTTTGAT CCTGGCTCAG AGTGAACGCT GGCGGCGTGC TTAACACATG
Lf-2.1 epsilon    AGAGTTTGAT CATGGCTCAG AGTGAACGCT GGCGGCGTGC TTAACACATG
LF-3.1 epsilon    AGAGTTTGAT CATGGCTCAG AATGAACGCT GGCGGCGTGC TTAACACATG
Lf-4.1 epsilon    AGAGTTTGAT CATGGCTCAG AGTGAACGCT GGCGGCGTGC TTAACACATG
Lf-1.1 gamma      AGAGTTTGAT CATGGCTCAG ATTGAACGCT GGCGGTATGC TTAACACATG
E.coli AY319394   AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG
                      55         65         75         85         95
LF-1.1 epsilon    CAAGTCGAAC GAGAACGGGT C-TGGCTTGC TAGATTGTCA GCTAAGTGGC
Lf-2.1 epsilon    CAAGTCGAAC GGTAAC-AGC A-GAGCTTGC TCC---GGCT GACGAGTGGC
LF-3.1 epsilon    CAAGTCGAAC GATGA--AGC C-TAGCTTGC TA----GGTG GATTAGTGGC
Lf-4.1 epsilon    CAAGTCGAAC GAGAACGGAT C-TAGCTTGC TAGATTGTCA GCTAAGTGGC
Lf-1.1 gamma      CAAGTCGAAC GGTAACATTG G--AGCTTGC TCCA---GAT GACGAGTGGC
E.coli AY319394   CAAGTCGAAC GGTAACAGGA AGCAGCTTGC TGCTT-CGCT GACGAGTGGC
                     105        115        125        135        145
LF-1.1 epsilon    GCACGGGTGA GTAATATATA GGTAATGTGC CTTGAAGATG GGGATAATTG
Lf-2.1 epsilon    GCACGGGTGA GTAATATATA GTTAATCTGC CTCGAAGACT AGGATAGCCA
LF-3.1 epsilon    GCACGGGTGA GTAATATATA GGTAACGTGC CTCTTAGTCT GGGATTGCCA
Lf-4.1 epsilon    GCACGGGTGA GTAATACATA GATAACATAC CTTTTAGTTT GGGATAACAG
Lf-1.1 gamma      GGACGGGTGA GTAATGCATA GGAAAT-TGC CGAATAGTGG GGGACAACAT
E.coli AY319394   GGACGAGTGA GTAATGTCTG GGAAAC-TGC CCGGTGGAGG GGGATAACTA
                     155        165        175        185        195
LF-1.1 epsilon    TTGGAAACGA CTTGTAAAAC CCGATATGCC TTTAATACAT AAGTATGCAA
Lf-2.1 epsilon    CTGGAAACGG TGATTAATAC TAGATAGTCC TTTCATTCAT AAGAATGATT
LF-3.1 epsilon    TTGGAAACGA TGATTAATAC TGGATACTCC TTCTAACTCT AATGTTAGTT
Lf-4.1 epsilon    TTGGAAACGG CTGCTAATAC CGAATATGCC TTTAATACTC AAGTATGAGA
Lf-1.1 gamma      GTGGAAACGC ATGCTAATAC CGCATACGCC CTACGGGGGA AAGATGGCCA
E.coli AY319394   CTGGAAACGG TAGCTAATAC CGCATAACGT CGCAAGACCA AAGAGGGGGA
                     205        215        225        235        245
LF-1.1 epsilon    GG-GAAAT-A TTTATAGCTT CAAGATCGGC CTGTACGGTA TCAGCTAGTT
Lf-2.1 epsilon    GG-TAAATGT TTTTTCGCTT TGAGATGAGA CTATATCCCA TCAGCTTGTT
LF-3.1 epsilon    GG-GAAA--G TTTTTCGCTA AGAGATCGGC CTATTTCCCA TCAGGTAGTT
Lf-4.1 epsilon    GG-GAAAG-A TTTATTGCTA AGAGATTGGT CTATGTCCTA TCAGTTAGTT
Lf-1.1 gamma      CTTCTTGAAA GCTATCGCTA TTTGATGTGC CTATGTCTGA TTAGCTAGTT
E.coli AY319394   C--CTTCGGG CCTCTTGCCA CCGGATGTGC CCAGATGGGA TTAGCTTGTT
                     255        265        275        285        295
LF-1.1 epsilon    GGTGAGGTAA TGGCTCACCA AGGCAATGAC GCCTAACTGG TTTGAGAGGA
Lf-2.1 epsilon    GGTAGTGTAA GAGACTACCA AGGCAATGAC GGGTAGCGGG TTTGAGAGGA
LF-3.1 epsilon    GGTAGTGTAA GAGACTACCA AGCCTATGAC GGGTAGCGGG TTTGAGAGGA
Lf-4.1 epsilon    GGTGAGGTAA AAGCTTACCA AGACTATGAC GGGTAGCTGG TTTGAGAGGA
Lf-1.1 gamma      GGTAGGGTAA AAGCCTACCA AGGCGACGAT CAGTAGCTGG TCTGAGAGGA
E.coli AY319394   GGTGAGGTAA CGGCTCACCA AGGCGACGAT CCCTAGCTGG TCTGAGAGGA
                     305        315        325        335        345
LF-1.1 epsilon    TGATCAGTCA CACTGGAACT GAGACACGGT CCAGACTCCT ACGGGAGGCA
Lf-2.1 epsilon    TGATCCGCCA CACTGGTACT GAGACACGGA CCAGACTCCT ACGGGAGGCA
LF-3.1 epsilon    TGATCCGCCA CACTGGTACT GAGACACGGA CCAGACTCCT ACGGGAGGCA
Lf-4.1 epsilon    TGATCAGCCA CACTGGTACT GAGACACGGA CCAGACTCCT ACGGGAGGCA
Lf-1.1 gamma      TGATCAGCCA CACTGGGACT GAGACACGGC CCAGACTCCT ACGGGAGGCA
E.coli AY319394   TGACCAGCCA CACTGGAACT GAGACACGGT CCAGACTCCT ACGGGAGGCA
                     355        365        375        385        395
LF-1.1 epsilon    GCAGTGGGGA ATATTGCACA ATGGACGAAA GTCTGATGCA GCAACGCCGC
Lf-2.1 epsilon    GCAGTGAGGA ATATTGCACA ATGGGGGAAA CCCTGATGCA GCAACGCCGC
LF-3.1 epsilon    GCAGTGAGGA ATATTGCACA ATGGAGGAAA CTCTGATGCA GCAACGCCGC
Lf-4.1 epsilon    GCAGTGGGGA ATATTGCACA ATGGAGGAAA CTCTGATGCA GCAACGCCGC
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Lf-1.1 gamma      GCAGTGGGGA ATATTGGACA ATGGGCGCAA GCCTGATCCA GCAATACCGC
E.coli AY319394   GCAGTGGGGA ATATTGCACA ATGGGCGCAA GCCKGATGCA GCCATGCCGC
                     405        415        425        435        445
LF-1.1 epsilon    GTGGAGGATG ACACATTTCG GTGCGTAAAC TCCTTTTATA TGAGAAGAAA
Lf-2.1 epsilon    GTGGAGGATG ACGCATTTCG GTGTGTAAAC TCCTTTTATA TGTCAAGAAA
LF-3.1 epsilon    GTGGAGGATG ACGCATTTCG GTGTGTAAAC TCCTTTTATA GGTCAAGAAA
Lf-4.1 epsilon    GTGGGGGATG ACACATTTCG GTGCGTAAAC TCCTTTTTTA TGGGAAGAAA
Lf-1.1 gamma      GTGTGTGAAG AAGGCTCGAG GGTCGTAAAG CACTTTCAAT AGTGAAGATT
E.coli AY319394   GTGTATGAAG AAGGCCTTCG GGTTGTAAAG TACTTTCAGC GGGGAGGAAG
                     455        465        475        485        495
LF-1.1 epsilon    A--------- ---------- -------TGA CGGTATCATA TGAATAAGCG
Lf-2.1 epsilon    A--------- ---------- -------TGA CGGTAGCATA TGAATAAGCA
LF-3.1 epsilon    A--------- ---------- -------TGA CGGTAGCCTA TGAATAAGCA
Lf-4.1 epsilon    A--------- ---------- -------TGA CGGTACCATA AGAATAAGCA
Lf-1.1 gamma      GGTTGTAAGG TTAATACCCT TATAGCTTGA CGTTAACTAT ACAAGAAGCA
E.coli AY319394   GGAT-TGTGG TTAATAACCG CAGTCATTGA CGTTACCCGC AGAAGAAGCA
                     505        515        525        535        545
LF-1.1 epsilon    CCGGCTAACT CCGTGCCAGC AGCCGCGGTA ATACGGAGGG CGCAAGCGTT
Lf-2.1 epsilon    CCGGCTAACT CCGTGCCAGC AGCCGCGGTA ATACGGAGGG TGCAAGCGTT
LF-3.1 epsilon    CCGGCTAACT CCGTGCCAGC AGCCGCGGTA ATACGGAGGG TGCAAGCGTT
Lf-4.1 epsilon    TCGGCTAACT CCGTGCCAGC AGCCGCGGTA ATACGGAGGA TGCAAGCGTT
Lf-1.1 gamma      CCGGCTAACT CAGTGCCAGC AGCCGCGGTA ATACTGAGGG TGCAAGCGTT
E.coli AY319394   CCGGCTAACT CCGTGCCAGC AGCCGCGGTA ATACGGAGGG TGCAAGCGTT
                     555        565        575        585        595
LF-1.1 epsilon    ACTCGGAATC ACTGGGCGTA AAGAGCGTGT AGGCGGGAAT TTAAGTTGGA
Lf-2.1 epsilon    ACTCGGAATC ACTGGGCGTA AAGGACGCGT AGGCGGGATG CCAAGTCTGA
LF-3.1 epsilon    ATTCGGAATC ACTGGGCGTA AAGGACGCGT AGGCGGGAAG CCAAGTCTGA
Lf-4.1 epsilon    ACTCGGAATC ACTGGGCGTA AAGAGCATGT AGGCTGGATA ATAAGTCAGA
Lf-1.1 gamma      AATCGGAATT ACTGGGCGTA AAGCGCGCGT AGGCGGTAAG TTAAGTTGGA
E.coli AY319394   AATCGGAATT ACTGGGCGTA AAGCGCACGC AGGCGGTCTG TCAAGTCGGA
                     605        615        625        635        645
LF-1.1 epsilon    AGTGAAATCC TATGGCTCAA CCATAGAACT GCTTCCAAAA CTGAATACCT
Lf-2.1 epsilon    TGTGAAATCC TATGGCTTAA CCATAGAACT GCATTGGAAA CTGGTAACCT
LF-3.1 epsilon    TGTGAAATCC TATGGCTCAA CCATAGAACT GCATTGGAAA CTGGTTACCT
Lf-4.1 epsilon    TGTGAAATCC TATCGCTTAA CGATAGAACT GCATTTGAAA CTGTTTTCCT
Lf-1.1 gamma      TGTGAAATCC CTAAGCTCAA CCTAGGAACT GCATTCAAAA CTGGCTCACT
E.coli AY319394   TGTGAAATCC CCGGGCTCAA CCTGGGAACT GCATTCGAAA CTGGCAGGCT
                     655        665        675        685        695
LF-1.1 epsilon    AGAATATGGG AGAGGTAGAT GGAATTTCTG GGGTAGGGGT AAA-TCCGTA
Lf-2.1 epsilon    AGAGTATGGG AGGGGGAGAT GGAATTAGTG GTGTAGGGGT AAAATCCGTA
LF-3.1 epsilon    AGAGTATGGG AGGGGGAGAT GGAATTAGTG GTGTAGGGGT AAAATCCGTA
Lf-4.1 epsilon    AGAGTATGGA AGGGGCAGAT GGAATTAGTG GTGTAGGGGT AAAATCCGTA
Lf-1.1 gamma      AGAGTATGGT AGAGGCAAGT GGAATTCCAG -TGTAGCGGT GAAATGCGTA
E.coli AY319394   TGAGTCTCGT AGAGGGGGGT AGAATTCCAG GTGTAGCGGT GAAATGCGTA
                     705        715        725        735        745
LF-1.1 epsilon    GAGATCAGAA GGAATACCGA TTGCGAAGGC GATCTACTGG AACATTATTG
Lf-2.1 epsilon    GATATCACTA GGAATACCTA AAGCGAAGGC GATCTCCTGG AACAATACTG
LF-3.1 epsilon    GATATCACTA GGAATACCTA AAGCGAAGGC GATCTCCTGG AACAATACTG
Lf-4.1 epsilon    GAGATCACTA GGAATACCGA AAGCGAAGGC GATCTGCTGG TACATAACTG
Lf-1.1 gamma      GATATTTGGA G-AACATCAG TGGCGAAGGC GACTTGCTGG GCCAATACTG
E.coli AY319394   GAGATCTGGA GGAATACCGG TGGCGAAGGC GGCCCCCTGG ACGAAGACTG
                     755        765        775        785        795
LF-1.1 epsilon    ACGCTGAGAC GCGAAAGCGT GGGGAGCAAA CAGGATTAGA TACCCT-GGT
Lf-2.1 epsilon    ACGCTAAGGC GTGAAAGCGT GGGGAGCAAA CGGGATTAGA TACCCC-GGT
LF-3.1 epsilon    ACGCTAAGGC GTGAAAGCGT GGGGAGCAAA CGGGATTAGA TACCCCCGGT
Lf-4.1 epsilon    ACGCTAAGAT GCGAAAGCGT GGGGAGCAAA CAGGATTAAA TACCCT-GGT
Lf-1.1 gamma      ACGCTGAGGT GCGAAAGCGT GGGTAGCAAA C-GGATTAGA TACCCC-GGT
E.coli AY319394   ACGCTCAGGT GCGAAAGCGT GGGGAGCAAA CAGGATTAGA TACCCT-GGT
                     805        815        825        835        845
LF-1.1 epsilon    AGTCCACGCC -CTAAACGAT GTACATTAGT TGTTGCGATG CTAGACATTG
Lf-2.1 epsilon    AGTCCACGCC -TTAAACGAT GAACACTAGT CGTCGGGATG CTTGTCATCT
LF-3.1 epsilon    AGTCCACGCC CTTAAACGAT GAACACTAGT CGTCGGGATG CTAGTCATCT
Lf-4.1 epsilon    AGTCC-CGCC -CTAA-CGAT GGACACTAGT CGTCGGAAAT TTA---ATTT
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Lf-1.1 gamma      AGTCCACGCC -GTAAACGAT GTCAACT-AG CCGTTGGGCC CGTTAGGTTT
E.coli AY319394   AGTCCACGCC -GTAAACGAT GTCTACTTGG AGGCTGTGCC CTTGAGGCGT
                     855        865        875        885        895
LF-1.1 epsilon    CAGTAATGCA GTTAACACAT TAAATGTACC GCCTGGGGAG TACGGTCGCA
Lf-2.1 epsilon    CGGTGATGCA CTTAACAGAT TAAGTGTTCC GCCTGGGGAG TACGGTCGCA
LF-3.1 epsilon    CGGTGATGCA CTTAACAAAT TAAGTGTTCC GCCTGGGGAG TACGGTCGCA
Lf-4.1 epsilon    CGGTGATGCC CTTA-CAAAT -AAGTGTCCC GCCTGGGGAG T-CGGGCNCA
Lf-1.1 gamma      AG-TGGCGCA GCTAACGCAT TAAGTTGACC GCCTGGGGAG TACGCCGGCA
E.coli AY319394   GGCTTCCGGA GCTAACGCGT TAAGTAGACC GCCTGGGGAG TACGGCCGCA
                     905        915        925        935        945
LF-1.1 epsilon    AGATTAAAAC TCAAAGGAAT AGACGGGGAC CCGCACAAGC GGTGGAGCAT
Lf-2.1 epsilon    AGATTAAAAC TCAAAGGAAT AGACGGGGAC CCGCACAAGT GGTGGAGCAT
LF-3.1 epsilon    AGATTAAAAC TCAAAGGAAT AGACGGGGAC CCGCACAAGT GGTGGAGCAT
Lf-4.1 epsilon    -GATTAA--C TCAAGGATAA C--------- ---------- ----------
Lf-1.1 gamma      ACGGTAAAAC TCAAAGGAAT TGACGGGGGC CCGCACAAGC GGTGGAGCAT
E.coli AY319394   AGGTTAAAAC TCAAATGAAT TGACGGGGGC CCGCACAAGC GGTGGAGCAT
                     955        965        975        985        995
LF-1.1 epsilon    GTGGTTTAAT TCGACGATAC GCGAAGAACC TTACCTGGCC TTGACATACC
Lf-2.1 epsilon    GTGGTTTAAT TCGAAGATAC GCGAAGAACC TTACCTAGCC TTGACATTGA
LF-3.1 epsilon    GTGGTTTAAT TCGAAGATAC GCGAAGAACC TTACCTAGCC TTGACATTGA
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      GTGGTTTAAT TCGATGCAAC GCGAAGAACC TTACCTACCC TTGACATTGA
E.coli AY319394   GTGGTTTAAT TCGATGCAAC GCGAAGAACC TTACCTGGTC TTGACATCCA
                    1005       1015       1025       1035       1045
LF-1.1 epsilon    AAGAAACTAA TAGAGATATA AGTGTGCTAG TTTACTAGAA CTTGGATACA
Lf-2.1 epsilon    TTGAATTCTG TAGAGATACG GAAGTGCC-- TTTCGGGGAA CATGAAAACA
LF-3.1 epsilon    TTGAACGGAC CAGAGATGGA CCGGTGCC-- CTTCGGGGAA CATGAAAACA
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      CAGAACCTCG TAGAGATACT TGGGTGCC-- --TTCGGGC- CTGGAAAACA
E.coli AY319394   CGGAAGACTG CAGAGATGCG GTTGTGCC-- --TTCGGGAA CCGTGAGACA
                    1055       1065       1075       1085       1095
LF-1.1 epsilon    GGTGCTGCAC GGCTGTCGTC AGCTCGTGTC GTGAGATGTT AGGTTAAGTC
Lf-2.1 epsilon    GGTGCTGCAC GGCTGTCGTC AGCTCGTGTC GTGAGATGTT GGGTTAAGTC
LF-3.1 epsilon    GGTGCTGCAC GGCTGTCGTC AGCTCGTGTC GTGAGATGTT GGGTTAAGTC
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      GGTGCTGCAT GGCTGTCGTC AGCTCGTGTC GTGAGATGTT GGGTTAAGTC
E.coli AY319394   GGTGCTGCAT GGCTGTCGTC AGCTCGTGTT GTGAAATGTT GGGTTAAGTC
                    1105       1115       1125       1135       1145
LF-1.1 epsilon    CT-GCAACGA GCGCAACCCT CGTGT-TTAG TTACTAACAG -TTCGGCTGA
Lf-2.1 epsilon    CC-GCAACGA GCGCAACCCT CGTCC-TTAG TTGCCAGCAG GTTAAGCTGG
LF-3.1 epsilon    CC-GCAACGA GCGCAACCCT CGTCA-ATAG TTGCCAGCAG GTTAAGCTGG
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      CC-GCAACGA GCGCAACCCC TATCC-TTAG TTGCTAACA- -TTCAGTTGA
E.coli AY319394   CCCGCAACGA GCGCAACCCT TATCCCTTTG TTGCCAGCGG -TCCGGCCGG
                    1155       1165       1175       1185       1195
LF-1.1 epsilon    GGACTCTAAA CATACTGCCT GGG-CAACCA GGAGGAAGGT GAGGACGACG
Lf-2.1 epsilon    GTACTCTAAG GAGACTGCCT TCG-CAAGGA GGAGGAAGGT GAGGACGACG
LF-3.1 epsilon    GCACTTTATT GAGACTGCCT TCG-CAAGAA GGAGGAAGGT GAGGACGACG
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      GAACTTTAAG GAGACTGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG
E.coli AY319394   GAACTCAAAG GAGACTGCCA GTGATAAACT GGAGGAAGGT GGGGATGACG
                    1205       1215       1225       1235       1245
LF-1.1 epsilon    TCAAGTCATC ATGGCCCTTA CGGCCAGGGC TATACACGTG CTACAATGGG
Lf-2.1 epsilon    TCAAGTCATC ATGGCCCTTA CGGCTAGGGC TACACACGTG CTACAATGGG
LF-3.1 epsilon    TCAAGTCATC ATGGCCCTTA TGGCTAGGGC TACACACGTG CTACAATGGG
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      TCAAGTCATC ATGGCCCTTA TGGGTAGGGC TACACACGTG CTACAATGGG
E.coli AY319394   TCAAGTCATC ATGGCCCTTA CGACCAGGGC TACACACGTG CTACAATGGC
                    1255       1265       1275       1285       1295
LF-1.1 epsilon    GTATACAAAG AGCCGCAATA CCGCGAGGTG GAGCAAATCT CATAAAATAT
Lf-2.1 epsilon    GCGTACAGAG TGTTGCGATA CCGCGAGGTG GAGCCAATCA CTTAAAGCGT
LF-3.1 epsilon    GCGTACAGAG AGTTGCGATA CCGCGAGGTG GAGCCAATCT CATAAAGCGT
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
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Lf-1.1 gamma      CAATACAGAG GGTCGCAAAC TCGCGAGAGT AAGCCAATCC CACAAAATTG
E.coli AY319394   ATATACAAAG AGAAGCGACC TCGCGAGAGC AAGCGGACCT CATAAAGTAT
                     1305       1315       1325       1335       1345
LF-1.1 epsilon    CTCCCAGTTC GGATTACACT CTGCAACTCG AGTGTATGAA GTTGGAATCG
Lf-2.1 epsilon    CTCTCAGTTC GGATTGGAGT CTGCAACTCG ACTCCATGAA GCTGGAATCA
LF-3.1 epsilon    CTCTCAGTTC GGATAGCAGT CTGCAACTCG ACTGCTTGAA GCTGGAATCA
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      TTCGTAGTCC GGATTGAAGT CTGCAACTCG ACTCCATGAA GTTGGAATCG
E.coli AY319394   GTCGTAGTCC GGATTGGAGT CTGCAACTCG ACTCCATGAA GTCGGAATCG
                     1355       1365       1375       1385       1395
LF-1.1 epsilon    CTAGTAATCG TAGATCAGCA ATGCTACGGT GAATACGTTC CCGGGTCTTG
Lf-2.1 epsilon    CTAGTAATCG TAGATCAGCA ATGCTACGGT GAATACGTTC CCGGGTCTTG
LF-3.1 epsilon    CTAGTAATCG TAGATCAGCA ATGCTACGGT GAATACGTTC CCGGGTCTTG
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      CTAGTAATCG TGAATCAG-A ATGTCACGGT GAATACGTTC CCGGGCCTTG
E.coli AY319394   CTAGTAATCG TGGATCAG-A ATGCCGCGGT GAATACGTTC CCGGGCCTTG
                     1405       1415       1425       1435       1445
LF-1.1 epsilon    TACTCACCGC CCGTCACACC ATGGGAATTG AATTCATTCG AAGCGGGGAT
Lf-2.1 epsilon    TACTCACCGC CCGTCACACC ATGGGAGTTG ATTTCACCCG AAATTGGGAA
LF-3.1 epsilon    TACTCACCGC CCGTCACACC ATGGGAGTTG ATTTCACCCG AAATTGGGAA
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      TACACACCGC CCGTCACACC ATGGGAGTTG GTTGCTAAAG AAGTGGGTAG
E.coli AY319394   TACACACCGC CCGTA----- ---------- ---------- ----------
                     1455       1465       1475       1485       1495
LF-1.1 epsilon    GCTAAAAT-- ----AGCTAC CTTCCACAGT GGATTTAGTA ACTGGGGTGA
Lf-2.1 epsilon    GCTAACCTTC GGGAGGCTAC CACTTACGGT GGAATTAGCG ACTGGGGTGA
LF-3.1 epsilon    GCTAACCTTC GGGAGGCTAC CACTTACGGT GGAATTAGCG ACTGGGGTGA
Lf-4.1 epsilon    ---------- ---------- ---------- ---------- ----------
Lf-1.1 gamma      ACTAACCTTC GGGAGGTCGC TCACCACTTA GTGATTAATG ACTGGGGTGA
E.coli AY319394   ---------- ---------- ---------- ---------- ----------
                     1505
LF-1.1 epsilon    AGTCGTAACA
Lf-2.1 epsilon    AGTCGTAACA
LF-3.1 epsilon    AGTCGTAACA
Lf-4.1 epsilon    ----------
Lf-1.1 gamma      AGTCGTAACA
E.coli AY319394   ----------
```

Appendix 4.2

Summary of identical matches (number of hits) between the FISH probes (5^1 to 3^1 direction) designed in this study and GenBank sequences. The clone groups Lf-1 to 3 epsilon at both positions 128-147 and 643-662 (relative to *Escherichia coli*) were not specific. However, the probes Lf-1.gamma and Lf-4 epsilon were specific and did not match GenBank sequences (BLAST: October 15, 2005). Identical matches are indicated as number of hits. The accession number for all hits are indicated.

Positions 128-147	Positions 643-662
Lf-1 epsilon128	***Lf-1 epsilon643***
CCATC-TTCAA-GGCAC-ATTAC	**CCCAT-ATTCT-AGGTA-TTCAG**
2 hits (AF468749, AF468786)	3 hits (AF468749, AF468786, AB110879)
Lf-2 epsilon128	***Lf-2 epsilon643***
TAGTC-TTCGA-GGCAG-ATTAA	**CCCAT-ACTCT-AGGTT-ACCAG**
1 hit (AF468743)	7 hits (AY263717, AF468743, AY171381, U46506, AF121088, AF224795, AF224780)
Lf-3 epsilon128	***Lf-3 epsilon643***
CAGAC-TAAGA-GGCAC-GTTAC	**CCCAT-ACTCT-AGGTA-ACCAG**
6 hits (AY2116702, AY211665, AF468781, AY360511)	17 hits (AY360489, AY360510, AY360511, AY2116702, AY211658, AY211665, AY354147, AY197379, AF468736, AY075129, AY547990-3, HVU15106)

CHAPTER 5

Influence of habitat on the persistence and morphology of a symbiosis between a gastropod and gamma-Proteobacteria

Abstract

The limpet-shaped gastropod, *Lepetodrilus fucensis* McLean, is a very abundant species at hydrothermal vents on the Juan de Fuca Ridge. One hypothesis is that this limpet benefits from an epibiotic association with gamma-Proteobacteria on its gill, however, the nature of this symbiosis is unknown. To assess the persistence and morphology of the symbiosis, I examined *L. fucensis* gill tissue from specimens collected in different habitats using microscope techniques. Symbionts were present on animals from multiple vent sites and substrata and from a range of *L. fucensis* size classes thereby illustrating a highly persistent relationship. In addition, bacteria were distributed similarly among lamellae from different regions of the gill. However, the morphology of the gill lamellae varied. The surface area of the blood space and bacteria-hosting epithelium on lamellae were positively related to bacterial abundance. In addition, animals with abundant symbiont populations had significantly higher tissue condition. Gill morphology and the abundance of the symbionts are directly related to fluid flux; in animals transplanted 50 cm away from a vent source, symbiont abundance and average filament diameter decreased while the host adopted the gill morphology and tissue condition typical of animals collected from low vent flux. To explain why prolonged durations in peripheral locations might decrease the persistence of the symbiosis, I hypothesized that the bacteria are thioautotrophic. An *in situ* experiment exposed *L. fucensis* to radiolabelled carbon dioxide in the presence and absence of reduced inorganic

sulphide. Carbon fixation in gill tissue was greater when sulphide was added and related to the amount of bacteria on the gills, suggesting that the bacteria oxidize inorganic sulphide. This study indicates that the plastic gill morphology of *L. fucensis* contributes to the persistence of the symbiosis under a range of environmental conditions and that the condition of limpets relates to the abundance of their gill symbionts. Hence, the symbiosis may benefit *L. fucensis* in warm vent flows where reduced sulphur is available.

INTRODUCTION

Nutritional symbioses between invertebrates and chemosynthetic bacteria are widespread in shallow and deep-sea marine habitats where reduced sulphur is present with oxygen. Symbiont-hosting invertebrates are phylogenetically diverse and exhibit an array of novel morphological and physiological adaptations to access chemical substrates from the environment and meet the metabolic requirements of their symbionts (e.g., *Riftia pachyptila*: Fisher 1990, Flores et al. 2005). In exchange, the host typically benefits from bacterial produced organic compounds (Felbeck 1981, Minic et al. 2002). One goal of symbiosis research is to understand how environmental variables influence the relative benefit to each symbiotic partner and, ultimately, the stability of these associations (Muller-Parker and Davy 2001). Studies aiming to characterize the persistence of symbioses in different environmental regimes can contribute to this goal.

Hydrothermal vent habitats are an excellent system to study the response of symbioses to different abiotic parameters because abundant symbiotic fauna colonize the mixing zone between vent fluids and deep-sea water where steep gradients in reduced sulphur, oxygen and temperature occur at small spatial scales (Sarrazin et al. 1999,

Urcuyo et al. 2003, Bates et al. 2005). The distribution of symbiont-hosting invertebrates along these fluid gradients permits assessment of how environment influences symbioses. For instance, the mixotrophic mytilid mussel (*Bathymodiolus thermophilus*) hosts a gill endosymbiosis that responds to hydrothermal fluid activity. When mussels were transplanted to the periphery of a vent, host tissue condition and bacterial abundance declined (Smith 1985, Raulfs et al. 2004). An example of a morphologically flexible host is *Ridgeia piscesae*. This vestimentiferan tubeworm alters its tube morphology in relation to fluid chemistry (Urcuyo et al. 2003), presumably to access the chemical substrates necessary for thioautotrophy by its endosymbionts.

Although the majority of symbiotic associations are described from few specimens due to the technological challenges associated with sampling deep-sea environments (e.g., Goffredi et al. 2004), symbiont-hosting species that are easy to collect, abundant and occupy a variety of hydrothermal vent habitats can provide model organisms to assess the role that habitat plays in determining the incidence and morphology of symbioses. One such species is a limpet-shaped gastropod, *Lepetodrilus fucensis* McLean 1988 (Vetigastropoda) that hosts a gill episymbiosis with filamentous gamma-Proteobacteria (Chapter 4). *L. fucensis* is found in very high densities in multiple habitats from vents on the Juan de Fuca Ridge (Tsurumi and Tunnicliffe 2003). Although the gill bacteria are digested in lysosmes and presumably contribute to the host's nutrition (de Burgh and Singla 1984), *L. fucensis* may also suspension feed and graze (Fretter 1988), suggesting the symbiosis is facultative in nature. Enzymes indicative of chemoautotrophy are present in *L. fucensis* gill tissues at levels comparable to other symbiont-hosting bivalves (Fox et al. 2002) and the bacteria may use inorganic

sulphide as an energy source. However, limpets are also common in fluids at near-ambient temperatures (Bates et al. 2005) where sulphide levels are negligible. Thus, the *L. fucensis* episymbiosis is a good candidate to assess the influence of habitat on the association.

My objective was to characterize the persistence and nature of the *Lepetodrilus fucensis* gill episymbiosis by examining specimens collected from habitats in different hydrothermal fluid regimes. I determined the distribution of the symbiosis in several settings: (1) at multiple sites across the geographic range of the limpet, (2) at basalt and sulphide hosted vents within sites, (3) across a range of host size classes and (4) on all regions of the gill. I then characterized host gill morphology, the abundance of gill bacteria and the condition of both partners in collections from high and low vent fluid flux. I designed a transplant experiment to test if the loss of gill bacteria relates to a decline in the limpet's gill morphology and host condition. To determine if digestion of bacteria by the gill epithelium is an important nutritional mechanism, I counted the number of lysosomal bodies and bacteria present in transmission electron micrographs and predicted a positive relationship. To explain why prolonged exposures to vent fluids with low sulphide concentrations might influence the condition of the symbiosis, I hypothesized that the bacteria oxidize sulphide. Thioautotrophy was tested *in situ* by exposing *L. fucensis* to radiolabelled carbon.

METHODS AND MATERIALS

Hydrothermal vent fields were visited during summer months (May-September). Sites on the Juan de Fuca Ridge (1988 and 2001-03) were Endeavour Segment

(47°57'N,129°06'W; depth 2220 m), Co-Axial Segment (46°09'N, 129°48'W; depth 2050 m) and Axial Volcano (45°56'N 130°00'W; depth 1570 m). On Explorer Ridge (2002), vents at Southern Explorer Segment (49°45'N 129°42'W; depth 1850 m) were sampled.

All *in situ* collections were made by suction sampling at outflows diffusing through basalt or low sulphide mounds, using the Canadian remotely operated vehicle, ROPOS. Point measurements of maximum temperature were recorded for most sampled sites with a rugged transition joint probe (Adam's module thermistor, ±1°C).

Microscopy

Gill tissue was dissected from specimens, rinsed in water filtered at 0.2 μm and fixed in 2.5% glutaraldehyde buffered in 0.4 M sodium phosphate (pH = 7.4). Samples were stored in glutaraldehyde at 4°C for several weeks. Gill lamellae were post-fixed with osmium tetroxide and dehydrated in a graded ethanol series. Specimens selected for light and transmission electron microscopy were transferred to 100% propylene dioxide and embedded in Epon 812 resin (TAAB). Semi-thin transverse sections (500 μm) of individual filaments were treated with Richardson's stain and viewed using a Zeiss Universal compound light microscope. Thin sections (70 nm) were stained with lead citrate and uranyl acetate and imaged with a Hitachi H-7000 transmission electron microscope. Scanning electron micrographs of gill lamellae were generated on a Hitachi S-3500N microscope (tissues were critically point dried and sputter-coated in gold).

Symbiosis distribution

To characterize the distribution of the symbiosis, I verified the presence of filamentous bacteria in association with the bacterial-hosting epithelium from transverse

sections of gill tissue (126 specimens) using a compound light microscope (tissue was prepared as described above for light microscopy). Animals were collected at different vent sites and substrata, and represented a range of size classes (collections are described in Appendix 2.2: in addition 40 animals from an Endeavour flange (Crypto: 47°56.9'N, 129°05.9'W) and a Co-Axial chimney (Beard Chimney, 46°9.3'N, 129°48.6'W)). Different regions of the gill were also examined (Table 5.1).

Measured gill parameters

Gill parameters were measured on animals sampled at ten vents from three vent sites as described in Appendix 2.2 (Axial: Hell = C, N3 = D, Shepherd = G and Caspar = H, Endeavour: S&M 'site 1' = F, S&M 'site 2' = G; Explorer: Einstein = A, Tubeworm Chimney = B). In addition, Cloud Vent at Axial (98 Lava Flow: 45°56.0'N 129°58.9'W) was sampled and is labeled 'I'. At each vent a high and a low vent flux location was sampled based on distance from focused vent source and fluid temperature. In-vent locations were characterized as being exposed to shimmering water between 5 and 15°C and ranged 0 to 25 cm from a focused fluid source (Bates et al. 2005). Far-vent locations were from 51 to 75 cm from a vent, no shimmering was evident and temperatures were from 2 to 3°C (Bates et al. 2005). Distance was determined by ROPOS pilots using laser spots at 10 cm apart.

Gill morphology. The depth of the afferent side of the lamellae, the depth of mid blood space and the depth of the efferent side of the lamellae were measured on light micrographs as the length of a line traced (Image-Pro Plus© 4.5) along the axis of each region following the natural curves of the tissue (Figure 5.1D). The bacterial epithelium depth was measured as the length of the line made by tracing the surface of the

Table 5.1
Transverse sections through the specialized gill epithelium of 126 specimens from active vent flows were examined using light microscopy to determine the distribution of the *Lepetodrilus fucensis*-bacterial symbiosis at different locations and within the host population (individual specimens counted for different categories). Bacteria were found on (a) animals from a wide geographic area and multiple substratum types, and on (b) gills from a range of host size classes and both sexes. To determine the distribution of the symbiosis on the gill, ten high flow specimens (>3 mm shell length) were randomly selected and further sectioned. (c) All regions of the gill and the entire length of individual lamellae hosted bacteria.

a. LOCATION				
Vent n > 10	Explorer	Endeavour	Co-Axial	Axial
Substratum Type n = 40	Sulphide	basalt	tubeworms	
b. HOST				
Size n > 30	small 0.5-2.9 mm	medium 3-10 mm	large >10 mm	
Sex n = 50	female	male		
c. GILL				
Whole Gill n = 10	Anterior	middle	posterior	
Lamella n =10	Base	middle	tip	

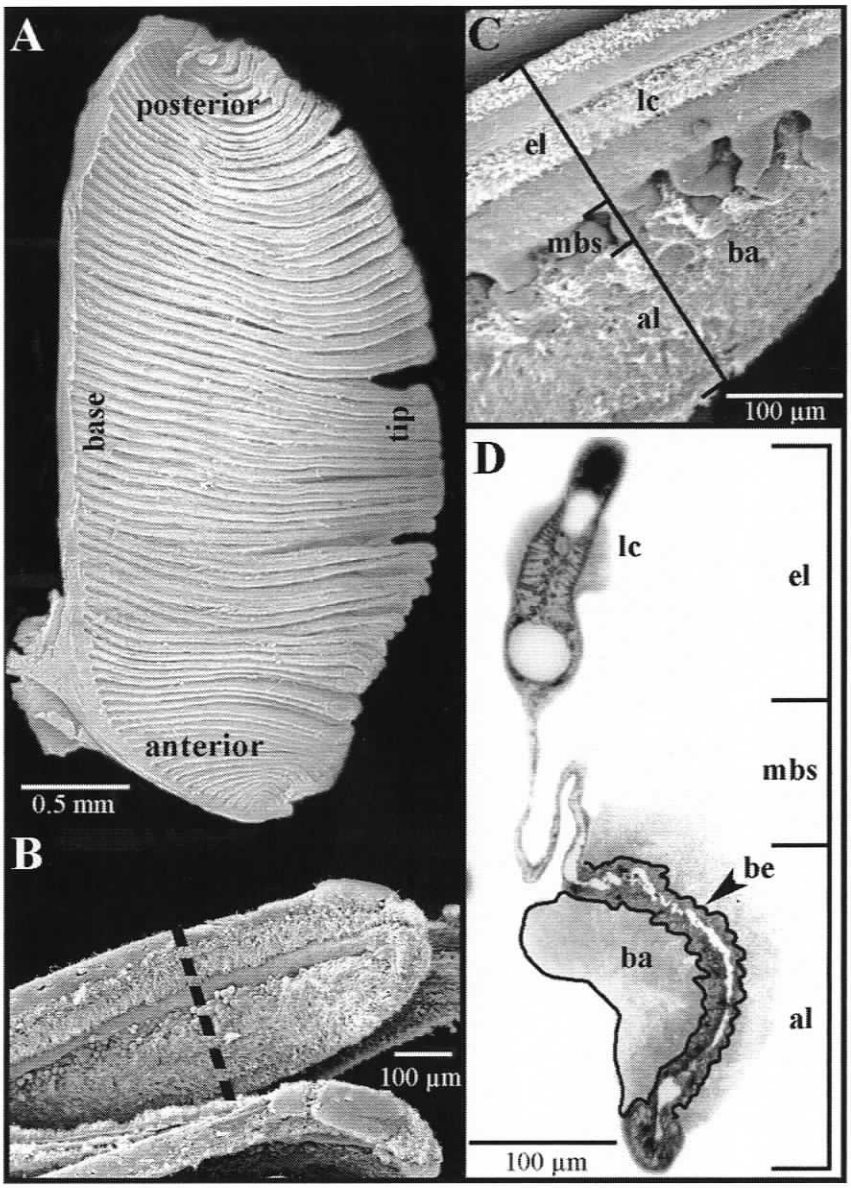

Figure 5.1
Scanning electron (a, b & c) and light (d) micrographs of *Lepetodrilus fucensis* gill lamellae. (A) Dorsal surface of an intact gill. Posterior and anterior lamellae are indicated. The base of each lamella attaches to the gill axis. Lamellae are free of the mantle from base to tip. (B) Three replicate lamellae were selected for sectioning in a transverse orientation (black dotted line). The lamellar 'depth' can be seen in transverse orientation. (C & D) Lamellae can be divided into the efferent (el), mid blood space (mbs) and afferent (al) regions. Bacteria (ba) are attached to a specialized epithelium (be) in the afferent region of the gill (see Fretter 1988). Lateral cilia (lc) are present in the efferent region. This specimen is from an in-vent location.

epithelium occupied by bacteria (as indicated by arrow "be" on Figure 5.1D). Differences in gill parameters between in- and far-vent were tested with a Monte Carlo Randomization routine (Manly 1991).

Bacterial abundance index. Three lamellae (selected from the gill mid-section) were dissected from 10 animals at each in- and far-vent location and prepared for light microscopy (tissue preparation described above). The morphology of different lamellae from one individual was virtually identical. To estimate bacterial abundance, I digitized the transverse section from a light micrograph and measured two variables on one representative lamella (Image-Pro Plus© 4.5): (1) the area (mm^2) occupied by bacteria (bac^{area}) (as indicated by the area labeled "ba" on Figure 5.1D), and (2) optical density of the area occupied by bacteria (bac^{opden}). These measures were divided, respectively, by the area occupied by the lateral cilia (lc^{area}) (standardized bac^{area} for size and fixation differences between samples) and the optical density of the lateral cilia (lc^{opden}) (standardized bac^{opden} for differential staining intensity between sections). Bacterial abundance index = $(bac^{area} / lc^{area}) * (bac^{opden} / lc^{opden})$. The relationships between different gill parameters and bacterial abundance were tested with a Pearson product moment correlation (r).

Three specimens at in- and far-vent locations were randomly selected for whole gill analyses from two vents (B and D). To compare bacterial abundance across the gill, I sectioned the base, mid and tip of lamellae from anterior, middle and posterior regions (Figure 5.1). Bacterial abundance was quantified as described above.

Bacterial-epithelial contacts. For three vents (A, C and D), I randomly selected seven of the ten gills examined with light microscopy at in- and far-vent locations for

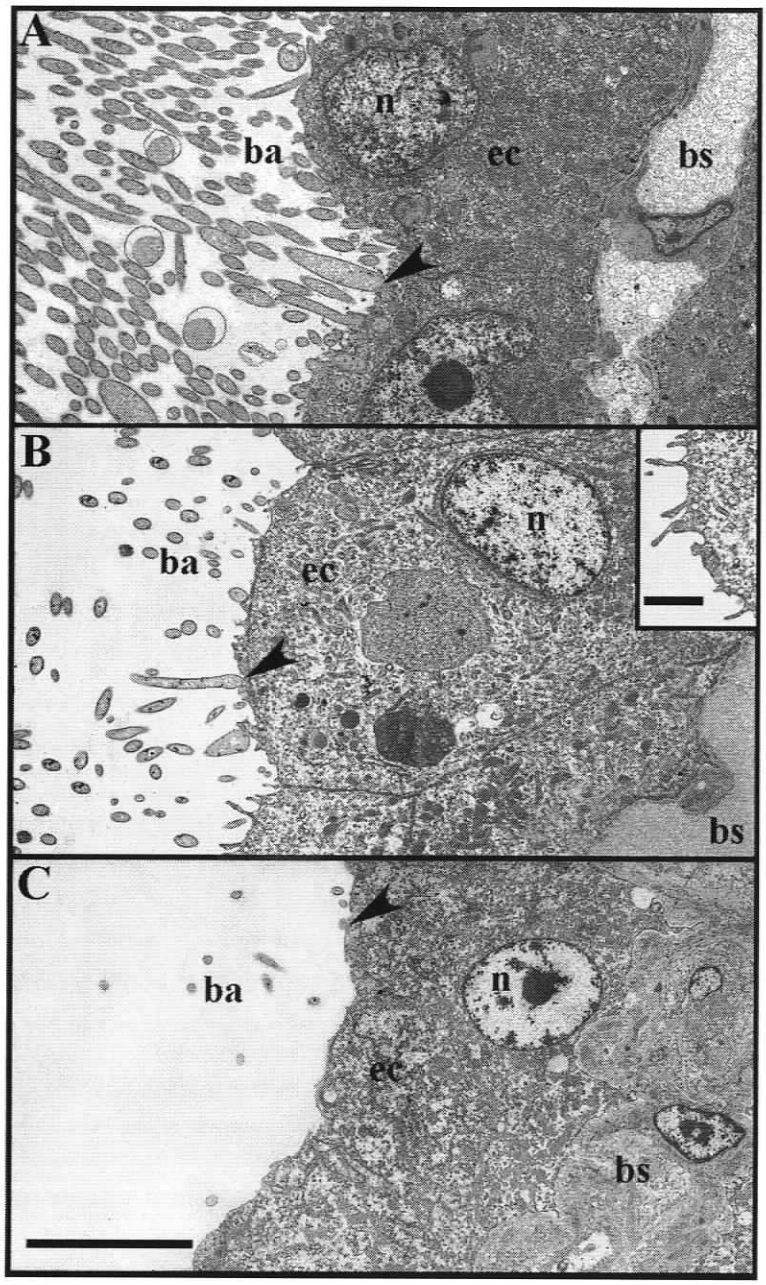

Figure 5.2
Transmission electron micrographs of the epithelium where filamentous bacteria attach on *Lepetodrilus fucensis* gills: bacterial epithelium (be). Bacteria (ba) are visible in the extracellular space in the mantle cavity. Contacts (arrow) between one end of the bacterial filament and the host epithelial cell (ec) are visible. (A) In- and (B & C) far-vent tissues are shown. Pit-like structures were observed on the host bacterial epithelium (inset: B). The nucleus (n) and blood space (bs) are present. Scale bars = 10 μm (A, B and C) and 2.5 μm (inset).

TEM (tissue preparation described above). Bacterial-epithelial contacts (Figure 5.2) along 25 μm of the bacterial epithelium (randomly selected) were counted from TEM micrographs. A Monte Carlo Randomization routine tested for differences in bacterial-epithelial contacts between in- and far-vent locations. To estimate the number of bacteria per gram wet weight of gill tissue, I scaled up the number of bacteria per 25 μm of epithelium to represent the whole gill based on average values for the following parameters: # full length lamellae per gill = 50, average lamella depth = 100 μm, average lamella length = 3 mm, gill wet weight = 0.003 g.

Lysosomes. For the TEM sections examined above (see bacterial-epithelial contacts), I counted the number of lysosomes present in the cytoplasm of cells that contributed to the 25 μm length of epithelium. Linear regression statistics determined if the number of lysosomes and bacterial-epithelial contacts were significantly related.

Host and symbiont condition

Thirty formalin-fixed *Lepetodrilus fucensis* (shell length = 5-8 mm) were selected from in- and far-vent locations at three vents (C, E, F). The tissue from each specimen was removed and dried in a 60°C oven for 12 hours. Dry weight was measured on an analytical balance (±0.0001 g). The shell volume of *Lepetodrilus fucensis* was estimated from shell dimensions based on the formula for cone volume (maximum length = height, 0.5 * aperture diameter = radius). A t-test for independent samples identified significant differences in the ratio of tissue dry weight to shell volume at in- and far-vent locations.

Specimens (5 mm in shell length) were selected from in- and far-vent (n = 6) at Vent C, frozen and stored at -80°C. Total lipids were extracted and analysis of

triacylglycerols (storage lipids) was quantified for each specimen. Methods for the lipid extraction procedure are described in Campbell et al. (2004). Briefly, animals were homogenized (ultrasonic homogenizer) in 2:1 (v/v) chloroform:methanol and cooled on a dry ice-ethanol bath. The extraction was completed at -15°C for 24 h after homogenization and then 0.9% NaCl solution was added to bring the extraction medium to 8:4:3 (v/v/v) of chloroform:methanol:water. The sample was then centrifuged. The lower phase was pipetted out, dried under argon gas and re-suspended in hexane. Analysis of lipid classes was performed using an Iatroscan MK-5 TLC/FID analyzer (see Campbell et al. 2004).

The diameter of bacteria found in contact with the host epithelium was measured from TEM micrographs for the same samples used to count bacterial-epithelial contacts (Vents A, C and D). Up to thirty bacteria were measured for each specimen (n = 7) collected at in- and far-vent locations. The mean bacterial diameter was calculated for each specimen and presented data are the mean of specimen means (±1 SE). Significance levels between in- and far-vent specimens were determined with a Monte Carlo Randomization routine.

Transplant manipulation

In (August) 2002, animals from a focused fluid source (maximum temperature over a 5 minute duration was 10°C) on a sulphide mound at Vent C (Axial Volcano: 45°55.9'N, 130°00.8'W, 1500m depth) were collected by suction and transported to the surface. Approximately 600 animals were deposited in a powder-coated stainless steel framed cage (30 x 25 x 25 cm, 2 mm polypropylene mesh) and placed 50 cm away from Vent C fluid flow to a location where no shimmering was visible (3°C fluid temperature).

Additional in-vent and far-vent limpets at the site of the transplant cage were collected at this time. The transplant cage was recovered after 12 months. 57 *L. fucensis* were alive, while the 549 empty shells were present (the calcium carbonate material had dissolved leaving a protein matrix). 10 animals were fixed for microscopy in 2.5% glutaradehyde, 30 were fixed in 7% buffered formalin and the remainder were frozen. Glutaraldehyde-fixed gills were prepared for microscopy. Bacterial abundance index, bacterial-epithelial contacts, gill parameters and symbiont diameter were measured as previously described. Shell volume to dry weight was measured from formalin-fixed animals (see methods for host shell volume to dry weight).

In 2003, a control comparison was attempted. I placed 500 *Lepetodrilus fucensis* collected from 14°C fluids in the transplant cage and re-positioned the cage at the vent fluid source. The mesh clogged and the animals died over the experiment duration (13 months).

Carbon dioxide fixation experiment

An experimental apparatus was designed to test the relationship between carbon fixation and bacterial abundance on *Lepetodrilus fucensis* gill tissue at an *in situ* location. Two 25 ml chambers (1 and 2) were made by drilling a 4 cm long by 1.3 cm wide hole through a 4 x 4 x 16 cm block of PVC that sealed against rubber gaskets (chambers were not airtight). Two spring-loaded syringes for fluid injection by ROV were attached to each chamber.

Lepetodrilus fucensis specimens were suctioned from a high flow (in-vent: 8-14°C) on a sulphide mound (Salut Vent, Endeavour Segment) and at the base of the mound (far-vent: 3°C). On the surface, animals of shell lengths 5-7 mm from in- and far-

vent collections were randomly selected. A sub-sample of in-vent animals was treated with antibiotic at 4°C for 12 hours; 400 ml of Penicillin-Streptomycin (HyClone©) (100X stock solution: 10,000 units of penicillin G and 10,000 μg of streptomycin sulphate) was diluted in 4 L seawater. In-vent (n = 7), far-vent (n = 7) and antibiotic exposed animals (n = 7) were added to Chambers 1 and 2. The different treatments were isolated in plastic mesh (1 mm) envelopes.

The experimental apparatus was returned to Salut Vent with animals (total surface time was 13 hours at 4°C) and positioned near a diffuse fluid source (4-5°C). After 24 hrs, ^{14}C-labeled sodium bicarbonate was injected into each chamber to achieve an activity of 30 μCi. At the same time, Chamber 1 was enriched with 1 ml of 1 mM sodium sulphide solution (pH = 8.5) by syringe injection (chamber concentration was <0.5 mM sulphide based on surface trials; sulphide was measured as in Cline (1969)). Sulphide levels in Chamber 2 remained at ambient levels. After 45 min, glutaraldehyde (~2.5% final concentration) was injected into both chambers to fix animals on the seafloor. The experimental apparatus was recovered. Animals were rinsed in filtered seawater (4°C), transferred to a fresh 2.5% glutaraldehyde solution (pH = 6.0) and transported to the lab for analyses.

The gill tissue was dissected from the animal and treated with 10% acetic acid in open containers for 12 hrs. It was not possible to analyze the tissues using autoradiography because the total acid-stable ^{14}C tissue levels were low. Instead, bacterial abundances were related to the total carbon fixed by the gill and foot tissue as follows. Three gill lamellae were removed for light microscopy (tissue preparation described above). The remaining gill lamellae and foot tissue were blotted on lens paper

and the wet weights determined using an analytical balance (±0.0001 g). The gill and foot tissues were then solubilized in 1 ml SOLVABLE™ (PerkinElmer) in glass scintillation vials at 40°C for 12 hours and subsequently cooled to room temperature. 19 ml of scintillation cocktail (ScintiVerse II; Fisher Scientific Chemicals) was added to each sample. Samples were kept in the dark overnight to minimize chemiluminescence and counted in a liquid scintillation counter. Background counts were measured from tissue treated as above, but removed from specimens fixed in glutaraldehyde that had not been exposed to ^{14}C-labeled sodium bicarbonate.

Total carbon fixed was estimated per gram of wet tissue by first converting CPM to µmol carbon (lambda = 2.30×10^{-10} min^{-1}). A conservative dilution factor (6) was used to estimate total carbon fixed based on a seawater CO_2 concentration of ~2000 µmol (Elderfield and Schultz 1996).

The bacterial abundance index was determined for each specimen (as above) and plotted against µmol carbon fixed per gram of wet gill and foot tissue. I calculated the Pearson product moment correlation (r) between bacterial abundance and fixed carbon by pooling data from in- and far-vent treatments (n = 14). A t-test for independent samples was used to determine significance levels between Chamber 1 and 2 (in- and far-vent data were pooled for each chamber). The antibiotic treatment is displayed for qualitative comparisons and was not included in the statistical analyses.

RESULTS

The gill symbiosis was observed on specimens from all vent sites (Axial, Co-Axial, Endeavour and Explorer) and from vents diffusing through basalt and sulphide

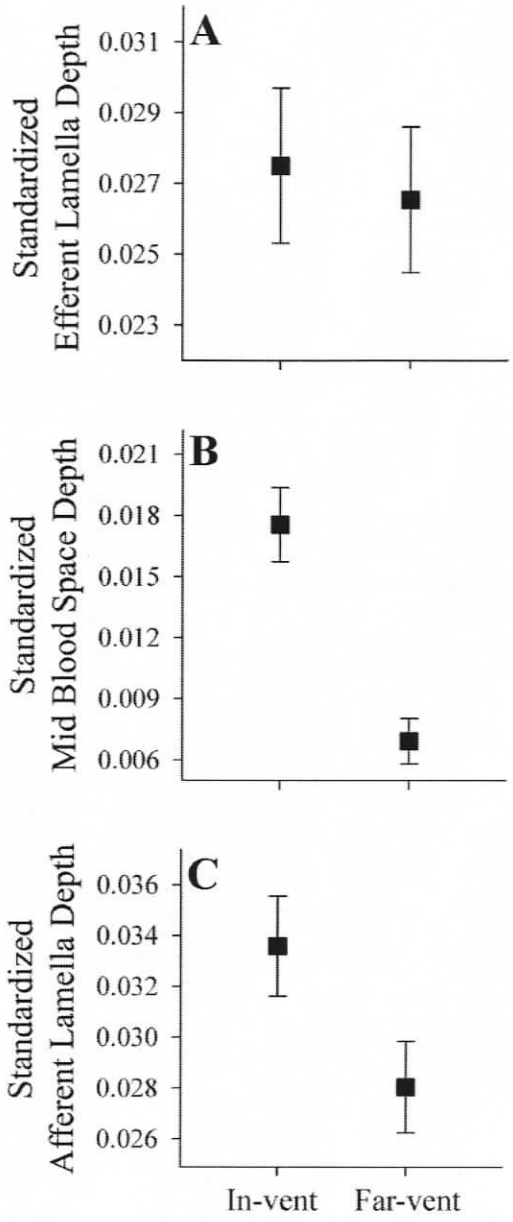

Figure 5.3
The morphology of gills from in- and far-vent specimens (n = 10) were compared at ten vents. The depths of three regions on gill lamellae were measured from transverse sections (Image-Pro Plus© 4.5): (A) afferent depth (mm), (B) mid blood space depth (mm) and (C) efferent depth (mm). All measurements were standardized by shell length (mm). Data are the mean (±1 SE) of means from several collections. The efferent lamella depth is similar for in- and far-vent gills. In-vent gills have significantly longer mid blood space and afferent lamella depth.

(Table 5.1). Animals of both sexes from 0.5 to 15 mm in size hosted gill bacteria. Bacteria were obvious on the bacterial epithelium of the gill lamellae in light micrographs. Certain regions of the gill showed variable morphology that related to habitat. While the mean efferent lamellae depth (standardized by shell length) was similar for animals collected from in- and far-vent (Figure 5.3A), the mid blood space depth and the afferent lamellae depth (standardized by shell length) were significantly reduced in far-vent locations (Monte Carlo Randomization, $p < 0.001$) (Figure 5.3B and C).

Comparisons of the bacterial abundance indices on lamellae from all regions of the gill (anterior, middle and posterior) at tip, mid and base positions on individual lamella returned ranges within the same order of magnitude (Table 5.2). There was an increasing trend in bacterial abundance from the tip to the base on in- and far-vent gills. Although this difference in bacterial abundance was not significant for anterior and middle lamellae, there were significantly fewer bacteria on posterior lamellae tips versus bases (in- and far-vent gills were pooled, paired t-test, $t < -3$, $p < 0.001$).

Bacterial abundance was significantly greater (Monte Carlo Randomization, $p < 0.001$) on gills collected from in- and far-vent locations at nine of ten vents (Figure 5.4A). Bacterial abundance was similar between animals collected from the substratum near the location of the transplant cage (far-vent) and animals left in the cage for one year (Vent C).

The number of bacteria in contact with the epithelium was also significantly greater in-vent (Figure 5.2 & 4B). In comparison, animals transplanted from in- to far-vent (Vent C) had similar numbers of bacterial-epithelial contacts to animals collected in

Table 5.2
Mean (±1 SD) bacterial abundance index on low and high flow gills (n = 4) at the base, mid and tip of lamellae from anterior, middle and posterior regions of the gill (Figure 5.1). Bacterial abundance increases from the lamella tip to the base for the three gill regions.

		Bacterial Abundance Index		
		Anterior	**Middle**	**Posterior**
a. Low Flow	**Tip**	1.8±1.5	2.4±1.9	1.9±0.9
	Mid	1.9±1.8	2.5±2.2	2.5±0.8
	Base	2.4±2.3	2.6±2.2	2.9±1.4
b. High Flow	**Tip**	4.8±3.6	5.4±2.9	4.6±2.3
	Mid	4.5±1.9	6.1±1.9	5.8±2.2
	Base	6.0±2.0	6.9±3.8	6.0±1.2

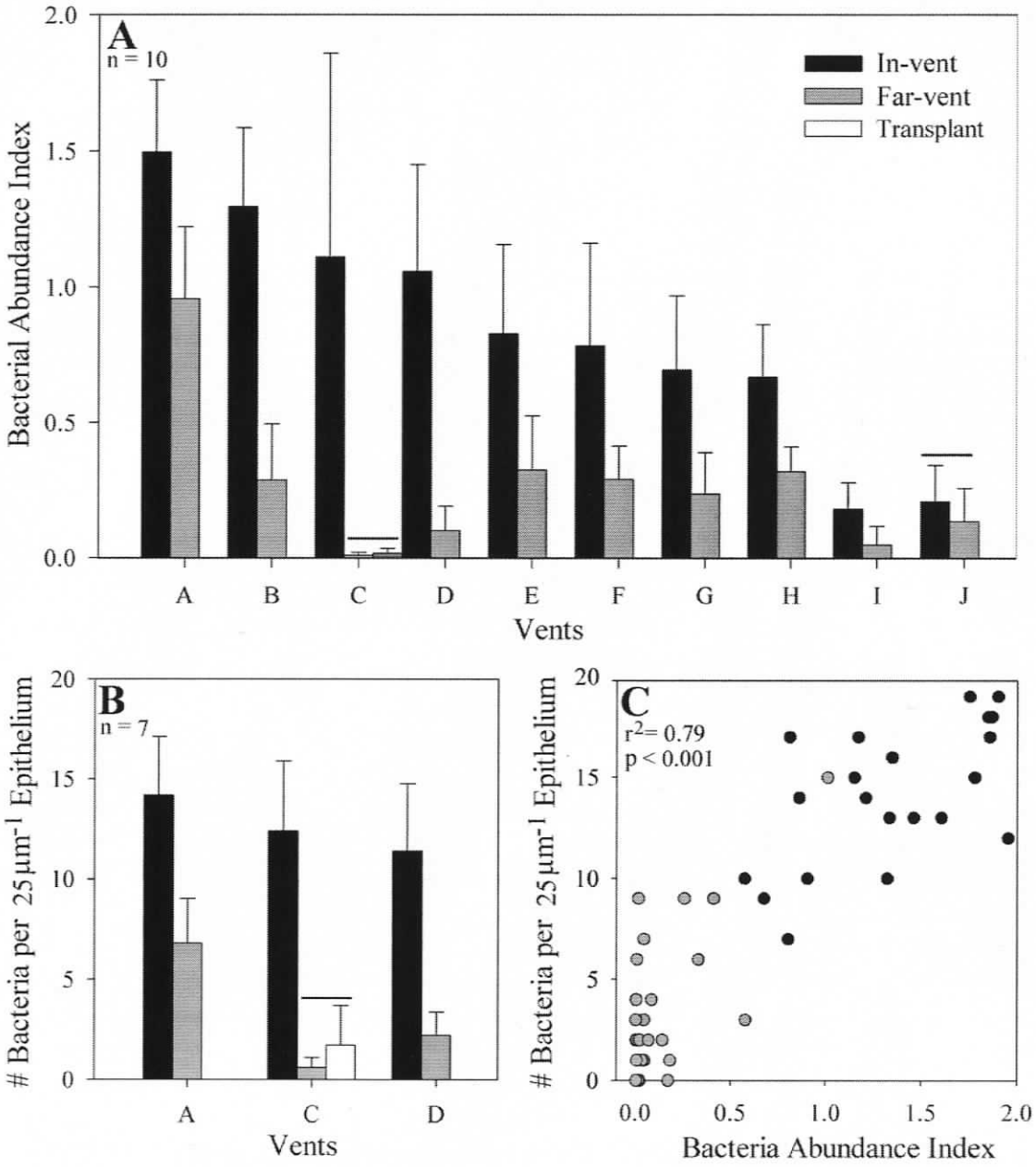

Figure 5.4
(A) Mean (±1 SD) bacterial abundance index compared between in- and far-vent locations at 10 vents. Gills (n = 10) were examined in transverse section using light microscopy. The transplant treatment (Vent C) represents animals moved from in- to far-vent for one year. A Monte Carlo Randomization test determined a significant difference between in- and far-vent sites at all vents except J ($p > 0.05$ is indicated by a bar). (B) The number of contacts between bacteria and 25 µm of gill epithelium was determined using transmission electron microscopy for specimens (n = 7) collected in- and far-vent at three vents. (C) The number of bacteria on the epithelium was positively related to bacterial abundance.

a nearby far-vent location (Figure 5.4B). There was a significant linear relationship (r^2 = 0.79, $p < 0.001$) between the total number of bacteria in contact with the epithelium (per 25 μm) and the bacterial abundance index (Figure 5.4C). The total number of bacteria present in 1 g (wet weight) of gill tissue was estimated from the number of bacteria in contact with the gill epithelium. Estimates ranged from 0 bacteria (note that symbionts were always found when additional regions of the bacterial epithelium were examined) to 10^9 bacteria per gram wet weight gill tissue. Pit-like structures (see Figure 5.2B inset) were observed on the epithelium of many low flow gills.

The variability in the morphology of the afferent lamella and mid blood space is related to bacterial abundance. The bacterial epithelium depth (standardized by efferent lamella depth) was significantly correlated with bacterial abundance on gills collected from in- and far-vent locations (Figure 5.5A & B). The same trend was observed when the bacterial epithelium depth was divided by the afferent lamella depth (Figure 5.5C & D); this measure provides information on the shape of the epithelium that hosts the bacteria. The bacterial epithelium reached up to three times the length of the afferent lamellae on several gills where the bacterial abundance index was high. This increase in the bacterial epithelium depth is due to the formation and morphology of the spherules (Figure 5.6A). In some gills, spherules were pronounced (Figure 5.6bI) while on others, they were reduced (Figure 5.6BII). The mid blood space depth also increased with bacterial abundance (Figure 5.5E & F).

The condition of animals from different flow regimes varied (Figure 5.7A & B). The dry weight to shell volume of animals in-vent was significantly greater than far-vent (three vents) (t-test for independent samples; $p < 0.001$). Animals transplanted away

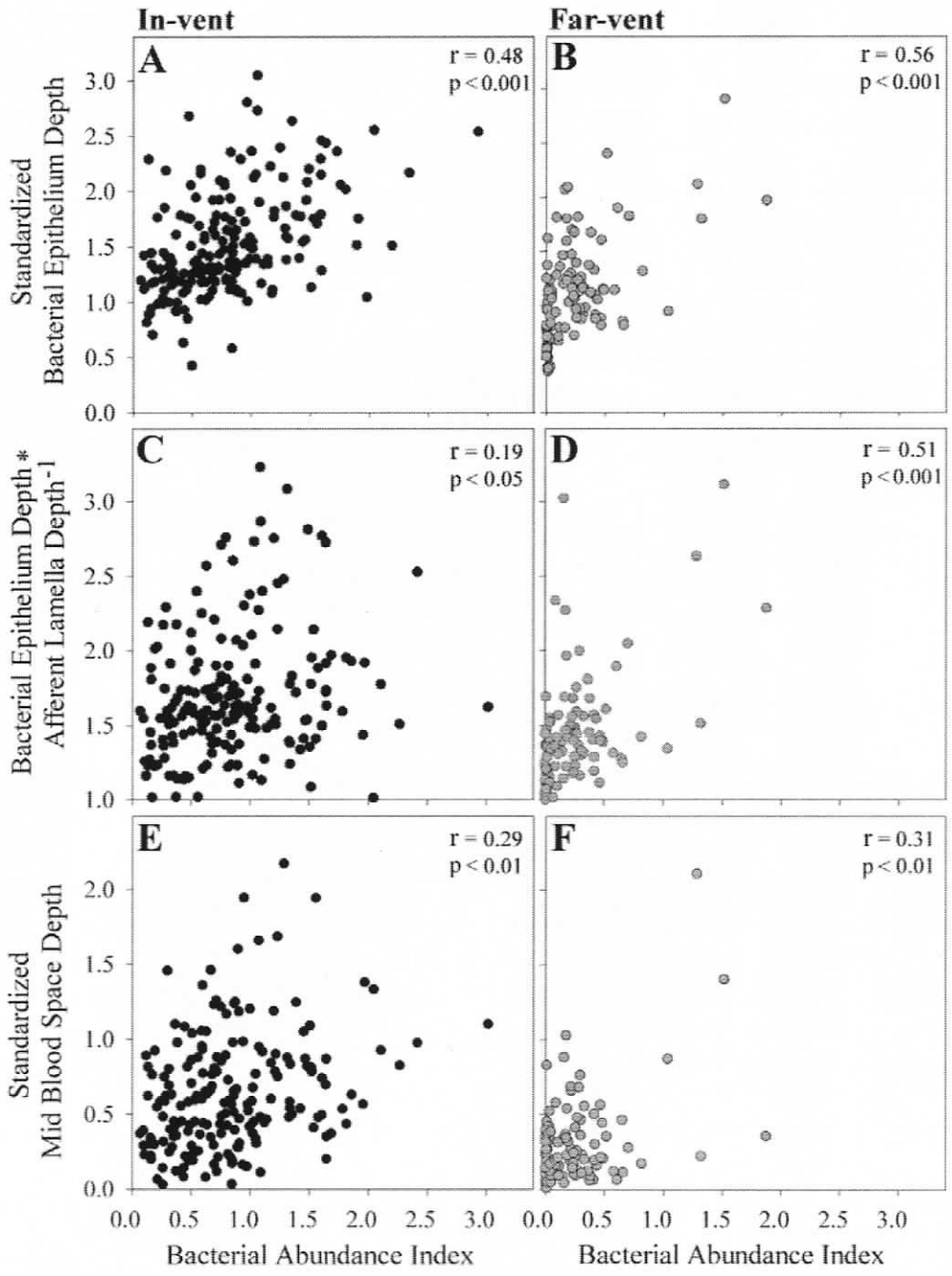

Figure 5.5
The morphology of *Lepetodrilus fucensis* gill lamellae was related to bacterial abundance for samples from a variety of vents and substrata. (A & B) Bacterial epithelium depth, (C & D) bacterial epithelium depth * afferent lamella depth^{-1} and (E & F) mid blood space depth versus bacterial abundance index for animals collected from 17 in-vent locations (n = 190) and 11 far-vent locations (n = 110) at Axial, Endeavour and Explorer. Bacterial epithelium depth and mid blood space depth are standardized by efferent lamella depth. There is a significant increase in the three different gill features with increasing bacterial abundance (Pearson correlation coefficient = r).

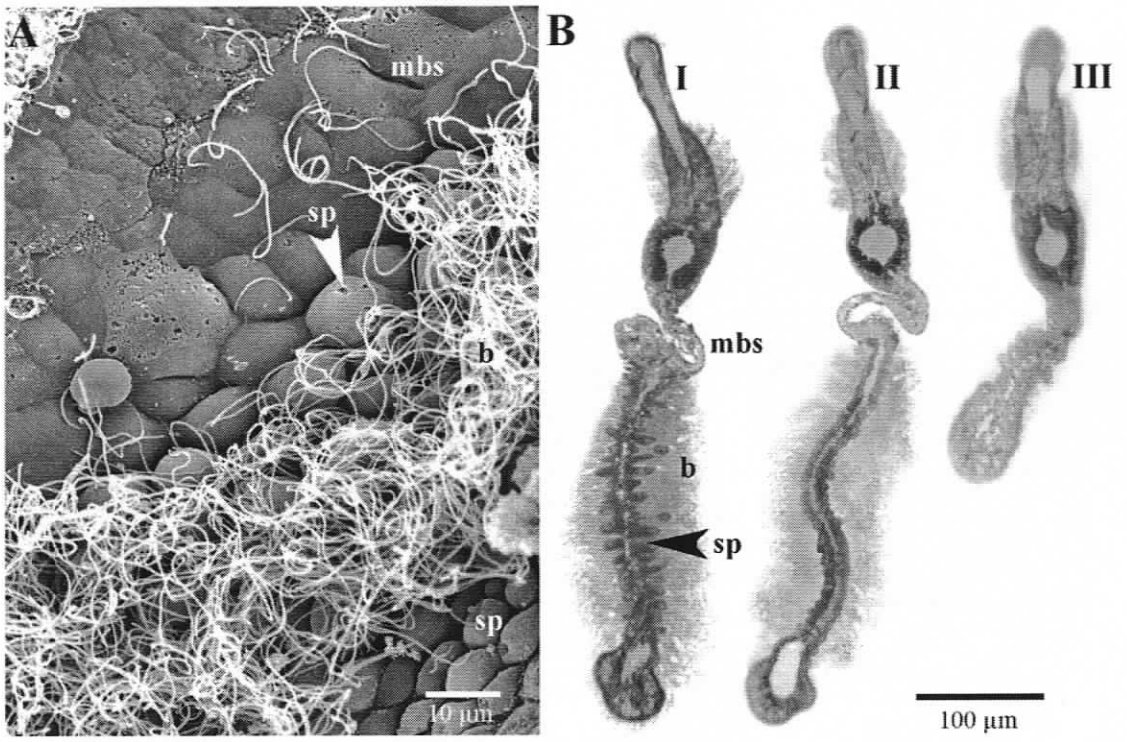

Figure 5.6
(A) Scanning electron micrograph showing the afferent lamellae of *Lepetodrilus fucensis* with low bacterial (ba) abundance. Spherules (sp) (sensu de Burgh and Singla 1988) are visible where bacteria are absent from the epithelium. (B) Light micrograph showing three gills with different morphotypes in transverse section. (I) Spherules are present on in-vent gills and increase the surface area for bacterial colonization. The convoluted mid blood space increases the surface area of this region of the gill. (II) On some in-vent gills, spherules were reduced. (III) Mid blood space and efferent lamella depth were highly reduced on the majority of far-vent gills and spherules were often absent.

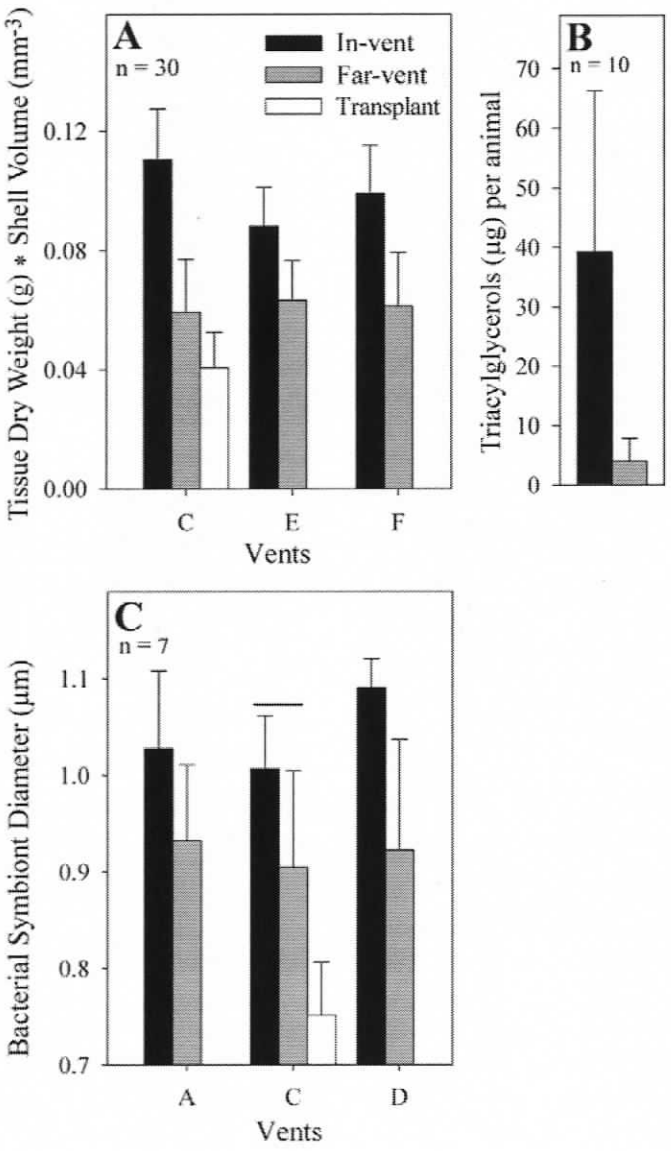

Figure 5.7
Estimates of *Lepetodrilus fucensis* and symbiont condition in high (in-vent) and low vent flow (far-vent). A transplant manipulation was completed at Vent C; a group of animals was moved away from flow. All paired observations without a bar are significantly different from each another. (A) Host condition index (tissue dry weight (g) * shell volume (mm^{-3})) at Vents C, E and F for in-, far-vent and transplanted animals. The condition indices of animals in-vent were higher than those from far-vent locations. Animals transplanted from in- to far-vent had significantly lower condition indices than the original in-vent population (t-test for independent samples, $n = 30$, $p < 0.001$). (B) The quantity of triacyglycerols (µg) per animal (Vent C) was significantly higher for in-vent animals (Monte Carlo Randomization, $n = 10$, $p < 0.001$). (C) The diameter of bacteria (µm) in contact with the host gill epithelium was larger on specimens collected in-vent. Bacteria on transplant gills had the smallest diameters (Monte Carlo Randomization, $n = 7$, $p < 0.001$).

from a vent source (Vent C) also showed a significant decrease in condition (Figure 5.7A). Storage lipid content (triacylglyeroles) was significantly higher for animals collected in-vent at Vent C; in comparison, far-vent animals had trace quantities of storage lipids (Figure 5.7B). TEM micrographs show that co-collected specimens from the same far-vent location (Vent C) harboured very few gill bacteria (see Figure 5.4A). Although tissues were fixed using identical protocols, mitochondria and golgi apparatus were consistently more difficult to distinguish in TEM micrographs of far-vent tissues and staining density was low.

Bacteria from far-vent locations at three vents were smaller than bacteria on in-vent gills, and at Vents A and D, this difference was significant (Figure 5.7C) (Monte Carlo Randomization; $p < 0.01$). Figure 5.2 shows a decreasing trend in bacterial diameter.

Lysosomal degradation of bacteria was only occasionally apparent in TEM micrographs. The number of lysosomal bodies along a section of epithelium (25 μm: 2-4 cells) from in- and far-vent specimens ranged between 1 and 13 (Figure 5.8). There was weak linear relationship between the number of lysosomes and the abundance of bacteria on the gill epithelium at Vent A (Figure 5.8A) ($r^2= 0.12$, $p < 0.05$), however, a relationship between these variables was not obvious at two additional vents (C and D) (Figure 5.8B & C).

The majority of animals collected for the carbon fixation experiment had low (~0) to medium (0.8: corresponds with $<10^9$ bacteria per gram wet weight) bacterial abundance indices (Figure 5.8). In the sulphide enriched chamber, carbon fixation was significantly higher than in the chamber where sodium sulphide was not added (Figure

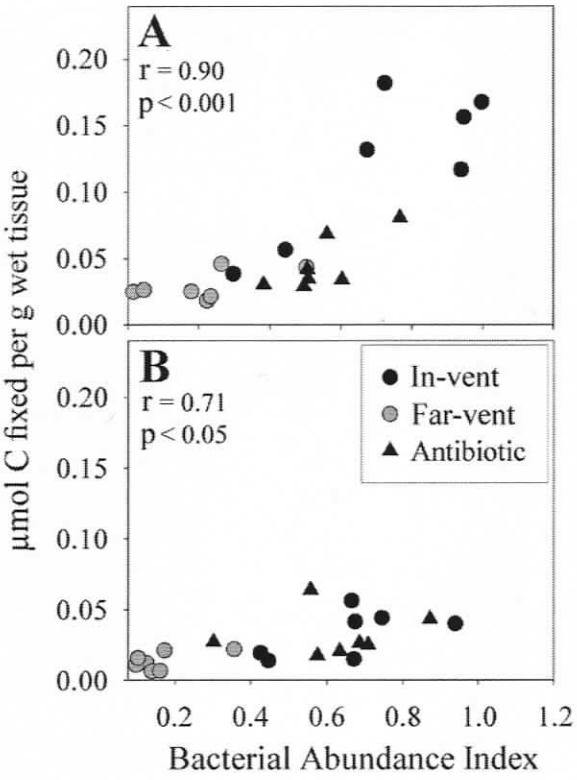

Figure 5.8
Total fixed carbon in gill tissues from in-, far-vent and antibiotic treated animals (n = 7) after 45 min. Values are µmol carbon fixed per gram of wet gill tissue (calculated by estimating the following parameters in the incubation media: inorganic carbon pool, activity of $NaH^{14}C0_3$ and acid-stable ^{14}C in the gill tissue). (A) Sodium sulphide was added to the chamber: sulphide enriched. (B) Sodium sulphide was not added. There is a significant positive correlation (Pearson's product moment correlation, r) between µmol carbon fixed (in- and far-vent data were pooled) and bacterial abundance index.

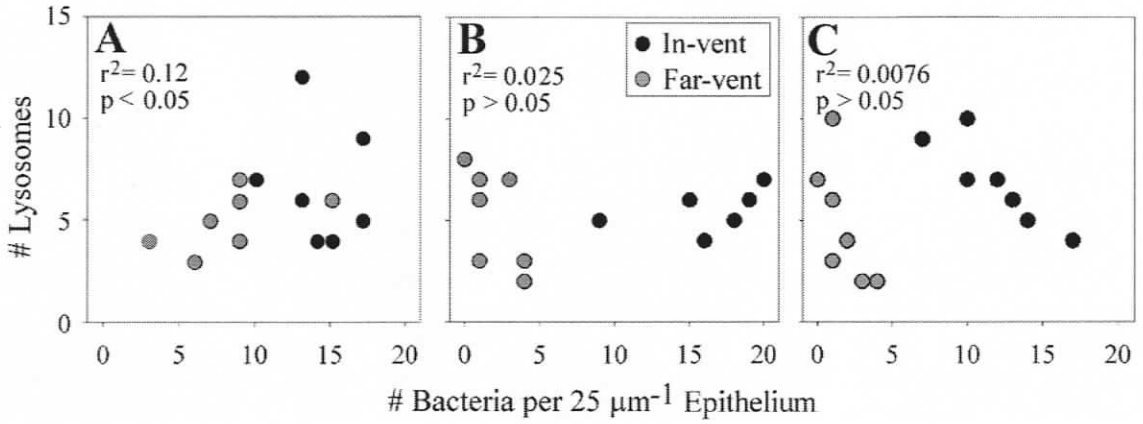

Figure 5.9
The number lysosomes present in epithelial cells versus the number of bacteria in contact with these same cells (a 25 μm length was examined) at three vents: (A) Vent A, (B) Vent C and (C) Vent D. Transmission electron micrographs for specimens (n = 7) collected in- and far-vent were examined (the samples are the same as in Figure 5.7). At Vent A, there was a significant positive linear relationship in the number of lysosomes and bacteria, while at Vents C and D, no relationship was observed.

5.9A & B). The total fixed carbon (μmol fixed C per gram wet tissue) by gills from specimens collected in-vent ranged from between 0.04 and 0.19 in the sulphide enriched chamber. In comparison, values from far-vent gills were from 0.01 to 0.05 and the antibiotic treated animals had intermediate values from 0.02 to 0.09. In the chamber where sodium sulphide was absent, total carbon fixed in the gill tissues of in-, far-vent and antibiotic treated animals were less than 0.05 μmol. In both chambers, there was a significant correlation between total fixed carbon and bacterial abundance (high and low flow animals were pooled) (Figure 5.9) ($p < 0.05$, $r > 0.7$, $df = 12$). The total carbon (μmol) fixed per gram of foot tissue (measured for comparison to gill tissue) ranged from 0.01 to 0.10 in the sulphide enriched treatment and from 0.01 to 0.03 when sodium sulphide was absent. Carbon fixation by foot tissues were not significantly correlated to bacterial abundance ($p > 0.05$, $r < 0.5$, $df = 12$), however, three in-vent specimens in the sulphide enriched treatment showed the highest fixation values (~0.10 μmol C fixed per gram of wet tissue).

DISCUSSION

The extent and morphology of the *Lepetodrilus fucensis* gill episymbiosis depends on whether the host occupies habitats in venting fluids. The better condition of *L. fucensis* and higher symbiont abundance in focused vent flows may drive its preference for in-vent habitats (Bates et al. 2005). The high incidence of the limpet-bacterial association across a broad geographic range suggest a persistent symbiosis that may be obligatory on the part of the limpet. Colonization of the gill by bacteria occurred in different habitats on animals as small as 0.5 mm in shell length. As protoconchs are ~0.1 mm, the association with bacteria probably forms soon after settlement. The bacteria

may be acquired from the surrounding environment because they are epibiotic; however, the mode of transmission is currently unknown for this symbiosis.

Gill morphology varied among specimens. While the shape and size of the efferent lamellae was independent of habitat, depth of both the mid blood space and the afferent lamellae increased in-vent where gill bacteria were abundant. Lower oxygen availability in higher temperature fluids with sulphides (Johnson et al. 1986) may initiate growth of the respiratory surface to facilitate the diffusion of oxygen across the gill epithelium (e.g. Hourdez & Jouin-Toulmond 1998). In addition, bacterial chemoautotrophy requires oxygen; bacterial consumption of oxygen possibly competes with host requirements and *Lepetodrilus fucensis* may modify its gill surface area in response to higher bacterial densities. The variability in the morphology of the afferent lamellae also relates to structures identified as spherules (sensu de Burgh & Singla 1989). All animals examined with shell lengths ~0.5 mm (n = 30 from in- and far-vent) had prominent spherules, suggesting that this morphotype might be important when the host is growing. Gills with spherules hosted abundant bacteria; these structures presumably increase the surface available for bacterial colonization on the afferent lamellae. However, spherules were not always present when bacteria were abundant. The mechanism driving the formation of spherules requires investigation.

The number of bacteria in contact with the epithelial membrane in TEM micrographs showed a strong linear relationship to bacterial abundance measured from light micrographs of the same tissues. Thus, light micrographs provide a good estimate of symbiont abundance. Both types of microscope techniques indicated that the abundance of bacteria on the gill increased with greater venting and was highest in

focused flows between 5 and 15°C. This finding supports the hypothesis that the bacteria are chemoautotrophic (Fox et al. 2002) and depend on a chemical substrate present in hydrothermal fluids. If so, the significant increase in bacterial abundance from the base to the tip of posterior lamellae might be due to a chemical gradient across the gill.

The bacteria probably oxidize sulphide as carbon fixation by *Lepetodrilus fucensis* gill tissue was positively related to bacterial abundance. Gills with abundant bacteria fixed twice as much carbon as foot tissues, supporting that the gill bacteria were incorporating $^{14}CO_2$ via autotrophy. Furthermore, animals treated with antibiotic exhibited relatively lower carbon fixation to antibiotic-free animals with similar bacterial abundances. Carbon fixation values, however, were an order of magnitude less than other symbiotic molluscs (e.g., Fisher and Childress 1986). Possible experimental problems include (1) physiological stress due to pressure changes; (2) non-conducive chemical conditions in the chambers; and (3) relatively lower bacterial densities compared to other animal hosts (e.g., *Riftia pachyptila*: $>10^9$ bacteria per gram of trophosome (Felbeck 1981)). In *Riftia pachyptila*, sulphide speciation (HS^- is used as the primary energy source) (Goffredi et al. 1997) and temperature (25 to 35°C) (Scott et al. 1994) create optimum conditions for symbiont carbon fixation. Sulphide species were not measured in the present study and temperature was around 3°C.

Anapleurotic host respiration may also be responsible for observed carbon fixation by the gill and foot tissues. Cavanaugh (1983) reports carbon fixation rates in the gill tissue of a non-symbiotic intertidal clam maintained at 22°C that are similar to the rates obtained for *Lepetodrilus fucensis* gill tissues at 3°C. Fixed carbon in the foot tissues may also be translocated from the gill. However, the total acid-stable ^{14}C tissue

levels obtained in the present study were too low to test this idea using autoradiography. A controlled experiment at higher temperatures conducted under pressure is needed to identify rates of thioautotrophy by the *L. fucensis* gill symbionts and the possibility of fixed carbon translocation from bacteria to host tissues.

Bacterial abundance decreased on *Lepetodrilus fucensis* gills moved from high to low vent fluid flux. After one year in low sulphide concentrations bacterial symbionts were rare on *L. fucensis* gills. Similarly, the mussel species, *Bathymodiolus thermophilus*, showed a rapid loss (on the scale of days) of gill endosymbionts when deprived of inorganic sulphide (Cavanaugh 1983, Raulfs et al. 2004, Kadar et al. 2005). However, Raulfs et al. (2004) suggest that the rate at which symbiont populations are lost is species specific. The rate of symbiont decline under low sulphide concentrations may also be related to morphology; the *L. fucensis* gill bacteria are episymbiotic and are bathed in ambient fluids while endosymbionts depend on chemical substrate delivery across membranes or through tissues.

Loss of bacteria on the *Lepetodrilus fucensis* gill was associated with pit-like structures on the bacteria-hosting epithelium. Similar pit structures occur on symbiotic bivalve gills and may provide free-living bacteria with access into bacteriocytes (Gros et al. 1998, Kadar et al. 2005). Likewise, these pit structures may be necessary for re-infection of the gill by the *L. fucensis* episymbiont but the shape, size and abundance of pits on low flow gills suggests extensive loss of gill bacteria.

A decrease in the diameter of persistent gill bacteria along the afferent gill epithelium occurred when *Lepetodrilus fucensis* was restricted from vent flow. Low concentrations of reduced inorganic sulphide and/or other required nutrients might limit

the size of the gill symbionts. For example, bacteria found in the trophosome of *Ridgeia piscesae* display a distinct size distribution that corresponds to a chemical gradient (de Burgh 1986). In low flux habitats, *L. fucensis* exhibited poor condition and may not be releasing metabolic compounds required by bacteria, such as ammonia. Alternatively, the environmental conditions in low flow habitats (e.g., sulphide, temperature and pH) may not promote bacterial proliferation. It is also possible that the smaller bacterial morphotype represents a different phylotype as invertebrate-bacterial chemoautotrophic symbioses are more flexible and less specific than previously suggested (e.g., Dubilier et al. 2001).

The transplant experiment showed that host morphology, the condition of both partners and the abundance of gill bacteria respond to a reduction in vent flux, indicating that the symbiosis is dynamic. The extent of the association appears to depend on the host accessing hydrothermal fluids; a decrease in the afferent lamellae depth corresponded with loss of the gill bacteria in far-vent locations. *Lepetodrilus fucensis* may actively reduce the bacteria-hosting region of the gill when vent flux is minimal. For instance, maintenance of the bacterial epithelium presumably incurs a cost that might not be balanced by the benefit of hosting symbionts under certain conditions. Studies of coral-algal symbioses show that hosts can limit symbioses when algal productivity is low (Bruno and Edmunds 1997), presumably because the net benefit from symbiosis varies with environmental conditions (e.g., Muller-Parker and Davy 2001, West et al. 2002).

Although the function of the symbiosis is unknown, this study indicates that the symbiosis is flux-dependent; vent fluids appear critical to the health of the host and generate dense symbiont populations. Several factors suggest that the bacteria serve a

nutritional function. Animals with the best tissue condition occurred in vent flows where gill bacteria were abundant. Conversely, animals with minimal bacteria had poor condition and trace quantities of storage lipids. Although grazing may be an important feeding mechanism employed by peripheral limpets, co-occurring grazing gastropods of other species have healthy tissues. Furthermore, *L. fucensis* transplanted to far-vent suffered high mortality and those animals still alive had poor tissue condition, probably because the number of animals in the transplant cage could not be sustained away from a vent source by grazing alone. In a similar study, Raulfs et al. (2004) used ultrastructural evidence to show that the tissue condition of symbiotic mussels transplanted to the periphery of a vent declines. Although I did not measure ultrastructural features of limpet tissues, I observed that the chromatin in epithelial nucleii was typically clumped and the cytoplasm contents stained less densely in far-vent tissues than animals collected in-vent (see Figure 5.2).

Determining the nature of the *Lepetodrilus fucensis* gill symbiosis is an important next step. Although endocytosis of bacteria by the gill epithelium followed by lysosomal digestion is a possible nutritional link (de Burgh and Singla 1984), lysosomal bodies with bacteria were rare in TEM micrographs. In addition, lysosome number was not related to bacterial abundance, suggesting that endocytosis of the bacteria may not be an important nutritional mechanism employed by the host. Indeed, the majority of chemosynthetic symbioses show evidence of bacterial degradation by lysosomal bodies; however, the relative contribution of this digestive process to the nutrition of the host may vary among species (Boetius and Felbeck 1995).

Summary

The persistence of the symbiosis between *Lepetodrilus fucensis* and gamma-Proteobacteria relates to the abundance patterns and condition of the host in different levels of hydrothermal flux. The prominent limpet stacks observed in active vent flows (Bates et al. 2005) may provide access to reduced sulphur for bacterial thioautotrophy as these animals harbored abundant symbiont populations. The gamma-Protoeobacteria appear to benefit their host because animals with the most symbiotic bacteria also had the highest tissue condition, while animals with sparse symbiont populations exhibited very low tissue condition. In addition, bacterial colonization increased with the surface area of the bacteria-hosting and gas exchange region of the gill. This morphological plasticity may enable the limpet to survive low oxygen conditions in vent fluids by decreasing diffusion distances across the gill epithelium and may encourage bacterial colonization of the gill when environmental conditions favour the symbiosis. These findings are consistent with the hypothesis that the *L. fucensis*-gill bacterial symbiosis contributes to the limpet's nutrition, although the data presented here do not indicate a mechanism.

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CHAPTER 6

Feeding strategy, morphological specialization and the presence of bacterial episymbionts in lepetodrilid gastropods from hydrothermal vents

Abstract

Hydrothermal vent gastropods use diverse feeding mechanisms in a variety of niches such as grazing, suspension feeding and by harbouring bacterial symbionts in their gill tissues. The aim of this study was to determine the feeding mechanisms employed by a prominent Juan de Fuca Ridge gastropod, *Lepetodrilus fucensis* McLean 1988, that hosts dense colonies of episymbiotic gamma-Proteobacteria on its gill. Morphological comparisons among *Lepetodrilus* species with (n = 2) and without these symbionts (n = 5) revealed that the gills of the two symbiont-hosting species exhibit specialized features consistent with suspension feeding gastropods: the lamellae are enlarged, are free of the mantle, do not narrow towards the tip and are stabilized laterally by ciliary junctions. In other gastropods, these modifications increase the gill's surface area and fluid current velocity. The radula length, radula tooth size and stomach volume of the two symbiont-hosting species are also significantly reduced in comparison to congeners, suggesting that these species may employ a different feeding strategy. Suspension feeding by *L. fucensis* was confirmed using shipboard observations in pressure vessels. Stained particles accumulated at the lamellar tips and were sorted into accepted and rejected material. Furthermore, the gamma-Proteobacterial symbionts may be farmed for food as FISH probes documented the presence of the symbiont with detached food material on the gill. The gill lamellar traits, and reduction in radula and stomach volume, are consistent with a specialization towards suspension feeding and/or symbiont farming, a novel feeding strategy in Class Gastropoda. However, grazing may also occur, demonstrated by

specimens that removed food particles from the sides of pressure vessels with their radula. The remarkable abundances reached by *L. fucensis* in a variety of vent habitats may be due to its use of multiple feeding mechanisms.

INTRODUCTION

Gastropods exhibit great variety in feeding strategy and morphology that enables food acquisition in a wide array of niches. The radula functions in tasks as diverse as harvesting biofilms, tearing algae, drilling shells and manipulating particle-filled mucous nets (see review by Kohn 1983). Several gastropod families also suspension feed by using their gills to concentrate and sort particles suspended in seawater, and these families exhibit convergent gill specializations, such as more numerous and longer lamellae (reviewed in Declerck 1995). Some species also use a combination of grazing and suspension feeding to exploit different food sources, thus maximizing their food intake and overall nutrition (as suggested by Chaparro et al. 2002). For instance, *Crepidula fecunda* is an intertidal limpet that can rasp biofilms from surfaces with its radula and ingest food particles entrained and processed by the gill (Chaparro et al. 2002).

Hydrothermal vents are a unique ecosystem because chemosynthetic production by bacteria is the major food resource available to primary consumers. Bacterial densities on surfaces and suspended in vent effluent are relatively high (Giere et al. 2003) and the majority of vent gastropods exploit these food sources. In addition, endosymbiotic chemosynthetic bacteria are harboured in the tissues of some species and contribute to the nutrition of their host through carbon translocation and/or digestion by

lysosomal bodies (reviewed in Cavanaugh et al. 2004). In other species, e.g., rimicarid shrimp (Casanova et al. 1993), episymbionts are cultivated and ingested by their host, a feeding mechanism unknown in gastropods. The symbiotic bacteria require simultaneous access to oxygen (in deep-sea water) and reduced chemicals (e.g., hydrogen sulphide: in hydrothermal fluids) and this dependence on specific chemical substrates tends to restrict the host's distribution to vent fluid conditions that promote symbiont growth (as suggested by Hessler et al. 1985).

Comparative morphological studies can highlight differences among species in feeding strategy (Wainwright 1991, Honkoop et al. 2003). For instance, bacteria-hosting tissues and structures are typically modified to increase nutritional gain from the symbiosis. For example, the vent shrimp, *Rimicaris exoculata*, cultivates dense colonies of epibacteria for food on the inner sheet of the carapace in an enlarged gill chamber, a specialization thought to increase the area available for symbiont growth (Casanova et al. 1993). Likewise, related species with identical feeding capabilities but differential dependence on specific feeding modes often exhibit distinguishing morphological traits. For instance, one endosymbiont-hosting gastropod (Family Provannidae) from the western Pacific possesses a larger gill (increases area for symbiotic bacteria) and reduced digestive organs (decreases food ingestion capability) in comparison to a close relative that is less dependent on its symbionts for nutrition (Windoffer & Giere 1997).

The *Lepetodrilus* genus (Order Vetigastropoda) presents an excellent model to study the relationship between feeding strategy and morphological specialization because different feeding modes may occur in different species. The distribution of the genus is global and species are common in the vent assemblages where they occur (Fretter 1988,

McLean 1993). Several *Lepetodrilus* species show morphological evidence of suspension feeding (e.g., modified frontal pads on lamellar tips) and grazing (e.g., radular wear) (Fretter 1988). However, two species from the northeast Pacific, *L. fucensis* McLean 1988 from vents on the Juan de Fuca and Explorer Ridges and *L. gordensis* n. sp. from the Gorda Ridge, host a symbiosis with a filamentous bacteria found partially embedded in the gill epithelium (Chapter 4). This association may be nutritional in nature although a mechanism is not obvious (Chapter 5).

The behaviour of *Lepetodrilus fucensis* suggests that it may rely more heavily on suspension feeding than other species in its genus, in addition to gaining nutrition from its symbiont, because it forms prominent stacks in warm vent flows where it reaches densities an order of magnitude greater than congeners and co-occurring gastropods (Bates et al. 2005). Its stacking behaviour may be driven by a requirement to access vent fluids in order to suspension feed and sustain its bacterial symbionts (Chapter 5). However, small *L. fucensis* (<1 mm in shell length) are most common in waning and peripheral habitats (Marcus & Tunnicliffe 2002, Chapter 3) where suspended particle densities and reduced sulphur concentrations are relatively low. In these habitats, *L. fucensis* may graze for nutrition.

I hypothesized that *Lepetodrilus fucensis* possesses a specialized feeding strategy in comparison to non-symbiont hosting congeneric species and predicted that its feeding structures would exhibit functional modifications. I designed a comparative study among *Lepetodrilus* congeners to identify a range of morphological features and/or specialized traits that might indicate innovative feeding mechanisms. Gill, radula and digestive tract dimensions were measured for seven *Lepetodrilus* species with and without gill

symbionts from different vent sites in the Pacific ocean. To determine the feeding capabilities of *L. fucensis*, I observed live animals feeding in shipboard pressure vessels in the presence of suspended and attached food particles. Last, microscope techniques and fluorescent *in situ* hybridization were used to characterize the detached food material present on the gill.

METHODS AND MATERIALS

Morphological comparisons

Collections. Specimens of seven *Lepetodrilus* species from the east and west Pacific were collected by remote operated vehicle (ROPOS) and manned submersible (ALVIN) and preserved in either 7% buffered formalin or 2.5% glutaraldehyde. Collection information includes Ridge, vent field, latitude, longitude and depth for each species: *L. fucensis* (Juan de Fuca Ridge, Endeavour Segment, 47°57'N 129°06'W, 2220 m), *L. gordensis* n. sp. (described in Johnson et al. in press) (Gorda Ridge, SeaCliff Site, 42°45'N 126°42'W, 2750 m), *L. guaymasensis* McLean 1988 (Guaymas Basin, Southern Guaymas, 27°1'N 111°40'W, 2000 m), *L. aff. nux* (Mariana Arc, NW Eifuku Seamount, 21°29'N 144°2'E, 1576 m), *L. elevatus* McLean 1988, *L. ovalis* McLean 1988 and *L. pustulosis* McLean 1988 (East Pacific Rise, 9N, 9°50'N 104°17'W, 2500 m). *L. aff. nux* has not been described, but resembles *L. nux* (A. Warén, pers. comm.).

Dissections. Thirty specimens of each *Lepetodrilus* species (shell length 5-7 mm) were transferred to 70% EtOH for dissection with a light microscope. I used an optical micrometer to measure the dimensions of several morphological features of the gill, radula and digestive tract. The lengths of the gill axis and the longest lamellae were

measured (Figure 6.1). The radula ribbon was removed from the buccal cavity by dissection and its length was determined. The length, width, and height of the digestive gland were measured to estimate volume (~rectangle shape) (I flattened the *L. aff. nux* digestive gland using tweezers during measurements because it was L-shaped). The digestive tract was excised by dissection and its length measured (from the point of entry to the exit from the digestive gland). Last, the length and diameter (at a middle position) of the stomach were measured to estimate volume (assuming a cylindrical shape).

Microscopy. Radulae were dissected from the buccal cavity and cleaned in a 1% bleach solution at room temperature for 15 min. The dorsal surface of excised gills and radulae from ten specimens (5 mm in shell length) of each *Lepetodrilus* species were prepared for scanning electron microscopy (SEM). Tissues were critically point dried and sputter-coated in gold to generate micrographs on a Hitachi S-3500N scanning electron microscope. Gill lamellae on a 1 mm length of gill axis were counted. The cusp areas of the central and lateral teeth were measured using Image-Pro Plus© 4.5. In addition, scanning electron micrographs of the dorsal surface of the intact gill and food mass of *L. fucensis* (n = 3) were generated from specimens with their shell and mantle removed.

Gill lamellae (n = 10 for each species) from anterior, middle and poster regions (Figure 6.1) were post-fixed with osmium tetraoxide, dehydrated in a graded ethanol series, transferred to 100% propylene dioxide and embedded in Epon 812 resin (TAAB). Semi-thin transverse sections (500 nm) of individual filaments were treated with 1% Methylene Blue (Richardson's stain) and viewed using a Zeiss Universal compound light microscope. Thin sections (70 nm) were stained with lead citrate and uranyl acetate and

imaged using a Hitachi H-7000 transmission electron microscope. Light and transmission election micrographs were examined to verify the presence or absence of gill bacteria and ciliary junctions, and to determine the morphology of gill lamellae for the seven *Lepetodrilus* species.

Feeding observations

Suspension feeding. Feeding observations were carried out on-ship. Ten *Lepetodrilus fucensis* at four Axial Volcano vents in 2001 at two distances from focused vent fluid flows: 0-25 cm (in-vent) and 50-75 cm (far-vent). Vents were Gollum, Hell and Mrk 33 (Appendix 2.2 summarizes collection information for these vents), and ROPOS (45°56.0'N, 130°00.8'W). Following collection, animals were placed in flow-through pressure vessels maintained at 13 789 kPa (2000 psi) by a Whitey backpressure regulator on the chamber outflow tubing; full pressure was achieved after ~15 min. I suspended carmine in seawater (5% solution) and injected this solution at a rate of 1 ml min^{-1} using a Beckman 110B high pressure liquid chromatography pump for one hour, followed by a 'chase' with filtered seawater that varied in duration for each vent: 1, 3, 7, and 11 hours. Animals were then preserved in 7% seawater formalin and dissected using light microscopy. I examined the gill, food mass, radula, stomach content and fecal pellets under light microscopy for the presence of carmine particles.

Grazing. Bacteria-like material from the shells of live animals was removed and stained red with carmine, and then smeared onto the inside of the viewing window in a pressure vessel. *Lepetodrilus fucensis* (n = 30) was placed in the pressure vessel in the presence of two grazing gastropods: *Depressigyra globulus* Warén and Bouchet 1989 (Order Neomphalida) and *Provanna variabilis* Warén and Bouchet 1989 (Order

Caenogastropoda). Pressure was maintained at 13 789 kPa (2000 psi) by a Whitey backpressure regulator on the chamber outflow tubing (full pressure was achieved after ~15 min). Filtered seawater was pumped into the chamber inlet with a Beckman 110B high pressure liquid chromatography pump at a rate of 2 ml min^{-1}. Five individuals of each species were observed on the viewing window for 15 min each for evidence of grazing. After observations were complete, I fixed the animals in 7% seawater formalin and dissected out the stomach from each individual to examine its contents using a light microscope.

Fluorescent *In Situ* Hybridization (FISH)

A FISH study helped to determine if the symbiont phylotype is found with the detached food material observed on the gill. Oligodeoxynucleotide probes were designed to target positions 128-147 and 643-662 (relative to *Escherichia coli*) of the *Lepetodrilus fucensis* symbiont rRNA molecule (methods are described in Chapter 4). Whole gills with the mantle tissue intact were excised from specimens collected at Axial Volcano and prepared for FISH (described in Chapter 4). The area between the lamellar tips and the mantle was examined for the presence of the symbiont phylotype.

RESULTS

Morphological comparisons. The gills of *Lepetodrilus fucensis* and *L. gordensis* were ~30% larger than non-symbiont hosting species and possessed several specialized traits (summarized in Table 6.1; Figures 6.1, 6.2 & 6.3). Individual lamellae were not markedly tapered, while congeners were wider at the base and narrowed toward the

Table 6.1. Summary of comparative observations for gill features among seven *Lepetodrilus* species. Specimens were whole (scanning electron microscopy, SEM; light microcopy, LM) or tissue sections (sect) (transmission election microscopy, TEM; LM). Gill lamellae (Lam) were examined for symbiotic bacteria and ciliary junctions (Cil Junc) (Fretter 1988) (pr = present, ab = absent). In all species where symbiotic bacteria were absent the mantle was attached (attach) to at least 30% of the dorsal surface of gill lamellae (except for the free anterior tip) and the base of each lamella was deeper than at the tip (tapered: tap). In the two species that host bacteria, the gill lamellae were entirely free of the mantle and were a similar depth at the tip and base (not tapered: not tap). Non-symbiotic species had less filaments for the same length of gill axis (n = 5; ~8 mm shell length).

	Bacteria TEM/SEM	**Cil Junc SEM**	**Mantle LM: whole**	**Lam Shape LM: sect**	**# Lam mm^{-1} SEM**
L. fucensis	pr	pr	free	not tap	1.3
L. gordensis	pr	pr	free	not tap	1.3
L. guaymasensis	ab	ab	attach	tap	1.1
L. affnux	ab	ab	attach	tap	1.1
L. elevatus	ab	ab	attach	tap	1.1
L. ovalis	ab	ab	attach	tap	1.1
L. pustulosis	ab	ab	attach	tap	1.1

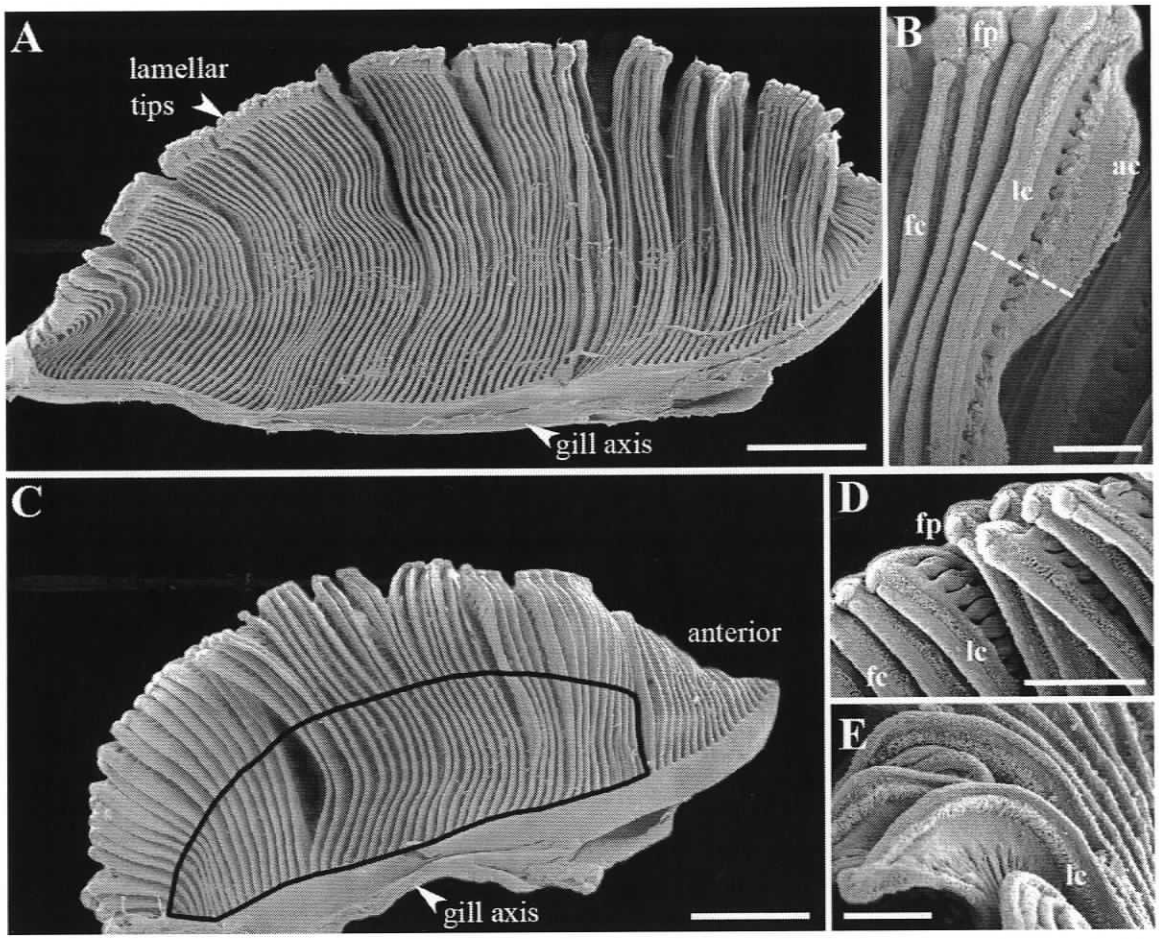

Figure 6.1
Scanning election micrographs showing the dorsal surface of the gill (A) and lamellae (B) of *Lepetodrilus fucensis*. The ciliated frontal pads (fp) and frontal (fc), lateral (lc) and abfrontal cilia (ac) are labeled. The orientation of tranverse sections through the gill lamellae is indicated by the dotted white line; the length of the dotted line is 'lamella depth'. The gill (C) and lamellae (D) of *L. guaymasensis* and lamellae from *L. elevatus* (E) are shown for visual comparisons. Gills were removed from specimens 8 mm in shell length. The gill axis length and lamellae length are greater in *L. fucensis*. Lamellae of *L. fucensis* were free of the mantle along their entire length (B). In *L. guaymasensis* and all aposymbiotic congeners, the dorsal surface of lamellae (opposite to the surface shown in C), were attached to the mantle for at least one third their length (except the anterior lamellae), as indicated by the black outline (C). The free lamellar tips are shown in D & E. Scale bars are 1 mm.

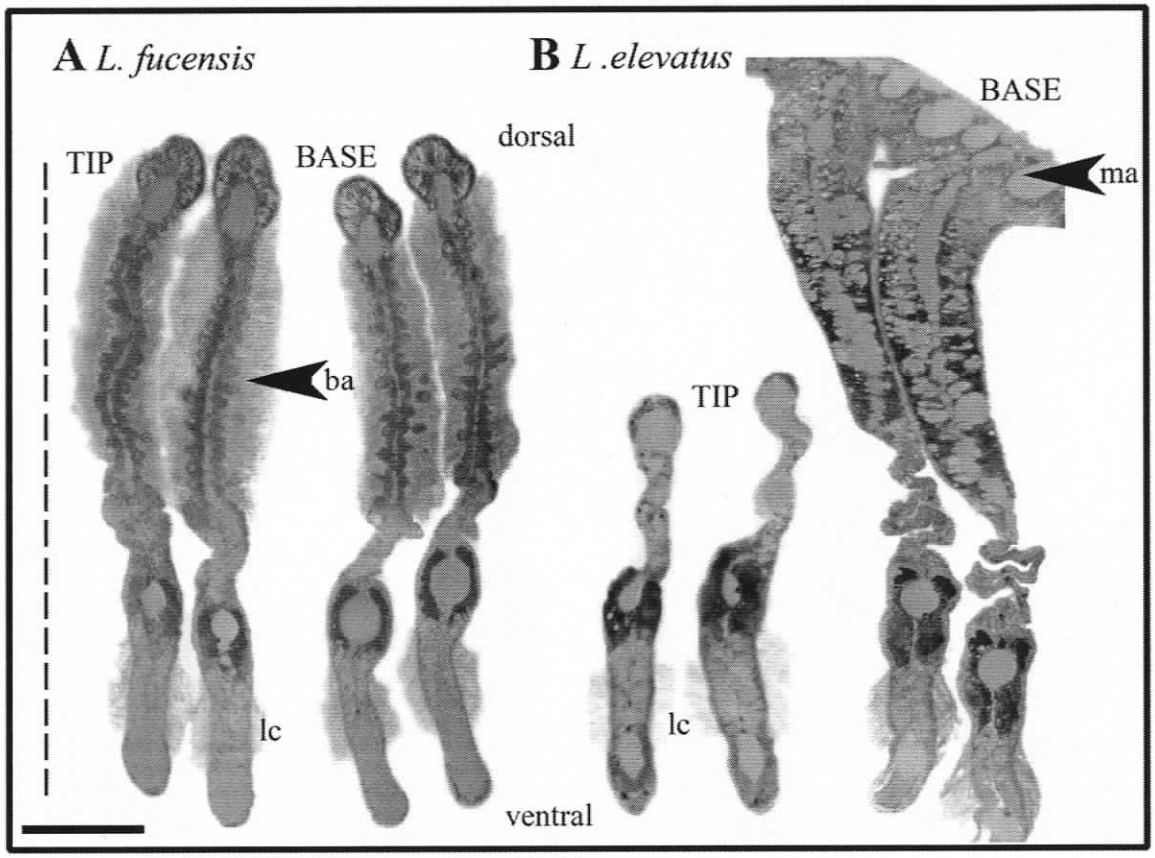

Figure 6.2
Light micrographs showing gill lamellae in transverse section from *Lepetodrilus fucensis* (A) and *L. elevatus* (B). The lamella depth (indicated by the dotted black line) of *L. fucensis* is similar at the tip and base (see Figure 6.1 B) and lamellae are free of the mantle (*L. gordensis* is similar). In *L. elevatus* (and all other aposymbiotic species), lamellae taper toward the tip. The dorsal base of lamellae are attached to the mantle (ma) for at least one third of the lamella length (except for the anterior lamellae: see Figure 6.1C). Specimens are from animals 8 mm in shell length. Lateral cilia = lc. Scale bar is 250 μm.

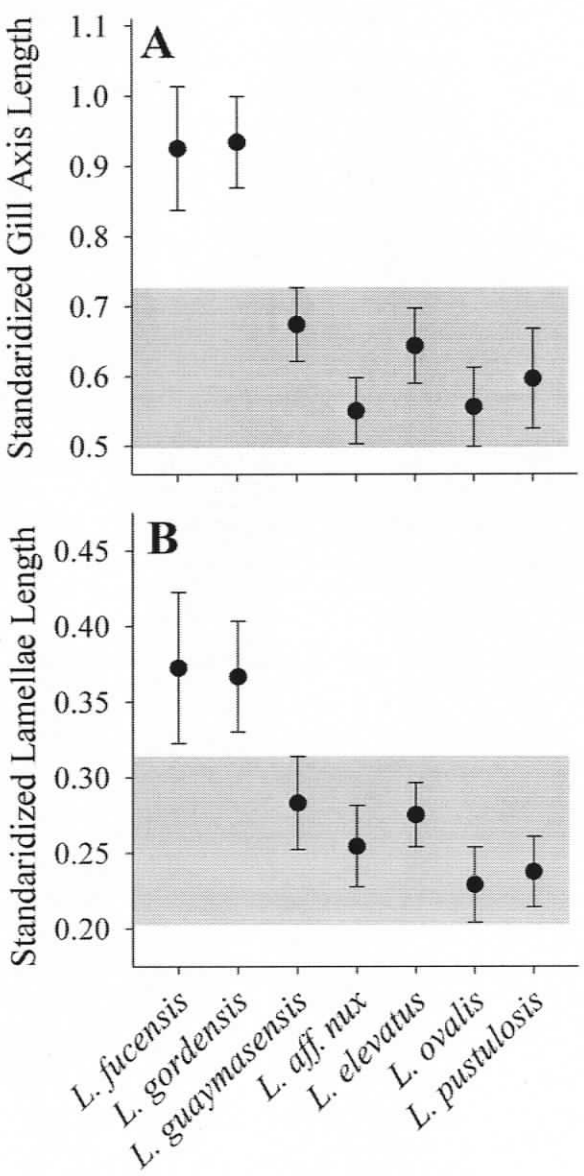

Figure 6.3
Measures of gill (A) and lamellae (B) length among *Lepetodrilus* congeners (A) standardized gill length (gill length (mm) / shell length (mm)) and (B) standardized filament length (filament length (mm) / shell length (mm)) among *Lepetodrilus* congeners. Values are mean ±1 SD. The data range for the non-symbiont hosting species is shaded in grey.

lamellar tip (compare the depth of the lamellar tips between *L. fucensis* and *L. elevatus* in Figure 6.2). Bacteria were visible on the afferent region (sensu Fretter 1988) of lamellae for the two symbiont-hosting species, but were absent from the corresponding region in congeners (Figure 6.2). The lamellae of *L. fucensis* and *L. gordensis* were free of the mantle along the entire length of their dorsal surface (between the mantle and the abfrontal cilia) and were stabilized by lateral ciliary junctions (see also Fretter 1988). In comparison, the basal portion of the dorsal edge of lamellae (except anterior lamellae) of all non-symbiont hosting species was attached to the mantle roof (for at least one third the lamellae length) and ciliary junctions were not observed (Table 6.1, Figs. 6.1 & 6.2). Lamellae were more densely spaced in *L. fucensis* and *L. gordensis* by two more lamellae for every ~10 mm of gill axis (compare *L. fucensis* to *L. guaymasensis*: Fig 6.1A & C) and the lengths of the gill axis and lamellae were >25% longer than congener lamellae (planned comparison ANOVA, $t > 26$, $p < 0.001$).

The radulae of the symbiont-hosting species were smaller than aposymbiotic congeners (compare *L. fucensis* to *L. elevatus*: Figure 6.4). The length of the radula was reduced by ~25% (Figure 6.5A); likewise, the areas of the cusps on the central and lateral teeth were reduced by ~50% (Figures 6.5C & B). These differences are significant between symbiont and non-symbiont hosting species (planned comparison ANOVA, $t < -10$, $p < 0.001$).

While the length of the gut and digestive gland volume of *Lepetodrilus fucensis* and *L. gordensis* fell within values obtained for congeners, the stomach volume was significantly smaller (reduced by ~50%) than the congener group (Figure 6.6A) (planned comparison ANOVA, $t < -3$, $p < 0.05$). In addition, species-specific stomach volumes

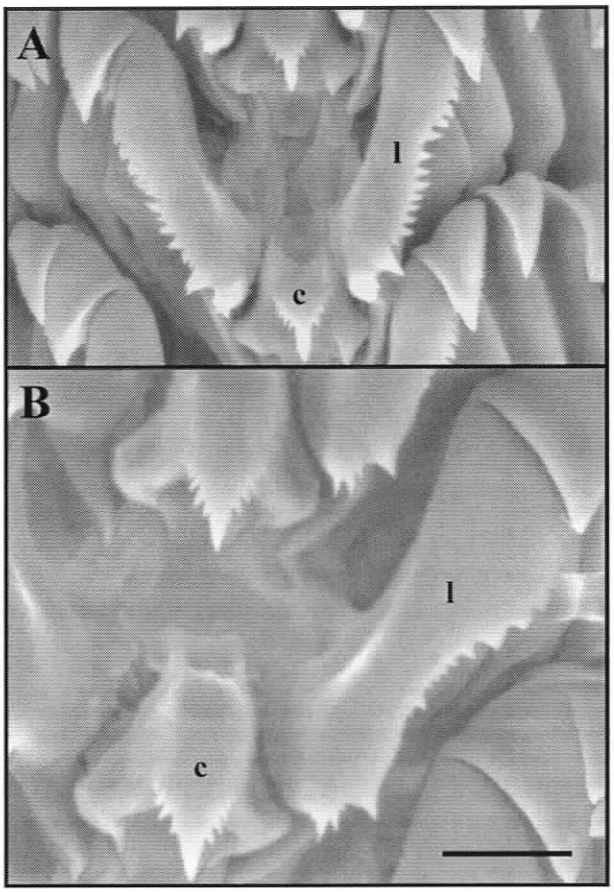

Figure 6.4
Scanning electron micrographs of radulae from *Lepetodrilus fucensis* (A) and *L. elevatus* (B). Animals were 8 mm in shell length. The central (c) and lateral (l) tooth cusps are visible. The radular teeth of *L. fucensis* were smaller than congeners. Scale bar is 25 μm and applies to both images.

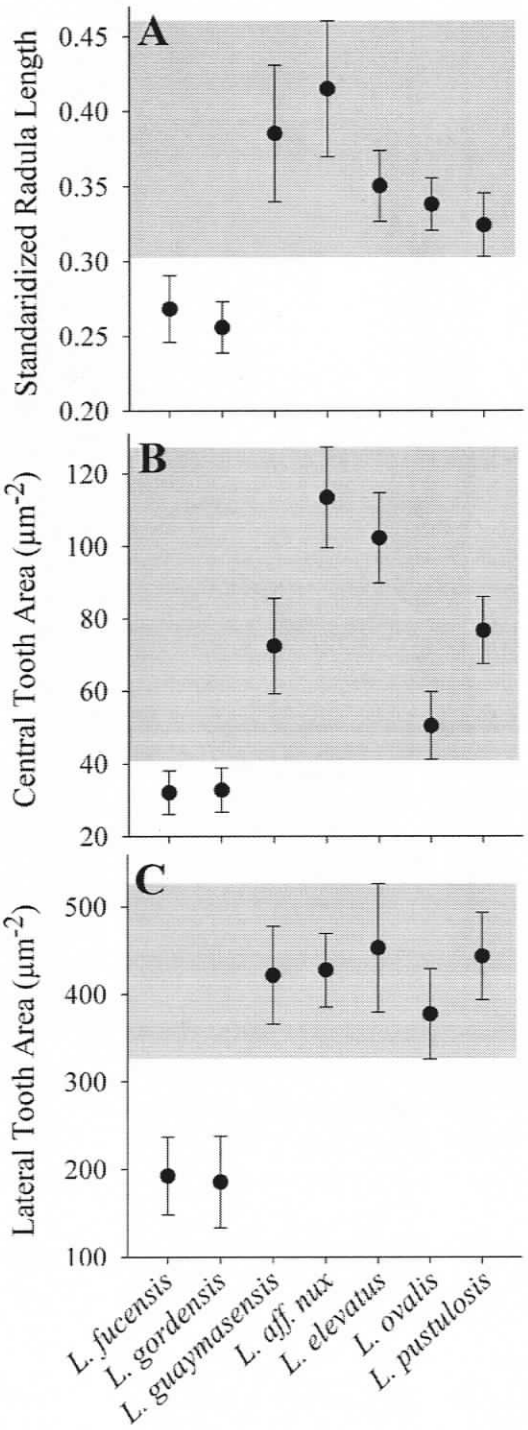

Figure 6.5
Measures of radula length and tooth area among *Lepetodrilus* congeners. (A) Standardized radula length (radula length (mm) / shell length (mm)), (B) central tooth area (μm^2) and (C) lateral tooth area (μm^2) among *Lepetodrilus* congeners. Values are mean ±1 SD. The data range for the non-symbiont hosting species is shaded in grey.

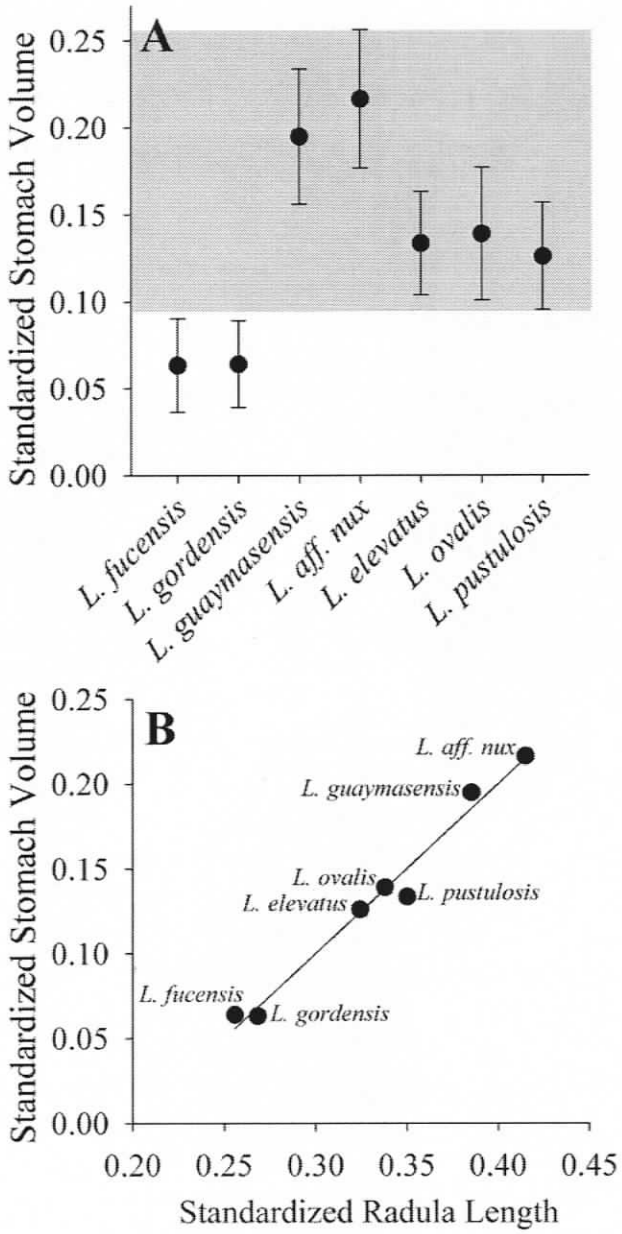

Figure 6.6
(A) Standardized stomach volume (stomach volume mm^{-3} / digestive gland volume mm^{-3}) among *Lepetodrilus* congeners (note: digestive gland volume was similar among congeners). Values are mean ±1 SD. The data range for the non-symbiont hosting species is shaded in grey. (B) Standardized stomach volume of each species is significantly correlated ($p < 0.001$, Pearson's $r = 0.98$) with standardized radula ribbon length (see Figure 6.5).

(standardized) and radula lengths (standardized) were significantly correlated (Fig 6.6B) (Pearson product moment correlation: $p < 0.001$, $r = 0.98$).

Suspension feeding. All sixty *Lepetodrilus fucensis* from habitats in different hydrothermal regimes concentrated carmine on their gills into a food mass (sensu Fretter 1988) that was ingested by the mouth. After 2 and 4 hours, food material at the lamellar tips contained carmine particles (Figure 6.7A & B). The food mass was transported to the neck (Figure 6.7C) in a ciliated tract that splits into an accepted and rejected tract (see Figures 6.7D & 6.8). The particles in the accepted tract were transported to the right of the cephalic tentacle to the mouth (Figure 6.7D) and, by 4 hours, carmine particles were present on the radular teeth and in the stomach. After 8 hours, fecal pellets contained carmine (Figure 6.7E). Scanning electron micrographs of the ciliated tracts with accepted and rejected material showed a different composition (Figure 6.8). The accepted tract contained high abundances of filaments, similar to the morphology of the bacterial gill symbiont, and lesser numbers of spheroid shapes (Figure 6.8A). The rejected material contained debris-like particles and relatively fewer filaments and spheroids (Figure 6.8B). Based on whole-animal examinations, fluids appear to enter the mantle cavity anteriorly and deposit larger particles on the ventral surface of the gill (Figure 6.9). The morphology of the frontal and lateral cilia suggests they push water between the gill lamellae and particles over to the right side of the gill (A. Warén, pers. comm.). Small particles were transported by the abfrontal cilia (Figure 6.8) along the entire dorsal lamellar length and appeared to be incorporated into the food material at the lamellar tips (Figure 6.7B).

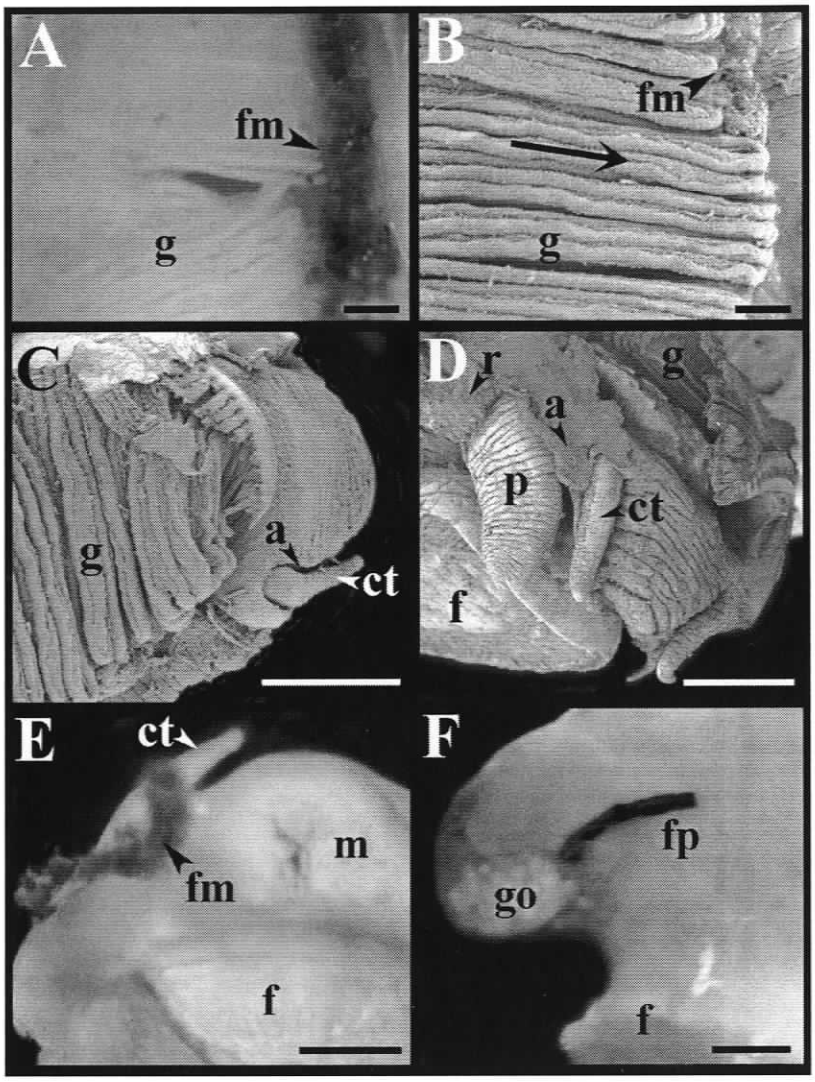

Figure 6.7
Light (A, E, F) and scanning electron (B, C, D) micrographs documenting the accumulation of particles at the lamellar tips (arrow) into a food mass (fm) (sensu Fretter 1988). (A) Carmine particles were removed from suspension and were accumulated into the food mass. (B) Small particles were observed on the ventral surface of the gill (g) and were transferred to, and incorporated into, the food mass. (C & D) The food mass was then transported to the neck and sorted into two ciliated tracts: the accepted material (a) passes the right of the cephalic tentacle (ct), while rejected material (r) passed to the right of the penis (p) (in the case of males). (E) The accepted material continued to the mouth (m) (see also Fretter 1988). Animals protracted their radula to ingest the food mass. (F) Carmine was observed in stomach contents and fecal pellets (fp). f = foot, go = gonad. Scale bars = 1 mm.

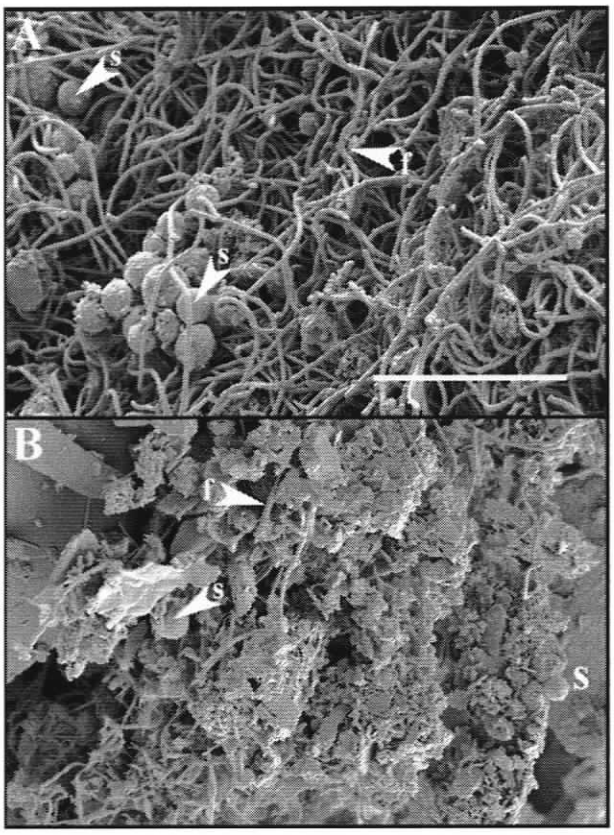

Figure 6.8
Scanning electron micrographs of the particles in accepted and rejected ciliary tracts on the neck of *Lepetodrilus fucensis*. Scale bar = 200 μm. Images were provided by A. Warén, Senior Curator, Swedish Museum of Natural History.
(A) Accepted food mass showing spheroid (s) and filamentous (f) shapes that resemble bacteria. The filaments were similar in length and diameter to the *Lepetodrilus fucensis* symbiont.
(B) Rejected material that passes to the right of the cephalic tentacle. Amorphous and mineralized particles are more common.

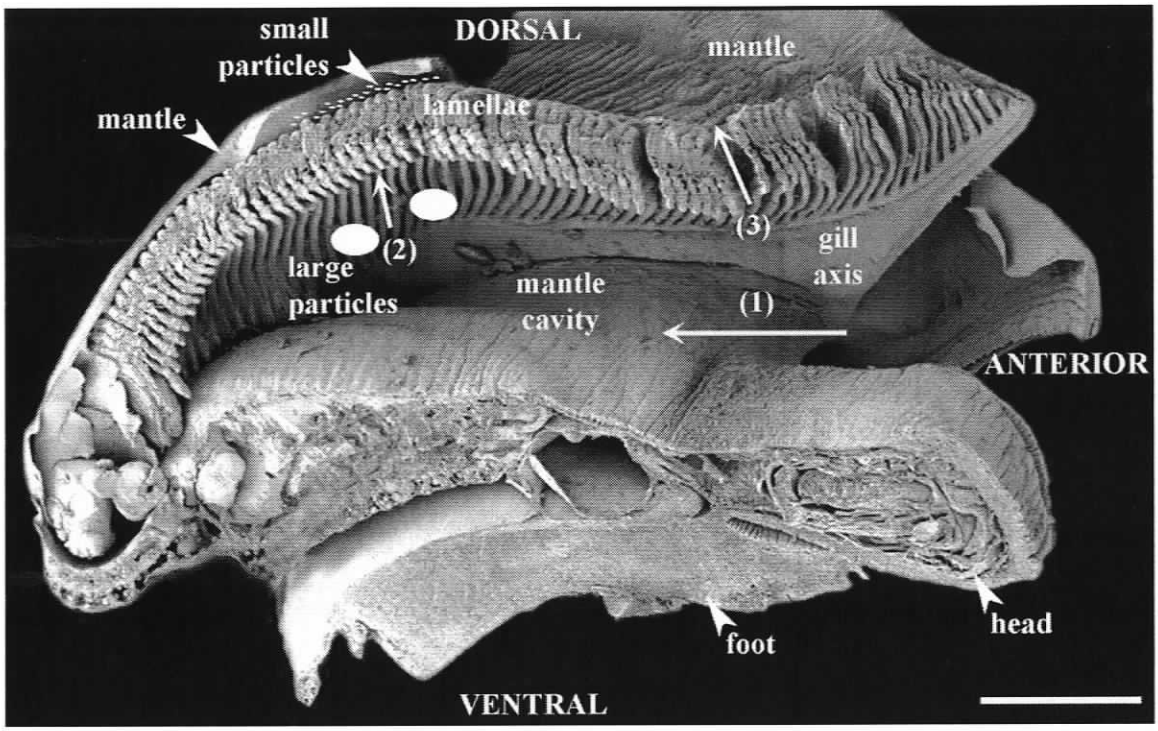

Figure 6.9
Lepetodrilus fucensis in a whole animal longitudinal section after critical point drying (courtesy of A. Warén, Senior Curator, Swedish Museum of Natural History). Based on dissections of animals at varying time intervals following exposure to carmine, fluids entered the mantle cavity anteriorly (arrow #1). The frontal cilia on the ventral surface of the gill accumulated large-sized particles into mucous chords that were transported at right angels to the lamellae (arrow #2); this material was then transported to the lamellar tips and incorporated into the food mass (see Figure 6.7A & B). Water appears to be pushed from the ventral surface in between the lamellae (arrow #3) by the lateral cilia. Small particles also accumulated on the dorsal surface of the gill between the mantle and the lamellar abfrontal cilia and were moved at right angels to the lamellae and coalesced into the food mass shown in Figure 6.7A & B. The food mass was then transported to the neck region and sorted into accepted and rejected ciliated tracts (see Figure 6.8). Scale bar = 1 mm.

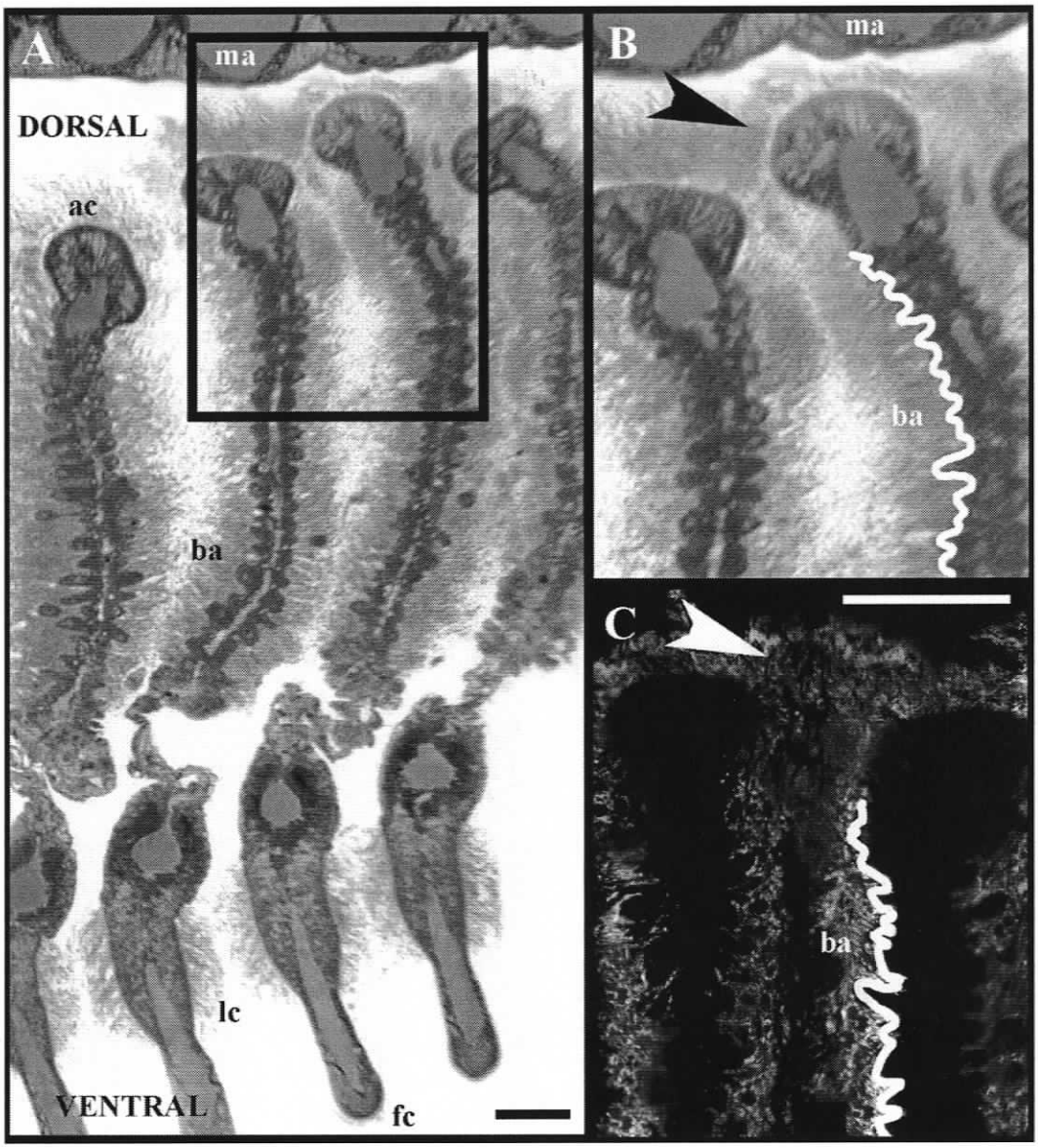

Figure 6.10
(A) Light micrograph of *Lepetodrilus fucensis* gill lamellae in transverse section with the mantle (ma) present. Lateral cilia = lc, frontal cilia = fc. (B) Epibiotic bacteria (ba) are present in between the lamellae and are attached to the gill epithelium along the white line. Detached bacteria-like filaments (arrow) are found between the abfrontal cilia (ac) on the dorsal edge of lamellae and the mantle. C. Fluorescent *in situ* micrograph showing the symbiont phylotype (arrow) in the space coinciding with the detached bacteria between the lamellae and the mantle. Scale bars = 10 μm.

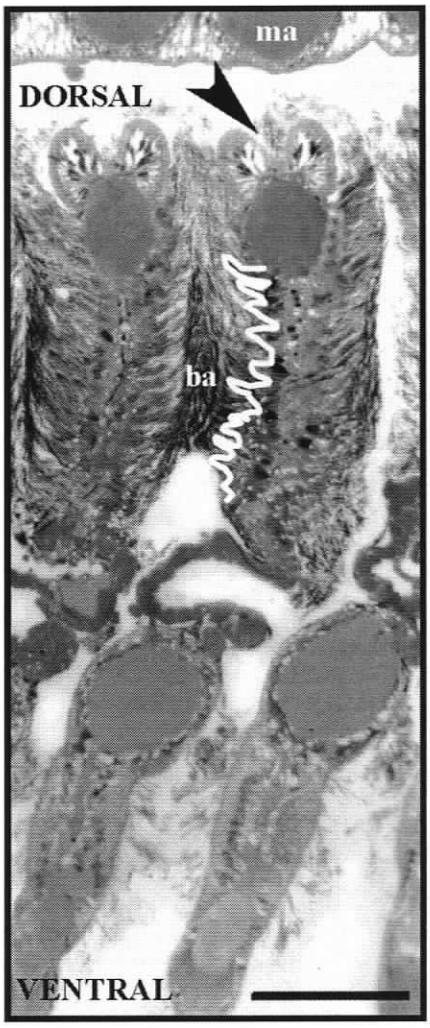

Figure 6.11
Light micrograph of *Lepetodrilus gordensis* gill lamellae in transverse section with the mantle (ma) present (orientation is the same as *L. fucensis* in Figure 6.10). Epibiotic bacteria (ba) are attached to the gill epithelium (white line) and are similar to *L. fucensis* in morphology, but appear denser. Detached bacteria-like filaments are also present between the abfrontal cilia on the dorsal edge of lamellae, and within a furrow (arrow) that is absent from *L. fucensis* lamellae. Scale bar = 20 μm.

Grazing. During grazing experiments, *Lepetodrilus fucensis* occasionally protracted their radulae to remove particulates from the surface of the pressure vessel window (~once every 15 minutes). In comparison, *Depressigyra globulus* and *Provanna variabilis* frequently rasped particulates from the glass with their radulae (~once per minute). The stomach contents of two *L. fucensis* specimens (out of 30) were stained red in comparison to all 30 *D. globulus* and *P. variabilis*.

Symbiont farming. *Lepetodrilus fucensis* hosts epibiotic bacteria attached to the gill epithelium that appear to be bathed in fluids pushed from the ventral to the dorsal surface by the lateral cilia (Figure 6.10). Detached bacteria-like filaments were also present dorsally in the region between the mantle and the abfrontal cilia on *L. fucensis* gills (Figures 6.10A & B). These detached bacteria are dominated by the symbiont phylotype, identified by fluorescent *in situ* hybridization experiments (Figure 6.10C). This material is transported to the food mass (Figure 6.9). Likewise, *L. gordensis* also accumulated similar filaments between the abfrontal cilia and mantle where a furrow is present on the lamellae (Figure 6.11) (see also Johnson et al. in press). In other *Lepetodrilus* species without gill bacteria, the dorsal surface of the lamellae is partially fused to the mantle (Figures 6.1 & 6.2) and where the lamellae were free from the mantle, dense particles were not observed.

DISCUSSION

Species in the *Lepetodrilus* genus exhibit morphological and behavioural traits characteristic of suspension feeding and grazing. For example, *Lepetodrilus* species possess enlarged frontal and abfrontal ciliary pads at the free tip of the lamellae; radular

tooth wear is also commonly observed and indicates grazing (Fretter 1984). However, comparisons of the gill morphology within the *Lepetodrilus* genus reveal that the two symbiont-hosting species possess additional gill specializations that have also evolved independently in several other suspension feeding gastropod families (Declerck 1995). Relative to their congeners, *L. fucensis* and *L. gordensis* have longer gill axes and longer, more densely spaced, lamellae. Furthermore, their gill lamellae do not taper, are connected by lateral ciliary junctions and are free of the mantle along their entire dorsal surface. In other gastropods, greater numbers of longer lamellae that are free of the mantle increase the velocities of the fluid currents across the gill (Vogel & Gutmann 1980) and surface area, thus enhancing particle delivery and processing capacity (reviewed in Declerck 1995). Hence, the gill specializations of *L. fucensis* and *L. gordensis* suggest that suspension feeding may be a more efficient feeding mode for these two species in comparison to their congeners.

In addition to suspension feeding, *Lepetodrilus fucensis* may farm its gill bacteria for food as FISH studies documented the symbiont phylotype with material that is incorporated into a food mass. The high abundance of symbiont-like bacteria in with food material on the gill (this study) and gut (de Burgh & Singla 1984) further supports that the symbionts are cultivated on the gill, transported to the mouth and ingested. If so, the same factors thought to select for the gill modifications that enhance suspension feeding capabilities (see above) also favour symbiont farming: (1) increased fluid velocities facilitate greater gas exchange and bacterial proliferation, (2) particle sorting concentrates the bacteria and (3) greater surface area increases symbiont biomass. Therefore, although the gill traits of the two bacteria-hosting species are consistent with

the morphological specializations exhibited by other suspension feeding gastropods, these suite of specializations also promote prolific symbiont populations. Increased access to suspended particles and symbiont cultivation are probably additive selective forces driving the gill modifications reported here. However, as only the symbiont-hosting species show these specializations, it may be that the symbiosis with bacteria are a key innovation that has lead to changes in gill morphology (Liem 1974, Wainwright 1991, Vermeij 2001).

The radula and stomach of the two symbiont hosting species were reduced in size. One explanation for this reduction is that these species obtain organic carbon from their symbionts directly through the gill epithelium, thus, supplementing ingested material. In other molluscs where carbon translocation is important, the entire digestive tract is reduced (e.g., Windoffer & Giere 1997). However, the gut length and digestive gland volume of *L. fucensis* and *L. gordensis* were similar to their aposymbiotic congeners. Thus, food digestion remains an important feeding mechanism for *L. fucensis* and *L. gordensis*, suggesting that the gill symbionts may not contribute significantly to their nutrition via carbon translocation or phagocytosis the gill epithelium (as suggested by de Burgh & Singla 1984).

An alternate explanation for the reduction in radula and stomach volume relates to the amount of inorganic content present in the ingested material. For example, in a guild of grazing intertidal gastropods, the cusp size of radular teeth increased with the proportion of inorganic material in the feces (Black et al. 1988). The food material ingested by *L. fucensis* is sorted on the gill and, therefore, likely contains less inorganic content than grazed material, thus the radula and stomach can be smaller in size.

Conversely, this reduction may limit the ability of *L. fucensis* to gain sufficient nutrition from grazing alone and provides an explanation for why animals on the periphery of vents exhibit poor condition where biofilms are a primary food resource (Chapter 5).

The relative sizes of radula and stomach may prove an important indicator of feeding strategy within the *Lepetodrilus* genus as radula and stomach size showed a tight linear relationship for the seven species examined. In particular, the radula size and stomach volume were large in *L. guaymasensis* and *L. aff. nux*. These two species may rely more heavily on grazing than the other five species or substratum type may be important. For instance, animals grazing primarily on basalt might need larger radular cusps than those grazing on animal surfaces. These ideas remain to be tested.

The feeding mode used by *Lepetodrilus fucensis* is likely dependent on food availability. In active vent flows, suspended particles (Giere et al. 2003) and symbiont biomass are high (Chapter 5) and, in these habitats, *L. fucensis* appears to rely on suspension feeding and symbiont farming by forming stacks at vent outflows and remaining sessile (Chapters 2). This behaviour presumably promotes suspension feeding in bacteria-rich fluids and the productivity of symbiont populations by providing increased access to sulphide-rich fluids (Chapter 5). In waning flows and on the periphery of vents, the concentration of suspended particles is relatively low and gill symbionts exhibit low densities (Chapter 5). In these habitats, *L. fucensis* does not stack (Bates et al. 2005) and grazing is probably the primary feeding mode. However, peripheral animals greater than 3 mm in shell length had poor tissue condition (Chapter 5) and their gonads were often empty (Chapter 3), indicating that nutrition from grazing alone in habitats with low hydrothermal flux is not sufficient to maintain healthy tissues

and reproductive output. However, small *L. fucensis* are abundant in peripheral habitats (Marcus & Tunnicliffe 2002, Bates et al. 2005) and grazing may provide adequate nutrition for this size class. Whether *L. fucensis* shows size or habitat specific variation in feeding remains an open question.

The ecology of *Lepetodrilus gordensis*, a newly described sister species to *L. fucensis* (Johnson et al. in press), has not been investigated but its morphology suggests it uses the same feeding modes as *L. fucensis*. Nevertheless, differences in the gill morphologies of these two species suggest that their feeding efficiency may vary. *L. gordensis* gill lamellae possessed a furrow on the dorsal surface that may relate to particle processing; in other suspension feeding species, ruffling of the gill lamellae increases food capture and processing efficiency (Declerck 1995). Furthermore, *L. gordensis* hosted remarkably dense colonies of bacteria and other debris in comparison to the same area of *L. fucensis* gills. The significance and generality of these observations are unknown.

The positions of particles on *Lepetodrilus fucensis* gills indicate possible directions of fluid flow and the sequence of particle processing that can be tested in future studies. First, based on the orientation and length of the frontal and lateral cilia, fluids are probably pushed in between the lamellae, presumably providing the bacterial symbionts with access to chemical substrates. Fluids are also probably pushed at right angles to the lamellae on the ventral surface of the gill (A. Warén, pers. comm.). These proposed directions of fluid flow are consistent with observations of particles on the gills of animals fixed at different intervals after exposure to suspended carmine; large particles accumulated on the ventral surface and were transported to the lamellar tips, and small

particles (similar in morphology to the *L. fucensis* symbiont) were found on the dorsal surface between the abfrontal cilia and the mantle (Figure 6.8). Similarly, in the suspension feeding limpet, *Crepidula fecunda*, material on the gill's ventral surface is comprised of larger particulates that settle from fluids as current passes in between the lamellae; these particles are subsequently rejected (Chaparro et al. 2002). Second, particles were not always evident in the rejection tract of *L. fucensis*. The concentrations of inorganic and organic particles suspended in fluids may influence whether material is sorted and rejected prior to ingestion, or is simply ingested and processed as feces, as in the suspension-feeding limpet, *C. fecunda* (Chaparro et al. 2004). Third, because *L. fucensis* everted its radula to move the food particles into the mouth, its feeding rate may be limited by how much and how quickly the radula can process material (Chaparro et al. 2004).

In summary, *Lepetodrilus fucensis* shows morphological and behavioural evidence of both suspension-feeding and grazing, in addition to what may be the first description of symbiont farming for the purposes of ingestion by a gastropod. The gamma-Proteobacteria associated with the gill appear to be sloughed into a food mass that is transported along a ciliated tract and then moved from the gill to the mouth with the radula. In addition, de Burgh and Singla (1984) suggest that phagocytosis of the bacteria by the gill epithelium, followed by lysosomal digestion may contribute to the limpet's nutrition. *L. fucensis* presumably uses a combination of these different feeding modes, which may explain its persistence in habitats with varying levels of hydrothermal fluid flux (Tsurumi & Tunnicliffe 2003). Although its gill specializations are consistent with well studied suspension feeding gastropods, and are thought to increase surface area

for particle capture and fluid flow across the gill, these specializations also increase symbiont abundance. It is possible that the symbiosis may be a key innovation in some species of the *Lepetodrilus* genus that has driven the gill modifications reported here. Grazing may be a less important feeding mode because the radula and stomach of *L. fucensis* are reduced in comparison to congeners, and may limit the relative inorganic content of the food material. *L. fucensis* may prefer active vent flows because these habitats offer suspended particulates and encourage symbiont biomass. Studies aiming to examine the relationship between feeding and the abundance of suspended particles, bacterial symbionts and biofilms are necessary to further characterize the feeding strategy of this limpet.

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CHAPTER 7

CONCLUSIONS

Introduction

Understanding how and why animals select certain habitats has been central to explaining species distributions. In particular, studies have aimed to determine the influence of abiotic variation on the biology of organisms to tease out factors important in habitat selection. For example, environmental conditions, such as temperature, typically vary among habitats and lead to differences in the energy required to maintain physiological processes, as well as restricting habitat occupation when species' tolerance levels are challenged (Huey 1991). Hydrothermal vent ecosystems are excellent models for habitat studies because they present extreme abiotic variability to animal populations at small spatial scales. We are just starting to explain processes that drive observed species patterns at vents by characterizing the habitat selection and biological characteristics of some the dominant, but little studied, fauna.

My thesis is the first attempt to link habitat selection by vent gastropods with their behavioural responses to abiotic parameters using *in situ* and shipboard experiments. Three abundant hydrothermal vent gastropods from vents on the Juan de Fuca and Explorer Ridges in the northeast Pacific Ocean were selected for study because little was known about their ecology: a limpet, *Lepetodrilus fucensis* McLean 1988 (Vetigastropoda, Lepetodrilidae), and two coiled species, *Depressigyra globulus* Warén and Bouchet 1986 (Neomphalida, Peltospiridae) and *Provanna variabilis* Warén and Bouchet 1986 (Caenogastropoda, Provannidae).

I further examined characteristics of the most abundant gastropod, *Lepetodrilus fucensis*, to isolate factors that might explain its high numbers in a variety of habitats. This work is novel because I examined multiple biological features of populations across environmental gradients, thus profiling small-scale habitat use by this species. Here, I endeavor to integrate the aspects of each data chapter to describe the optimal habitat conditions for *L. fucensis* by relating its food availability and feeding strategy to recruitment patterns, reproductive potential and survivorship.

I first review the major species patterns of the three gastropods and conclusions from behavioural studies designed to isolate the role of thermal conditions in driving observed patterns. I then provide an overall picture of habitat use by limpet populations across spatial and temporal gradients in vent fluid flux. Next, I suggest directions for future research that were indicated by my results. Last, I summarize the major conclusions from this work.

Habitat selection

Fluid temperature plays a central role in habitat selection by *Lepetodrilus fucensis*, *Depressigyra globulus* and *Provanna variabilis* (Bates et al. 2005). Adults of each species selected habitats in vent flows less than 15±2°C. The variability presented by higher temperature flows may pose physiological constraints on their habitat occupation. Locations along a 75 cm transect away from flow hosted all three species, however, their abundance patterns varied across this small-scale environmental gradient. *L. fucensis* and *D. globulus* were most abundant in-vent (0 to 25 cm from flow) where fluids were warm (10±5°C), but also occurred near- (26 to 50 cm) and far-vent (51 to 75 cm) at lower temperatures (< 5°C); however, *L. fucensis* showed a higher degree of

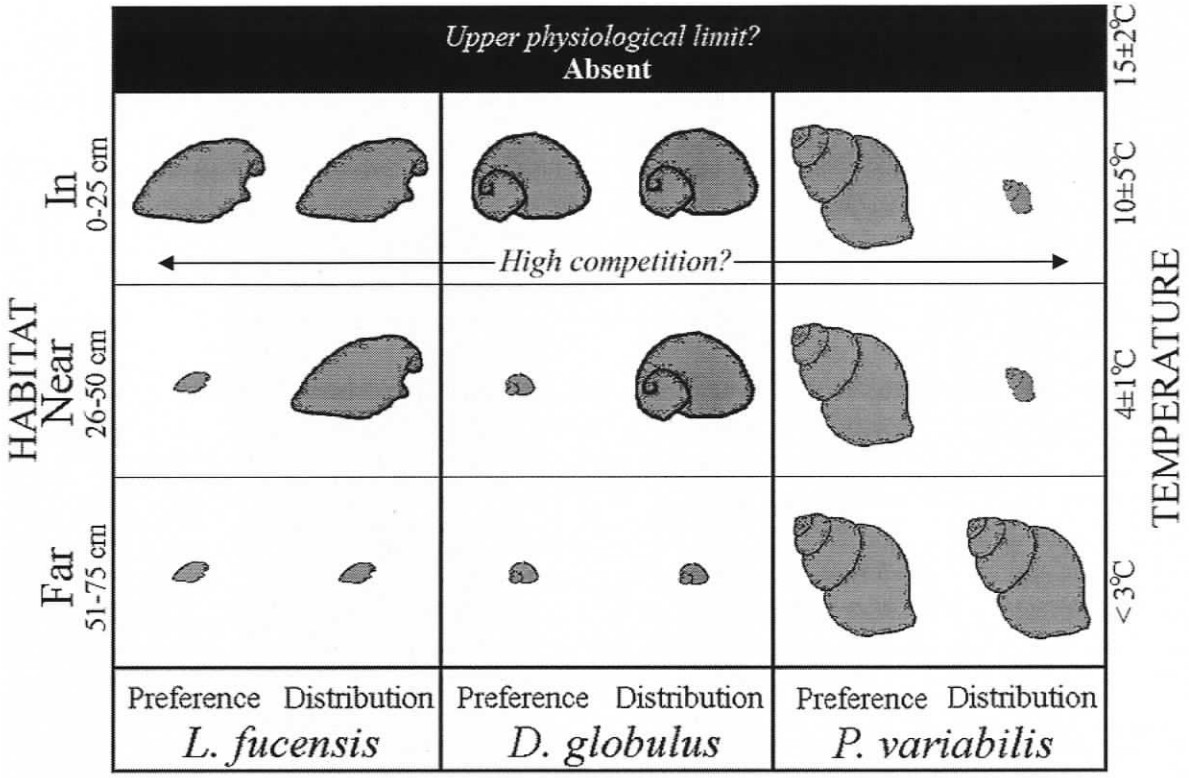

Figure 7.1
General model comparing the abundance patterns of the three gastropod species in three distance categories near a vent source to their respective habitat and temperature preferences. Large symbols represent high abundance; small symbols represent low abundance. *Lepetodrilus fucensis* and *Depressigyra globulus* prefer in-vent habitats and temperatures between 5 and 15°C, but are also common near-vent where fluid temperatures range from 3 to 5°C. *Provanna variabilis* is relatively more abundant far-vent, but does not show a distinct preference for temperatures typical of these habitats. Competition for space and/or food may limit individuals from occupying habitats closest to vents. We found that gastropods avoid sustained exposure to temperatures greater than ~15°C, suggesting that animals respond to temperature in order to maintain their position within a favourable environmental regime.

habitat specialization because it occurred at densities one to two orders of magnitude higher than in near- and far-vent locations. The high numbers of *L. fucensis* and *D. globulus* in-vent can be explained by their behaviour: both preferred in-vent locations and the fluid temperatures typical of these habitats (Bates et al. 2005) (Figure 7.1). In comparison, *P. variabilis* was common far-vent and occurred at lesser abundances near- and in-vent, and did not exhibit distinct habitat and fluid preferences (Figure 7.1). A reasonable conclusion is that it does not compete effectively for space in-vent, thus partitioning habitat near vent sources.

Small-scale habitat use by *Lepetodrilus fucensis*

Aggregation by adults in-vent. Adult *Lepetodrilus fucensis* preferred habitats nearest warm vent fluids where they reached high densities (up to 290 000 individuals dm^{-2}) by stacking (Bates et al. 2005, Chapter 3). Observations of animals *in situ* in time lapse images and video imagery indicate that limpets in these stacks remain stationary. The position of limpets in some of these stacks must be stable for at least several days, as epibionts were often absent from the top surfaces of shells where the foot of an overlying limpet was planted (Figure 7.2). Animals in these stacks do not appear to graze but lift up their shells to access venting fluids (Bates et al. 2005). This behaviour is common in suspension feeding gastropods (e.g., *Crepidula fecunda*: Chaparro et al. 2005) and probably facilitates fluid circulation across the gill to remove suspended organics delivered by vent fluids and to supply the gamma-Proteobacterial symbionts with reduced sulfide and oxygen for thioautotrophy (Chapter 5 & 6). Detached symbionts (identified by FISH) occur with food particles on the gill (Chapter

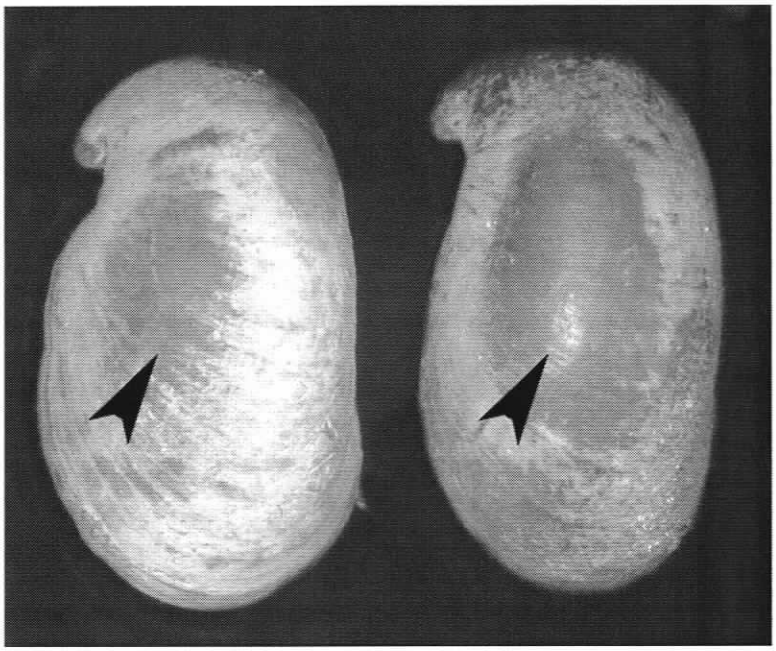

Figure 7.2
Shells of two *Lepetodrilus fucensis* specimens that were in stacks showing oval-shaped patches (black arrows) where no epibionts are present. This bare patch corresponds with the foot position of an overlying limpet. Limpet length is ~10 cm.

6), suggesting that these dense colonies of gill bacteria are farmed, transferred to the mouth in ciliated tracts and ingested.

The preferences by *Lepetodrilus fucensis* for active fluid flow are probably driven by its feeding strategy: this species, compared to congeners, exhibited modified gill, radula and stomach features consistent with suspension feeding and symbiont farming as main feeding mechanisms. These mechanisms appear to sustain healthy, dense in-vent populations with high condition indices, healthy tissue appearance in transmission electron micrographs (Chapter 5) and gonads full of gametes (Chapter 3). The larger animals in habitats with active fluid flux tended to be female, which implicates size as a potential factor determining the outcome of intraspecific competition for optimal habitat space (Chapter 3). The majority of fertilized eggs are likely released into the water column from active fluid flux habitats, judging from the full gonads of resident females and the relatively high numbers of larger females (Figure 7.3). Therefore, the majority of larvae are probably produced in-vent, where adult limpets are most abundant, females aggregate, animal health and survivorship are higher and food acquisition is possible through multiple feeding mechanisms.

In comparison to active vent flows, far-vent locations were suboptimal habitats for adult *Lepetodrilus fucensis*. Limpets actively avoided locations on the periphery of vent flow (Bates et al. 2005) and these locations were sparsely populated by adults. Further, *L. fucensis* in far-vent locations were solitary, suggesting that suspension feeding may not be an important feeding mechanism for far-vent animals. Bacterial symbionts were also far less abundant on the gills of these adults and their gill lamellae surface area was reduced, thus the symbionts do not provide a major food resource in peripheral

habitats (Chapter 5). Grazing may be the primary mode of feeding when suspended particles and symbiont biomass are low. However, the diet of *L. fucensis* surviving in far-vent locations was minimal because peripheral limpets exhibited low condition indices and poor tissue appearance, and their gonads were rarely full of eggs or sperm (Chapters 3 & 5). Larger far-vent animals also tended to be male and this pattern is likely related to competition between the sexes: more males occupy the suboptimal habitats where they experience lower mortality due to a lesser cost of reproduction, and are thus over-represented in populations with low survivorship (Chapter 3). Because far-vent populations support few adults that were unhealthy, dominated by males and suffer high mortality, these locations are unlikely to contribute significantly to the larval pool.

Recruitment to far-vent habitats. While the bulk of adult biomass occurs in active vent flows that are optimal for survivorship and reproductive output, far-vent locations are important because they foster early post larval populations. *Lepetodrilus fucensis* recruitment occurred at the periphery of hydrothermal vent sources where the density of animals less than 1 mm in shell length were two orders of magnitude greater (up to 500 000 m^{-2}) than in active venting (Chapter 3). Furthermore, larval protoconchs occurred exclusively in far-vent locations (Figure 7.3). It seems that early post larval stages grow in peripheral locations and migrate to active venting (Figure 7.3) at sizes larger than ~1 mm, thus explaining the drastic increase in the size profile between far- and in-vent habitats (Chapter 3). It is not likely that small *L. fucensis* are suspension feeding due to (1) constraints on the size and quantity of food particles that can be processed by small gills (e.g., Montiel et al. 2005) and (2) the low availability of suspended organics away from vent sources. It is more probable that these small limpets

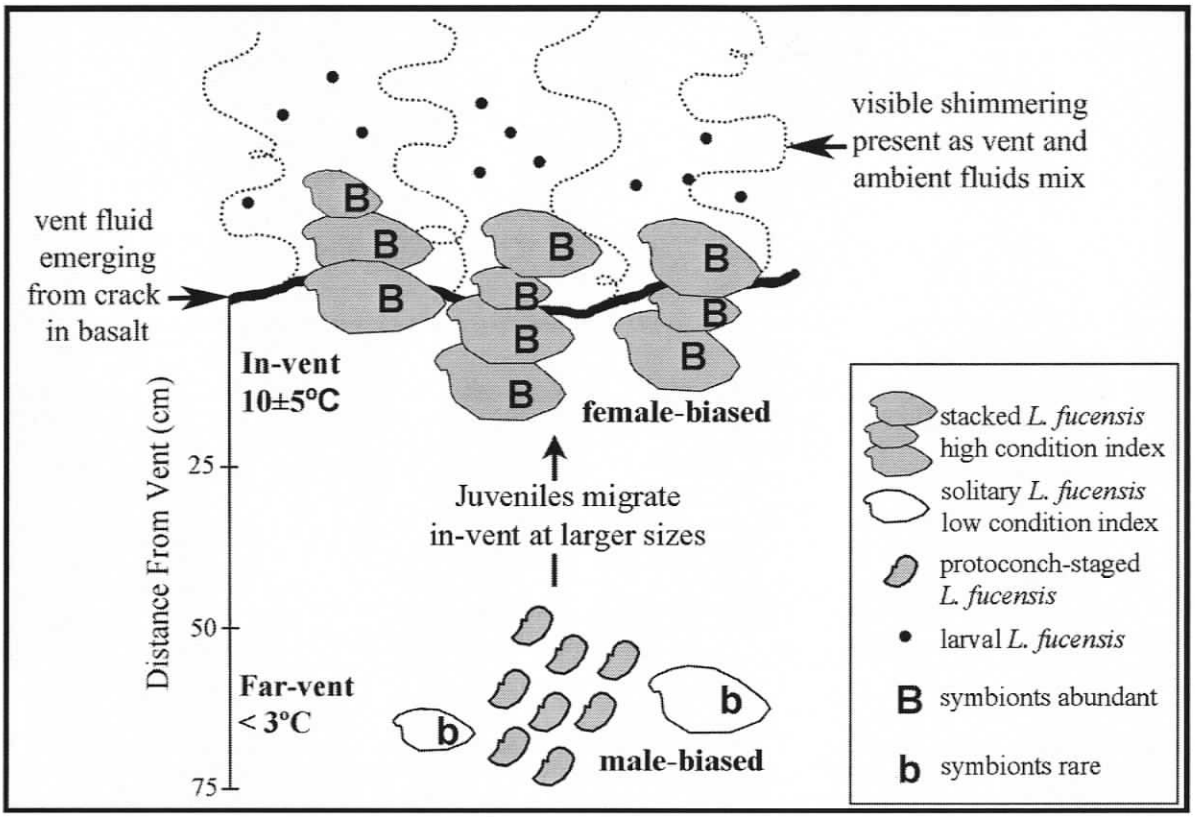

Figure 7.3
Small-scale habitat use by *Lepetodrilus fucensis* at opposite ends of a environmental gradient near a warm hydrothermal vent with focused fluid flow. In-vent populations are characterized by high abundances of animals in dense stacks where larger animals tend to be females. In-vent limpets host abundant symbiont populations and both sexes have gonads full of sperm or eggs. Far-vent populations include newly settled protoconchs and are dominated by early post larval limpets. Larger animals in these locations occur at low densities and tend to be male; these animals have low condition indices and their gill symbiont populations are sparse.

are grazing biofilms present on the substratum; indeed, *Provanna variabilis* co-occurs with *L. fucensis* juveniles and is sustained by grazing. In addition, the gills of juveniles ~0.5 mm in shell length in peripheral habitats hosted bacterial symbionts, although at low abundances, and it is not known if these symbionts contribute to the nutrition of peripheral juveniles. Far-vent locations may present a refuge from predation for juveniles, but are little studied.

Temporal patterns. Spatial patterns in the condition of *Lepetodrilus fucensis* populations across environmental gradients correspond with temporal shifts from active to waning vent flow. *Lepetodrilus fucensis* populations from senescent tubeworm bushes were male biased and animals exhibited poor tissue appearance in transmission electron micrographs, near-empty gonads and almost no gill symbionts (Chapters 3 & 5). As venting wanes, bacterial productivity and sulphide decline, thus food availability is probably limited and limpets must rely on grazing, although their relatively small radula and stomach volume may limit the feasibility of this feeding mechanism (Chapter 6). The dominance of *L. fucensis* in senescent tubeworm bushes (Tsurumi & Tunnicliffe 2003) is probably attributable to their abundance in these same locations when fluids were active. Senescing habitats hosted male biased populations, presumably because these populations persist in low vent flux where the mortality of females is relatively higher than that of males. In addition, the poor tissue condition and gonad fullness scores indicate that, as in far-vent locations, reproduction by *L. fucensis* at waning vents is minimal.

Limitations to *Lepetodrilus fucensis*

There are many possible factors that might limit *Lepetodrilus fucensis*. For

example, the condition indices of limpets in peripheral locations reported herein suggest that sulphide availability may be important to survivorship. Predation may also limit the distributions of protoconch-stages (see Pechenik et al. 2004), thus explaining the absence of *L. fucensis* protoconchs from in-vent locations where grazer densities were high. However, the mechanism limiting protoconch-stages from in-vent locations is unknown. Predation by deep-sea species may also restrict the limpet, as its shells were found in the gut contents of a deep-sea octopus observed foraging at a Juan de Fuca vent (Voight 2000).

In addition, dissections of *Lepetodrilus fucensis* revealed infestations of a copepod that may be related to a parasitic family (Huys 2002) (Figure 7.4). The copepod formed branching structures into the major aortas on the ventral surface of the mantle roof and was positioned over ~25% of the gill. Up to 20% of animals investigated from locations in active vent flow hosted this copepod. Thus, the copepod's density can probably exceed 20 000 individuals m^{-2} based on the densities of its host (Bates et al. 2005). Although the impact of this copepod on the reproduction and food acquisition of the limpet has not been quantified, my observations of the gill tissue suggest that the lamellae in contact with the copepod are tattered or absent, thus its presence may limit suspension feeding, the symbiosis and gas exchange by *L. fucensis*, in addition to posing a physical obstruction to fluid flow across the gill.

Directions for future research

Experiments. One of the most important achievements of my work was to discover that gastropods brought up to the surface, placed in an experimental apparatus and then re-positioned on the seafloor, exhibit apparently normal behaviour. This

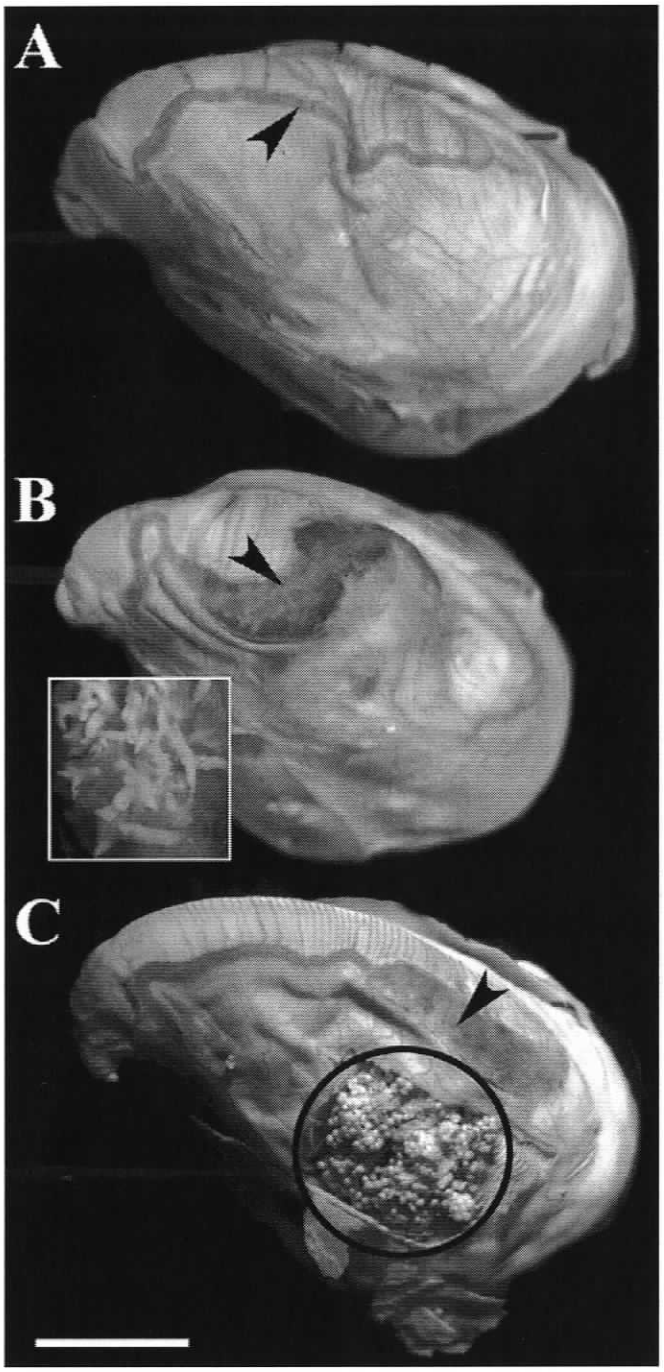

Figure 7.4
Light micrographs showing the dorsal surface of the mantle in critical point dried *Lepetodrilus fucensis* with their shells removed. (A) Uninfected specimen: the arrow shows a major aorta running through the mantle tissue. (B & C) Infected specimens: the arrows show the infected portion of the aorta where 'rootlet systems' are visible (inset). The mantle was removed to reveal densely packed copepod larval nauplii. Scale bar = 2 cm.

protocol greatly expands the realm of experimental possibility and allowed me to test species-specific habitat preferences *in situ*. Further research relating other abiotic and biotic factors to habitat selection by vent fauna may benefit by using similar experimental designs. However, collection depth determined animal activity. Gastropods from depths less than 1600 m were active almost immediately when placed in experimental pressure vessels shipboard and were active after longer durations (1-3 days) when returned to the seafloor. In comparison, gastropods from depths greater than 2000 m showed high morbundity in shipboard experimental vessels and animals from greater depths may not be ideal candidates for behavioural experiments.

Species patterns. Studies aiming to determine if the variability inherent to higher temperature vent flows (for example, erratic temperature, pH, oxygen and/or sulphide fluctuations (Johnson et al. 1988)), limit the physiological capabilities of these three gastropods might help explain why they avoid higher temperature flows (Figure 7.1). For example, behavioural experiments that present fluctuating gradients may be more representative of *in situ* conditions than stable gradients.

The habitat and thermal preferences of *Lepetodrilus fucensis* and *Depressigyra globulus* also did not explain why animals were common near-vent or why *Provanna variabilis* selects far-vent locations (Figure 7.1). Clearly, factors other than temperature must also influence the abundance patterns of these three species. For example, within or between species competition for habitats adjacent to vents may force the use of more distant habitats by *L. fucensis* and *D. globulus* (Figure 7.1) and studies that test for density-dependent habitat selection are an important next step. In addition, *D. globulus* reached high densities (up to 90 000 individuals m^{-2}) at the same locations where *L.*

fucensis were most abundant (Bates et al. 2005) and the characterization of positive interactions between these two species may refine our understanding of their abundance patterns. For example, the presence of *L. fucensis* in active flows may facilitate grazing by *D. globulus* because they are mobile and fit interstitially between limpet shells.

I found similar abundances of *Lepetodrilus fucensis* and *Depressigyra globulus* >1 mm in shell length in three collections from Southern Explorer (Appendix 2.3). *D. globulus* abundances <1 mm were also greater than similar sized *L. fucensis* (Appendix 2.3). Hence, the abundance patterns of these two species on the Explorer Ridge may prove to be different than the Juan de Fuca Ridge where *L. fucensis* densities were an order of magnitude higher than *D. globulus* in both small and large size classes. As mentioned above ('Limitations to *Lepetodrilus fucensis*'), factors such as sulphide availability and predation may pose limitations to limpet populations. Sulphide to heat ratio may also restrict the limpet (Marcus 2003). Another curiousity was the absence of gastropod recruit-stages from four far-vent locations (Axial = 1 vent; Explorer = 3 vents) at the base of chimneys (Appendix 2.3); perhaps settlement is restricted at chimneys by factors such as fluid flow patterns.

Juvenile biology. The survivorship of juvenile intertidal gastropods commonly relates to differential abiotic and biotic parameters among microhabitats (Montiel et al. 2005, Pechenik et al. 2002). However, factors governing the survivorship of *Lepetodrilus fucensis* juveniles (greater than ~0.5 mm) in different fluid fluxes has not been quantified, but is presumably influenced by different factors. For example, abiotic variability may pose greater limits on in-vent juveniles as small gastropods tend to be less able to withstand abiotic variability than larger stages (Vermeij 1972). Competition may also

limit recruitment in-vent where gastropod densities are an order of magnitude higher than far-vent (Bates et al. 2005). In comparison, peripheral juveniles may be limited by food availability and predation by invading deep-sea species.

Symbiosis. Studies aiming to first characterize the transmission of the symbiont and then locate the *Lepetodrilus fucensis* symbiont in the environment are critical to building on our understanding of this symbiosis. The epibiotic nature of the *L. fucensis* symbiosis suggests that the bacteria are probably acquired from the environment. If so, the presence of the symbiosis in newly settled *Lepetodrilus fucensis* indicates that the symbiosis may be established at the time of settlement in far-vent locations. Determining the distribution of the gamma-Proteobacteria in the free living bacterial community may also reveal limitations to the symbiosis. By comparison, the *Riftia pachyptila* symbiont is common environmentally in multiple habitats and was found on experimental plates and substrates from sites that range from on- to off-axis, suggesting that acquisition of the symbiont by juvenile tubeworms may not be limited by its availability (T. Harmer, pers. comm.).

Determining whether limpets at different sizes gain nutrition by endocytosing and digesting their symbionts in lysosomes (de Burgh & Singla 1984) and/or by absorbing bacterial produced fixed carbon through their gill epithelium is also open to further study. If the symbionts contribute to their host's nutrition via these alternate pathways, the symbiosis may contribute the bulk of the limpet's nutrition in conditions where the bacteria are thriving.

Understanding what drives the evolution of intracellular symbioses is one important goal of symbiosis research. *Lepetodrilus fucensis* exhibits a preliminary

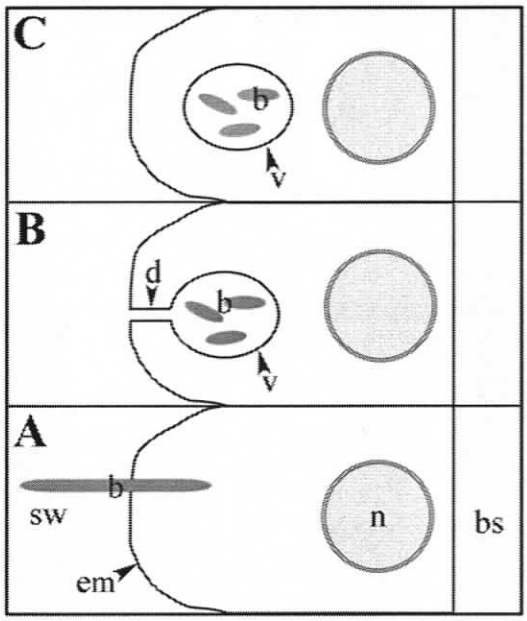

Figure 7.5
Diagram depicting proposed evolutionary trend from extra- to intracellular in the gill morphology of bivalve and gastropod mollusc symbioses. The *Lepetodrilus*-hosted bacterial (b) symbiosis exhibits a novel morphotype where the bacteria are partially embedded in the epithelial membrane (em) and extend as filaments into the seawater (sw) circulating across the gill (A); this association may represent a preliminary evolutionary stage. (B) Provannid gastropods host bacteria in apical vacuoles (v) retain ducts (d) to the seawater. (C) Intracellular symbioses, where bacteria are completely enclosed in a vacular membrane, are hosted by several clam families and mussels. Nucleus (n), blood space (bs).

stage in the progression of extra- to intracellular symbioses (Figure 7.5), hence ecological studies of *L. fucensis* may help to isolate factors that select for integration between host and symbiont. For example, symbiont-hosting invertebrates tend to select optimal habitats for the growth of their symbionts and as a result, exhibit constrained habitat selection (Hessler & Smithey 1985). Therefore, *L. fucensis* may prefer warm vent flows to foster a prolific symbiont populations, indeed, animals with abundant symbionts exhibited higher reproductive potential than those with sparsely populated gills. This habitat preference may drive the success of the symbiosis and select for further integration between partners.

Summary

Habitat selection by three Juan de Fuca Ridge hydrothermal vent gastropods is governed by their responses to the thermal environment, indicating that abiotic conditions are a key factor determining their abundance patterns. Small-scale environmental gradients and temporal trends in vent flux also influenced the persistence of the gill-hosted bacterial symbiosis and the biology of *Lepetodrilus fucensis*, the dominant limpet-shaped gastropod. Vent flows between 5 and 15°C are optimal habitats; *Lepetodrilus fucensis* clearly preferred these locations in experiments where they occurred at maximum *in situ* densities, and exhibited high tissue condition and reproductive potential. Although *L. fucensis* were also abundant in senescent tubeworms bushes and peripherally (Marcus & Tunnicliffe 2002, Tsurumi & Tunnicliffe 2003, Bates et al. 2005), adults in these habitats were unhealthy, suffered high mortality and consequently, may contribute minimally to the larval pool. Therefore, the high relative abundance of adult *L. fucensis* at senescent sites is a relic of its success in active venting. The high

abundances of limpets in peripheral locations are because these areas sustain high abundances of post larval recruits and appear to offer small distances from in-vent locations (~50 cm) for their recruitment into the larger size classes.

The flexible feeding strategy of *Lepetodrilus fucensis* diversifies food acquisition in the highly ephemeral vent environment, thereby maximizing survivorship and reductive output: phagocytosis of the bacteria by the epithelial membrane followed by lysosomal digestion (as suggested by de Burgh & Singla 1984), symbiont farming and suspension feeding in active vent flows sustain healthy adult populations, while grazing is the likely feeding mechanism used by peripheral animals. The symbiosis between *L. fucensis* and its epibiotic gamma-Proteobacteria was ubiquitous, persistent and highly specific across broad geographic range and may represent a key innovation. The habitat preferences and stacking behaviour of *L. fucensis* provide the gill symbionts with access to venting fluids for thiooautotrophy. In addition, the gill lamellar morphology exhibited modifications that increase the area available to symbionts for attachment and maximize fluid delivery across the gill.

In summary, the success of *Lepetodrilus fucensis*, as indicated by its high density, is at least partly attributable to its innovative ability to gain nutrition from suspension feeding and its symbionts while maintaining sessile stacks in active vent flows where its survivorship and reproductive output are highest.

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