GUT-ASSOCIATED MICROBIAL SYMBIONTS OF THE MARSH FIDDLER CRAB, *UCA PUGNAX*

By

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ABSTRACT

Digestive associations between marine invertebrates and resident (attached) microbial communities may play a critical role in host physiology and involve previously unidentified microbial species. The overarching goal of this thesis was to characterize the ecology and genetic diversity of resident gut microbes to advance our understanding of their interactions with their host, the marsh fiddler crab, *Uca pugnax.* Furthermore, we assessed whether microbes benefit the host by contributing extracellular enzymes along the digestive tract. This is the first report of the eccrinid protists, *Enteromyces callianassae* and *Enterobryus* sp., inhabiting *U. pugnax*. The greatest abundances of both bacteria and protists were documented in the host stomach and hindgut. For these sections, we have described morphologies, measured abundances and characterized the genetic diversity (bacteria) of resident microbes. Presence and abundance of the Eccrinales protists depends on host molt stage as all eccrinid biomass is shed with the host's molt. In intermolt crabs, both bacterial and protozoan symbionts appear to be consistent features of the stomach and hindgut. Furthermore, bacterial diversity patterns seem to be comparable among individuals and over time, as assessed by denaturing gradient gel electrophoresis (DGGE). Community composition, however, does differ between stomach and hindgut populations, as resolved by DGGE and clone libraries of the 16S rRNA gene. Many recovered clones were most closely related to other symbiotic or gut-associated bacteria. Few identified clones, however, shared more than 95% 16S rRNA gene sequence similarity with their nearest known relatives, indicating that this environment may support novel bacterial phylotypes. An exception was the *U. pugnax* hindgut phylotype most closely related to a phylotype identified from hindguts of the detritivorous shrimp *Neotrypaea californiensis*. This finding suggests that detritivorous crustacean hindguts may provide an ecological niche for specific bacterial phylotypes. Functionally, resident bacteria, particularly in the hindgut, may contribute to total enzyme activity in the gut of their host.

TABLE OF CONTENTS

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Chapter 1: INTRODUCTION

Over the past few decades, the scientific community has begun to recognize how individual organisms are rarely solitary entities, but often hosts of veritable ecosystems of microbes. While these microbial associates sometimes compete with or harm their macroscopic hosts, they frequently contribute to the host's fitness through various diverse and complex relationships. Nutritional interactions are particularly widespread and highly developed, especially among herbivorous and detritivorous hosts (Mackie 2002). Although there has been considerable research into nutritional symbioses in terrestrial invertebrates (i.e. termites), little is known about the presence or activities of gut microbes in marine invertebrates, specifically deposit-feeding marine invertebrates. These types of associations have the potential to impact host physiology as well as environmental microbiology and geochemistry and therefore demand further attention.

Gut microbial communities in marine invertebrates have been predicted to benefit the host in a mutualistic association, although it is possible that they are commensal or pathogenic (Harris 1993). Gut microbes may contribute to the host's fitness by: 1) enzymatically increasing the pool of organic matter accessible to digestion, 2) fermenting organic matter and producing absorbable concentrations of short chain fatty acids (SCFAs), 3) supplying vitamins or essential amino acids, 4) favorably altering the geochemistry of the gut environment, 5) being directly digested via grazing, and/or 6) preventing the proliferation of pathogenic bacteria (Harris 1993).

In the environment, microbes can be limited in their mobility, vulnerable to changing environmental conditions, and dependent on episodic input of substrates. By associating with the digestive tract of active deposit feeders these limitations could be successfully overcome. Specifically, the host may aid resident gut microbes by providing: 1) a supply of substrates in the form of the

host's digestive products, 2) a mixed environment that increases diffusive exchange, 3) a relatively constant environment, and/or 4) protection from (other) predators (Plante et al. 1990). Alternatively, microbes may be commensals, thriving on the host's un-absorbed digestive products without contributing to the host's fitness. Finally, microbes may be pathogenic if they cause disease or absorb substrates at the cost of the host's condition. Although some or all of these interactions have been predicted, no consistent patterns of host-microbiota interaction have emerged among marine invertebrates (Harris 1993).

Fiddler crab significance

Much of the research on the gut microbiota of marine invertebrates has focused on crustaceans (Deming et al. 1981; Dempsey and Kitting 1987; Harris 1992; Lau et al. 2002; Pinn et al. 1997; Sochard et al. 1979). Marine crustaceans are especially interesting subjects by way of analogy with terrestrial arthropods, many of which host complex and abundant gut microbial communities (Breznak 1982; Dillon and Charnley 2002). Among crustaceans, gut microbes have been identified in multiple species, but few studies have thoroughly described the association in terms of colonization sites, morphologies, frequency of occurrence, microbial densities, and genetic diversity. There is a need for complete descriptions of such associations to better understand the nature of the interactions and potential environmental impacts. Fiddler crabs are an ideal group for a thorough study because of their widespread distribution (Crane 1975), concentrated activity in environmentally important habitats (Teal 1958), knowledge of their feeding behaviors (Miller 1961; Robertson and Newell 1982a) and resultant environmental impacts (Hoffman et al. 1984; Shanholtzer 1973). As a group, fiddler crabs have a global distribution and are active in coastal marshes and mangroves (Crane 1975), both highly productive and sensitive ecosystems. The genus *Uca* includes selective, surface deposit feeders that influence the geochemistry and biotic composition of marsh

environments (Montague 1982; Reinsel 1995). These crabs are present in high densities, often over 200 individuals m^{-2} (Bertness 1985), and are found in most marshes along the U.S. Atlantic coastline from Cape Cod to Florida (Teal 1958).

Fiddler crab feeding and digestion

Fiddler crabs preferentially ingest the smaller, lighter fraction of the sediment by exploiting a flotation feeding mechanism. Water from the gill chambers is used, in coordination with a scrubbing motion, to suspend organic matter associated with the ingested substrate (Miller 1961). Benthic unicellular algae, bacteria, fungi, protozoa and detritus are separated from the mineral fraction on the basis of size and density (Miller 1961; Robertson and Newell 1982b).

Fiddler crabs, and crustaceans in general, have a highly specialized digestive tract and digestive process (Icely and Nott 1992). A chitin-lined foregut (esophagus, cardiac and pyloric stomachs) and hindgut typify crustaceans (refer to Chapter 2, Fig. 2.1, pg. 23). Selected material is passed into the cardiac stomach where the chitinous 'teeth' of the gastric mill masticate the gut contents and enzymes from the hepatopancreas initiate chemical breakdown. The pyloric stomach regulates the movement of solids and filtrates between the foregut and midgut. Very fine particles (< 2 μm) are passed into the hepatopancreas for intracellular and extracellular digestion (Brunet et al. 1994). Larger particles are passed directly in the midgut. At the junction of the hepatopancreas and midgut, glands secrete a peritrophic membrane around the food bolus (Bignell 1984). This membrane is made of chitin microfibrils embedded in a matrix of protein and glucosaminoglycans and has a pore size from 6-100's of nanometers (Jarial and Engstrom 1997). Peritrophic membranes have been variously predicted to function in: 1) preventing microbial attachment, 2) protecting the midgut from abrasion, and/or 3) concentrating digestive products between the membrane and midgut lining (Bignell 1984; Tellam et al. 1999; Terra 2001). The midgut receives

membrane-bound material from the hepatopancreas and pyloric stomach and functions to adsorb residual digestive products. Upon passage into the hindgut, the peritrophic membrane is thought to be eroded by chitinous projections and muscular contractions (Bignell 1984). The chitin-lined hingut is likely involved in ion regulation, water re-absorption and, possibly, uptake of small nutrients such as amino acids and SCFAs (Dall and Moriarty 1983; Hogan et al. 1985).

Theoretical basis of association

Mutualistic associations between attached microbes and detritivorous invertebrate hosts have been predicted to occur in particular gut sections (Plante et al. 1990). Based on cost-benefit analysis, resident microbes are expected to colonize the hindgut. Dade et al. (1990) suggested that animals should optimize digestive efficiency by processing food until returns were reduced, then ingest fresh material. Consequently, material in the hindgut is expected to include unabsorbed digestive products and undigested material. Microbes may exploit this hindgut residue and avoid exposure to enzymatic digestion. If hindgut microbes supply beneficial enzymes or products that can be utilized by the host, these microbial associates may form a mutualism with their host.

In most detritivores, the foregut (stomach) and midgut are expected to be sites of competition between host and microbes (Plante et al. 1990). This competition is based, in part, on the theory that attached microbes would occlude critical absorptive sites along the gut. We expect that competitive interactions dominate in the non-chitin lined midgut and hepatopancreas. However, in crustaceans, the chitin-lined stomach is not expected to be absorptive (Brunet et al. 1994). This characteristic may influence the nature of interactions between attached microbes and crustaceans. In the stomach, concentrations of digestive products are high, however so are host-enzyme activities, which may potentially damage microbial cells. If microbes can survive in these conditions, and if they release valuable enzymes or other products, they may form a mutualistic

association in the stomach. Yet resident microbes may compete with the host if they are only absorbing nutrients.

Possible environmental impact

A poorly understood role of gut microbes is their capacity to influence environmental microbiology and geochemistry. Because marine deposit feeders ingest, selectively process and egest microbes, a digestive association should influence the microbiology of the marine sediments (Plante and Jumars 1993). Resident microbes may be transferred from gut populations to egested material, seeding marine sediments with a particular microbial community. This transfer process has been studied in the terrestrial arthropod *Folsomia candida*, and shown to influence microbiology of the feces (Thimm et al. 1998). Furthermore, if resident bacterial activities modify the geochemistry of material passing through the crab gut, this effect may factor into salt-marsh geochemical cycles.

Goals of this study

The general goal of this thesis was to characterize the ecology and genetic diversity of resident gut microbes in order to advance our understanding of interactions between microbial associates and their host, the marsh fiddler crab, *Uca pugnax*. Initially we intended to study only the interaction between *U. pugnax* and its bacterial associates. However, early investigations revealed the presence of eukaryotic gut residents, the Eccrinales. This group has been reported in numerous species of marine and freshwater arthropods, yet little is known about their physiology or ecology. We sought to characterize the presence, abundance and species identity of these resident eukaryotic microbes, as well as that of the bacterial community. The presence of bacterial gut microbiota stimulated questions on the extent of bacterial diversity, as well as consistency of this diversity among individuals and over time. Finally, we

examined the relationship between dissolved extracellular enzyme activity and microbial abundances.

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Chapter 2: Eccrinales symbionts of the marsh fiddler crab, *Uca pugnax*

ABSTRACT

We have identified and studied two species of protists (Class Mesomycetozoa, Order Eccrinales) that colonize digestive tracts of the marsh fiddler crab, *Uca pugnax*. *Enteromyces callianassae* colonizes the cardiac stomach and *Enterobryus* sp. populates the hindgut. Both *E. callianassae* and *Enterobryus* sp. are consistent features of the crab gut: *E. callianassae* was present in > 50% of crabs in the intermolt phase and *Enterobryus* sp. was present in > 90%. Extent of colonization increases with time since last molt of the host. Within individual hosts, total lengths of thalli for *E. callianassae* versus *Enterobryus* sp. are directly correlated. The hindgut species, *Enterobryus* sp., is present in two morphological forms: a long, spiraling form ($\leq$ 4.0 mm in length) in the anterior hindgut and a typically shorter form (0.1-0.2 mm in length), that develop as bushy clusters towards the posterior hindgut. Phylogenetic analysis of the 18S rRNA gene of *E. callianassae* confirms its grouping as a protist in the class Mesomycetozoa, rather than as a fungal species of the class Trichomycetes.

INTRODUCTION

The order Eccrinales comprises a diverse group of organisms that are symbionts associated with digestive tracts of marine, freshwater, and terrestrial mandibulate arthropods. Eccrinales are typically found in detritivorous, algivorous and omnivorous, mandibulate arthropods, belonging to the groups Crustacea, Insecta and Diplopoda. They have been documented in hosts from habitats as disparate as deep-sea hydrothermal vents (Van Dover and Lichtwardt 1986) to tropical streams (Lichtwardt and Williams 1990) and are known from locations around the globe. This group of organisms lives only in association with their hosts and in this context have developed complex life histories. The Eccrinales-arthropod symbiosis is thought to be ancient: congruence in timing of speciation suggests that they have co-evolved with their hosts for over the past 200 million years (Lichtwardt 1986).

Members of the order Eccrinales were first described by Leidy in 1848, and were classified as plants living within digestive tracts of several species of

arthropods (Leidy 1849; Leidy 1853a). Until very recently, the accepted phylogeny classified eccrinids as an order within the fungal class Trichomycetes (Misra 1998), and we initially investigated them as such. Molecular analysis of the 18S rRNA gene has since revealed that they are more closely related to a class of protists, the Mesomycetozoa (Cafaro 2003). This newly established phylogenetic group comprises predominantly symbiotic organisms, including fish pathogens and crustacean gut microbiota, as well as saprotrophic microbes (Mendoza et al. 2002). This class includes many species previously thought to have other phylogenetic affliliations, but recent results from molecular sequencing strongly support the Mesomycetozoa as monophyletic.

The order Eccrinales is distinguished by unbranched, non-septate, coenocytic thalli in which growth is subapical. Asexual reproduction is accomplished by spores produced in terminal sporangia. Six types of spore morphologies have been described (Lichtwardt 1954), and among these morphologies are two functional categories: primary, uninucleate sporgangiospores that are passed into the environment to colonize other hosts and secondary, multinucleate sporangiospores that develop within the same host. Spore producing and recolonizing abilities are especially important for success of these species, because their hosts regularly shed their carapaces and all associated eccrinid thalli with every molt. A better understanding of the physiology and ecological significance of this group has been limited by the failure to culture successfully any representatives (Lichtwardt 1986).

Even though Eccrinales are known to colonize many host species around the globe, relatively little is know about their ecology and their interactions with their hosts. Eccrinales are thought to be commensal symbionts (Moss 1979) that obtain nutrition from the material passing through the host gut, but not to the detriment of the host. However, there are no definitive case studies investigating the nature of their relationships with their hosts (but see Kimura et al. 2002). Some, or all, eccrinids could be parasitic if they compete with their hosts for

absorption of nutrients within the digestive tract. Alternatively, eccrinids could benefit their hosts if they confer a nutritional advantage via enzyme or vitamin contribution.

Eccrinales species have been reported in numerous decapod crustaceans, including the fiddler crab *Uca pugilator* (Mattson 1988; Tuzet and Manier 1962; Wagner-Merner 1979). In marine crustaceans, these symbionts are most commonly found attached to the chitinous hindgut lining, but are also found on surfaces of the chitin-lined stomach (both cardiac and pyloric sections). There are no reports of eccrinid species adhering to the midgut or the hepatopancreas, both of which are lined with tissue rather than chitin.

Detritivorous and burrowing crustaceans such as *Uca* spp. have significant influence on the biogeochemistry and hydrology of coastal ecosystems (Bertness 1985; Howes et al. 1981; Montague 1982). They are important ecosystem engineers and, as a population, are constantly reworking the top few centimeters of marsh substrate (Miller 1961). Any impact that resident eccrinids might have on host physiology or on the geochemical composition of material passing through the gut could have subsequent effects on the entire marsh ecosystem.

Too little is known, however, about the natural history of Eccrinales symbionts to define their interactions with their hosts or constrain speculations about their impact on marsh ecosystems. The objective of the present study is to describe and quantify key aspects of Eccrinales life histories and interactions with an arthropod host. We have chosen to study the Eccrinales of the fiddler crab *Uca pugnax* because this host is important ecologically and typically harbors at least one species of Eccrinales. Our characterization of distributions of Eccrinales species within the gut and their recolonization after host molting is designed to identify potential interactions with the host. Specific questions that we address are: What species of Eccrinales are present and where; what proportion of crabs is colonized; how does abundance of Eccrinales correlate

with host size and host molt stage; and, is there any correlation between abundances of two Eccrinales species within individual hosts?

METHODS

Crab collection

Marsh fiddler crabs, *Uca pugnax*, were collected regularly from an intertidal salt marsh in Barnstable Harbor, Massachusetts, USA (41°42'31 N, 70°18'17 W) from July, 2001 to September, 2003. To avoid possible seasonal effects, only crabs collected during summer months (June-September) were used in the present analyses. Crabs were kept in cooled containers (~15°C) and brought to the laboratory within 2 h after collection. Gender, molt stage (see below), and carapace width and length of each specimen were recorded. Stomach, hepatopancreas, midgut and hindgut sections were removed with sterile dissecting tools, and each section was examined to determine the presence and morphotype of Eccrinales symbionts. Adult green crabs, *Carcinus maenus*, (n = 5) were collected from the same location in August 2002 and were investigated in the same manner.

Electron Microscopy

Immediately after dissection, hindgut, midgut, hepatopancreas and both the cardiac and pyloric stomachs of four crabs were fixed in 3% gluteraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 3 h. Samples were washed three times in sodium cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h, and washed another three times before dehydrating in a series of ethanol dilutions. Samples were critical-point dried using carbon dioxide as the transitional fluid, mounted on aluminum stubs, coated with gold palladium and examined in a JEOL JSM-840 scanning electron microscope (SEM).

Eccrinales Identification

We observed two distinct morphotypes of Eccrinales: one which appeared to be *E. callianassae,* in the host's cardiac stomach, and the other appeared to belong to the genus *Enterobryus*, along the hindgut. Species identification was based on morphology. *Enteromyces callianassae* is characterized by dimorphic, unbranched thalli that form tufts with a common holdfast, and produces both uni- and multinucleate sporangiospores (Misra and Lichtwardt 2000). *Enterobryus* sp. has unbranched coenocytic thalli and does not form tufts, but individually attached thalli (Misra and Lichtwardt, 2000). We refer to this symbiont as *Enterobryus* sp. because it lacks sufficient morphological characters to assign it unambiguously to a particular species. Althought two morphotypes of *Enterobryus* sp. were found (long and short forms), they were treated as a single species in this study.

Eccrinales Length

Each section of the digestive tract was stained with lactophenol cotton blue (0.5% v/v) in 0.1 M sterile phosphate buffer (Lichtwardt 1954). To improve visualization, *Enteromyces callianassae* tufts were removed from the cardiac stomach and the chitinous lining of the hindgut with attached *Enterobryus* sp. was separated from host tissue before staining. Wet mounts were photographed immediately after staining with a Sony 120X Digital Camera attached via a phototube to a Zeiss Axiostar plus (either 250, 500, or 1,000X magnification). Images were mosaicked in Adobe Photoshop and the lengths and widths of each thallus were measured with a Matlab-based image analysis program (Digitizer 0.99). The presence or absence of both species, the number of tufts of *E. callianassae* and the molt stage of 66 individuals were determined. For 12 crabs, total thallus length of both species was measured. Additional measurements were made of total length of both eccrinid species, but from different individual

crabs [*E. callianassae* (n = 3, *U. pugax* hosts) *Enterobryus* sp. (n = 5, *U. pugax* hosts)]. To compare total length of the two forms of *Enterobryus* sp., we measured lengths of anterior and posterior *Enterobryus* sp. forms in six crab hosts. A comparison of the total length of *E. callianassae* and *Enterobryus* sp. was made on a set of intermolt and premolt crabs from a single collection date (n = 12), in which both stomach and hindgut eccrinid lengths were measured. This approach allowed us to determine if there was a correlation between total lengths of the two species within individual hosts. Furthermore, by comparing only crabs from a single collection date we eliminate any possible variation due to seasonality or daily, weekly, or monthly changes.

Molt Stage Analysis

Molt stage of individual crabs was determined by examining characteristics of pleopod and abdominal setae as described in Vigh and Fingerman (1985). Four periods of molt staging were identified: postmolt, intermolt, premolt and ecdysis (Drach 1939). Setae were removed from crabs with fine dissecting forceps, immediately wet mounted on slides in 0.1 M NaCl buffer and photographed with a Sony 120X Digital Camera attached via a phototube to a Zeiss Axiostar plus (500 or 1,000X magnification). Molt stage was determined for all crabs dissected and all crabs used for Eccrinales length measurements.

DNA extraction

DNA was extracted from isolated Eccrinales thalli in 1X CTAB (hexadecyltrimethyl-ammonium bromide; Sigma-Aldrich) buffer. Samples had been frozen at –80°C prior to extraction. Samples were subjected to manual grinding and at least three freeze-thaw cycles (liquid nitrogen and 65°C water bath) before adding one volume of chloroform, vortexing and centrifuging (13,000 rpm; 15 min). Supernatants were removed and precipitated in one volume of

100% isopropanol (-20°C; 16 h) before centrifuging (13,000 rpm; 15 min) and washing the resulting pellet twice with 70% ethanol. Isolated DNA was resuspended in 35 µl sterile H_2O and kept at -20°C until use. DNA extraction attempts using standard extraction kits (DNeasy Tissue Kit, Qiagen and UltraCleanSoil DNA Kit, Mo Bio Laboratories, Inc.) were unsuccessful.

PCR amplification

Extracted DNA was amplified with universal fungal primers for the ribosomal 18S gene (NS1, NS2) (White et al. 1990). Fungal primers were used because the Eccrinales had been classified within the fungal class Trichomycetes. The PCR reaction mixture included: 200 µM of each dNTP, 10 mM of each primer, 10% of 10X buffer, 25 mM $MgCl_2$ and sterile, double-distilled water. Taq DNA polymerase (Promega Corp., Madison, Wisconsin) was added at 1 unit per 50 µl reaction. Amplifications were performed in an Eppendorf Mastercycler Gradient thermal cycler.

Phylogenetic analysis

Sequence data from *E. callianassae* were compared with 18S rRNA gene sequences from other Eccrinales species (Cafaro pers. comm.), as well as known representatives of the Mesomycetozoa and Fungi obtained from GenBank (Benson et al. 1997). Species belonging to the group Stramenopila were used as an outgroup because the Eccrinales were once thought to be a type of oomycete fungi within the Stramenopila. Even with multiple attempts, we were unable to amplify DNA successfully from *Enterobryus* sp., so only data from *E. callianassae* are presented. The symbiont sequence was checked by referring to predicted protist secondary structures available from the Comparative RNA Website (http://www.rna.icmb.utexas.edu/members). Sequences were aligned with Autoassembler sequence editor (Version 2.1) and phylogenetic trees were constructed with the aligned sequences using PAUP version 4.0b10 (Swofford

1993). All nucleotide positions that could be unambiguously aligned for all taxa were included in the analysis. Two large inserts (~100 bp) that did not exist in any other eccrinid or fungal species were removed before analysis. A consensus tree was constructed using parsimony analysis and bootstrapping of 1000 replicates.

Statistical Analysis

Differences in total thallus length among crab molt stages were tested with a two-way ANOVA, with molt stage and eccrinid species as treatments. Differences in pH values among crab gut sections were tested using a one-way ANOVA. The Bonferroni multiple comparisons test was used for post hoc, pairwise comparisons. All statistical analyses were performed with Systat, Version 10 (SPSS, Inc.).

RESULTS

Of 45 intermolt crabs investigated, 26 had *Enteromyces callianassae* in their cardiac stomachs and 41 had *Enterobryus* sp. in their hindguts; thus 57.8% and 91.1% were respectively colonized (Table 2.1). All six premolt crabs

Table 2.1 Comparison of host species and presence of *Enteromyces callinanassae* and *Enterobryus* sp.

Host species Gut section	Collection site	Proportion with *E. callianassae*	Average # of tufts per gut (SD)	Range of # of tufts	Proportion with *Enterobryus* sp.	Average # of thalli (SD)
Uca pugnax[1]	Barnstable, MA					
cardiac stomach		57.8% (n=45)	2.0 (2.1)	0-7	0%	0
hindgut		0%	0	0	91.1% (n=45)	227 (86)
Nihonotrypaea harmandi[2]	Kyushu, Japan					
foregut		51.4% (n=31)	8.4 (11.4)	1-36 (n=22)	0	0
Upogebia affinus[3]	Beaufort, NC					
foregut		16.2% (n=37)	nd	nd	0	0
hindgut		0%	0	0	0% (n=37)	nd

[1]This study, only intermolt crab results included in this table

[2] Kimura et al. 2002

[3] McCloskey and Caldwell 1965

nd = not determined

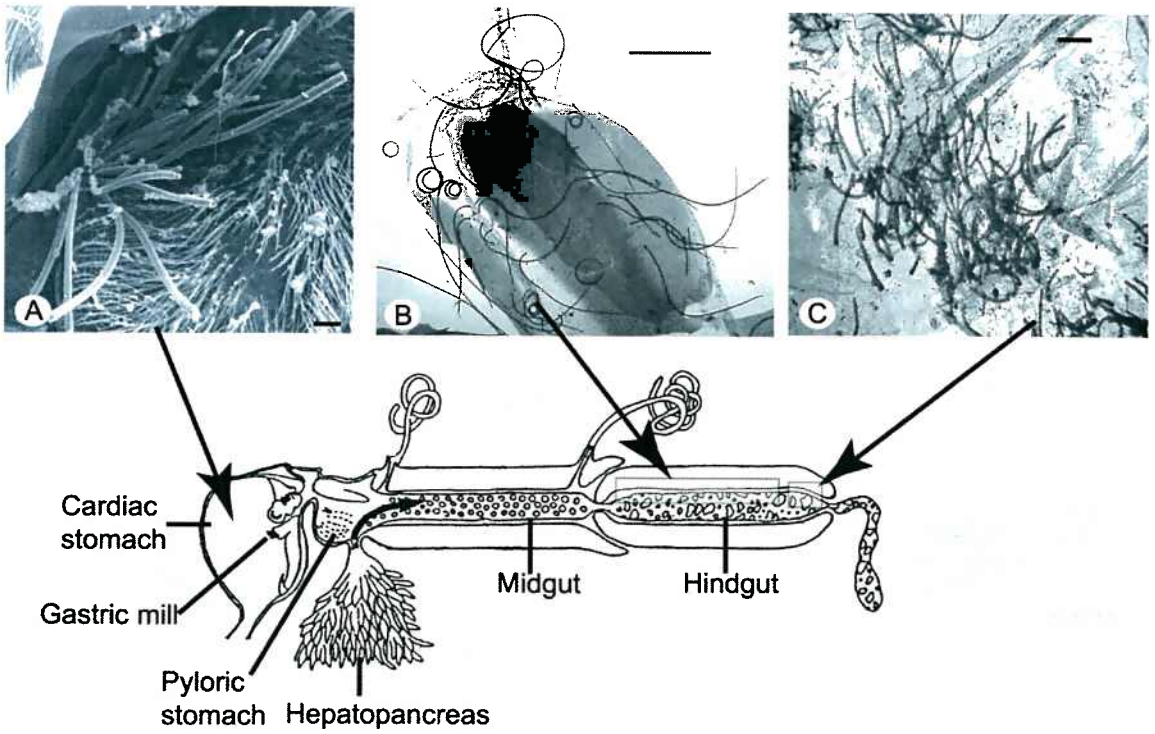

Figure 2.1 Schematic of generalized crustacean digestive tract (modified from Hopkin and Nott 1980). Grey boxes indicate relative regions of the hindgut that are populated by each form of *Enterobryus* sp. A. SEM image of a single tuft of *E. callianassae* attached to the chitinous lining of the cardiac stomach (Scale bar = 100 μm). B. Light microscopy image of anterior hindgut lining and attached long, spiraling form of *Enterobryus* sp. (Scale bar = 1 mm) C. Light microscopy image of posterior hindgut lining with attached short, bushy form of *Enterobryus* sp. (Scale bar = 100 μm).

collected were colonized by both *E. callianassae* in their cardiac stomachs and by *Enterobryus* sp. along their hindguts. None of the 15 postmolt crabs had eccrinid thalli in any gut section. Furthermore, no Eccrinales spores or thalli were observed in the midgut or in the hepatopancreas of any crab. A single-holdfast morphotype of *E. callianassae* was found in the pyloric stomach of two individuals, but will not be addressed further in this paper. The presence of eccrinid symbionts did not cause any obvious harm to the host in terms of maximal size or activity level, even with extensive colonization. None of the

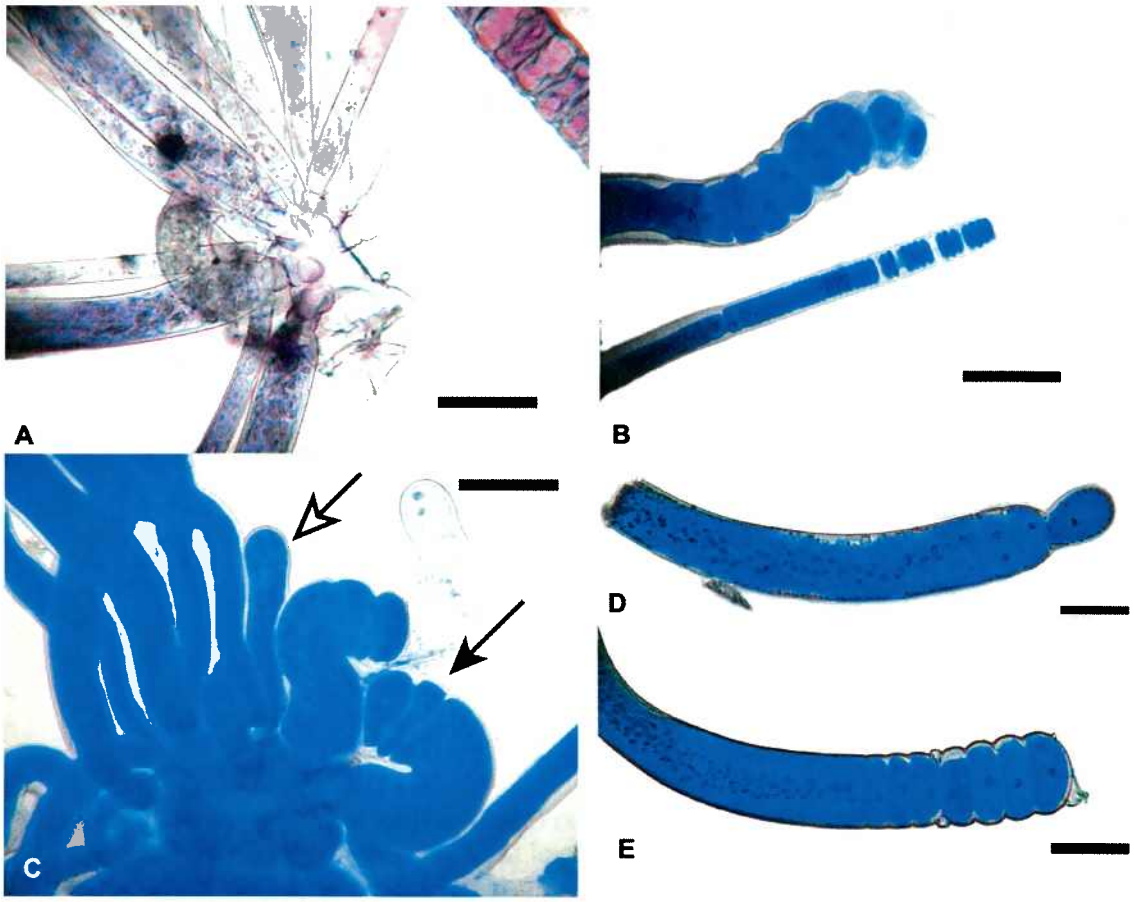

Figure 2.2 Images of *Enteromyces callianassae* from *Uca pugnax* cardiac stomach. All scale bars are 50 µm. A. *E. callianassae* holdfast, detached from the cardiac stomach. B. Comparison of uninucleate macro- and micro-thalli. C. Holdfast with mother thallus producing multinucleate spores (solid arrow) and immature, undifferentiated thalli with scattered nuclei (hollow arrow). D. Straight thallus with uni- and bi-nuceate spores with intact spore case. E. Thallus with bulging uninucleate spores without original spore case.

green crab, *Carcinus maenus,* individuals observed had any Eccrinales thalli or spores in the stomach, midgut, hepatopancreas or hindgut.

Enteromyces callianassae attaches to chitinous surfaces of the cardiac stomach and forms tufts of both macro- and micro thalli (Fig. 2.1A). Tufts adhered to the surface by means of a secreted, common holdfast (Fig. 2.2A). Macro-thalli averaged 0.046 mm wide (SD = 0.008), were up to 1.6 mm long, and averaged 1.01 mm long (SD = 0.4; n = 411). Micro-thalli were narrower but not

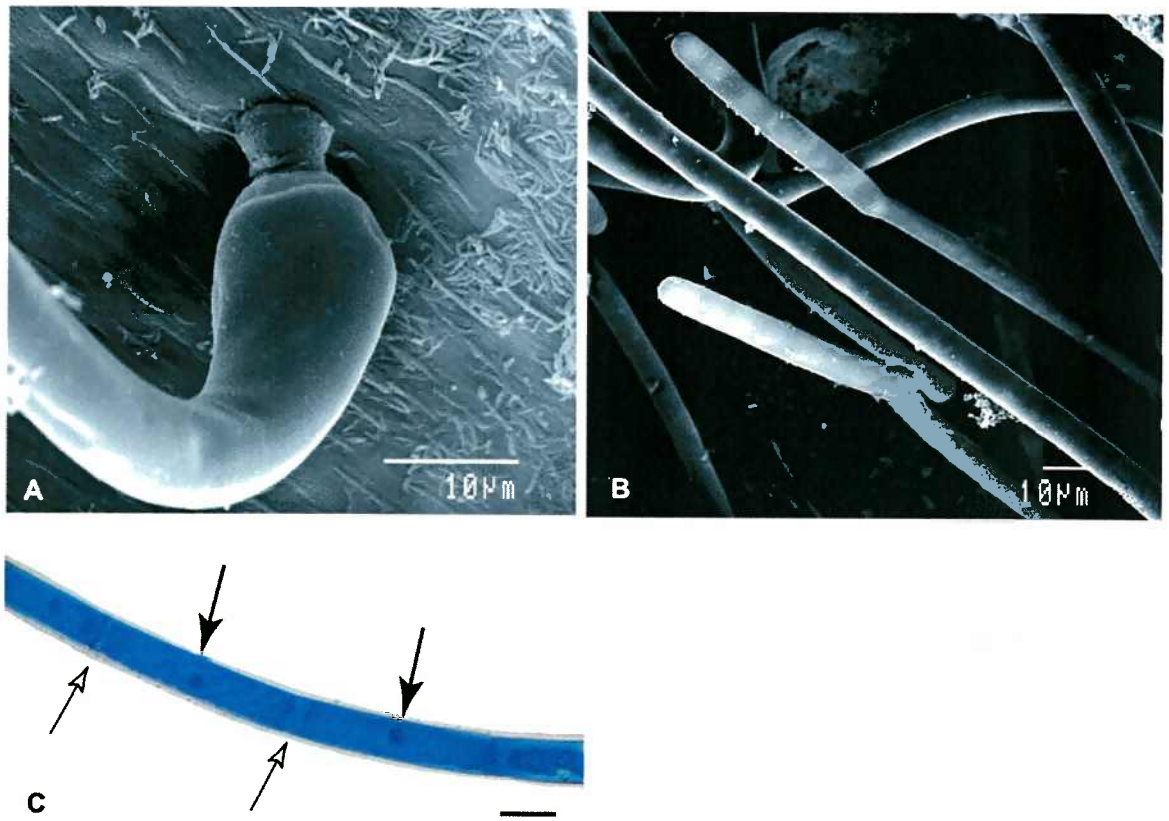

Figure 2.3 Images of *Enterobryus* sp. from *Uca pugnax* hindgut. A. SEM image of individual holdfast. B. SEM image of *Enterobryus* sp. thalli and terminal angled spore case. C. Light microscopy image of stained uninuclear spores from posterior form of *Enterobryus* sp. Solid arrows point to nuclei. Hollow arrows point to ends of a single spore (Scale bar = 10 µm).

necessarily shorter, averaging 0.014 mm wide (SD = 0.004), and 0.89 mm long (SD = 0.38; n = 218). Both macrothalli and microthalli produced uninucleate spores that were oval or discoidal (Fig. 2.2B). Multinucleate spores were formed by a curved 'mother thallus' (Fig. 2.2C). These spores re-attach to the original holdfast and allow multiple thalli to develop from a single holdfast (Hibbits 1978). They then develop into thalli with haphazardly arranged nuclei (Fig. 2.2C). As

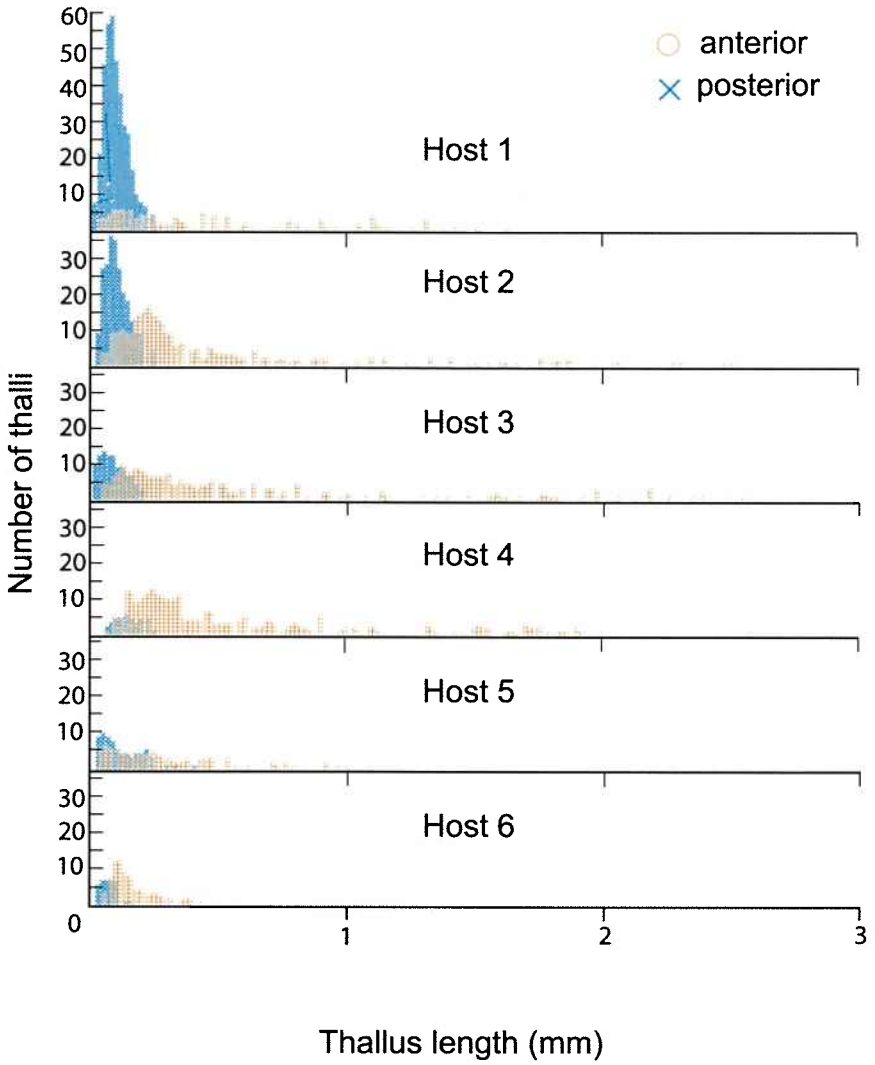

Figure 2.4 Dot density plot of individual thallus lengths for anterior long form and posterior short, bushy form of *Enterobryus* sp. Each thallus measured is represented by a single symbol. All hosts (1-6) were in intermolt phase.

the thallus matures, nuclei assemble along the axis and uninucleate or occasionally binucleate spores are produced (Fig. 2.2D, 2.2E). The most distal spores tend to bulge out from the thallus wall (Fig. 2.2E), while proximal, and probably immature, spores are constrained by the thallus wall (Fig. 2.2D). Within an individual crab, tufts were in various stages of development, having anywhere

from four short, immature (undifferentiated) thalli to over 50 long, sporulating thalli. The average number of tufts in intermolt crabs was 2.0 (SD = 2.1) (Table 2.1) but a single cardiac stomach can be colonized by up to 20 well developed tufts (observed in a molt exuvium). For premolt crabs (n = 6) we found an average of 3.3 (SD = 1.2) tufts. Tufts were often observed attached to the anterior dorsal ridge in the cardiac stomach, but were occasionally observed attached to the gastric mill and seemed to be able to attach to most chitinous surfaces in the cardiac stomach. Tufts were not found in the esophagus or the pyloric stomach.

In crabs colonized by *Enterobryus* sp., thalli were observed along the entire length of the hindgut. In contrast with *E. callianassae*, *Enterobryus* sp. formed single thalli attached by individual holdfasts rather than tufts (Fig. 2.3A). There were two general morphologies in the hindgut: a longer, often spiraling form in the anterior hindgut (Fig. 2.1B) and, commonly in the posterior hindgut, a shorter form that develops dense clusters of thalli (Fig. 2.1C). Here we define the anterior hindgut as the forward approximately 8 mm of the hindgut and the posterior hindgut as the terminal 1-2 mm of the hindgut, according to where each form exists. Both forms typically have an angled, terminal spore case at the distal tip (Fig. 2.3B). This spore is believed to be the original spore from which the observed thallus developed (Hibbits 1978). In the observed *U. pugnax* hosts, both uninucleate (Fig. 2.3C) and multinucleate spores were present. Anterior-form thalli averaged 423 μm (SD = 375) in length and 11.0 μm (SD = 4.3) in diameter (n = 775), but thalli up to 4 mm long were observed. The posterior, bushy form averaged 114 μm (SD = 56) long and 8.3 μm (SD = 2.9) in diameter (n = 577). Total length of the short posterior thalli composed 3.3 - 42% of total thallus length in individual crabs (Fig. 2.4). All but one crab measured for Eccrinales length had both the anterior and bushy posterior form present (data not shown). Total length of Eccrinales present in the gut, in both cardiac

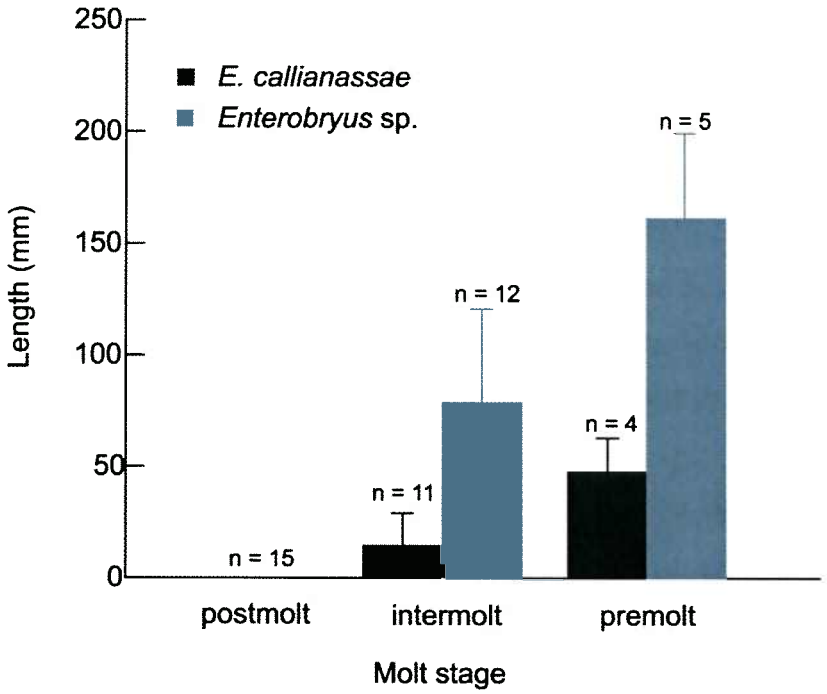

Figure 2.5 Molt stage and total thallus length in hosts in various molt stages for both eccrinid species. Results of 2-way ANOVA and Bonferroni post hoc test: Postmolt < Intermolt < Premolt ($P < 0.01$) Underlined terms represent molt stages with significantly different thallus lengths.

stomach and hindgut, corresponded with the host molt stage. In the postmolt crabs we inspected, there was no evidence of Eccrinales colonization (Fig. 2.5). Intermolt crabs were colonized to varying degrees, with anywhere from 0 - 37.0 mm (*E. callianassae*) and 11.8 – 135.6 mm (*Enterobryus* sp.) of total thallus length. Premolt crabs had the greatest quantity of Eccrinales thalli in both their cardiac stomachs and hindguts. There was a significant difference in total Eccrinales length between molt stages for both eccinids (2-way ANOVA, $F = 21.31$, $df = 2,46$, $P < 0.01$).

In comparing abundances of *E. callianassae* and *Enterobryus* sp. within individual hosts, we found significant correlation ($P < 0.05$) in total thallus length between the two species (Fig. 2.6). No significant relationship was found

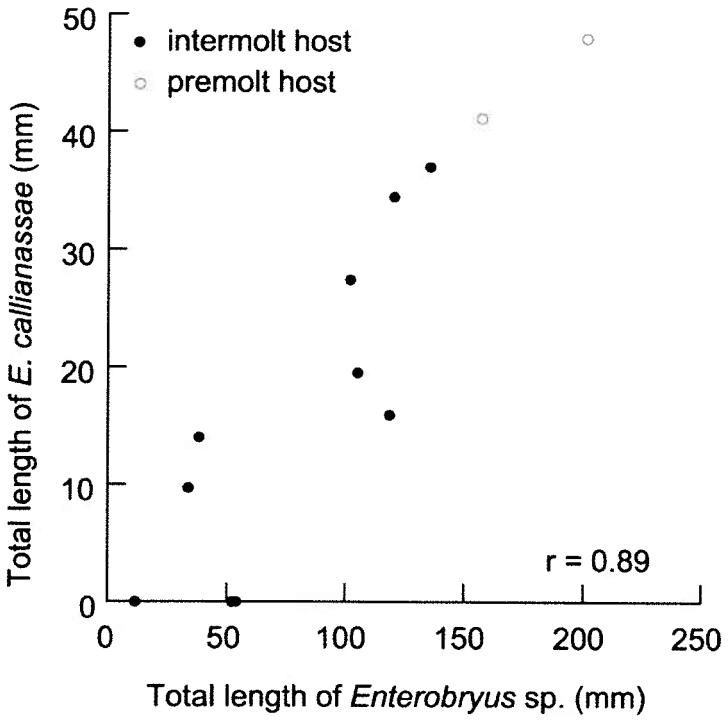

Figure 2.6 Correlation between total length of *Enterobryus* sp. and *E. callianassae*, within individual hosts. A significant Pearson product moment correlation coefficient (r = 0.89; $P < 0.05$) is reported.

between crab size (carapace width) and total length of either *E. callianassae* (r = 0.26; $P > 0.05$) or *Enterobryus* sp. (r = 0.21; $P > 0.05$) (Fig. 2.7).

The product amplified for the 18S rRNA gene with primers NS1 and NS2 was 715 base pairs (bp) in length (complete eccrinid 18S rRNA gene ~ 1900 bp). After excluding 2 large inserts (~ 100 bp each) and other ambiguous regions, 452 base pairs were aligned with other Fungal, Mesomycetozoea, and Stramenopila (outgroup) sequences to create a phylogenetic tree (Fig. 2.8). Distance and parsimony analyses of these sequences unambiguously placed *E. callianassae* within the class Mesomycetozoa, not in the fungal class Trichomycetes. *Enteromyces callianassae* was most closely related to a group of eccrinales

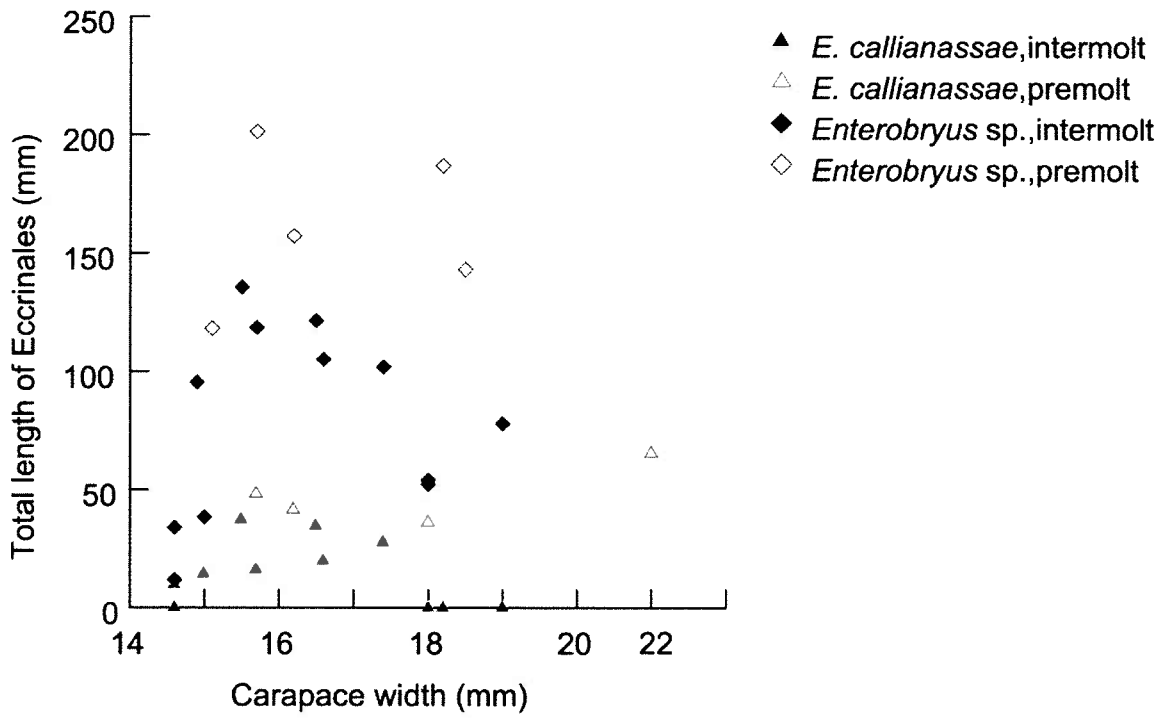

Figure 2.7 Total thallus length of both species of Eccrinales, in crabs of different sizes (carapace width) and molt stages. Pearson product moment correlation coefficients for both *E. callianassae* (r = 0.26) and *Enterobryus* sp. (r = 0.21) were not significant ($P > 0.05$).

symbionts (*Taeniellopsis* sp., *E. sexuale*, *T. carcini*, and *E. callianassae* from *N. californienesis*) all associated with marine crustacean hosts (Galt 1971; Johnson 1966). Interestingly, *E. callianassae* from *U. pugnax* shares only 94% sequence similarity (of a 452 bp fragment of the 18S rRNA gene) with *E. callianassae* reported in *Neotrypaea* (formerly *Callianassae*) *californiensis*.

DISCUSSION

Distribution along host digestive tract

Eccrinales appear to select specific gut sections along the digestive tract of *U. pugax* and other hosts. In this study, the two eccrinids never co-occurred

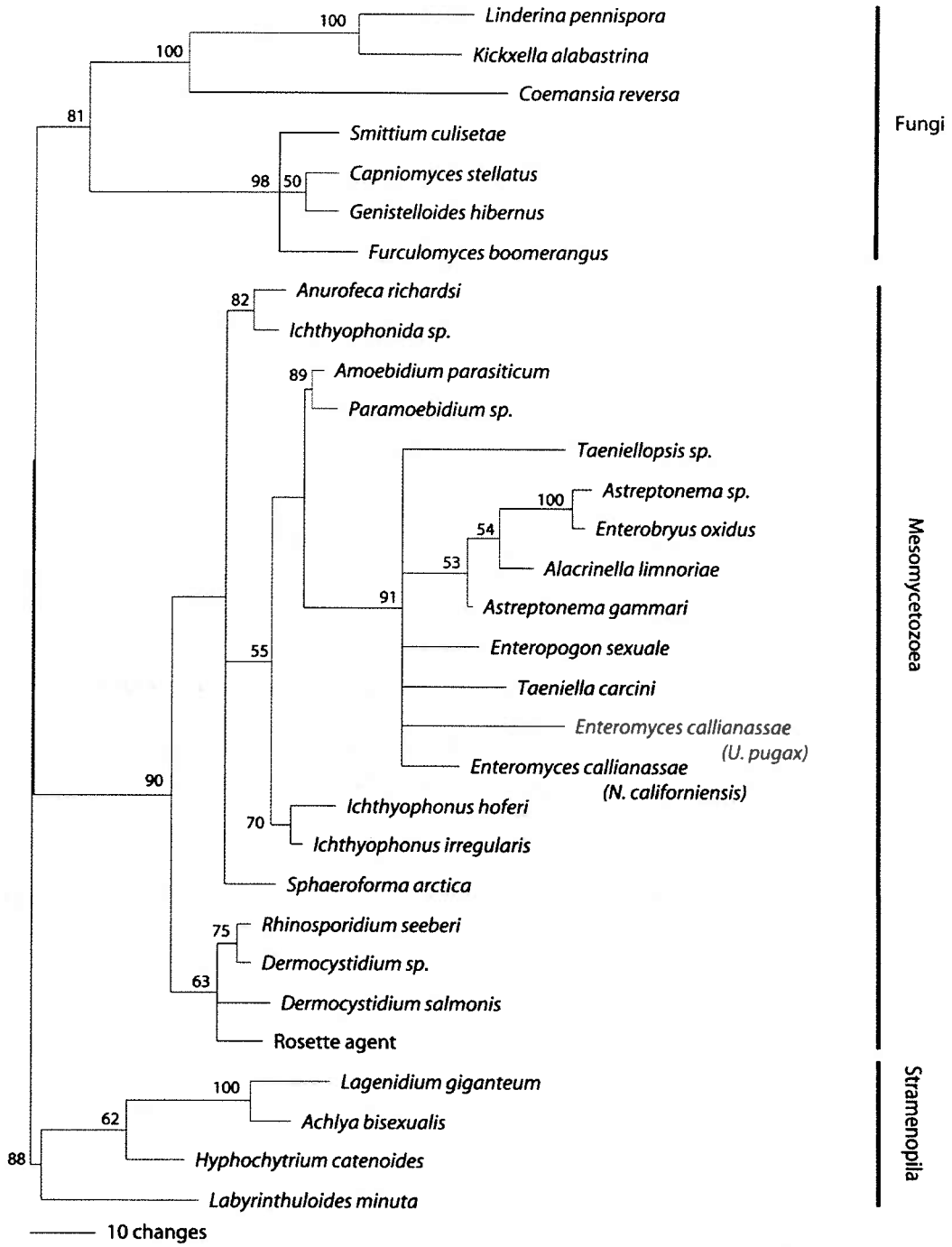

Figure 2.8 Phylogenetic relationships based on the partial 18S rRNA sequence of *E. callianassae* from *Uca pugnax*. Evolutionary distance tree was constructed by parsimony analysis; bootstrap values are based on 1000 replicates and are shown for values > 50%.

within the same section of the fiddler crab gut. *Enteromyces callianassae* was observed only in the cardiac stomach and *Enterobryus* sp. was observed only along the hindgut. In other studies, *E. callianassae* is most commonly reported in the cardiac stomach or foregut region (Tuzet and Manier 1962; Hibbits 1978; Kimura et al. 2002), although it is also known to colonize the esophagus and pyloric stomach in the thalassinid *Neotrypaea* (formerly *Calllianassa*) *gigas* (Hibbits 1978). In marine crustacean hosts, *Enterobryus* sp. is typically reported to attach to the hindgut lining (Mattson 1988; Wagner-Merner 1979). In one case, however, *Enterobryus* sp. thalli were observed in the stomach of an unspecified species of *Uca* (Lichtwardt 1961). There may be some site-specific conditions along the gut that serve as germination signals to control where *E. callianassae* and *Enterobryus* sp. attach. Alternatively, certain gut sections may prohibit development. Alkalinity is unlikely to be a cue because pH does not vary significantly between the stomach and hindgut (Chapter 5; this thesis).

Although we don't know what the cue might be, we can speculate that the driving force behind habitat preference is niche selection and that each species has adapted to its specific region of the digestive tract. At least in the stomach, competitive exclusion of *Enterobryus* sp. by *E. callianassae* is probably not a factor because there were multiple cases of an individual with a colonized hindgut and an uninhabitated stomach. Any differences in the nature of the gut contents between the two regions, due to sequential stages of digestive processing, could be a selection factor.

Host molt stage and Eccrinales colonization

Both eccrinids have had to adapt life cycles that accommodate periodic molting of the host, during which the entire chitinous surface of the digestive tract (i.e. stomach and hindgut lining) is shed and expelled into the environment. In all postmolt crabs studied, gut linings were devoid of any Eccrinales thalli or spores, suggesting that all Eccrinales biomass is shed upon molting. Ingested spores

appear unable to colonize the gut during this molting stage. Lichtwardt (pers. comm.) has suggested that there might be some quality of the newly exposed chitin lining which is not amenable to attachment. The postmolt cuticle is soft and fragile and Eccrinales spores might not attach to a surface from which they could be easily torn. The molt stage following postmolt, i.e. intermolt, is the longest stage at 25-171 d (Vigh and Fingerman 1985) and has the greatest range of Eccrinales colonization (0 - 135.6 mm $crab^{-1}$). We suspect that extent of colonization could be a function of duration of intermolt, where likelihood of spore encounter and *in situ* Eccrinales reproduction would both be time-dependent variables.

Significant correlation in total thalli length between *E. callianassae* and *Enterobryus* sp. (Fig. 2.6) could be a consequence of time in intermolt phase. If a host has been in intermolt phase for short duration it is likely to have a lesser amount of total Eccrinales thalli, and a correspondingly greater amount the greater the length of intermolt phase. Premolt crabs have the greatest amount of both *E. callianassae* and *Enterobryus* sp. and, presumably, have had the longest period of exposure to Eccrinales spores and the longest potential development time. Alternatively, this pattern could be explained if, for some unknown reason, a crab gut which is favorable for *E. callianassae* growth is also favorable for *Enterobryus* sp. growth.

The observation that all Eccrinales thalli associated with the stomach and hindgut are shed upon molting has been noted by other researchers (Lichtwardt 1954; Mccloskey and Caldwell 1965). This is the first quantitative study, however, to compare Eccrinales thalli abundance with particular host molt stages. Other papers have contrasted total abundance of an Eccrinales species among different hosts (Mattson 1988) or have reported percent of hosts colonized (Kimura et al. 2002; Mccloskey and Caldwell 1965) yet have not differentiated hosts by molt stage. High variation in colonization observed within a single host species in the present study suggests that accurate comparisons

between hosts can be made only if the hosts are in comparable molt stages. Furthermore, if only post-molt individuals are examined, the absence of Eccrinales could be inferred mistakenly for all molt stages of that host.

Host size and Eccrinales colonization

There was no clear correlation between host size and total length of Eccrinales (Fig. 2.7). We expected to detect a direct correlation between crab size and total amount of Eccrinales because the larger the crab, the greater the surface area of the stomach and hindgut, therefore the more surface area available for colonization. Also, larger, older crabs tend to molt less frequently (Passano 1960) so there could be more time for Eccrinales growth and reproduction between molt periods. It is likely that we did not observe a direct correlation due, again, to the uncertainty of timing within intermolt. We have no way to gauge how long an individual host has been in intermolt phase. Thus we are potentially grouping individuals that just entered intermolt phase with those preparing to enter premolt.

Host diet type and Eccrinales colonization

There appears to be a connection between host food preference and Eccrinales colonization. Eccrinid species are present in a range of detritvorous, algivorous, and omnivorous crustaceans, but Mattson (1988) found that they are absent from the guts of many carnivorous and scavenging species, including *Callinectes sapidus*, *Neopanope texana*, *Panopeus herbstii*, *Eurypanopeus depressus*, and *Menippe mercenaria*. In the present study we inspected digestive tracts of the carnivorous green crab (*Carcinus maenus*) and found no evidence of Eccrinales colonization. It seems that eccrinids: a) aren't ingested by carnivores, b) aren't cued to germinate, or c) are not able to survive in digestive tracts of carnivores. Most carnivorous crustaceans do ingest some sediment along with their target prey, and thereby could consume sediment-associated

eccrinid spores. Lichtwardt (1986) suggested that carnivores lack some crucial enzyme(s) or other substrates necessary for germination or growth of spores.

Both *E. callianassae* and *Enterobryus* sp. are known to colonize hosts with a variety of feeding strategies, including both filter and deposit feeders, as well as detritivores, algivores and omnivores. *Enteromyces callianassae* has been reported from both deposit-feeding thallassinids (*N. harmandi*) and filter-feeding thallassinids (*Upogebia affinus*) (McCloskey and Caldwell 1965; Kimura et al. 2002) as well as the detritivorous fiddler crab *U. pugilator* (Tuzet and Manier 1962). *Enterobryus* sp. is known from detritivorous and omnivorous fiddler crabs (*U. pugilator, U. rapax, U. longisignalis*) (Mattson 1988). Thus the mode of food acquisition does not appear to select for or against Eccrinales colonization. Diet composition, specifically the presence of detritus and/or plant matter, could be an important criterion determining host suitability. Carnivores ingest a diet rich in protein and readily digested starches (Stevens and Hume 1995), have sufficient endogenous enzymes to process their diets and therefore have been predicted to have competitive interactions with gut microbes, at least in the foregut (Hungate 1976; Mackie 2002). Yet hosts that consume diets high in fiber and carbohydrates could benefit from a resident microbiota breaking down refractory ingested matter and are expected to have cooperative relationships (Hungate 1976).

Phylogenetic position of E. callianassae

Our finding that *E. callianassae* is more closely affiliated within the newly coined protist class, the Mesomycetozoa, is corroborated by the results of Cafaro (2003). Cafaro (2003) sequenced the 18S rRNA gene for numerous Eccrinales species (including *E. callianassae* from the ghost shrimp *Neotrypaea californiensis* and *Enterobryus* sp. from a millipede (class Diplopoda)) and found that they all aligned within the Mesomycetozoa. Previously, the order Eccrinales was grouped with a class of arthropod-associated fungi, the Trichomycetes,

because they shared certain characters, including production of sporangiospores, attachment via an acellular holdfast and an obligate association with mandibulate arthropods. Eccrinids have been classified as fungi due to the coenocytic nature of their thalli, formation of holdfasts, and the presence of cellulose in their cell walls (Lichtwardt 1961). However, this group has not always been considered fungal. They were initially described as nonphotosynthetic plants (Leidy 1853b), and since have been described as either oomycete fungi (Kingdom Stramenopila) or protists (Galt 1971). Some of the characters that supported classifying the Eccrinales within the Trichomycetes, and therefore with the Fungi, could be adaptations to a common lifestyle, rather than true phylogenetic similarity. Both the Eccrinales and the Trichomycetes are only known to live within the gut lumen of arthropods. Development of holdfasts may only be a shared adaptation of unrelated organisms to a habitat where secure attachment is necessary for survival (Moss 1979). Another arthropod gut microbe, the *Arthromitus* stage of *Bacillus cereus*, also forms spores and develops a holdfast, yet as a eubacterium, is related to neither the Eccrinales nor the Trichomycetes (Margulis et al. 1998).

The comparision of our 18S rRNA gene sequence for *E. callianassae* from *U. pugnax* with the sequence for *E. callianassae* from *N. californiensis* (Cafaro 2003), revealed that the two sequences were divergent, and had only 94% of a 452 base-pair fragment in common. There is no commonly accepted definition of what constitutes a genetic protist species, but if we apply the definition of genetic bacterial species as those that share at least 97% of their small subunit rRNA gene (16S rRNA) sequence (Stackebrandt and Goebel 1994), we would assign these two sequences to two distinct species. Our analysis, however, was based on only a short fragment, representing approximately 24% of the entire SSU rRNA gene (452/~1900 bp). Consequently, our sequence information on *E. callianassae* is too limited to evaluate the possibility of cryptic species.

Host specificity and extent of colonization

Enteromyces callianassae and *Enterobryus* sp. have not been reported before together in *U. pugnax*. These two eccrinids are not host-specific, and each inhabits multiple species of crustacean hosts. *Enteromyces callianassae* is commonly found in the foregut of thallassinid burrowing shrimp (Table 2.1) and has been reported in the stomach of the fiddler crab *U. pugilator* (Tuzet and Manier 1962). From these observations it appears that *E. callianassae*, as defined morphologically, is not host specific, but our sequence data suggest that genetically distinct organisms may be associated with different host species. However, for the present study, we classify *E. callianassae* based on morphology, to compare colonization levels to other studies.

The frequency of occurrence of *E. callianassae* that we documented in *U. pugnax* was similar to that found in *Nihonotrypaea harmandi* and substantially greater than that reported in *Upogebia affinus*. However the other reports did not categorize hosts by molt stage, and could have included postmolt individuals; possibly reducing the number of observed hosts with *E. callianassae* as compared with those from our intermolt hosts. Even so, the burrowing shrimp, *N. harmandi*, averaged 8.4 tufts and had up to 36 tufts in a single host foregut (Kimura et al. 2002). The greater number of tufts, and presumably greater eccrinid biomass, could result if the host provides a better environment for *E. callianassae* growth, if there is a greater surface area for successful attachment and growth, or if there is a greater number of ingested spores. Although both hosts are deposit feeders, diet quality could vary substantially, among hosts and among habitats. *Nihonotrypaea harmandi* lives in estuarine intertidal sandflats and *Uca pugnax* (from this study) inhabits the banks of salt marshes. *Nihonotrypaea harmandi* is a much larger crustacean than *U. pugnax*, and has a correspondingly larger foregut, with more area for attachment. Spore abundance was not quantified, but presumably a higher level of colonization would result in a

greater number of released spores, more spores in the hosts' habitat, and possibly more ingested spores.

Enterobryus sp. has been reported in hindguts of multiple species within the genus *Uca* (Mattson 1988), as well as in the mole crab, *Emerita talpoida* (Cronin and Johnson 1958). However the extent of host specificity for *Enterobryus* sp. is difficult to interpret without accurate species identification. If all *Enterobryus* sp. found in marine crustaceans constitute a single species, then *Enterobryus* sp. does not demonstrate host specificity. Furthermore, if the anterior long form and posterior bushy form are actually distinct species, it is possible that multiple species of *Enterobryus* sp. coexist within individual hosts. We will consider both forms of *Enterobryus* sp. and literature reports of *Enterobryus* sp. all as a single species to facilitate the comparison.

The total thallus length of *Enterobyrus* sp. that we measured in *U. pugnax* is in the upper range reported in other marine crustacean hosts. In the only other published paper of *Enterobryus* sp. lengths, Mattson (1988) used an intersection method (Olson 1950) to estimate total thallus length in decapods. He found that mean total thallus lengths in the hindguts of the fiddler crabs *Uca longisignalis* and *Uca rapax* (both from Tampa Bay, FL) were 7 and 35 mm per crab, substantially less than the average total length in both intermolt and premolt *U. pugnax* (Fig. 2.5). Yet Mattson (1988) did not distinguish crabs based on molt staging and these averages include numerous individuals with no eccrinid thalli. Total thallus length for individual crabs ranged up to 120 mm in *U. longisignalis* and up to 315 mm in *U. rapax*. We found that *U. pugnax* individuals had up to 135.6 mm per crab (intermolt phase) and 201.3 mm per crab (premolt phase). So in *U. pugnax* (Barnstable, MA) *Enterobryus* sp. had a greater average length of total thallus, when post molt individuals were excluded, yet the hindgut Eccrinales in the fiddler crab *U. rapax* (Tampa Bay, FL) can have greater abundances on an individual basis.

Possible functional role of Eccrinales

Almost nothing is known about the nature of the interaction between Eccrinales and their hosts. As a member of the class Mesomycetozoa, the Eccrinales are now considered most closely related to various fish and crustacean pathogens, including *Ichthyophonus* spp. and *Dermocystidium* spp. (Mendoza et al. 2002). By phylogenetic affiliation, an argument could be made for considering the Eccrinales as pathogens of their crustacean hosts. Mesomycetozoa also includes the Amoebidiales, however, a group of protists that associate with freshwater crustaceans and insects and are believed to be commensals (Misra 1998). Furthermore, because we did not observe any apparent detriment to the host, in terms of activity level or maximal size, we suggest that the association is not deleterious, but might be classified as a commensalism or, possibly, a mutualism. Other researchers have noted no observable impairment to the hosts, even in cases of highly colonized individuals (Hibbits 1978). In the only published study addressing the functional role of the Eccrinales, Kimura et al. (2002) found that digestive fluid from ghost shrimp *Nihonotrypaea harmandi* with *E. callianassae* released significantly more enzymatically hydrolyzable amino acids (EHAA) from natural field sediment as compared with a population of *N. harmandi* without *E. callianassae*. However, the total environmental sediment EHAA concentrations were also greater from the habitat of the population with *E. callianassae*. Therefore the higher EHAA concentration and predicted nutritional gain might not have been solely, or even partially, a result of the presence and presumed contribution of *E. callianassae*.

Eccrinales symbionts must derive some essential nutrient or condition from their hosts; otherwise they would exist in a free-living form (Moss 1979). Because these organisms are found only in hosts that consume live or decaying plant matter, there could be some component(s) of this diet that are crucial for Eccrinales growth and development. Without any eccrinids in culture, however, we can only speculate about what these components might be. Also, we have

no evidence of what, if any, compound(s) *E. callianassae* and/or *Enterobryus* sp. might provide their hosts. The mesomycetozoan parasite, *Perkinsus marinus*, has been reported to release a number of extracellular proteins including highly potent proteases (La Peyre et al. 1995). This parasite relies on extracellular proteases to degrade host matrix proteins, allowing it to propagate within host tissue. However, different extracellular proteases released into the gut might provide a benefit to the host. If eccrinid species produce extracellular proteases that breakdown organic matter in the gut lumen, the products might be incorporated by the host. Alternatively, some essential compound, such as a vitamin, could be released by the Eccrinales and absorbed by the host. Further, explicit studies of host-Eccrinales interaction are needed to assess the true nature of the association and possible environmental implications. The most useful type of study would correlate specific measures of host gain or harm (i.e. egg production, host growth rate, host size, enzyme production or metabolic activity level) with the extent of eccrinid colonization.

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Chapter 3: Bacterial gut microbiota of the marsh fiddler crab *Uca pugnax*: bacterial morphologies and distributions

ABSTRACT

Via scanning electron microscopy (SEM), we investigated digestive tracts of the marsh fiddler crab, *Uca pugnax,* to assess locations and morphologies of resident (attached) bacteria. The greatest abundances occurred on the pyloric fingerlets and along the hindgut. Scattered coccoid bacteria colonized the cardiac stomach and rod-shaped bacteria almost completely coated the pyloric fingerlets of the pyloric stomach. The midgut contained coccoid bacteria associated with the epithelium and remnants of the peritrophic membrane. Two distinct morphologies of rod-shaped bacteria colonized the chitinous hindgut: curved rods in the anterior hindgut and mats of rods in the posterior hindgut. Using direct counts, we confirmed that stomach and hindgut sections contained the greatest numbers of resident bacteria. Transient bacteria were approximately twice as abundant per section as resident bacteria in these regions. Attached bacteria, however, are consistently present among individuals, are more likely to be active and, therefore are expected to play a greater role in host physiology.

INTRODUCTION

Nutritional symbioses between metazoans and microbes are widespread in both marine and terrestrial environments and are characterized by high microbial population density and complex interactions. In terrestrial environments, nutritional interactions between microbes and arthropods such as termites, cockroaches and locusts have been studied extensively and are known to influence host physiology as well as impact the local and global environment (Brauman et al. 1992; Breznak 1982). In termites, hindgut microbes release gases (CH_4, CO_2 and H_2) at sufficient rates to factor into estimates of global greenhouse gas emissions (Breznak and Brune 1994). Hosts typically consume detritus, wood or other plant material, and their microbiota assist in degrading the ligno-cellulose rich fraction of the diet by depolymerizing cellulose and fermenting the resulting carbohydrates to short-chain fatty acids. Hosts absorb these

products, thereby increasing their dietary carbon and energy gain. Gut microbial activities can also impact the environment via chemical alteration of egesta. Associations between terrestrial arthropods and their gut microbiotas are often specific, stable and highly evolved (Breznak 1984; Dillon and Charnley 2002). By housing microbial communities with specific and complementary metabolisms, hosts are able to utilize ecological niches unavailable to them without digestive symbionts.

In the marine environment, digestive associations have been found most commonly among invertebrate detritivores and herbivores, specifically animals that consume diets low in concentration of labile organic matter and/or high in concentration of refractory substances (Harris 1993; Plante et al. 1990). Because of their diets and phylogenetic proximity to terrestrial hosts, marine detritivorous arthropods are likely candidates to host gut microbial communities. There is growing evidence that many marine invertebrates, and arthropods in particular, harbor resident gut microbes that may contribute to nutritional mutualisms (Harris 1993; Harris et al. 1991; Lau et al. 2002; Pinn et al. 1997). However, a clear understanding of the specificity and nature of these associations is lacking. Early literature reported the presence of microbes in digestive tracts of marine arthropods, but often failed to distinguish between food-associated microbes and more permanent microbial communities (Barlocher et al. 1989; Beeson and Johnson 1967).

Gut-associated microbes can be classified into two general categories: transient, food-associated microbes that are digested or pass through the gut, and resident bacteria that attach to the gut lining. Types of possible host interactions with transient bacteria could range from competition for food to direct digestion (Harris 1993). Resident bacterial communities are expected to have greater stability than transient bacterial communities and are more likely to have evolved a symbiotic relationship with the host. Here we define a stable microbial association as one in which the same bacterial strains are found among

individuals of a host species and over time. Quantifying abundances of resident and transient bacteria in each gut section is a necessary first step to assess potential significance of each population to the host.

Multiple species of detritivorous fiddler crabs harbor particular morphologies of resident microbes in their hindguts (Harris 1992). It is unknown, however, if resident bacteria colonize other gut sections. Different gut sections may have particular chemical environments, each with the potential to support distinct bacterial communities. Furthermore, each bacterial community may have a unique digestive function in host physiology. Identifying morphologies, location(s), and abundances of resident microbes is essential to develop an understanding of possible bacterial-host interactions.

In this study, we describe and quantify abundances of resident bacterial communities along the digestive tract of a marine detritivore, the fiddler crab *Uca pugnax.* We have chosen *Uca pugnax* because it is known to host attached hindgut bacteria (Harris 1992) and is important ecologically. We limit this study to an investigation of bacterial microbiota (as opposed to eukaryotic microbes) to allow detailed examination of one component of the gut microbiota. Specific questions that we address include: 1) Where are resident bacteria located along the gut and 2) How do resident bacterial abundances compare with transient bacterial abundances?

METHODS

Crab collection and dissection

Adult marsh fiddler crabs, *U. pugnax*, were collected in August 2003 from an intertidal salt marsh in Barnstable Harbor, Massachusetts (41°42'31 N, 70°18'17 W) during low tide. Eight male crabs were collected for bacterial counts of transient and resident bacteria. Eight crabs (four males and four females) were collected for scanning electron microscopy and light microscopy of thin sections. Males and females were inspected initially to confirm that bacterial

morphologies and attachment locations were consistent among the sexes. Only males were used for the transient and resident counts to avoid any possible sex-based bias in bacterial abundances. Crabs were kept in cooled containers (~15°C) and brought to the laboratory within 2 h after collection. Gender, molt stage (see below), carapace width and length of each specimen were recorded. For electron and light microscopy preparations, crabs were allowed to depurate their gut contents for 3 h in 0.2-μm filtered flowing seawater at the approximate temperature and salinity of their collection. Crabs collected for transient and resident bacterial counts were dissected upon arrival in the lab. Eight samples (~1 g wet weight each) of surficial (top 1 cm) habitat sediment from eight separate cores were collected in August 2003, during crab collection.

Before dissection, crabs were thoroughly washed with sterile, filtered, 0.1 M phosphate buffer (pH 8.1) and cold-anesthetized (0°C for 8-10 min). We dissected stomach, hepatopancreas, midgut and hindgut sections from anesthetized crabs using sterile tools on a sterilized aluminum surface. Gut sections were removed from the organism and measured for length and diameter (for midgut and hindgut samples) and for wet weight (hepatopancreas). Gut contents were collected, transferred to sterile 1.7-ml tubes, and wet weighed. Stomach samples included both pyloric and cardiac stomach sections. Midgut samples included the region between anterior and posterior midgut ceca. The hindgut was considered to be the length of gut from the posterior midgut cecum to the anus. Tissue samples, gut contents and sediment samples were each preserved in 1 ml sterile 3% gluteraldehyde in 0.1 M phosphate buffer. All samples were kept at 4°C until processed.

Molt stage analysis

Molt stages of individual crabs were determined by examining characteristics of pleopod and abdominal setae as described in (Vigh and Fingerman 1985). Four molt stages were identified: postmolt, intermolt, premolt

and ecdysis (Drach 1939). Setae were removed from crabs with fine dissecting forceps, immediately wet-mounted on slides in 0.1M NaCl buffer and viewed with a Zeiss Axiostar Plus Microscope (500 or 1,000X magnification). Only intermolt crabs were used for this study.

Electron and light microscopy

Immediately after dissection, hindgut, midgut, hepatopancreas and both the cardiac and pyloric stomachs of eight crabs were fixed in 3% gluteraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 3 h. Samples were washed three times in sodium cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h, and washed another three times before dehydrating in a series of ethanol dilutions. Samples were critical-point dried using carbon dioxide as the transitional fluid, mounted on aluminum stubs, coated with gold palladium and examined in a JEOL JSM-840 scanning electron microscope (SEM). For thin sectioning, samples were fixed as described for SEM up to the ethanol dehydration step, when individual samples were embedded in an Araldite 502-Epon mixture. Thin sections (< 1 μm) were stained with Toluidine Blue (1%) and examined with a Zeiss Axiostar Plus microscope (1,000X).

Hindgut bacterial densities were estimated from SEM images. Densities in both anterior and posterior hindgut regions were determined. For three crabs, bacterial abundances in five haphazardly selected quadrats (10 x 10 μm), were averaged, for each hindgut region. Total hindgut surface area (mm^2) was estimated for each crab collected for SEM from measured hindgut lengths and widths by approximating the hindgut as a cylinder. Total midgut and hindgut volumes (ml) were estimated for each crab collected for bacterial counts in the same manner (Table 3.1). Resident bacterial counts were normalized per ml gut volume to compare measured bacterial densities with literature reports.

Sonication

To dislodge bacteria from gut linings, gut contents and sediments, we sonicated, centrifuged and collected sequential supernatants from samples, as described by Hymel and Plante (1998) with the following modifications. Samples (1 ml each) were centrifuged at 6000 x *g* for 12 min, and 0.8 ml of gluteraldehyde was removed before adding phosphate buffer (4.8 ml, 0.1 M) with 0.05% Triton X-100 (to enhance dislodgement). Removal of gluteraldehyde fixative increases cell counts and reduces count variability (Hymel and Plante 1998). These samples (5 ml each) were vortexed for 30 s, sonicated on ice for two 30-s bursts and allowed to cool for 30 s between bursts (Branson Ultrasonifier 250; nominal power output of 15 W). After 10 s of vortexing, samples were centrifuged (400 x *g*) for 8 min at 10°C to settle large particles. Four milliliters of supernatant from each sample was pipetted into separate sterile 14-ml tubes. Four ml of fresh 0.1 M phosphate buffer with 0.05% Triton X-100 was added to the residue, and this series (vortex, sonicate, vortex, centrifuge) was repeated a total of three times, such that 12 ml of supernatant was collected for each sample. Sediment and gut-content samples were dried at 60°C for 16 h and re-weighed to obtain dry weights.

Staining and counting

Each 12-ml supernatant was well mixed before taking two, 1.0 ml or 2.0 ml aliquots. These aliquots were incubated with 10 µl DAPI (5 $\mu g\ ml^{-1}$) and 1.0 ml sterile filtered phosphate buffer for 15 min in darkness (Tso and Taghon 1997). Samples were filtered onto 0.2 µm black polycarbonate membrane filters (25 mm; Poretics) backed by 0.45 µm filters (Millipore) and rinsed with 2 ml of 0.2-µm filtered milliQ water (vacuum pressure < 90 mm Hg). Filters were placed on glass slides with immersion oil and examined at 1250 or 1500X magnification using a standard Zeiss DAPI filter combination (365-nm excitation filter, 400-nm

barrier filter) with a Zeiss Axiovert S100. Negative controls consisted of sterile 0.1 M phosphate buffer and sterile milliQ water (same volumes as those added to gut samples) sonicated, stained, and prepared in the same manner described above. At least 10 haphazard fields were counted (for a total of > 200 cells counted) and counts from negative controls (average of 10 fields) were subtracted from total counts. Total bacterial counts were reported as numbers of bacteria per gut section (resident and transient), per gram dry sediment (transient and habitat sediments), per mm gut length (resident) or per ml gut volume (resident).

Statistical analysis

Prior to statistical analyses, data were tested for homogeneity of variances and for normal distribution. Bacterial counts were square-root transformed to meet these assumptions. A two-way analysis of variance (ANOVA) was used to assess whether total bacterial numbers varied among gut sections and between categories [resident versus transient (gut content-associated)]. One-way ANOVA tested for differences in transient bacterial densities (per gram dry weight) among gut contents and habitat sediment. If treatment effects were significant, post hoc comparisons were carried out with a Bonferroni test. All statistical tests were performed in SYSTAT (version 10).

RESULTS

Bacterial morphology and distribution within the gut

We observed dense populations of apparent bacteria attached to pyloric fingerlets (Fig. 3.1A,B) in the pyloric stomach and the hindgut chitin lining (Fig. 3.1C- F) of all four crabs inspected by SEM. We refer to forms observed by SEM as bacteria if they had common bacterial morphologies (i.e. rods, cocci). Bacteria did not colonize all surfaces, nor all projections in the pyloric stomach, but appeared restricted to chitinous pyloric fingerlet projections (Fig. 3.1A), where

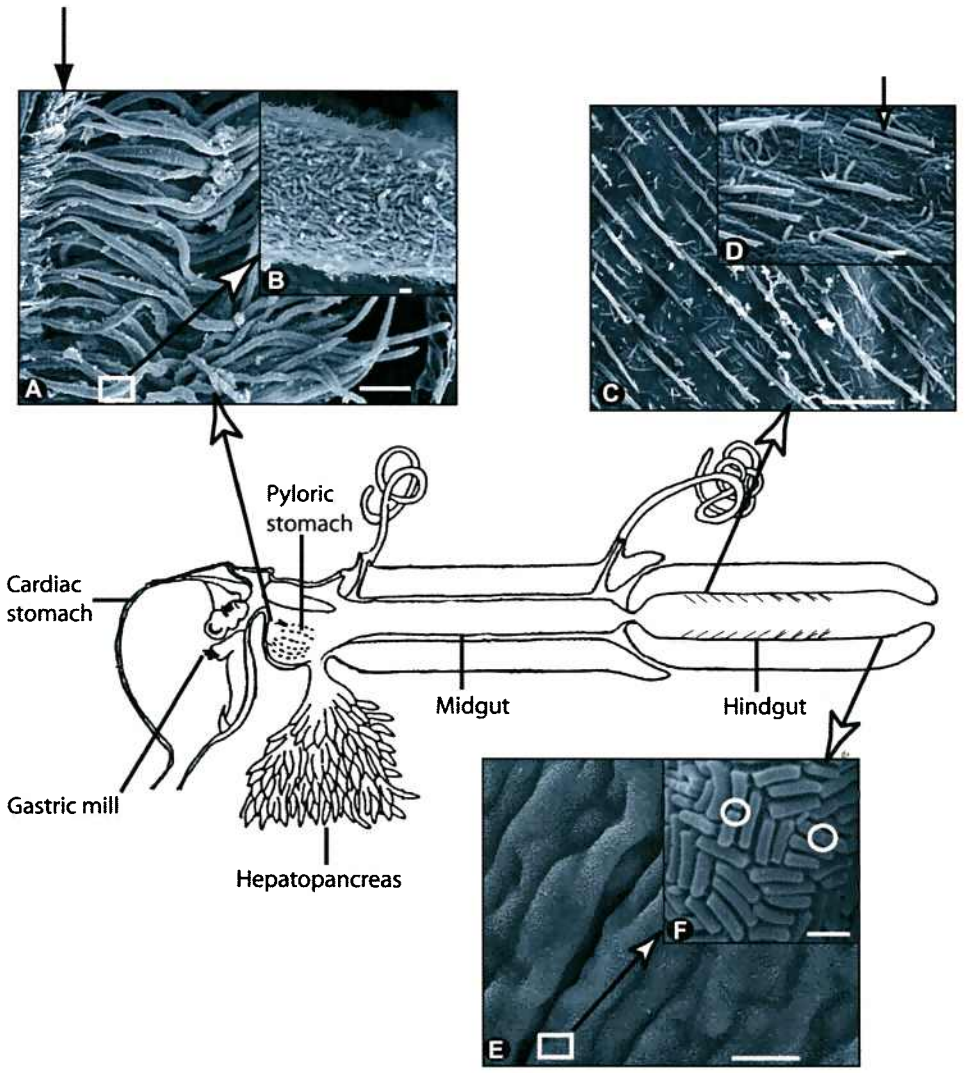

Figure 3.1 SEM images of bacteria in *U. pugnax* gut, with schematic of generalized crustacean gut (modified from Hopkin and Nott 1980) for reference. A. Pyloric fingerlets in pyloric stomach, small, solid arrow designates smaller projections at the base of the fingerlets. B. Close-up of an individual fingerlet with fine setae and associated bacteria. C. Forward segment of anterior region of chitin-lined hindgut with curved rod-shaped bacteria and projections. D. Close-up of rear segment of anterior region with dimpled chitin and associated bacteria and projections. Small arrow indicates crab chitin projections. E. Posterior region of hindgut lined with smooth, furrowed chitin and dense mat of attached rod-shaped bacteria. F. Close-up of attached rods and apparent glycocalyx adhering bacteria to chitin lining. Glycocalyx consists of web-like fibers between individual cells and chitin lining. Probable sites of recent bacterial divisions are circled. Scale bars A) 100 μm; C, E) 10 μm; B, D, F) 1 μm

they colonized from base to tip with approximately uniform density. Rod-shaped bacterial cells (1-2 x 0.4 μm) were attached to the fingerlets, to fine spines on the

fingerlets and to other bacteria. Smaller projections, directly at the base of the fingerlets had very few attached bacteria (Fig. 3.1A, solid arrow).

In the cardiac stomach, curved and spiral-shaped bacteria were attached to thalli of the resident protist *Enteromyces callianassae* (Fig. 3.2A) and coccoid bacteria were observed on the chitinous cardiac lining (Fig. 3.2B). Images of semi-thin sections revealed coccoid bacteria (< 1.6 μm diameter) in hepatopancreatic tissue (Fig. 3.2C) in two of the four crabs inspected. Contrary to observations in hepatopancreas tissues of some arthropods (Drobne et al. 1999), these cells were neither contained within bacteriocytes nor detected in all

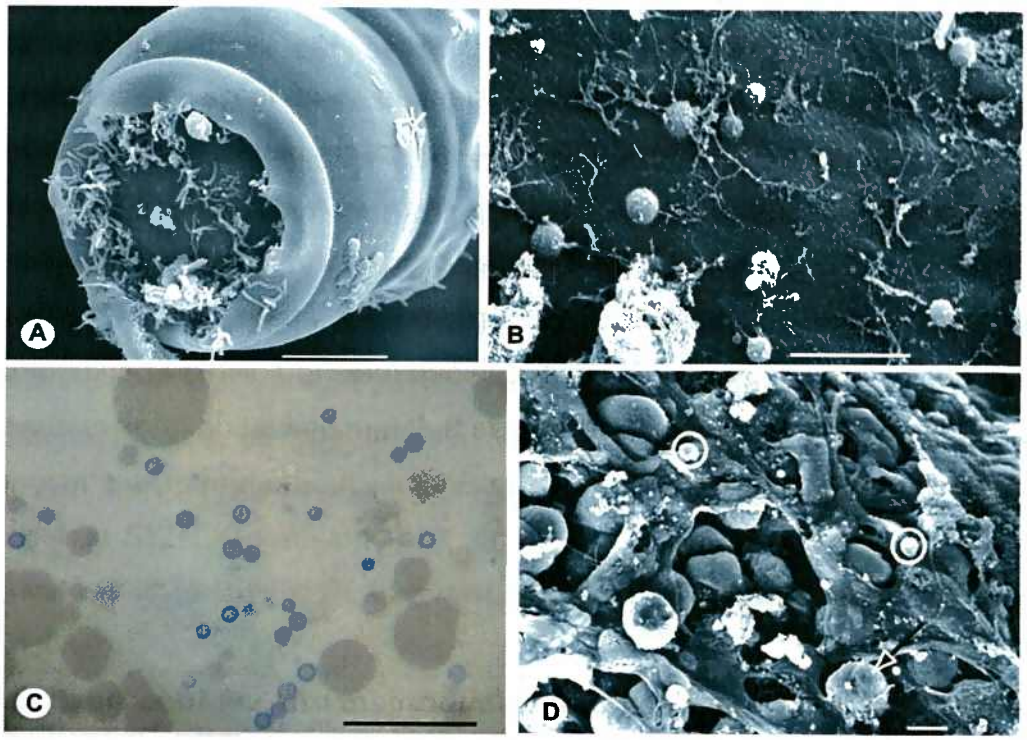

Figure 3.2 SEM and thin section images of bacteria in *U. pugnax* cardiac stomach, hepatopancreas and midgut. All scale bars - 10 μm. A. Bacteria associated with attached resident protist *Enteromyces callianassae* (large, bulbous projection) in the cardiac stomach. B. Coccoid bacteria attached to the chitin lining of the cardiac stomach. C. Cross section of hepatopancreas tissue with associated bacteria (blue cells). D. Midgut epithelium with secretory cells (arrow), remnants of peritrophic membrane and associated bacteria (circled).

thin sections of an individual hepatopancreas. Along the midgut, a few bacterial cells were attached to remnants of an apparent peritrophic membrane (Fig. 3.2D). The non-chitinized midgut epithelium consists of an anterior microvillous lining and a posterior epithelial layer with secretory cells (Fig. 3.2D).

At least two distinct bacterial morphologies colonized the hindgut lining, each consistently observed within a specific region. The anterior region (~ 65-70%) of the hindgut lining had regularly spaced projections that pointed posteriorly. Within this anterior hindgut region, the forward segment was covered with individual projections (Fig. 3.1C) and the rear segment had clusters of two to four projections (Fig. 3.1D). Curved, rod-shaped bacteria (2-3 x 0.2 μm) were associated with the anterior hindgut region. These irregularly shaped bacteria typically adhered to the lining by one end or lengthwise. In this anterior hindgut region, the chitin lining appeared finely dimpled (Fig. 3.1D). The lining might be naturally dimpled or this appearance may result from the activities of chitinoclastic bacteria. The posterior region (~30-35%) of the hindgut had a smooth, furrowed chitinous lining (Fig. 3.1E). Dense aggregations of rod-shaped bacteria (0.8-1.2 x 0.3 μm) colonized this posterior region. This form consistently attached lengthwise and appeared to have a web-like glycocalyx sheath affixing it to the lining. These bacteria appeared to reproduce by dividing into two

Table 3.1 Midgut and hindgut dimensions and estimated volumes for crabs collected for transient and resident bacterial counts

	Midgut			Hindgut		
Crab	length (mm)	diam. (mm)	vol. (ml)	length (mm)	diam. (mm)	vol. (ml)
1	4.0	1.5	0.0188	9.0	1.50	0.0424
2	3.3	1.2	0.0124	10.9	1.65	0.0566
3	3.0	1.5	0.0145	10.5	1.70	0.0561
4	3.6	1.7	0.0192	9.0	1.40	0.0340
5	4.9	1.6	0.0246	12.0	1.70	0.0641
6	3.8	1.2	0.0141	11.0	1.40	0.0484
7	3.0	1.4	0.0132	11.2	1.40	0.0493
8	4.5	1.6	0.0226	10.5	1.65	0.0544

collinear but shorter rods as inferred from apparent recent cleavages (Fig. 3.1F) and seemed to be actively reproducing, based on the number of recent divisions observed. As calculated from SEM images, average densities of posterior hindgut bacteria (x = 1.89 x 10^6, SD 1.23 x 10^5 mm^{-2}) were approximately four times as dense as the anterior form (x = 4.59 x 10^5, SD 6.30 x 10^4 mm^{-2}).

Transient and resident bacterial abundances

To investigate abundances of resident and transient bacteria, we compared total numbers of attached bacteria and total numbers of content-associated bacteria in each section (Fig. 3.3). Two hepatopancreas samples were uncountable due to tissue interference with DAPI staining, and three crabs did not contain any midgut sediment. With these exceptions, counts of resident and transient bacteria were made on all gut sections of all eight crabs. To

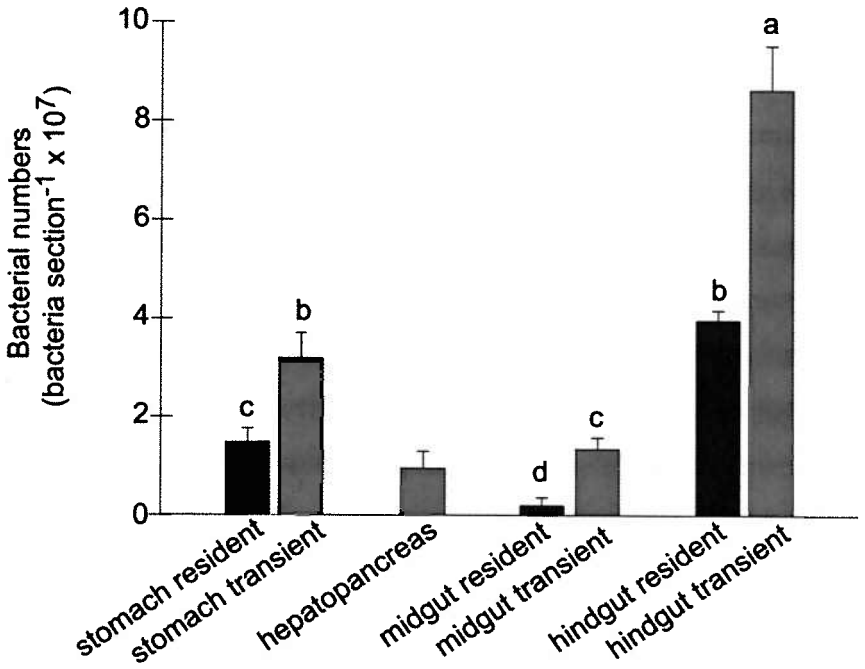

Figure 3.3 Total resident and transient (contents-associated) bacterial numbers (average +SE; n = 6-8) in each gut section. Letters indicate groupings with significant differences in ANOVA and Bonferroni post hoc test ($P < 0.05$).

increase comparability among hosts, only intermolt crabs were used for the presented analyses. Data are presented as bacterial numbers per gut section to resolve which gut section(s) harbored the greatest total abundances of resident bacteria. Section(s) with relatively high resident bacterial abundances are most likely to be sites of bacterial-host interaction. To account for small differences in midgut and hindgut sizes among crabs, we normalized bacterial densities in the tubular midgut and hindgut sections by per mm gut length and by per milliliter gut volume (approximating the gut as a cylinder) (Table 3.1). Resident midgut bacterial densities averaged 2.71×10^5 (SD 1.34×10^5) mm^{-1} gut length and 5.81×10^7 (SD 2.58×10^7) ml^{-1} gut volume. Hindgut bacterial densities of resident bacteria averaged 3.79×10^6 (SD 5.01×10^5) mm^{-1} gut length and 8.04×10^8 (SD 1.69×10^8) ml^{-1} gut volume. Ideally, we also would normalize hepatopancreas and stomach counts; however, it was not feasible to estimate the lumen volume or surface area of these sections due to their irregular shapes.

Abundances of resident bacteria varied significantly among gut sections (Fig. 3.3, Table 3.2). Because we had only one category for hepatopancreas counts, this section could not be included in the full 2-way ANOVA and was excluded from this analysis. Resident bacterial abundances were significantly different among sections. Bacterial abundances were greatest in the hindgut, followed by the stomach and the least in the midgut ($P < 0.05$; Bonferroni post hoc test). Transient bacterial numbers mirrored the resident abundance pattern and were significantly different between each pair of sections ($P < 0.05$; Bonferroni post hoc test). These transient numbers, however, were assessed

Table 3.2 Two-way ANOVA comparing bacterial abundances among gut sections and between transient and resident populations (category).

Source	*df*	MS	*F*	*P*
Section	2	106,456,000	135.95	< 0.01
Category	1	66,495,300	84.92	< 0.01
Section x category	2	1,354,679	1.73	0.19
Error	39	783,072		

from varying amounts of sediment and indicate only that the total number of transient bacteria varied with gut section. The number of transient bacteria was significantly greater than the number of resident bacteria for the stomach, midgut and hindgut sections ($P < 0.01$; Bonferroni post hoc test). Numbers of resident bacteria equaled just 8% of the average transient number in the midgut, but nearly equaled half the average transient numbers in the stomach and hindgut.

Density of bacteria (g^{-1} dry wt) in sediment from the host's habitat was significantly greater than that in all gut content sections (1-way ANOVA, F = 16.58, $df = 3$, $P < 0.01$) (Fig. 3.4). Within the gut, average transient bacterial

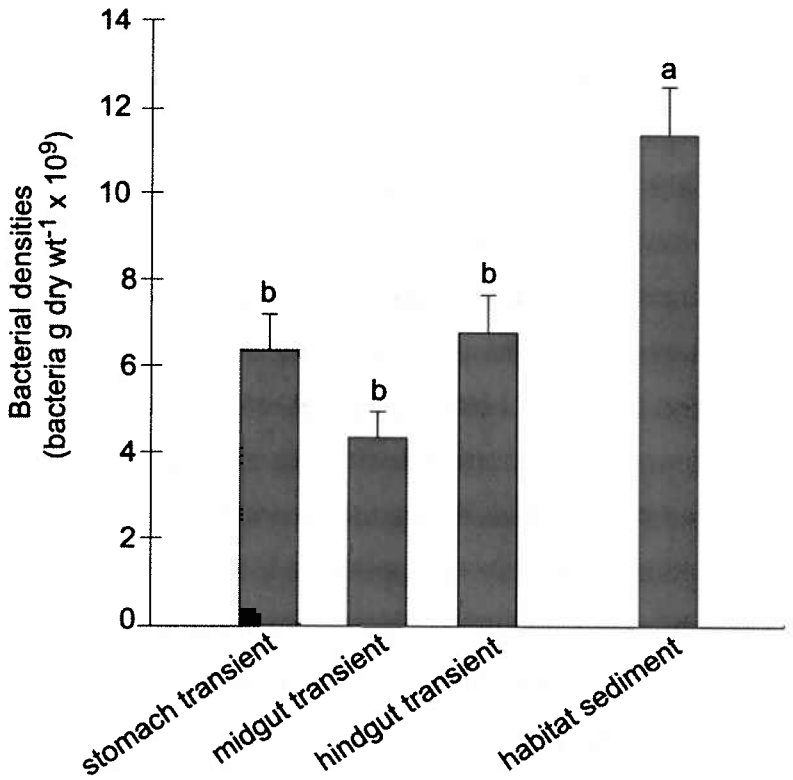

Figure 3.4 Transient bacterial densities in gut contents and habitat sediment (average +SE; n = 8) on a per gram dry weight basis. Letters indicate groupings with significant differences ($P < 0.05$).

densities (number gram^{-1} dry sediment) followed the same qualitative trend found for total transient abundances with greatest density in the hindgut, followed by the stomach and lowest density measured in the midgut section. However, differences in transient bacterial densities were insignificant among the three gut sections ($P > 0.05$; Bonferroni post hoc test).

DISCUSSION

Significance of bacterial locations and morphotypes

Distinct morphologies of attached bacteria are consistently associated with different regions of the *U. pugnax* gut, including the pyloric fingerlets and along the anterior and posterior hindgut. Bacteria resembling those we observed in the posterior hindgut of *U. pugnax* have been reported in other marsh fiddler crabs and detritivorous thalassinid prawns (Harris 1992; Harris et al. 1991; Pinn et al. 1999). Both *U. pugnax* and *U. minax* collected from Sapelo Island, Georgia harbor morphotypes of hindgut bacteria identical to those in *U. pugnax*: curved rods in the anterior projection region and dense mats of rods covering the smooth hindgut lining (Harris 1992). Hindgut bacteria similar in appearance to the dense mats of posterior hindgut rods in *U. pugnax* have been observed in species of detritivorous thalassinid shrimp, including filter feeders *Upogebia africana and U. pugettensis,* and deposit feeders *Neotrypaea* (formerly *Callianassa*) *californienesis* and *N. kraussi* (Harris 1991, 1992; Pinn 1997). Although we cannot draw any conclusions about which or how many bacterial species are present based on morphology, these hindgut morphotypes seem conserved among fiddler crab species from different locations and among both filter-feeding and deposit-feeding, detritivorous mud shrimp.

Length-wise attachment would increase attachment surface area for each cell, but it would appear to be the least advantageous orientation for diffusive exchange if the attachment surface were impermeable (Murray and Jumars

2002). Such bacterial-mat monolayers develop on relatively permeable cuticles (Bignell 1984), however, in which fluid transfer across the chitin surface would alleviate boundary-layer depletion for the bacteria. On-end attachment orientation occurs on less permeable surfaces and exposes the cell to greater diffusive gains (Murray and Jumars 2002). If these cuticle permeability generalizations apply to the fiddler crab gut, the observed bacterial arrangement suggests that the posterior cuticle is the most permeable. Consequently, bacterial products that accumulated along the hindgut could be absorbed rather than egested.

Posterior hindgut bacteria appear to use glycocalyx coatings to adhere to the chitin lining. In addition to having a role in attachment (Dunne 2002), this coating is thought to immobilize extracellular enzymes, thereby increasing local product concentrations. In rumen bacteria, attachment of cellulose fibers to glycocalx coatings facilitates enzymatic breakdown of cellulose (Roger et al. 1990), likely by limiting diffusion distances of product (Vetter et al. 1998). Additionally, glycocalyx coverings might protect cells from detrimental substances, such as surfactants and digestive enzymes.

Dense aggregations of attached bacteria within the pyloric stomach have not been reported previously in marine crustaceans. Furthermore, no resident foregut bacteria were observed with electron microscopy in any of the nine thalassinid shrimp species or the three fiddler crab species studied previously (Harris 1992; Pinn 1999). Observed pyloric stomach bacteria might be transient bacteria that are collected on the pyloric fingerlets before they are ingested. However, we propose that this pyloric stomach microbiota is a typical and resident feature of *U. pugnax* guts. Four intermolt individuals examined by SEM had pyloric fingerlet bacteria, and all these crabs had voided any visible gut contents. Pyloric fingerlets are an adaptation of particle-feeding crustaceans (Icely and Nott 1992), and we suspect that this region is colonized in other detritivorous crustaceans. Other researchers might have observed, as we did,

an absence or low abundance of bacteria on cardiac stomach surfaces and smaller pyloric projections and concluded that the entire stomach was free of attached bacteria.

Different regions along the crab gut are expected to have unique chemical environments and flow regimes due to specialized digestive processing in each section (Dall and Moriarty 1983). Each of these microenvironments might select for particular bacterial species and metabolisms. Bacteria associated with the pyloric stomach have access to ingested food and breakdown products before the host can absorb this material. At the same time they are exposed to digestive enzymes that may cause cellular damage. Fluid circulation and filtration within the crustacean stomachs and hepatopancreas is exceedingly complicated (Dall and Moriarty 1983; Icely and Nott 1992). Here we present a simplified description of digestion in *U. pugnax* with the goal of clarifying what materials are passed through the pyloric fingerlets. The host ingests a diet composed predominantly of benthic, unicellular phytoplankton, detrital organic matter (predominantly marsh grass) and associated bacteria (Miller 1961; Shanholtzer 1973). The light, organic-rich fraction is separated from denser mineral grains (see Miller 1961 for detailed description) and crushed by the gastric mill in the cardiac stomach. Host digestive enzymes are secreted from the hepatopancreas into the pyloric and cardiac stomachs and initiate organic matter breakdown (Dall and Moriarty 1983; Icely and Nott 1992). Very fine (< 2 μm) particles are passed directly into the pyloric stomach and filtered into the hepatopancreas. Coarser material is retained on filters and compressed by the pyloric fingerlets, thereby increasing the efficiency of fluid extraction. Fine setae on the fingerlets (Fig. 3.1B) collect particles that are then transferred towards the midgut, while fluid passes into the lumen channel of the hollow fingerlets and moves towards the hepatopancreas for absorption. Thus enzymes and breakdown products circulate across the pyloric fingerlets and flow past attached bacteria as the host processes food. These bacteria are ideally situated for

maximal exposure to digestive products, as long as the cells can withstand digestive enzyme activities. Furthermore, the host may absorb any released bacterial products, or bacteria themselves, along the midgut or in the hepatopancreas.

Bacteria along the hindgut do not necessarily have access to the same high-quality digestive products as the pyloric fingerlet bacteria, but avoid exposure to the same levels of active and potentially harmful digestive enzymes. Along the hindgut, resident bacteria have access to 'left-overs', that the host has processed but presumably still have some nutritional value for bacteria. Digestion theory predicts that detritivores increase their nutritional (absorptive) gains by ingesting fresh food before all (or even most) nutritional value has been removed from their gut contents (Dade et al. 1990). Therefore hindgut contents are likely to retain substantial nutritional value for bacteria.

As in the pyloric stomach, the perimeter of the hindgut is a hypothesized zone of re-circulation. Some crustacean species can take up water from the environment through the anus and move it forward via reverse peristalsis along the hindgut and midgut, at times as far anterior as the hepatopancreas (Dall 1967; Fox 1952; Lovett and Felder 1990). If hindgut bacteria produce fermentation products or host-beneficial enzymes, this mechanism could transport both bacterial products and detached bacteria toward the midgut and hepatopancreas for absorption. Fox (1952) observed that reverse peristalsis was most vigorous just before defecation, during the host's final opportunity to obtain any nutritional or energetic gain from its gut contents. Mayer et al. (2001) make the analogy to a rinse cycle in doing laundry to remove residual material. Alternatively, or in addition to the flushing mechanism, bacterial products may cross the hindgut cuticle for host absorption, as occurs in termites and other insects (Hogan et al. 1985; Maddrell and Gardiner 1980). Passive, cross-cuticle transport may occur whenever the concentration gradient of product is in the right direction, and reverse peristalsis may occur intermittently and likely just before

egestion. Resident bacteria in both the pyloric stomach and hindgut may benefit from inhabiting fluid recirculation zones. Fluid and digestive product recirculation would provide greater solute flux and might reduce the thickness of the boundary layer surrounding the bacteria (Plante et al. 1990).

Although we did not test for fermentation activities in this study, we observed black patches in the gut contents frequently, especially on the exterior of the food bolus in the pyloric stomach and hindgut. The pyloric fingerlets themselves commonly had visible black spots. These black sections result from sulfate reduction and increased concentrations of precipitated iron sulfides (Madigan et al. 2003), suggesting that the region may experience anoxic conditions and could support fermentative metabolisms. Fermentation products such as short chain fatty acids (SCFAs) are a potential energy source for the host crab and can be absorbed without active transport, even across the hindgut cuticle (Hogan et al. 1985; Maddrell and Gardiner 1980). In the hindgut, uptake of SCFA would allow the host to recover some nutritional value from host-derived organic matter including peritrophic membrane material and sloughed midgut cells (Plante et al. 1990).

Resident bacterial abundances

Bacterial abundances calculated from direct counts (DAPI) indicated that the hindgut and stomach harbored the greatest numbers of resident bacteria per section (Fig. 3.3). Both of these gut sections are lined with chitin, which may provide a better surface for attachment than tissue epithelium. In the blue crab, *Callinectes sapidus,* (Huq et al. 1986) demonstrated that *Vibrio cholerae* attaches more promptly to the chitinous hindgut lining than to the non-chitinous midgut lining. Although this lining is shed periodically with the host molt, chitin surfaces probably offer greater stability than midgut tissue from which surface epithelial cells are continuously sloughed (Dall 1967).

Hindgut bacterial densities in *U. pugnax* (8.04×10^8 ml^{-1}) were within the range reported in four species of thalassinid crustaceans (*Neotrypaea subterranean, Jaxea nocturna, U. stellata* and *Calocaris macandreae*) (1.01 – 9.07 10^8 ml^{-1}) collected from the Clyde Sea off Scotland (Pinn 1995; Pinn et al. 1997). However per unit of gut length, bacterial densities in *U. pugnax* were almost an order of magnitude greater than those in the thalassinids. In these shrimp species, bacterial densities ranged from $0.5 – 4.6 \times 10^5$ mm^{-1} gut length (Pinn 1995; Pinn et al. 1997) as compared with averages of 3.79×10^6 mm^{-1} gut length in *U. pugnax*. The thalassinids presumably have longer but narrower guts than *U. pugnax*.

We had expected lower densities of bacteria in *U. pugnax* than in thalassinids because previous SEM observations showed lower bacterial densities in fiddler crab hindguts than in thalassinid hindguts (Harris 1992). Two methodological issues might explain why we obtained surprisingly high bacterial densities. Firstly, Pinn (1995) did not assess the molt stage of the studied thalassinid specimens. Therefore some individuals may have been in postmolt phase and have had a reduced density of resident bacteria due to the limited time for recolonization. Secondly, differences in methods (homogenization in Pinn (1995), versus sonication in the present study) would explain the difference if homogenization detaches fewer resident bacteria than sonication, as reported in previous studies (Ellery and Schleyer 1984; Epstein and Rossel 1995). Our results suggest that *U. pugnax* hindgut bacterial abundances are at least as great or greater than those reported for four other species of detritivorous crustaceans.

In contrast with the hindgut comparison, densities of midgut bacteria (mm^{-1}) in *U. pugnax* are similar to those reported in other crustaceans. Four thalassinid species (*N. subterranean*, *J. nocturna*, *U. stellata*, and *Calocaris macandreae*) described above had midgut bacteria at densities of $0.5 - 3.2 \times 10^5$ mm^{-1} (Pinn et al. 1997). Average abundance of resident midgut bacteria in *U. pugnax* fell within this range (2.71×10^5 bacteria mm^{-1} midgut).

Within *U. pugnax*, midgut bacteria were over an order of magnitude less abundant and less dense than hindgut bacteria. It has been suggested that a host may protect the midgut from bacterial attachment by radically altering the pH of absorptive gut sections, as observed in mammals (Drasar et al. 1969; Giannella et al. 1973). However, this mechanism probably does not occur in *U. pugnax* because our measures of along-gut pH indicate that all gut sections have average pH values between 6.6 and 7.3 (data not shown). These near-neutral pH measures are in accordance with published results from marine detritivorous annelids and echinoderms, which generally reflect environmental pH values (Plante and Jumars 1992) and supports the idea of marine detritivores having relatively 'open' gut systems (Plante et al. 1990). Recent studies have found that some insects secrete antibiotic proteins in the anterior midgut to prevent bacterial colonization in this region (Munks et al. 2001). The presence of endogenous antibiotic substances has not been studied in fiddler crabs, but the relatively low abundance of resident midgut bacteria observed in *U. pugnax* suggests this could be a viable hypothesis for further study.

Another mechanism predicted to reduce bacterial attachment in the midguts of insects (Bignell 1984), is the formation of a midgut peritrophic membrane encasing the food bolus. *Uca pugnax* does appear to form a peritrophic membrane (Fig. 3.2D) and we propose that this membrane, likely in conjunction with 'lytic enzymes' and midgut tissue sloughing, is responsible for preventing bacterial growth along the midgut. In the hindgut, cuticular projections (Fig. 3.1C,D) are thought to destroy this peritrophic membrane (Bignell 1984), allowing hindgut bacteria direct access to gut contents.

It was unclear whether bacteria observed in the hepatopancreas were resident or transient, possibly in the process of being digested. If these are resident bacteria and a regular feature of the fiddler crab hepatopancreas, we would expect them to be present in all crabs inspected, not only half (2/4). Yet, their bacterial cell membranes appear fully intact, signifying that they are

probably not being digested but possibly living within the hepatopancreas. Furthermore, if these were transient bacteria, we would expect to detect more than a single bacterial morphology. The hepatopancreas is the main site of crustacean digestion and any bacteria associated with this section may have substantial impact on the host and material passing through the gut. Further research on the presence and role of hepatopancreatic bacteria is needed to clarify possible functions of these bacteria. We chose to focus our study on the two gut sections with clear resident populations; the stomach and hindgut.

Transient bacterial abundances

Detritivorous fiddler crabs such as *U. pugnax* ingest bacteria-rich marsh sediments and are presumed to have high abundances of bacteria passing through their guts. These transient bacteria may potentially contribute greater quantities of extracellular enzymes or SCFA products or both compared with resident bacteria (Harris 1993) or they may compete with the host for absorption of digestive products (Plante et al. 1990). One of our goals was to compare the numbers of transient and resident bacteria in each gut section, to assess whether resident bacteria are likely to play an important role in host digestive physiology.

On a gut-section basis, transient bacteria were roughly twice as abundant as resident bacteria in both the stomach and hindgut, but these numbers may not reflect their relative influence on host digestion. At most forty percent of marine sedimentary bacteria are estimated to be active (Plante and Wilde 2001), and the actual proportion of active bacteria may be much lower. Furthermore, digestive enzymes may lyse and/or inactivate many transient bacteria, especially in the stomach and midgut where enzymatic activities are greatest (unpublished results). In contrast, we expect that resident bacteria have adapted to gut conditions and the majority should be active. Therefore the number of active resident bacteria may be similar to the number of active transient bacteria. Even if all transient bacteria were active, resident bacteria would still constitute approximately one third of all gut bacteria in the stomach and hindgut; an ample

quantity to contribute to bacterially mediated digestion. Also, resident bacteria form a stable community (Chapter 4; this thesis, Lau et al. 2002) and are likely to have evolved a more specialized relationship with the host, particularly in the stomach and hindgut regions.

We expected to find the greatest concentration of transient bacteria in the stomach because *U. pugnax* selects for and concentrates the most organic-rich, and presumably bacteria-rich, fraction of the sediment in its stomach (Miller 1961). Yet, digestive enzymes are also released into the stomach and may lyse bacterial cells, lowering observed bacterial counts. Sediment in the midgut had the lowest concentration of transient bacteria, but not significantly less than sediment in the stomach or hindgut. The midgut section is an absorptive region where the products of lysed bacteria could be absorbed. Most bacteria are expected to be lysed and digested between the stomach and hepatopancreas, whereas the midgut allows further absorption of digestive products (Icely and Nott 1992). Slight, but non-significant increase in bacterial concentration in the hindgut may be due to bacterial regrowth in the relative absence of active digestive enzymes (Chapter 5; this thesis), as found for *Abarenicola vagabunda* (Plante et al. 1989). Either resident bacteria shed from the hindgut lining or surviving transient bacteria could reproduce along the hindgut, possibly thriving in a habitat relatively rich in organic matter (compared with environmental sediments) and with reduced enzyme activity (compared with anterior gut sections).

CONCLUSION

We have demonstrated that the stomach and hindgut of *U. pugnax* harbor relatively high abundances of resident bacteria, compared with other gut sections and with other crustacean hosts. Furthermore we found that resident bacteria constitute almost half the total transient count in these sections. Yet, with this information we can still only speculate about what roles these bacterial

populations have in host digestion. The species composition and stability of these populations needs to be addressed before further tracer studies and *in situ* fluorescent probe work can be done to determine bacterial metabolisms and to correlate particular species with specific gut sections.

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Chapter 4: Genetic diversity of resident bacteria in the digestive tract of the marsh fiddler crab, *Uca pugnax*

ABSTRACT

We assessed phylogenetic diversity of resident (attached) bacterial communities in the detritivorous salt marsh crab, *Uca pugnax*, over time and among individual hosts. DGGE analysis of 16S rRNA genes from gut populations revealed distinct patterns of bacterial diversity that were relatively conserved among individuals. Gut community diversity appears to be distinct from that found in habitat sediments and appears to differ markedly between stomach and hindgut. Although some changes in diversity patterns were observed between summer and winter, resident communities were not entirely different. Hindgut bacterial densities, however, did vary significantly with season. Clone libraries of 16S rRNA genes were generated from both stomach and hindgut microbiota (pooled from seven individual hosts), as well as from each of three DGGE bands (from a single host). We identified six bacterial phylotypes in the stomach and 13 in the hindgut, indicating that the hindgut hosts a more diverse community than the stomach. Many clones were most closely related to other symbionts and gut-associated bacteria. Few identified clones, however, shared more than 95% 16S rRNA gene sequence similarity with their nearest known relatives. Interestingly, the most abundant hindgut clone was most closely related to a clone from the hindgut microbiota of the detritivorous shrimp *Neotrypaea* (formerly *Callianassa*) *californiensis*, suggesting that detritivorous crustacean hindguts may provide a niche for specific bacterial phylotypes.

INTRODUCTION

The combination of detritivore and microbial metabolisms in the form of a digestive association has enormous potential to impact habitat biogeochemistry, as well as affect host physiology. Carbon and energy flows in marine sediments are influenced, in part, by activities of both invertebrate detritivores and microbial communities. Resident (attached) gut bacteria have been identified in multiple species of detritivorous arthropods (Harris ét al. 1991; Pinn et al. 1997). Researchers have suggested that these bacteria form the basis of mutualisms in which bacteria contribute enzymes or fermentation products to host digestion

(Pinn et al. 1997; Lau et al. 2002), as well as alter the chemical composition of egested matter (Lau et al. 2002).

To understand the roles of gut microbiota in host digestion and habitat biogeochemistry, information about bacterial diversity should be evaluated in light of gut morphology, gut chemistry and spatial distribution of microorganisms along the digestive tract. Estimating bacterial diversity in each gut section is a necessary first step before more specific questions on microbial function can be addressed. Early studies on the microbiota of detritivores relied on culture-based techniques (Harris et al. 1991; Pinn et al. 1997) and probably underestimated community diversity due to difficulties in simulating gut lumen conditions (Amann et al. 1995). A recent study has applied culture-independent techniques to the hindgut communities of a detritivorous marine arthropod (Lau et al. 2002), providing the first insight into detritivore microbial diversity.

In this study, we investigate the diversity of bacterial communities among gut sections of the detritivorous fiddler crab *Uca pugnax*. Our goal is to assess bacterial diversity within and between individual hosts, as well as changes in composition and abundance over time. For the temporal abundance measurements, we focus on the hindgut because it is commonly colonized by bacteria in various crustacean hosts (Dempsey and Kitting 1987; Harris 1992; Lau et al. 2002; Pinn et al. 1997) and supports the greatest bacterial abundances in *U. pugnax* (Chapter 3; this thesis). To assess variability among individual crab hosts, we compare individuals with the fingerprinting technique, denaturant gradient gel electrophoresis (DGGE). To assess seasonal change, we compare bacterial diversity patterns among representative summer and winter samples. Finally, we investigate bacterial diversity by sequencing clone libraries of both stomach and hindgut communities and comparing our findings with those from other arthropod microbiotas. Given the strong potential for fiddler crab hosts and resident bacteria to have evolved digestive symbioses, we predict that resident bacterial communities are consistent among individuals and stable over time.

Furthermore, we expect that these resident bacteria are related to phylotypes from other crustacean gut microbiotas and to known symbiotic bacteria. For our purposes, we define a stable microbial association as one in which the same bacterial phylotypes are found among individual hosts and over time.

METHODS

Crab collection and dissection

Marsh fiddler crabs, *Uca pugnax*, were collected from an intertidal salt marsh in Barnstable Harbor, Massachusetts (41°42'31 N, 70°18'17 W) during low tide. In February and September 2003, male crabs were collected to assess winter and summer diversity of resident microbiotas (n = 3;8). Only males were studied to eliminate the possibility of confounding, gender-related differences. Feeding rates differ between male and female fiddler crabs (Weissburg 1992; Weissburg 1993) and may influence bacterial diversity. Four samples of habitat sediment (~ 10 g wet weight each) were collected in September 2003 during crab collection; each was homogenized separately by mixing and frozen at –20°C. For seasonal counts of hindgut bacteria, crabs were collected monthly (July 2001 – August 2002) during year 1 and then every other month (October 2002 – August 2003) during year 2 (n = 6-10 $month^{-1}$). Crabs were kept in cooled containers (~15°C) and brought to the laboratory within 2 h after collection. Gender, molt stage (see below), carapace width and length of each specimen were recorded. These crabs depurated their gut contents for 24 h in 0.2-μm filtered flowing seawater at the approximate temperature and salinity of their collection or were dissected immediately and any gut contents removed by rinsing with sterile 0.2-μm filtered 0.1 M phosphate buffer. We found no significant difference in bacterial density between these two approaches (unpublished data). For bacterial diversity work, crabs were allowed to depurate for 3 h.

Before dissection, crabs were washed thoroughly with sterile filtered 0.1 M phosphate buffer (pH 8.1) and cold-anesthetized (0°C for 8-10 min). Anesthetized crabs were measured with calipers, weighed, and dissected using sterile tools on a sterilized aluminum surface. Stomach, hepatopancreas, midgut and hindgut sections were removed from the organism, measured for length and diameter (for hindgut samples), transferred to sterile 1.7-ml tubes and weighed. For counts of resident hindgut bacteria, tissues were preserved in 1 ml filter-sterilized 3% gluteraldehyde in 0.1 M phosphate buffer.

Molt stage analysis

Molt stage of individual crabs was determined by examining characteristics of pleopod and abdominal setae as described in Vigh and Fingerman (1985). Four molt stages were identified: postmolt, intermolt, premolt and ecdysis (Drach 1939). Setae were removed from crabs with fine dissecting forceps, immediately wet-mounted on slides in 0.1 M NaCl buffer and viewed with a Zeiss Axiostar Plus Microscope (500 or 1,000X magnification). Molt stage was determined for all crabs dissected after February, 2002. Only intermolt crabs were used for phylogenetic analysis of gut microbial communities.

Sonication, staining, and counting

Samples were sonicated and counted as described in Chapter 3 (this thesis). Total bacterial counts were reported as number of bacteria mm^{-1} gut length. Due to longitudinal folds in the hindgut lining, it is difficult to measure accurately the diameter of empty crab hindguts. Therefore we chose to normalize bacterial abundances by gut length, which we were able to measure accurately.

DNA extraction

Stomach, hepatopancreas and hindgut sections were placed in 0.7 ml DNA extraction buffer composed of 100 mM Tris buffer (pH 8), 100 mM NaEDTA (pH 8), 100 mM phosphate buffer (pH 8), 1.5 M NaCl, and 1% CTAB (hexadecyltrimethyl-ammonium bromide; Sigma-Aldrich). To separate bacterial biomass from host tissue, samples were sonicated as described for DAPI counts (Chapter 3; this thesis) except that only 0.6 ml supernatant was collected after each sonication (two total). Complete cell removal was confirmed by microscopic inspection of DAPI-stained samples. Combined supernatants (1.2 ml) were collected for each gut section sample and flash frozen in liquid nitrogen. Supernatant samples were subjected to three freeze-thaw cycles (liquid nitrogen and 65°C water bath) before adding 10 µl filter-sterilized proteinase K (10 mg/ml) and incubating at 37°C for 30 min. Cell lysis was confirmed by microscope inspection of DAPI-stained samples. Next, 50 µl sodium dodecyl sulfate (20% solution) was added, and samples were shaken on a rotary shaker at 300 rpm, at 65°C for 2 h. Mixtures were transferred to pre-pelleted Phase Lock Gel I tubes (Eppendorf) before extracting DNA twice with an equivalent volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8), followed by three chloroform:isoamyl alcohol (24:1) extractions. Supernatants were removed and precipitated in two volumes of 100% isopropanol (-20°C; 16 h) before centrifuging (12,600 rpm; 30 min) and washing the resulting pellet twice with 70% ethanol. Isolated DNA was resuspended in 30 µl sterile water and kept at -20°C until use. DNA was extracted from four sediment subsamples (~ 0.2 g each) with the UltraClean Soil DNA kit (Mo Bio Laboratories) according to the manufacturer's instructions.

PCR amplification

Touchdown PCR was used to amplify the 16S rRNA gene for DGGE (primers 341-GC, 907R) as described by (Muyzer et al. 1998). PCR reaction mixtures included: 200 μM of each dNTP, 10 mM of each primer,10% of 10X buffer, 25 mM $MgCl_2$ and sterile, double-distilled water. Taq DNA polymerase (Promega) was added at 1 unit per 50 μl reaction. After a 5-min denaturation step (94°C), the annealing temperature was lowered from 65 to 55°C over 20 cycles, followed by 10 cycles performed at 55°C. For sediment samples, bovine serum albumin (Promega) was added to the PCR mixture at a final concentration of 2 mg ml^{-1} to prevent inhibition of enzymatic amplification by humic compounds associated with sediments.

For cloning, DNA was extracted from stomachs and hindguts, as well as from DGGE bands, and the 16S rRNA gene was amplified with universal bacterial primers (27F and 1492R; or 341F and 907R for excised DGGE bands). To reduce PCR bias we combined 10 replicate reactions of 15 cycles each (Polz and Cavanaugh 1998). Amplifications consisted of 3-min at 94°C followed by 15 cycles of 94°C for 1 min, 50°C for 45 s, 72°C for 1 min, with a final extension step of 12 min at 72°C, performed in an Eppendorf Mastercycler Gradient thermal cycler. Combined PCR products were purified with the Wizard SV purification kit (Promega) and resuspended in 30 μl sterile water. Formation of heteroduplexes was restricted by performing five further PCR cycles with the above conditions (Thompson et al. 2002). Final PCR products were purified with the Wizard SV purification kit and cloned immediately.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE gels were run on a DCode electrophoresis unit (Bio-Rad). Samples were electrophoresed for 18 h at 100 V on denaturing gradient gels (1 mm thick, 6% (wt/vol) polyacrylamide, 30-60% or 20-70% denaturant). Gels of 20-70% denaturant were used initially to assess the complete spectrum of

diversity patterns. A narrower gradient (30-60%) was used subsequently to provide better resolution of neighboring bands. Amplified 16S rRNA gene fragments from eight individual crabs from summer 2003 collection (stomachs and hindguts) were run on both 20-70% and 30-60% DGGE gels. Winter (collected February, 2003) stomach (n = 3) and hindgut samples (n = 3) and four samples of homogenized summer Barnstable marsh sediment were run only on 20-70% gels. Running buffer (1X TAE buffer; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) temperature was maintained at 60°C. Gels were stained with SYBR Green nucleic acid gel stain (0.01% in 1X TAE buffer, Molecular Probes) and gel images were taken with a ChemiImager Light Image system (Alpha Innotech) gel documentation system. All visible bands were sampled with a wide-bore micropipette tip, and these gel fragments were incubated in 50 µl sterile water for 24 h at 4°C before storing at –20°C. Aliquots (1 µl) of this water were used to re-amplify bands by PCR for cloning.

Cloning

One bacterial clone library from the stomach and one from the hindgut were generated from equal amounts of DNA combined from each of seven individual stomach and hindgut samples (crabs 1-7). In addition, three bands were cloned from the 30-60% DGGE for crab 4 (H-A, H-B, and S-D). Purified PCR products of the 16S rRNA gene (27F, 1492R primers) or DGGE fragments (341F, 907R primers) were cloned into the pCR 4 TOPO vector, and transformed into TOP10 *Escherichia coli* cells with the TOPO TA cloning kit for sequencing (Invitrogen). Clones were grown in SuperBroth with ampicillin (100 µg ml^{-1}) for 18 h at 37°C on a shaker table (250 rpm). Plasmids were purified with a RevPrep Orbit robot (Genemachines) and resuspended in 60 µl of sterile water. Clones were screened by restriction analysis with the enzyme *Eco*R1. Restriction digests included: 5 µl of each purified plasmid, 0.1 µl acetylated BSA (10 µg μl^{-1}), 3.5 µl sterile water and 0.4 µl *Eco*R1 (10 U μl^{-1}). Mixtures were

incubated at 37°C for 4.5 or 5 h and gel analyzed. Only clones that had a single restriction pattern and had appropriately sized inserts [either ~ 1.5 kb or ~ 550 bp (DGGE)] were selected to sequence. Each unique restriction pattern was well represented by selected clones. Nearly complete, double-stranded sequences of 16S rRNA genes (1,349 –1,528 bp) were sequenced on a 96-capillary 3730xl DNA analyzer (Applied Biosystems) with primers 27F, 341F, 907R, 1492R, T3F and T7R.

Phylogenetic tree construction

By aligning both forward and reverse strands, a consensus sequence for every clone was created with the program Sequencher (version 4.1.2, Applied Biosystems). Sequences were tested for the presence of chimeras with CHIMERA_CHECK in the Ribosomal Database Project RDP (Maidak et al. 1997) and with the Bellerophon server (Huber et al. 2004). The ARB software package was used to analyze sequence data and construct trees (version 2.5b; O. Strunk and W. Ludwig, Technische Universitat Munchen, Munich, Germany; **http://www.arb-home.de**). Gene sequences (16S rRNA) were aligned with Fast Aligner tool (version 1.03) and corrected manually. All clonal sequences were compared to 16S rRNA gene sequences in the BLAST database (Altschul et al. 1990), and related sequences were added to the alignment.

Phylogenetic analyses of full-length sequences were performed in PAUP, version 4.0b10 (Swofford 1993) and ARB. Neighbor-joining trees were constructed with the Jukes-Cantor correction, with base frequency filters (50-100% similarity) and were bootstrapped with 1000 replicates (bootstrapped in PAUP). For tree construction, 1,305 sequence positions were used. Related sequences significantly shorter than 1,500 bp (DGGE band and *N. californiensis* hindgut clone sequences) were added to the tree with the ARB parsimony tool, allowing the addition of short sequences without changing overall tree topology. Trees created in PAUP and ARB produced identical branching patterns.

Therefore, rather than present both a tree with all full-length sequences and bootstrap values (from PAUP) and a separate tree with partial sequences (from ARB), we appended the ARB tree with the PAUP bootstrap values, for all full-length sequences.

Diversity analysis

Diversity coverage of 16S rRNA gene clone libraries was analyzed with the analytical rarefaction approximation algorithm of Hurlbert (1971) and 95% confidence intervals were estimated as described by Heck et al. (1975). Rarefaction curves were constructed with the Analytical Rarefaction software (**http://www.uga.edu/~strata/software/**). Total number of phylotypes present was estimated with the nonparametric indicator, Chao-1 (Chao 1987; Hughes et al. 2001).

RESULTS

Molt stage

Molt stage was determined for all collected crabs from February 2002 to September 2003. To improve comparability among hosts, only intermolt crabs were used for the presented analyses from February, 2002 onward. We suspect that resident hindgut bacterial measures for July to September 2001 included some postmolt and possibly premolt crabs for two reasons. Firstly, numerous postmolt crabs were found in the field in following years. Secondly, hindgut bacterial densities were highly variable in these crabs (postmolt crabs usually have lower bacterial densities than intermolt crabs; unpublished data).

Hindgut bacterial counts

Hindgut bacteria densities show strong seasonality, being greatest in summer and lowest in winter. Monthly variation in bacterial densities was significant (1-way ANOVA, $F = 26.98$, $df = 19$, $P < 0.01$). The Tukey post hoc

test indicated that May to August densities are greater than those measured from October to April, for both 2002 and 2003 (see Fig. 4.1 for groupings). We suspect that the inclusion of some postmolt individuals in sample sets from July to September, 2001 was responsible for the relatively high standard deviations and, in August 2001, the particularly low densities measured.

DGGE profiles

All eight individual stomach microbiotas produced similar banding patterns on both 30-60% (Fig. 4.2A) and 20-70% denaturant gels [Fig. 4.2B (a single representative profile is shown)]. Similarly, seven hindgut microbiotas had similar patterns (one hindgut microbiota produced smears) on both gels (Fig. 4.2A,B), yet the typical hindgut profile was distinct from the typical stomach profile.

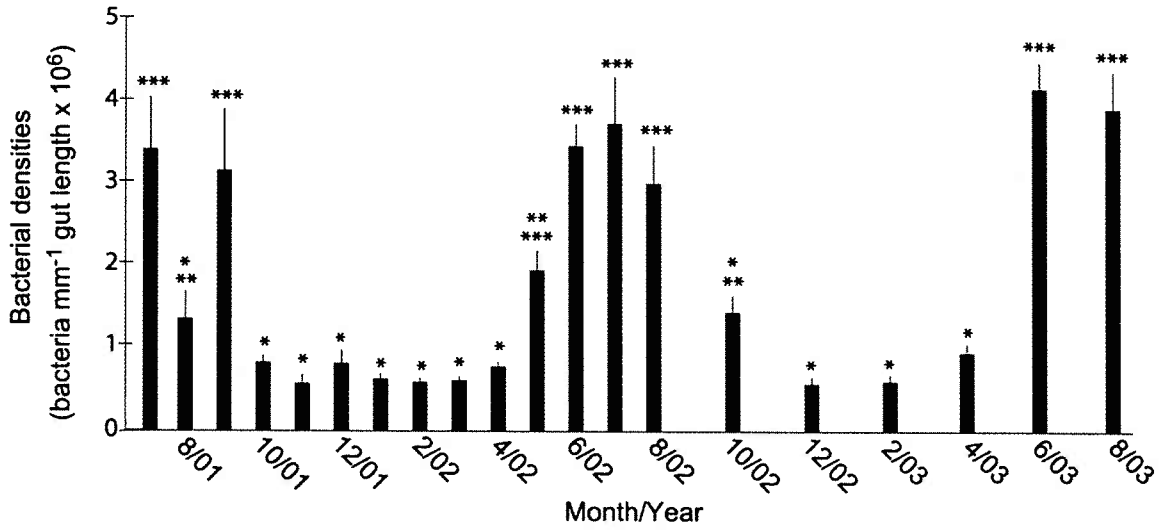


Figure 4.1 Seasonal counts of resident hindgut bacterial densities (average +SE; n = 6-10). Single, double and triple asterisks designate groupings with significant differences ($P < 0.05$) (One-way ANOVA and Tukey post hoc test).

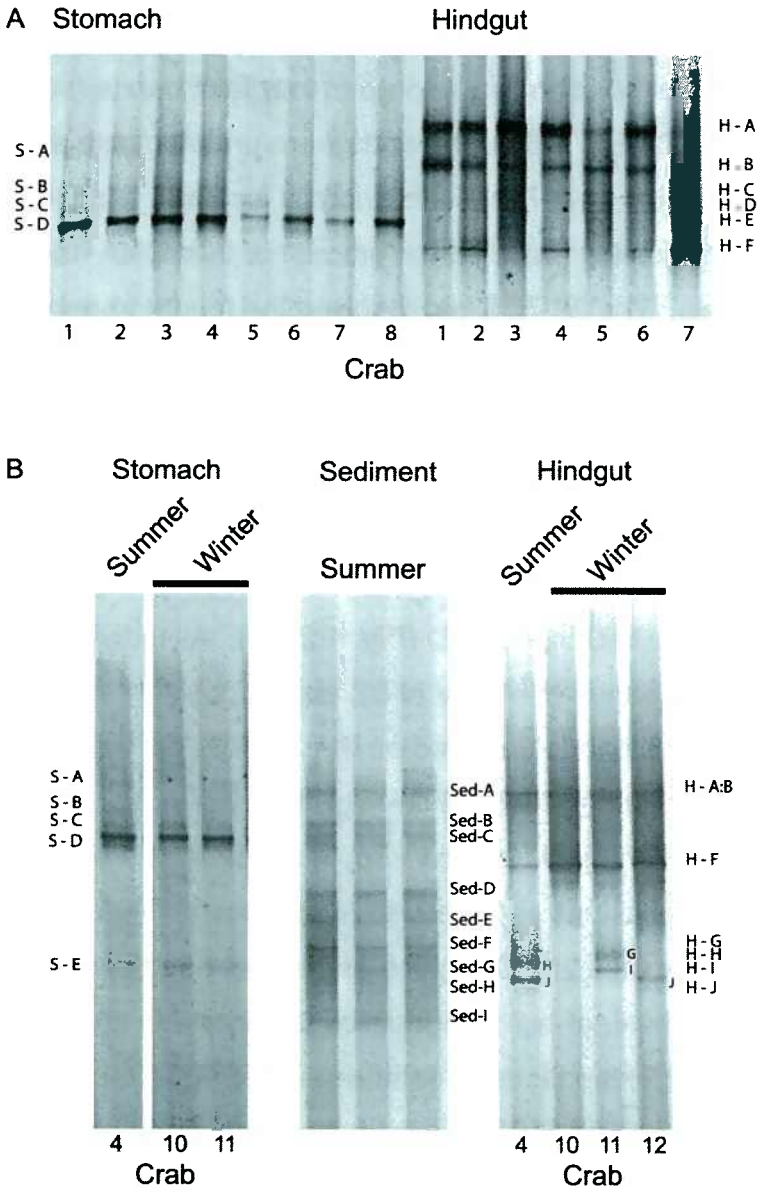


Figure 4.2 DGGE images A. Profiles (30-60%) of 8 individual crab stomach microbiotas and 7 individual crab hindgut microbiotas, from summer collections. Positions of apparent bands labeled S (stomach) A - D and H (hindgut) A - F. B. Profiles (20-70%) of summer habitat sediment and winter stomach and hindgut profiles compared with representative stomach and hindgut samples from crabs collected in the summer. Three representative sediment profiles are shown. Note that with this gradient of denaturant hindgut bands A and B converged to form a single band. Bands H-C,D,E were not detectable but bands H-G,H,I,J were resolved.

All stomach microbiotas, from winter and summer collections, exhibited a single intense band at position S-D and up to four faint bands (positions S-A, S-B, S-C, S-E) (Fig. 4.2A,B). Although bands were undetectable at positions S-A, S-B or S-C in crabs 5, 6, and 7 these individuals also had relatively weak bands at position S-D, suggesting that a lesser amount of total DNA had been loaded for these individuals. The 20-70% gel resolved the four stomach bands observed with the 30-60% gel, and, due to the broader range of denaturant, also revealed a lower band, S-E (Fig. 4.2B). This S-E band was present in the stomach microbiota from summer crabs 4-7, on 20-70% gels (not shown).

As determined from 30-60% and 20-70% gels, hindgut bacteria typically produced two intense bands (H-A, H-B), three strong bands (H-F, H-H, and H-J) and three faint bands (H-C, H-D, H-E) (Fig. 4.2A,B). All individuals appear to have the two intense bands (H-A and H-B), except possibly crab 7. For the hindgut profiles, not all bands detected on the 30-60% gel were evident on the 20-70%; in particular, bands A and B co-migrated (Fig. 4.2B). On the 20-70% gel, lower bands H-H and H-J were present in all summer crabs except crab 5, which had bands at positions H-G and H-I (not shown). DNA from excised bands (30-60% gel) was run on a 20-70% gel to confirm all band positions. None of the strong stomach and hindgut bands appeared to migrate to the same positions, as determined by both combinations of denaturant (Fig. 4.2A,B). Weak stomach band S-B and hindgut band H-C appear to share the same position.

Dominant bands found in typical summer profiles were also detectable in winter crabs (Fig. 4.2B), as indicated by three hindgut samples (crab 10, 11, 12) and two stomach samples (crabs 10, 11) from winter crabs (collected February 2003). One winter stomach DGGE sample (crab 12) was unreadable and not included in the results. Typical summer stomach bands S-D and S-E were visible, while bands S-A and S-C were barely detectable in winter crabs. Hindgut bands H-A:B and H-F were present in all three winter crab hindgut profiles. Lower bands showed greater variability in their presence and position and were

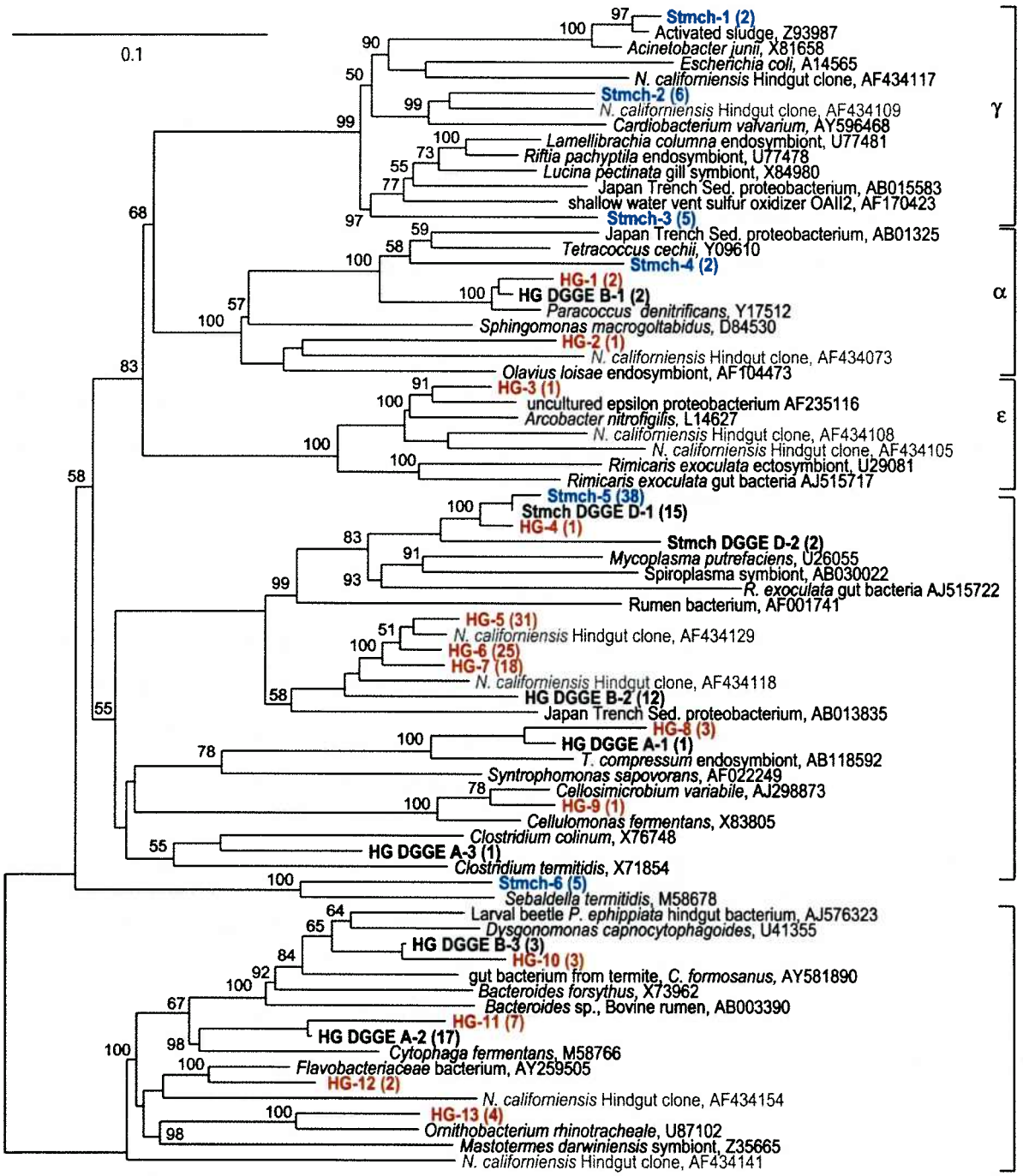
either absent (crab 10), consisted of double bands (crab 11, bands H-G and H-I) or had a single band (crab 12, band H-J). Interestingly bands H-G and H-I were also found in the hindgut profile from summer crab 5 (not shown).

Amplifying DNA from Barnstable marsh sediments proved difficult, possibly due to the presence of humic substances (Juniper et al. 2001; Tsai and Olson 1991). The occurrence of smeared bands may result from high bacterial diversity, in which the presence of many co-migrating sequences hinders resolution of distinct bands. These types of smeared bands are commonly observed in soils containing high bacterial diversity (Nakatsu et al. 2000). Only one attempt to visualize sediment samples on a DGGE gel was successful, and still produced only faint bands (Fig. 4.2B). In general, most stomach and hindgut bands do not seem to be detectable on the sediment profiles. The described extraction protocol with commercial kits (UltraClean Soil DNA Kit, Mo Bio Laboratories and DNeasy Tissue Kit, Qiagen) were unsuccessful in extracting any bacterial DNA from the hepatopancreas. Hepatopancreas tissue is very rich in lipids (Icely and Nott 1992) that may have hindered our ability to extract bacterial DNA.

Clone libraries

Two clone libraries were generated from 16S rRNA gene fragments amplified from stomach and hindgut DNA, each pooled from seven individual host crabs. We cloned pooled DNA in order to produce stomach and hindgut clone libraries representative of typical gut bacterial communities, rather than that of one individual. We justified pooling DNA from seven individuals based on the similarities in DGGE patterns. Three clone libraries were generated from DGGE bands: two hindgut bands (H-A and H-B) and one stomach band (S-D) from crab 4. Selected clones from the hindgut (100), stomach (58) and DGGE bands

Figure 4.3 Neighbor-joining tree with the Jukes-Cantor correction showing the positions of bacterial 16S rRNA gene sequences recovered from 7 grouped *U. pugnax* stomach and hindgut microbiotas. Partial DGGE sequences (~550 bp) and *Neotrypaea californiensis* hindgut clone sequences (~500 bp) were inserted into the tree using maximum parsimony criteria (ARB) without affecting the initial tree topology. Bootstrap values are based on 1000 replicates and are shown for values > 50%. Numbers of recovered clones are in parentheses after clone names. Scale bar represents 10% sequence difference. Accession numbers of reference sequences are indicated. Species used as the outgroup were *Archaeoglobus fulgidus*, *Methanomicrobium mobile*, and *Metallosphaera sedula*. CFB, Cytophaga-Flavobacterium-Bacteriodes phylum.


0.1
Stmch-1 (2)
Activated sludge, Z93987
Acinetobacter junii, X81658
Escherichia coli, A14565
N. californiensis Hindgut clone, AF434117
Stmch-2 (6)
N. californiensis Hindgut clone, AF434109
Cardiobacterium valvarium, AY596468
Lamellibrachia columna endosymbiont, U77481
Riftia pachyptila endosymbiont, U77478
Lucina pectinata gill symbiont, X84980
Japan Trench Sed. proteobacterium, AB015583
shallow water vent sulfur oxidizer OAII2, AF170423
Stmch-3 (5)
Japan Trench Sed. proteobacterium, AB01325
Tetracoccus cechii, Y09610
Stmch-4 (2)
HG-1 (2)
HG DGGE B-1 (2)
Paracoccus denitrificans, Y17512
Sphingomonas macrogoltabidus, D84530
HG-2 (1)
N. californiensis Hindgut clone, AF434073
Olavius loisae endosymbiont, AF104473
HG-3 (1)
uncultured epsilon proteobacterium AF235116
Arcobacter nitrofigilis, L14627
N. californiensis Hindgut clone, AF434108
N. californiensis Hindgut clone, AF434105
Rimicaris exoculata ectosymbiont, U29081
Rimicaris exoculata gut bacteria AJ515717
Stmch-5 (38)
Stmch DGGE D-1 (15)
HG-4 (1)
Stmch DGGE D-2 (2)
Mycoplasma putrefaciens, U26055
Spiroplasma symbiont, AB030022
R. exoculata gut bacteria AJ515722
Rumen bacterium, AF001741
HG-5 (31)
N. californiensis Hindgut clone, AF434129
HG-6 (25)
HG-7 (18)
N. californiensis Hindgut clone, AF434118
HG DGGE B-2 (12)
Japan Trench Sed. proteobacterium, AB013835
HG-8 (3)
HG DGGE A-1 (1)
T. compressum endosymbiont, AB118592
Syntrophomonas sapovorans, AF022249
Cellosimicrobium variabile, AJ298873
HG-9 (1)
Cellulomonas fermentans, X83805
Clostridium colinum, X76748
HG DGGE A-3 (1)
Clostridium termitidis, X71854
Stmch-6 (5)
Sebaldella termitidis, M58678
Larval beetle P. ephippiata hindgut bacterium, AJ576323
Dysgonomonas capnocytophagoides, U41355
HG DGGE B-3 (3)
HG-10 (3)
gut bacterium from termite, C. formosanus, AY581890
Bacteroides forsythus, X73962
Bacteroides sp., Bovine rumen, AB003390
HG-11 (7)
HG DGGE A-2 (17)
Cytophaga fermentans, M58766
Flavobacteriaceae bacterium, AY259505
HG-12 (2)
N. californiensis Hindgut clone, AF434154
HG-13 (4)
Ornithobacterium rhinotracheale, U87102
Mastotermes darwiniensis symbiont, Z35665
N. californiensis Hindgut clone, AF434141
γ
α
ε
Proteobacteria
Gram-positive
CFB

[Stmch D (17), HG A (19) and HG B (17)] were sequenced. One hindgut clone was identified as a chimera and was excluded from further analysis.

By assigning sequences with at least 98% sequence similarity to single phylotypes (16S rRNA sequence types), stomach and hindgut clones clustered into six and 13 different phylotypes, respectively (Tables 4.1,4.2). We chose to group phylotypes by 98% sequence similarity for two reasons. Firstly, this criterion is at least as stringent as the commonly applied bacterial species criterion of 97% 16S rRNA sequence identity (Kisand and Wikner 2003; Lau et al. 2002). Secondly, this criterion allows diversity comparison with another study of detritivore crustacean microbiota diversity that presented phylotypes of > 98% similarity (Lau et al. 2002).

All identified phylotypes belonged within the Proteobacteria (γ, α, or ε), gram-positive or *Cytophaga-Flavobacteria-Bacteroides* (CFB) phyla (Fig. 4.3). Half the identified stomach phylotypes (3/6) affiliated with γ-Proteobacteria. The greatest number of individual stomach clones (66%) belonged with the *Mycoplasma,* a group characterized by its lack of a cell wall. Hindgut clones

Table 4.1 Bacterial phylotypes identified from 16S rRNA gene sequences cloned from 7 crab stomachs.

Phylotype name	Length (bp)	# of clones	Within group % similarity	Nearest neightbor	% similarity to nearest neighbor[a]	DGGE band (# clones)
Stmch-1	1432	2	99.2%	*Acinetobacter junii*	97%	
Stmch-2	1387	6	99.4%	*Cardiobacterium valvarum*	92%	
Stmch-3	1421	5	99.9%	*Lucina pectinata* symbiont	89%	
Stmch-4	1349	2	99.1%	*Tetracoccus cechii*	92%	
Stmch-5	1494	38	98.9%	*Mycoplasma putrefaciens*	85%	Stmch D-1 (15) Stmch D-2 (2)
Stmch-6	1440	5	99.7%	*Sebaldella termitidis* (termite gut microbe)	90%	

[a] % similiarity is from BLAST results (Altschul 1990).

associated with the CFB, gram-positives, or α, or ε-Proteobacteria phyla. The gram-positive clones constituted the most frequent clone type in the hindgut library (79% of all clones) and within this group, three closely related firmicutes phylotypes (HG-5, HG-6, and HG-7), comprised 74% of all clones. Most stomach and hindgut phylotypes were not closely related to known bacterial species and typically shared only 86-94% sequence similarity with their nearest neighbors (fully sequenced phylotypes). HG-1, HG-3, and HG-9 and were exceptions, sharing 96%, 95% and 98% sequence similarity, respectively, with their nearest neighbors. Also HG-5, HG-6 and HG-7 were all 95% similar to a published partial sequence (~ 450 bp) of a firmicutes bacterial clone from hindguts of a detritivorous thalassinid shrimp, *Neotrypaea* (formerly *Callianassa*) *californiensis* (Lau et al. 2002).

DGGE clone libraries

Clones from the stomach DGGE band (S-D) clustered into two phylotypes, both of which were related to the *Mycoplasma-Spiroplasma* group within the Gram-positives (Fig. 4.3) and most closely associated with the most common stomach clone phylotype, Stmch-5 (Table 4.1). Phylotype Stmch DGGE D-1 is closely affiliated with Stmch-5, however Stmch DGGE D-2 is more distantly related and may correspond with another phylotype present in the crab stomach microbiota, not identified in this study.

The clone library of hindgut DGGE band H-A produced three different sequences (Table 4.2), two of which associated closely with particular clone phylotypes and one of which (HG DGGE A-3) may correspond with another phylotype present within the hindgut microbial community but not recovered by our clone library (Fig. 4.3). Hindgut DGGE band H-B clone library included three different sequences, each of which was attributed to identified phylotypes from the hindgut clone library. We expected that cloned sequences obtained from strong DGGE bands would associate with the most abundant phylotypes

Table 4.2 Bacterial phylotypes identified from 16S rRNA gene sequences cloned from 7 different crab hindguts.

Phylotype name	Length (bp)	# of clones	Within group % similarity	Nearest neightbor	% similarity to nearest neighbor[a]	DGGE band (# clones)
HG-1	1369	2	99.8%	*Paracoccus denitrificans*	96%	HG B-1 (2)
HG-2	1453	1	-	*Sphingomonas macrogoltabidus*	85%	
HG-3	1419	1	-	uncultured ε–proteobacterium (North Sea pelagic bacterium)	95%	
HG-4	1497	1	-	*Mycoplasma putrefaciens*	86%	
HG-5	1528	31	98.9%	*N. californiensis* hindgut clone	95%	
HG-6	1524	25	98%	*N. californiensis* hindgut clone	95%	HG B-2 (12)
HG-7	1526	18	99.7%	*N. californiensis* hindgut clone	95%	
HG-8	1472	3	99.3%	*Trimyema compressum* (anaerobic ciliate endosymbiont)	91%	HG A-1 (1)
HG-9	1420	1	-	*Cellulosimicrobium variabile* (termite hindgut symbiont)	98%	
HG-10	1437	3	98.8%	*Pachnoda ephippiata* gut bacterium (humus feeding beetle)	92%	HG B-3 (3)
HG-11	1485	7	99.5%	*Cytophaga fermentans* *Bacteroides* sp.	91% 91%	HG A-2 (17)
HG-12	1422	2	99.9%	*Flavobacteriaceae* bacterium	94%	
HG-13	1404	4	99.1%	*Ornithobacterium rhinotracheale*	93%	

[a] % similiarity is from BLAST results (Altschul 1990).

recovered from each gut section. Both methodologies, DGGE and clone libraries, are subject to PCR biases. Therefore amplified phylotypes frequencies do not necessarily reflect their natural abundances (Polz and Cavanaugh 1998; Suzuki and Giovannoni 1996). Nevertheless, phylotypes abundant in the crab microbiota and/or phylotypes with sequences favorably amplified by PCR should be relatively abundant in DGGE (as indicated by strong bands) and in the clone library (represented by most abundant phylotype). Indeed, the most abundant phylotype from hindgut DGGE band H-B corresponded with the three most abundant, and closely related, hindgut clone phylotypes (HG-5, HG-6, HG-7). The most common phylotype from hindgut DGGE band H-A corresponded with

HG-11, which was the next most abundant hindgut phylotype after the HG-5, HG-6, HG-7 cluster.

Many phylotypes were most closely related to other known symbiotic bacteria, and relatives of arthropod symbionts were particularly common. Both Stmch-5 and HG-4 clustered with the *Mycoplasma-Spiroplasma* group, which includes known arthropod gut symbionts (Whitcomb 1980; Williamson et al. 1997). Three hindgut phylotypes were most closely aligned with clones from the hindgut of the detritivorous shrimp, *Neotrypaea californiensis*. Three other hindgut phylotypes (HG-8, HG-9, HG-10) were closely related to known symbiotic bacteria from termites, beetles, and an anaerobic ciliate.

Diversity analysis

Due to an undersampling bias, clone library sequences might not reflect total community diversity (Von Wintzingerode et al. 1997). For hindgut and stomach clone libraries, diversity coverage was assessed with rarefaction analysis (Fig. 4.4). The slope of the curve indicates the extent of total diversity sampled, with low or zero slope signifying good coverage and steep slope indicating extent of unsampled diversity. The stomach clone library appeared to have reached saturation of unique phylotypes. The shape of the hindgut library curve suggests that more than 13 phylotypes were present in the hindgut. The Chao-1 estimator confirmed these results and calculated total diversity as six phylotypes in the stomach microbiota and 17 phylotypes in the hindgut microbiota.

DGGE profiles also offer an estimate of bacterial diversity. Ideally, each phylotype is manifested as a separate DGGE band. Stomach bacterial communities produced five total DGGE bands and hindgut bacterial communities produced 10 bands. Therefore both clone library results and DGGE patterns suggest that the hindgut communities are more diverse than those in the stomach. Yet DGGE patterns underestimate total diversity (as calculated by

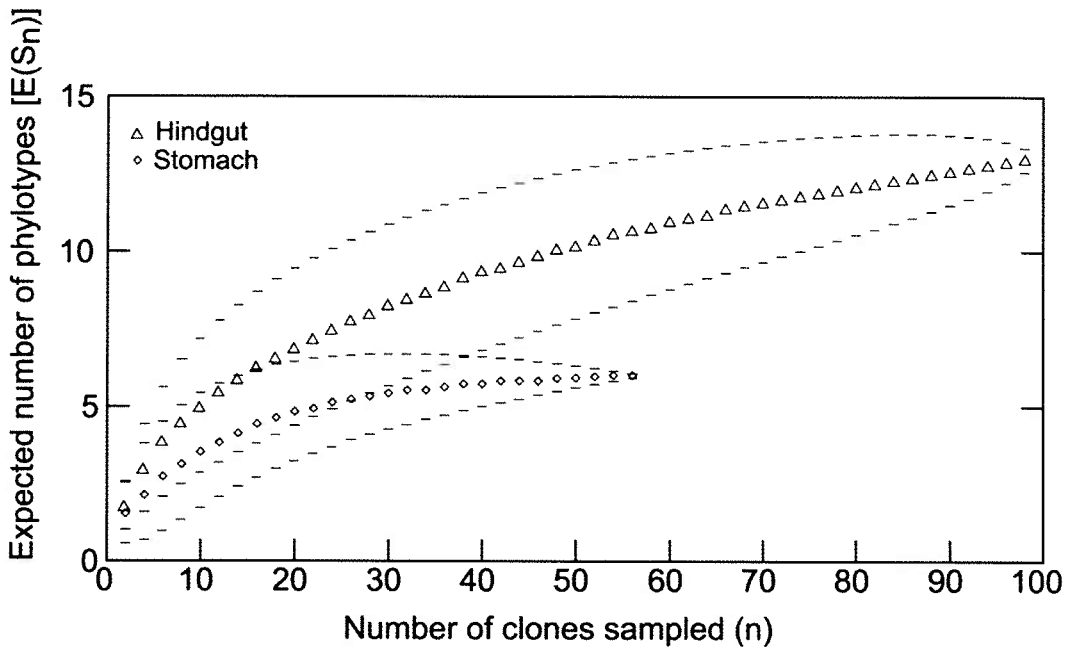


Figure 4.4 Rarefaction curves of observed phylotypes for 58 stomach 16S rRNA sequences and 99 hindgut sequences in which phylotypes are defined by >98% sequence similarity. The slope of the curves indicates the extent of total diversity sampled, with low or zero slope signifying good coverage and steep slope indicative of extent of unsampled diversity. Dashed lines delineate 95% confidence intervals.

Chao-1 estimators), due, most likely, to the occurrence of co-migrating bands as documented by our finding of two or more phylotypes in a single band.

DISCUSSION

Seasonal densities of hindgut bacteria

Resident hindgut bacteria in *U. pugnax* are present throughout the year (Fig. 4.1). Significantly greater abundances in summer may be a function of bacterial growth from increased food intake, improved food quality and/or elevated temperatures, compared with winter. Reduction in bacterial densities coincides with onset of dormancy (pers. observation) in which crabs remain in their burrows in a state of torpor, coming out to feed only on days when temperatures exceed ~ 10°C. Because crabs do not molt during winter, one might expect bacterial densities to increase with time, assuming that resident bacteria continue to divide. Yet without food passing through the gut regularly,

there are few nutritional sources available to the resident bacteria. It is possible that bacterial division rates slow or cease during winter and/or that crab hosts may digest dislodged bacteria. Resident microbes experience different thermal and nutritional conditions in winter compared with summer, and these different conditions might select for an entirely different community. Our DGGE results, however, suggest that winter microbiotas share at least two to three bands (Fig. 4.2B) with the typical summer microbiota. Therefore, although some bacterial phylotypes might be lost during winter dormancy, the community is not completely altered.

Bacterial community profiles among individuals

Distinct bacterial communities appear to associate with specific gut microhabitats. Finding a common DGGE pattern among individual crab stomachs and another DGGE pattern common among hindguts, suggests that resident microbial communities are not a haphazard mix of opportunistic bacteria from habitat sediment colonizing the gut lining. Although DGGE profiles of habitat sediment may not have resolved all potential bands, certain bands were distinct and clearly absent from hindgut and stomach profiles. This absence suggests that abundant and/or well-amplified sediment sequences were not present in the crab microbiota and corroborates reports of distinct bacterial communities in deposit-feeder guts compared with local sediment communities (Harris et al. 1991; Ward-Rainey et al. 1996). A reduction in bacterial diversity in the stomach and hindgut, compared with the sediment community, suggests that gut conditions favor growth of specific populations.

This difference between sediment and gut communities raises the question of how a particular gut microbiota persists though successive molts if not re-inoculated from local sediments. Resident bacterial populations may arise from bacterial strains present in marsh sediments in abundances too low to detect on the sediment DGGE profile. After molting and shedding their chitinous

gut linings, crabs take up bacteria with ingested sediment, and some of these strains may survive gut passage, attach and initiate colonization of the new lining. If only a limited number of sediment-associated strains are capable of surviving gut passage and attaching to the gut lining, bacterial diversity would be conserved among individuals, as suggested by our DGGE profiles. Presumably some resident gut microbes would exist in local sediments if neighboring crabs egest fecal material containing resident bacteria shed during gut passage. Alternatively, crabs may have obtained their resident microbiota early in development (i.e. during a pelagic larval stage; Lau et al. 2002) and microbial continuity could be maintained between molts if bacteria are retained within the gut lumen to reattach to the new cuticle. We think that the former, more parsimonious explanation is more likely.

Our conclusion that *U. pugnax* hosts share similar stomach communities and similar hindgut communities was based on common DGGE banding patterns. However, we found that three of these DGGE bands comprised more than one unique sequence. Our clone libraries revealed that the two most common hindgut bands, HG-A and HG-B, were composed of three phylotypes each, and the stomach band S-A contained two phylotypes. Therefore the presence of band HG-B might correspond with the existence of HG-1, HG-6, and HG-10 or any combination of them. Consequently, there may be more variability among individual microbiotas than our DGGE patterns indicate. Co-migrating phylotypes are not unprecedented and have been reported in bacterial communities (Kowalchuk et al. 1997). Alternatively, a single DGGE band may yield multiple sequences due to contamination from background DNA, as evidenced by the relatively high background smear. To resolve which phylotypes correspond with a particular DGGE band, amplified DNA from clones of these phylotypes should be run with the original DGGE band. Nonetheless, the general consistency of DGGE patterns and the differences from the sediment community suggests that bacterial microbiotas do not colonize at random.

Although some DGGE sequences were highly similar to particular phylotypes from clone libraries (< 2% sequence divergence), other DGGE sequences shared less than 92% sequence similarity (Fig 4.3). If these DGGE sequences all correspond with clone library phylotypes, we would expect each sequence to share at least 98% sequence similarity with a specific clone library phylotype. These sequence differences may result from UV alteration of DNA during DGGE visualization (Muyzer et al. 1998), uncorrected PCR errors (Polz and Cavanaugh 1998), and/or use of different sets of primers. Different primers vary in their binding efficiencies with clone sequences and therefore may influence the composition of recovered clones.

Furthermore, our rarefaction analysis indicates that bacterial diversity is reasonably well sampled for the hindgut and thoroughly sampled for the stomach. Hence, there is a low probability that we should recover any unique DGGE sequences. However Stmch DGGE D-2 and HG DGGE A-3 both appear to be unique sequences, unaffiliated with any recovered clone phylotypes. These DGGE sequences may represent phylotypes not recovered by the original clone libraries. Again the use of a different set of primers may have selected for different sequence recovery. Alternatively these two unique sequences may result from heteroduplex formation (Thompson et al. 2002).

Diversity and phylogenetic affinities in resident bacterial communities

The stomach bacterial community appears to be less diverse than the hindgut community, based on number of DGGE bands, clone phylotypes, rarefaction analysis and Chao-1 estimator values. This finding suggests that some aspect of stomach conditions limits the number of microbial strains able to survive. Higher enzyme activity levels in the stomach than the hindgut (Chapter 5; this thesis) may restrict stomach bacterial diversity.

Based on scanning electron microscope (SEM) images of the stomach (Chapter 3; this thesis), we speculated that the numerically dominant, rod-shaped

form would correspond with the strongest DGGE band and most abundant clone. However, the most abundant clone (Stmch-5) and corresponding DGGE band sequences (Stmch DGGE D-1, D-2) were all most closely related to *Spiroplasma* sp. and *Mycoplasma* sp., strains that do not have a cell wall and are characterized by either corkscrew-shaped (*Spiroplasma* sp.) or minute (< 1 μm) coccoid forms, respectively (Madigan et al. 2003; Whitcomb 1980). Either the observed rod-shaped bacteria were not the most abundant form or our methodologies do not reflect relative strain abundances. The latter explanation is possible because both DGGE and clone libraries are subject to PCR bias and do not necessarily reflect environmental abundances. These *Spiroplasma* sp.-related sequences may have originated from the corkscrew-shaped bacteria observed on the gut protist, *Enteromyces callianassae* (Chapter 3; Fig. 3.2A). On the other hand, if these gut phylotypes lack cell walls like their *Spiroplasma* sp. relatives, they may have been destroyed during SEM sample dehydration.

Strains from the *Spiroplasma-Mycoplasma* spp. group are known to inhabit terrestrial arthropod guts (Whitcomb 1980). *Spiroplasma* sp. have been found in digestive tracts and hemolymph of a broad range of plant-eating insects and, although generally considered pathogens, have been described as beneficial or commensal in some reports (Hackett et al. 1992). Another *Spiroplasma* sp.-related phylotype was identified in the gut of the Mid-Atlantic Ridge hydrothermal vent shrimp, *Rimicaris exoculata* (Zbinden and Cambon-Bonavita 2003). Based on the healthy appearance of the host specimens, the authors speculated that these gut bacteria were not pathogenic. Judging from the activity levels and generally fit appearance of *U. pugnax* hosts, we also expect that resident gut bacteria do not inflict harm on their hosts. We presume that this *Spiroplasma*-like phylotype originated from the bacterial community attached to the stomach lining; however, we cannot exclude the possibility that the crab hemolymph was the source. Before sonicating to detach resident bacteria, all visible tissue was removed from the stomach, yet bacteria from the

host hemolymph may have been present. Further research using phylotype-specific fluorescent probes and *in situ* hybridization is necessary to resolve the specific identity and attachment sites of this phylotype.

Closely related bacterial phylotypes were found in hindgut microbiotas of both fiddler crab *U. pugnax* from the Atlantic Ocean (this study) and detritivorous shrimp *Neotrypaea californiensis* from the Pacific (Lau et al. 2002). Although these two host species were collected from different oceans and different habitats (intertidal salt marsh and deep intertidal mud flats), they both host related resident phylotypes from within the gram-positive bacteria, α-Proteobacteria, and ε-Proteobacteria. In particular, the most abundant hindgut phylotypes, from both the clone and DGGE band B libraries, in *U. pugnax* (HG-5,-6,-7) were most closely related to two *N. californiensis* gram-positive clone phylotypes. Yet these clustered hindgut phylotypes were only distantly related to any other known bacteria (< 85% sequence similarity). We suggest that the presence of these similar phylotypes among different hosts supports the idea of a specific association between detritivorous crustaceans and their gut microbiotas. Furthermore, the abundance of recovered bacterial phylotypes with low similarity to known strains, from both host species, suggests that crustacean gut microbiotas may be a source of novel bacteria, as has been suggested for termite gut microbiotas (Ohkuma and Kudo 1996) and a crustacean gut microbial community (Lau et al. 2002).

Hindgut bacterial diversity in *U. pugnax* was similar to that found in the microbiota of other crustaceans. In the resident hindgut microbiota of *N. californiensis*, Lau et al. (2002) identified 12 phylotypes (> 98% sequence similarity) and total diversity was estimated to include 13 phylotypes (Chao-1 estimator; our calculation). These phylotypes were found among nine individuals exposed to different feeding treatments (9 clone libraries: 30 clones each). In the hydrothermal vent shrimp, *R. exoculata*, only seven phylotypes were identified in a gut microbiota clone library (Zbinden and Cambon-Bonavita 2003). Total

diversity was estimated to include eight phylotypes (Chao-1 estimator; our calculation). This clone library was created from the entire digestive tract (fore-, mid- and hindgut) of two individuals. Because all gut sections and both transients and residents were grouped, this library might have been expected to contain greater diversity than that found in *U. pugnax* stomach and hindgut libraries combined. Two factors may help to explain the lower diversity observed in vent shrimp. Firstly, some *R. exoculata* phylotypes were grouped by a less stringent criterion (> 92% similarity). Secondly, this shrimp species is not a detritivore and is thought to obtain nutrition by ingesting epibiotic bacteria (Rieley et al. 1999), vent chimney surface bacteria, and/or incorporating organic matter produced chemoautotrophically by gut bacteria (Polz et al. 1998; Pond et al. 1997). These three cases highlight the potential for different interactions between host and gut microbiota, even within marine crustacean hosts. We speculate that *U. pugnax* and *N. californiensis* are likely to have similar types of interactions with their resident gut microbiotas, based on similarity in hindgut phylotypes and similar levels of diversity.

Possible roles of bacterial microbiota

Resident bacteria of *U. pugnax* may be involved in a nutritional mutualism, particularly if bacteria provide the host with useful enzymes or fermentation products. Many recovered gut clones were related to other known symbionts, some identified as having autotrophic, fermentative or cellulose-degrading metabolisms that may provide nutritional energy to their hosts. Bacterial strains represented by the observed phylotypes do not necessarily have the same metabolisms as their closest relatives. Nevertheless, this information can help refine expectations of possible metabolic activities. In particular one hindgut phylotype (HG-9) was closely related (98% sequence similarity) to the facultative anaerobe, *Cellulosimicrobium variabile*, a cellulytic and fermentative bacterium isolated from the hindgut of termite *Masototermes darwiniensis*. *Paracoccus*

denitrificans was the closest relative (96% sequence similarity) of another hindgut phylotype and a DGGE band sequence. This strain is a facultative anaerobe that can grow autotrophically on hydrogen and carbon dioxide with nitrate as the electron acceptor (White 1995). If the related gut phylotype is also capable of autotrophic production, the host may benefit from uptake of any leaked substrates.

Multiple phylotypes belonging to the *Cytophaga-Flavobacterium-Bacteroides* phyla were recovered from the hindgut library and from DGGE bands. *Cytophaga* spp. are known for their abilities to degrade polymers such as chitin and cellulose and often associate with surfaces (Delong et al. 1993) such as nematode cuticles (Polz et al. 1999). *Bacteriodes* strains are commonly the numerically dominant gut microbiota member found in phylogenetically diverse hosts, including humans, termites, and ruminants (Ohkuma and Kudo 1996; Suau et al. 1999; Whitford et al. 1998). The *Bacteroides* group includes mostly strict anaerobes involved in starch degradation (Mccarthy et al. 1988). Furthermore, *Sebaldella termitidis*, previously *Bacteroides termitidis,* produces acetate and therefore the related stomach phylotype may be important to host carbon and energy budgets (Potrikus and Breznak 1980). The abundance of phylotypes related to facultative anaerobes offers some support for our expectation that the gut may experience occasional anoxic conditions (Chapter 3; this thesis).

The absence of certain typical salt marsh and crustacean-associated strains from both stomach and hindgut libraries supports our hypothesis of a non-random assemblage of gut microbiota. The δ-Proteobacteria subdivision is characteristic of salt-marsh sediments (Burke et al. 2002), yet no representatives were identified in any clone library. In particular, anaerobic, sulfate-reducing bacteria (SRB) of the δ-subdivision of Proteobacteria are common salt-marsh bacteria (Devereux et al. 1996; Klepac-Ceraj et al. 2004) and important to marsh geochemistry, but were not present in gut microbiota libraries.

Vibrio spp. are typical of estuarine waters and sediments and have been detected in association with crustacean hosts from around the globe, yet were conspicuously absent from all clone libraries in this study. *Vibrio* spp. commonly associate with chitin carapaces and gut linings and have been identified both with SEM and by culturing approaches. *Vibrio* spp. were the dominant forms found on anal plates of the amphipod *Boeckosimus affinus* (Atlas et al. 1982), in stomachs and hindguts of the brown shrimp *Penaeus aztecus* (Dempsey and Kitting 1987; Dempsey et al. 1989), and in guts of the deep-sea royal red shrimp, *Pleoticus robustus* (Dilmore and Hood 1986). Vibrios have been shown to attach to the chitinous hindgut lining of the blue crab, *Callinectes sapidus*, but not to the midgut (Huq et al. 1986). The presence of gut-associated *Vibrio* spp. could depend on diet composition. Most of these identified *Vibrio* spp. hosts are carnivores or scavengers, whereas *U. pugnax* and *N. californiensis* are detritivores. Gut chemistries differ between these feeding guilds. Alternatively, detritivores might be able to lyse and digest *Vibrio* spp.

CONCLUSION

We have demonstrated that the stomach and hindgut of *U. pugnax* harbor distinct bacterial associates, and that these communities differ from those associated with their habitat sediment. A study of habitat bacterial diversity is needed to address our hypothesis that resident bacteria arise from low-abundance sediment populations. Also, to understand better the nature of the association, specific bacterial morphologies and attachment sites need to be connected with particular bacterial phylotypes and metabolisms. A study with phylotype-specific probes for fluorescent *in situ* hybridization (FISH) and metabolic tracers would best address these questions. These types of studies will give further insights into the role resident bacterial communities play in host digestive physiology and environmental biogeochemistry.

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Chapter 5: Microbial contributions to digestive enzyme activity in the marsh fiddler crab, *Uca pugnax*

ABSTRACT

The stomach and hindgut of the salt-marsh fiddler crab, *Uca pugnax*, harbor diverse and abundant microbial communities. These microbes may release extracellular enzymes along the digestive tract, thereby benefiting their host. The main goal of this research was to determine if the presence and abundance of resident microbes correspond with dissolved extracellular enzyme activity. We found that antibiotic treatments typically reduced both total enzyme activity and total bacterial abundances in the stomach and hindgut. Bacterial and Eccrinales abundances, however, did not correlate significantly with enzyme activities in the stomach. On the other hand, hindgut bacterial abundances correlated with multiple enzymes, generating highly positive correlation coefficients. Hindgut *Enterobryus* sp. lengths correlated positively with esterase and protease activities. We suggest that these bacteria, and possibly *Enterobryus* sp., may be degrading the remnants of the chitin- and protein-rich peritrophic membrane. These enzymes or bacterial products may benefit their host if they are transferred anterior-ward by fluid flow. We also found that, using the described methodology, essentially all measured activity was derived from dissolved extracellular enzymes, rather than from particle-associated or cell-associated enzymes.

INTRODUCTION

Fiddler crabs may benefit from enzymes and other products generated by microbes inhabiting their digestive tract, as demonstrated for termites and other insects (Breznak and Brune 1994). The marsh fiddler crab, *Uca pugnax,* ingests surficial marsh sediment, and is adept at selecting for benthic algae and the organic-rich fraction of the sediment (Miller 1961). Yet detritus, and in particular refractory marsh-grass detritus, composes the bulk of the crab gut contents (Shanholtzer 1973). These fiddler crabs, therefore, subsist on a relatively low quality diet, compared with carnivores, and would benefit from any nutritional input increasing their total metabolic gain. Microbial enzymes may make a

substantial contribution to the host's energy budget. In this partnership, resident (attached) microbes would lose a fraction of their potential metabolic products, however, this loss might be offset by the provision of a stable habitat and constant food supply from the host.

Microbial enzyme contribution is a commonly suggested basis of interaction between marine invertebrates and resident gut microbes (Dempsey and Kitting 1987; Erasmus et al. 1997; Pinn et al. 1997; Vitalis et al. 1988). In *U. pugax* this simple prediction is complicated by the presence of both resident bacteria and Eccrinales protists in the stomach and the hindgut (Chapter 2, this thesis). Furthermore, transient (sediment-associated) microbes may be lysed in the gut, conceivably releasing active enzymes, or may able to survive gut passage and release enzymes into the gut lumen (Harris 1993). Any or all of these microbial populations might contribute a novel enzyme not produced by the host or make a quantitative contribution of an endogenously-produced enzyme. Therefore, any enzyme activity detected along the digestive tract may have been produced by the host, bacteria (resident and/or transient), and/or symbiotic protists. One of the goals of this study is to measure enzyme contributions from these various sources.

In *U. pugnax*, hindgut microbes (bacteria and protists) are attached to the chitinous hindgut lining and stomach microbes are attached to the pyloric fingerlets or cardiac stomach chitin surfaces (Chapters 2, 3, this thesis). Presumably, these microbes benefit from attachment by avoiding washout and by residing in relatively nutrient-rich locations. However, their attached position necessarily limits resident microbes' exposure to substrates. Firstly, these microbes are hindered from attaching directly to food particles. Secondly, resident microbes, and bacteria in particular, sacrifice absorptive surface area and likely lose access to diffusive transport by attaching to the gut lining (Murray and Jumars 2002).

Attached microbes are expected to produce extracellular enzymes that act on organic material passing through the host's gut. For direct absorption of substrates, bacteria are limited by the dimensions of pores in their cell walls (porins) to hydrophilic molecules smaller than approximately 600 Da (Schirmer 1998; Weiss et al. 1991). Therefore, production of extracellular enzymes allows microbial access to relatively large particles of organic matter. These enzymes may be attached to the cell surface or may be released into the lumen (cell-free extracellular enzymes). Attached enzymes have the benefit of concentrating the product in close proximity to the cell, yet have a very limited physical range to access organic matter. For the producing cell, released extracellular enzymes have the advantage of a greater physical ambit and correspondingly greater likelihood of contact with potential substrates, but also suffer increased likelihood of loss of enzyme and product (Vetter et al. 1998).

Most active bacterial enzymes are thought to be cell-associated (Chróst 1991). Yet, in some marine environments, substantial dissolved extracellular enzyme activity has been documented and attributed to bacterial production (Overbeck 1991). Vetter et al. (1998) predicted that enzymes could be active and useful to the producing cell across a distance of 10 μm and up to 500 μm in certain cases. Based on these results, the authors predicted that dissolved extracellular enzymes would provide a valuable feeding mechanism in fluid environments rich in organic matter and with high surface area. In nutrient enriched, confined spaces, like the crab gut, the energetic benefits obtained from releasing extracellular enzymes likely outweigh the costs of some enzyme and product loss.

Moreover, released extracellular enzymes are the form of enzyme most likely to benefit the host crab. Any enzyme or product 'lost' to the microbiota, may potentially benefit the host. Specifically, enzymes released from stomach microbes may act on organic matter along the digestive tract and be absorbed by the host in the hepatopancreas or midgut (See Chapters 2 and 3 for detailed

discussions of digestive process). In the hindgut, the products of microbial extracellular enzymes (i.e. amino acids, short chain fatty acids) may be absorbed across the hindgut lining, if they are small molecules (Hogan et al. 1985), or may be passed forward by reverse peristalsis (Dall 1967; Fox 1952; Lovett and Felder 1990) to be absorbed in the midgut.

The main goal of the present study is to determine if the presence of resident microbes correlates with dissolved extracellular enzyme activity. The specific questions we address include: 1) Are activities of extracellular enzymes reduced with a reduction of bacterial and/or Eccrinales abundance; 2) Does bacterial abundance and/or Eccrinales biomass (length) correlate with enzyme activity; and 3) Is the measured enzyme activity particle-associated?

METHODS

Crab and sediment collection

Marsh fiddler crabs, *Uca pugnax*, were collected for pH measurements from an intertidal salt marsh in Barnstable Harbor, Massachusetts, USA (41°42'31 N, 70°18'17 W) in October 2002. Both crabs and surficial (top 1 cm) sediments were collected for enzyme experiments in August, 2003. Shortly after collection, all sediments were thoroughly homogenized before freezing in ~1 kg batches. Crabs were kept in cooled containers (~15°C) and brought to the laboratory within 2 h after collection. Gender, molt stage (see below), and carapace width and length of each specimen were recorded.

Molt stage analysis

Molt stage of individual crabs was determined as described in Chapter 2 (this thesis).

Gut pH measurements

Immediately after dissection, all four gut sections (stomach, hepatopancreas, midgut and hindgut) from seven feeding fiddler crabs were

placed on a wax tray. A needle pH microelectrode (0.89 mm; Diamond General Develop Corp) with pH meter (Orion Model 720A) was inserted into the gut lumen to measure the pH of full gut sections after calibration with reference solutions (pH 4.0, 7.0, and 10.0). For each gut section, three replicate measurements were taken.

Treatments - Rationale

Four different feeding and molt stage treatments were designed to determine if the presence of bacteria or Eccrinales increases extracellular enzyme activity in the crab digestive tract. Antibiotics were used to reduce the abundances and activities of gut-associated bacteria. No comparable substances are known to eliminate Eccrinales protists from fiddler crab stomachs and hindguts, but recently molted crabs (postmolt stage) have been observed to be free of Eccrinales symbionts (Chapter 2, this thesis). Therefore, postmolt crabs treated with antibiotics should have no, or substantially reduced, enzyme contributions from any remaining gut microbiota. A comparison of enzyme activities in postmolt crabs, treated with antibiotics (PA, Table 5.1), with activities in intermolt crabs, not treated with antibiotics (IR), was intended to reveal how activity changed when both bacteria and Eccrinales were removed.

Yet, both molt stage (Peters et al. 1999) and antibiotic exposure (Donachie et al. 1995) might influence the host and endogenous enzyme production. Therefore we devised two other treatments, to function dually as controls and efforts to isolate potential bacterial contributions from those of

Table 5.1 Treatments for enzyme experiment. Expected effect of treatment on bacterial and Eccrinales abundances are indicated by (NC) for presence at typical abundances (no change), (-) for reduced abundance and (- -) for greatly reduced abundances.

Treatment name	Antibiotics	Molt stage	Eccrinales abundance	Bacterial abundance
IR	no	intermolt	NC	NC
IA	yes	intermolt	NC	-
PR	no	postmolt	-	-
PA	yes	postmolt	-	- -

Eccrinales. We compared enzyme activities in intermolt crabs not treated with antibiotics (IR) and intermolt crabs treated with antibiotics (IA) (Table 5.1) to determine if reducing only bacterial abundances decreases enzyme activity levels in the stomach and hindgut sections. To connect enzyme activities with Eccrinales presence and biomass, we compared activities in postmolt crabs (fed regular sediment) (PR) with activities measured in intermolt crabs, both with and without antibiotic treatments (IR and IA).

Treatments

For antibiotic treatments, habitat sediment was dosed with an antibiotic mixture of equal amounts of penicillin, streptomycin and ampicillin for a total of 10 g antibiotics kg^{-1} sediment mass, as described in Zimmer et al. (2001). The same combination of antibiotics was added to filtered (0.2 μm) seawater (500 mg l^{-1} each antibiotic). These antibiotics, individually or in combination, have been shown to reduce significantly abundances of gut-associated bacteria in marine invertebrates (Donachie et al. 1995; Erasmus et al. 1997; Wainwright and Mann 1982). These antibiotics destroy or inactivate both gram-negative and gram-positive bacteria by two different mechanisms. Streptomycin inhibits prokaryotic protein synthesis while both penicillin and ampicillin inhibit bacterial cell wall synthesis. Ampicillin and streptomycin inactivate gram-negative and gram-positive bacteria, whereas penicillin affects only gram-negative bacteria.

Immediately after collection, crabs were gut-evacuated in 0.2 μm-filtered seawater in individual containers for 3 h (Fig. 5.1). Over this period, crabs were removed to fresh sterile containers twice (approx. every 1.5 h). Crabs (IA and PA) were then exposed to antibiotic treatments, again in individual containers, for 24 h. At the same time, crabs for non-antibiotic treatments (IR and PR) were fed untreated habitat sediment and kept in 0.2 μm-filtered seawater. For both antibiotic and regular feeding treatments, crabs were transferred to fresh sediments and seawater every 8 h.

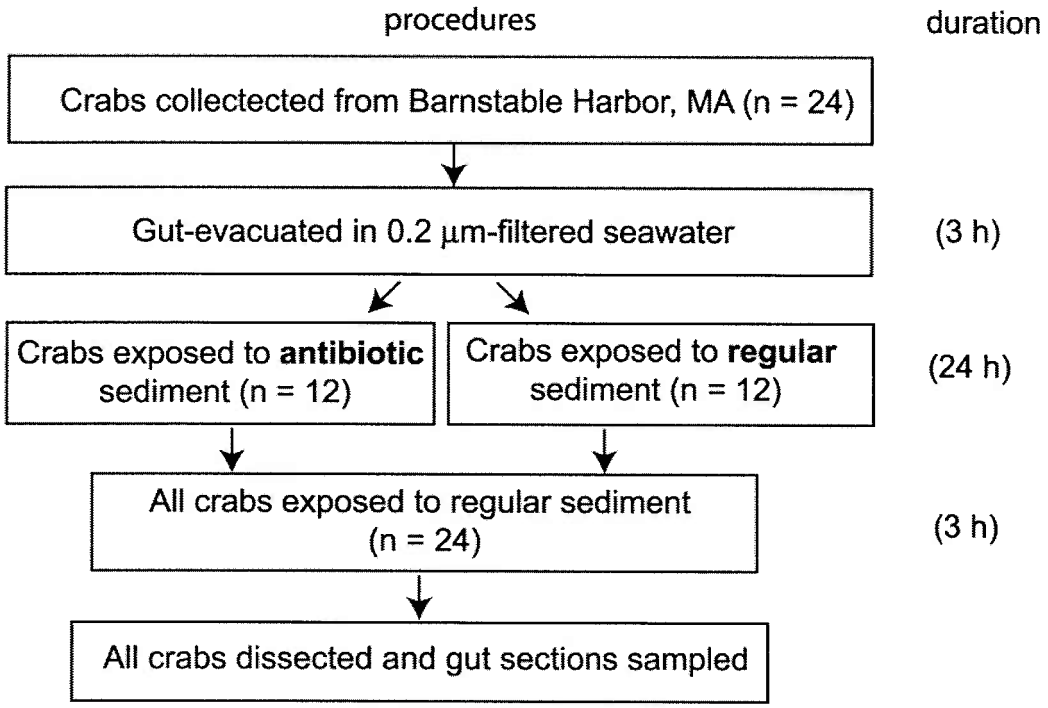

Figure 5.1 Summary of crab collection and sediment exposure procedures for antibiotic and regular treatments.

After exposure to treatment sediment (antibiotic or regular), all crabs were presented with regular sediment for 3 h before dissecting and collecting gut sediments (Fig. 5.1). Only feeding crabs were used, as confirmed by observing feeding behaviors and monitoring fecal pellet production.

During this procedure, crabs treated with antibiotics were presented with regular sediment, not dosed with antibiotics, rather than antibiotic sediment or no sediment at all, for multiple reasons. Firstly, in crustaceans, types and activity levels of enzymes produced depend on the presence of a food stimulus in the gut (Donachie et al. 1995; Vogt et al. 1989). Hosts and/or resident microbes may only be stimulated to produce their typical suite of enzymes when natural food, and therefore transient, food-associated microbes, are present. However, if we

measured the enzyme activity of crabs with antibiotic-dosed sediment in their guts and compared this with crabs feeding on regular sediment, we would not be able to discriminate between effects on resident bacteria and transient bacteria. We might observe a reduction in enzyme activity due to inactivation of transient bacteria and resultant reduction in overall enzyme activity, rather than solely due to the impact on resident bacteria. Finally, undissolved antibiotics have fluorescent properties that might interfere with our enzyme activity measurements (see below).

Another concern was that crabs treated with antibiotics would re-acquire their gut microbiotas after exposure to sediment-associated bacteria. We conducted preliminary tests to ensure that bacterial counts were not increased significantly by 3 h exposure to natural (albeit previously frozen) sediments (results not shown).

Sample preparation and enzyme activity measurement followed procedures in Mayer et al. (1997) with the following modifications. Crab stomach, hepatopancreas, midgut and hindgut sections were removed with sterile dissecting tools. Gut contents and associated digestive fluids were placed in 1.7-ml sterile tubes. One milliliter of 0.2 μm-filtered 0.1 M phosphate buffer was added to gut contents and this mixture was vortexed for 30 s and centrifuged for 4 min (3,000 rpm). Supernatants were collected, either 400 μl (hepatopancreas) or 700 μl (all other samples), and each sample was aliquoted among three tubes. Tubes with pelleted gut contents and sediments were dried at 60°C for 18 h to obtain dry weight measurements.

In order to assess the extent of particle-associated activity in the collected digestive fluid samples, we compare activities in paired filtered and unfiltered samples. One aliquot for each sample was filtered (0.2 μm) and all aliquots were frozen at -80°C until use. If a substantial fraction of active enzymes were particle-associated, we would expect filtered activities to be categorically reduced as compared unfiltered activities. On the other hand, if a significant fraction of

active enzymes were either inactivated by attachment to particles or attacking particle-associated organic matter (rather than synthetic substrates), we would expect to find greater activities in filtered over unfiltered samples.

Enzyme measurements

To measure crab enzymatic activity, diluted digestive fluids were incubated with substrate monomers bonded to fluorophores with the appropriate linkage (Sigma-Aldrich; Table 5.2). The fluorophores used, methylumbelliferone (MUF) and methylcoumarinyl amide (MCA), can be detected at low levels, have a wide range of detection and have been shown to be a reliable method of measuring extracellular enzymatic activity in many environments, including within the guts of deposit feeders (Mayer 1989; Mayer et al. 1997). These synthetic substrates are relatively non-fluorescent, whereas the hydrolytic products (fluorophores) are highly fluorescent. Digestive fluids (paired filtered and unfiltered samples) were diluted with 0.1 M phosphate buffer of appropriate pH (6.6 for hepatopancreas; 7.3 all other gut sections, see pH results). Midgut and hindgut fluids and sediments were diluted with buffer to a ratio of 1:25, stomach and hepatopancreas to 1:80. Various dilutions were tested and these dilutions were selected because they produced linear activity rate measurements over the measured time-course, indicating that enzymes were saturated with fluorophore-linked substrates. In 96-well plates diluted digestive fluid (180 µl) was combined with 20 µl of enzyme substrate (100 µM) and vortexed. All reactions were run in

Table 5.2 Description of synthetic enzyme substrates, fluorophores and bond types used in this study.

Fluorophore	Monomer	Target enzyme activity	Bond
MCA	alanine	protease	peptide
MUF	palmitate	lipase	ester
MUF	butyrate	esterase	ester
MUF	glucopyranoside	glucosidase	β–glucosidase
MUF	diacetylchitobioside	chitinase	β–glucosidase
MUF	cellobioside	cellobiosidase	β–glucosidase

triplicate. The hydrolysis reaction was measured by the evolution of the cleaved fluorophore via fluorescence (λ_{ex} 360 λ_{em} 460) on a CytoFluor Multiwell plate Reader 4000 (PerSeptive Biosystems). Fluorophore release over time was plotted and slopes were converted to hydrolysis rates in terms of μM MCA or MUF released min^{-1}. All activities were corrected for fluorescence quenching by adjusting slopes by the fluorescence of 1 μM solutions of unbound MCA or MUF in the same diluted solutions of digestive fluid. Activities were also expressed as μM MCA or MUF released g^{-1} min^{-1} dry gut contents in order to normalize activity to the mass of material in the gut. Hosts and/or microbes may upregulate enzymes in response to the presence of 'food' in the gut (Brunet et al. 1994). Although all crabs measured were feeding, gut volume and 'fullness' varied somewhat. By normalizing to the mass of material in the gut, we can account for variations in gut fullness and subsequent, possible variations in enzyme activity.

Enzyme activities measured in the hepatopancreas were analyzed independently of the other gut sections because the hepatopancreas samples were unique in multiple ways. Digestive fluids collected from entire homogenized hepaptopancreas sections do not represent one gut volume's worth of released and active enzymes, as digestive fluids collected for the stomach, midgut and hindgut do, but likely include all available enzymes stored in this gut section. Hepatopancreatic tissues are believed to generate all endogenous enzymes (Brunet et al. 1994; Dall 1967; Icely and Nott 1992) and these cells may be disrupted by our centrifugation step, releasing any stored enzymes. Secondly, the gut lumen of this section contains a filtrate, rather than solid material. It is unfeasible to separate this filtrate from the rest of the hepatopancreas without rupturing the tissue. Therefore, we could not report activities in terms of μM MCA or MUF released g^{-1} dry gut contents min^{-1}. Instead hepatopancreas activities were reported μM MCA or MUF released min^{-1}. Finally, in terms of digestion, the hepatopancreas is a unique section because digestion can occur via both intracellular and extracellular mechanisms (Brunet et al. 1994; Icely and Nott

1992). Measuring only extracellular activity, therefore, does not give a reliable measure of total digestive ability. Nevertheless, it is important to attempt some measure of hepatopancreas enzyme activity because all enzymes produced by the host crab are thought to be generated in this section. Furthermore, comparisons of relative activities of different enzymes and treatment affects in the hepatopancreas should offer insight into the host's endogenous enzyme production.

Sonication, staining and bacterial counts

Samples were sonicated, DAPI stained, and counted as described in Chapter 3 (this thesis). Total bacterial counts were reported as number of bacteria per gut section.

Eccrinales identification and length measurement

Eccrinales species, *E. callianassae* in the stomach and *Enterobryus* sp. in the hindgut, were identified and lengths were measured as described in Chapter 2 (this thesis). All crabs were inspected for the presence of Eccrinales and all Eccrinales hyphae were measured for total length.

Statistical analyses

Prior to statistical analyses, data were tested for homogeneity of variances and for normal distribution. All bacterial counts and enzyme activities, except hepatopancreas and sediments, were logarithmically (ln +1) transformed to meet these assumptions (normality, homoscedasticity).

Analyses of stomach, midgut and hindgut enzyme data were performed with a two-way MANOVA (Systat version 11), testing effects of treatment (four levels) and gut section (three levels) on enzyme activity. Two-way ANOVAs tested significance of these effects among individual enzymes. Tukey post hoc tests were used to ascertain which means differed significantly.

Separate one-way MANOVAs tested effects of treatment on hepatopancreas and sediment enzyme activities. One-way ANOVAs tested significance of these effects among individual enzymes. Tukey post hoc tests were used to ascertain which means differed significantly. Data for pH results and bacterial abundances were assessed separately with one-way or two-way ANOVAs and Bonferroni or Tukey post hoc tests.

Pearson product-moment correlation coefficients were calculated to assess the relationship between filtered and unfiltered activities, as a group and for each enzyme. Relationships between both Eccrinales lengths and bacterial abundances and enzyme activities were also evaluated with pearson product-moment correlation coefficients. Significance of these correlations was tested after the Bonferroni adjustment for multiple comparisons was performed.

RESULTS

Gut pH measurements

The mean pH values of stomach, hindgut, midgut, and hepatopancreas sections were 7.4 (SD = 0.3), 7.3 (0.4), 7.1 (0.4), 6.6 (0.3), respectively. ANOVA and Bonferroni post hoc tests indicated that the hepatopancreas pH values were significantly less ($F = 6.51$, $df = 3$, $P < 0.05$) than those in the stomach and hindgut. The stomach, hindgut and midgut sections were not significantly different from each other. Therefore, we used the mean pH value from all stomach, midgut and hindgut measurements (mean pH = 7.3) for enzyme analyses of these sections. For hepatopancreas enzyme analyses, we used the mean pH measured in hepatopancreas samples (mean hepatopancreas pH = 6.6). Enzyme activities are sensitive to sample pH, and often have narrow pH optima (Dall and Moriarty 1983; Lehninger et al. 1993). By measuring activities at physiological pH *in vitro*, we expect that these activities most closely reflect *in vivo* activities.

Comparison of filtered and unfiltered enzyme activities

The slope generated by plotting enzyme activities of filtered and unfiltered samples (Fig. 5.2), approximated a slope value of 1.0, indicating that filtering did not influence total enzyme activity. Enzyme activities of filtered and unfiltered samples were significantly correlated in a direct association, as measured both for all activities ($r = 0.997$; $P < 0.01$) and by individual enzymes ($P < 0.05$) (Table 5.3). Significant, direct correlations persisted for comparisons among treatments and among sections (results not shown). For further analyses, activities measured in unfiltered samples are reported.

Cellulase activity

We attempted to measure cellulase activity in the various gut sections and sediments with the synthetic substrate of cellulobiosidase attached to MUF, however none of these samples produced a non-zero slope. We ran representative samples for longer durations (30 min) without observing any positive slopes, indicating significant activity. Initially we confirmed that MUF-cellulobiosidase was a reasonable proxy for cellulase activity via incubations with purified cellulase (results not shown).

Table 5.3 Pearson coefficient for paired filtered and unfiltered samples (n = 24). Significant correlations ($P < 0.05$), as adjusted by Bonferroni adjustment for multiple comparisons, are bolded.

Enzyme	Correlation coefficient
chitinase	**0.83**
glucosidase	**0.71**
esterase	**> 0.99**
lipase	**0.86**
protease	**0.91**

Table 5.4 MANOVA and univariate ANOVAs comparing enzyme activities, among four treatments (IR, IA, PR, PA) and among three sections (stomach, midgut, hindgut).

		MANOVA			Univariate ANOVA					
					chitinase			esterase		
Source	df	Wilks' λ	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Treatment	3	0.05	7.74	< 0.01	4.13	4.68	< 0.01	2.83	4.05	0.017
Section	2	0.03	20.69	< 0.01	75.96	86.24	< 0.01	38.39	54.96	< 0.01
Treatment x Section	6	0.31	0.99	0.49	1.39	1.58	0.19	1.63	2.34	0.059
Crab(Treatment)	18	0.01	2.18	< 0.01	0.75	0.85	0.64	2.51	3.59	< 0.01
Error	28				0.88			0.70		

		Univariate ANOVA								
		glucosidase			lipase			protease		
Source		MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Treatment	3	2.92	6.01	< 0.01	10.01	11.76	< 0.01	0.97	7.51	< 0.01
Section	2	78.87	162.17	< 0.01	17.83	20.96	< 0.01	16.22	125.29	< 0.01
Treatment x Section	6	0.86	1.78	0.14	1.45	1.70	0.16	0.19	1.43	0.24
Crab(Treatment)	18	0.43	0.89	0.59	1.96	2.31	0.02	0.18	1.42	0.19
Error	28	0.49			0.85			0.13		

Logarithmic transformations [ln (x+1)] were performed on all data to validate assumptions of normalacy and homoscedascity. For tests with a significant treatment or section effect ($P < 0.05$), Tukey post hoc tests indicate means that are not significantly different ($P > 0.05$), connected with underlines. (Sections are designated as S (stomach), M (midgut), and H (hindgut)).

Chitinase:
S-PR S-IR S-PA S-IA M-PR M-IA M-PA M-IR H-PR H-IR H-PA H-IA

Esterase:
S-IR S-PA S-PR S-IA M-IA M-PR H-PR H-IR H-IA M-IR M-PA H-PA

Glucosidase:
S-IR S-IA S-PR S-PA M-IA M-IR M-PR M-PA H-IR H-PR H-PA H-IA

Lipase:
S-IR S-PR H-PR S-PA M-PR M-IA S-IA M-IR M-PA H-IR H-IA H-PA

Protease:
S-PR S-IR S-PA S-IA M-PR M-IA M-IR M-PA H-IR H-PR H-PA H-IA

Treatment effects on enzyme activity

Enzyme activities varied significantly among treatments and among gut sections (MANOVA ; $P < 0.05$; Table 5.4). These treatment and section effects were also significant for individual enzymes, as indicated by separate univariate ANOVAs. For both esterase and lipase, variation among replicate crabs within a

treatment was significant, as might be expected due to the many variables that may influence individual crab enzyme production and activity levels.

In general, enzyme activities in crabs from antibiotic treatments (IA, PA) were reduced compared with those from regular sediments (IR, PR), in the stomach and hindgut sections of both intermolt and postmolt individuals, as well as in the midguts of postmolt individuals (Fig. 5.3). Post hoc tests indicated that differences between IR and IA as well as PR and PA were significant for some comparisons within lipase and protease (Table 5.4), however the relatively high variability among individual samples precluded a greater number of significant outcomes. In intermolt crabs, midgut activities were not significantly different between treatments, still, the reverse pattern of elevated IA activity over IR activity was observed for most enzymes (chitinase, esterase, glucosidase and protease).

Activities in postmolt individuals were not consistently reduced, as compared with intermolt individuals. We had anticipated that IR crabs would have the greatest activities and that PA crabs would have the lowest activities. Although activities of intermolt regular crabs were greater than those of postmolt antibiotic crabs for all enzymes, in both the stomach and hindgut, this trend was not significant for any pairs (Table 5.4). Furthermore, for some enzymes, the greatest activities measured were in postmolt individuals. Surprisingly, we observed the greatest overall rates of chitinase and protease activity in postmolt crabs, in the stomach and hepatopancreas sections (Fig. 5.3, 5.4). These enzymes may be upregulated by the host during molt and postmolt stages in conjunction with the molting process.

MANOVA and ANOVA analyses indicated significant differences among gut sections (Table 5.4). Among the stomach, midgut and hindgut, activities were typically greatest in the stomach and least in the hindgut. Most stomach activities were significantly greater than those in the hindgut and some stomach activities were significantly greater than those in the midguts (see post hoc

comparisons; Table 5.4). All stomach activities were significantly greater than all hindgut rates measured for chitinase, glucosidase and protease.

Hepatopancreas enzyme activity

Hepatopancreas activities varied significantly with treatment ($P < 0.05$; Table 5.5). For all enzymes, activities were consistently greater in regular treatments as compared with antibiotic treatments (IR vs IA, PR vs PA). Also, intermolt regular activities were typically greater than those of postmolt antibiotic activities, yet these differences were significant only for one comparison within glucosidase (Table 5.5). Also, for chitinase and protease, the greatest activities were measured in postmolt regular treatments while, for glucosidase and lipase, intermolt regular treatments had the greatest activities.

Esterase was excluded from hepatopancreas analysis because for many samples esterase was not saturated with substrate, as indicated by the observed non-linear slope for fluorescence change. Although we had tested initially for

Table 5.5 Hepatopancreas: MANOVA and univariate ANOVAs comparing enzyme activities, among four treatments (IR, IA, PR, PA).

	MANOVA				Univariate ANOVA					
					chitinase			glucosidase		
Source	df	Wilks' λ	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Treatment	3	0.164	3.27	< 0.01	2.93	4.04	0.02	6.74	4.09	0.02
Error	28				0.73			1.65		

	lipase			protease		
	MS	*F*	*P*	MS	*F*	*P*
	36.18	1.55	0.24	2.49	2.39	0.10
	23.31			1.04		

For tests with a significant treatment or section effect ($P < 0.05$), Tukey post hoc tests indicate means that are not significantly different ($P > 0.05$), connected with underlines.

Chitinase:
PR PA IR IA

Glucosidase:
IR IA PR PA

Table 5.6 Sediment: MANOVA and univariate ANOVAs comparing enzyme activities among two treatments (antibiotic and regular).

		MANOVA			Univariate ANOVA					
					chitinase			glucosidase		
Source	df	Wilks' λ	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Treatment	1	0.02	8.92	0.025	< 0.01	0.10	0.76	0.02	12.12	0.01
Error	6				< 0.01			< 0.01		

		Univariate ANOVA								
		lipase			protease			esterase		
Source	df	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Treatment	1	0.05	33.29	< 0.01	0.01	10.05	0.02	0.22	16.90	0.01
Error	6	< 0.01			< 0.01			0.01		

appropriate dilutions to ensure that hepatopancreas enzymes were saturated with synthetic substrates, activities do vary among individuals. We did not have sufficient numbers of accurate esterase activity measurements to report esterase activity.

Sediment activities

Sediment activities varied significantly with treatment ($P < 0.05$; Table 5.6). Sediment activities were significantly greater in the antibiotic treatment as compared the regular treatment (Fig. 5.5) for all enzymes except chitinase (Table 5.6).

Bacterial numbers and enzyme activity

A two-way ANOVA of bacterial numbers along the gut and among treatments indicated a significant treatment and section effect, but also a significant interaction term (Table 5.7). This significant interaction term most likely resulted from the lack of a consistent pattern in the midgut and hepatopancreas, as compared with the stomach and hindgut. If we exclude the midgut and hepatopancreas from the analysis, we no longer generate a significant interaction term. Nevertheless, each section-treatment pair was

Table 5.7 Two-way ANOVA on bacterial abundances with treatment and section as factors.

Source	df	MS	*F*	*P*
Treatment	3	2.20	5.09	< 0.01
Section	3	15.72	36.48	< 0.01
Section x Treatment	9	1.49	3.45	0.002
Error	63	0.43		

Logarithmic transformations [ln (x+1)] were performed to validate assumptions of normalacy and homoscedascity. Post-hoc Tukey tests indicate means that are not signficantly different ($P > 0.05$) connected with underlines.

H-IR H-PR S-IR H-IA HP-PR H-PA HP-PA HP-IA HP-IR S-PR M-PA M-PR S-IA M-IR S-PA M-IA

analyzed individually to test for significance, by means of post hoc tests (Table 5.7).

For the stomach and hindgut, bacterial abundances were greatest in the IR treatment (Fig. 5.6) and were significantly greater than those in the IA treatments, for the same sections (Table 5.7, post hoc comparisons). Comparing intermolt and postmolt stages for these same sections, IR abundances were significantly greater than PR in the hindgut, but not in the stomach.

Abundances were not significantly different among treatments in the hepatopancreas and midgut and no clear pattern emerged for these two sections.

Bacterial abundances and enzyme activity correlations

In all gut sections, no enzyme activities showed significant positive correlation with bacterial abundance (Table 5.8). Though, in the hindgut, most enzyme activities correlated positively with hindgut bacterial abundances. In particular, chitinase, glucosidase and protease activities indicated high positive correlations with hindgut bacterial abundances for both IR and PR treatments. Many PR stomach activities were highly negative, but again, not significantly.

Only crabs exposed to regular treatments were used for correlation analysis of bacterial abundances and enzyme activities. Antibiotic treatments may lyse bacteria, increasing extracellular enzyme activities. Also, many bacteria may be inactivated and no longer producing extracellular enzymes, but

Table 5.8 Pearson coefficient values from bacterial abundances and enzyme activity correlations (n = 6). After Bonferrroni adjustment for multiple comparisons, no correlations were significant ($P > 0.05$).

Treatment	Enzyme	Section			
		stomach	hepatopancreas	midgut	hindgut
IR					
	chitinase	-0.32	0.54	0.39	0.77
	esterase	0.49	-	0.16	-0.85
	glucosidase	0.03	0.14	0.12	0.76
	lipase	0.78	-0.53	-0.02	0.51
	protease	-0.36	0.33	-0.13	0.83
PR					
	chitinase	-0.83	-0.21	0.06	0.78
	esterase	0.40	-	0.36	0.17
	glucosidase	-0.82	-0.60	0.33	0.61
	lipase	0.35	-0.61	-0.31	0.07
	protease	-0.76	-0.003	0.27	0.66

still counted via DAPI counts. These two possibilities may decouple extracellular enzyme activity from bacterial abundances.

Eccrinales length and enzyme activity

Correlation coefficients for *Enteromyces callianassae* lengths and enzyme activities were not significant (Table 5.9; $P > 0.05$). Many intermolt crabs, however, were uncolonized by *E. callianassae* (Fig. 5.7A). Of the crabs that were colonized, measured Eccrinales lengths varied only from 33.3 – 44.2 mm. Therefore our comparison for stomach activity and (non-zero) Eccrinales length is limited to five samples and restricted range of lengths. Considering only colonized crabs, there appears to be a weakly positive trend between *E. callianassae* length and both protease and esterase activities.

In the hindgut, we measured a broad range of *Enterobryus* sp. lengths (Fig. 5.7B). We found significant positive correlation between *Enterobryus* sp.

Table 5.9 Pearson coefficient values from Eccrinales length and enzyme activity correlations. A significant correlation ($P < 0.05$), as adjusted by Bonferroni adjustment for multiple comparisons, is bolded.

Enzyme	Length	
	E. callianassae	*Enterobryus* sp.
chitinase	0.03	-0.48
glucosidase	0.37	-0.07
esterase	-0.13	**0.82**
lipase	-0.69	-0.38
protease	0.08	0.44

lengths and esterase activity (Table 5.9; $P < 0.05$). For protease activity we found a weakly positive correlation with total *Enterobryus* sp. lengths.

DISCUSSION

Finding a consistent, albeit rarely significant, antibiotic treatment effect in the stomach and hindgut supports our proposition that bacteria may contribute to total extracellular enzyme activity in these regions. Not only were enzyme activities reduced after exposure to antibiotics, but bacterial counts in intermolt antibiotic treatments were significantly reduced as compared with intermolt regular treatments, in these two sections. Furthermore, most enzyme activities measured in the hindgut correlated positively with bacterial abundances, at least for intermolt crabs.

Filtered and unfiltered activities

In this study, we found no appreciable differences in enzyme activities among filtered and unfiltered samples. Our methodology was devised to collect and measure dissolved, extracellular enzyme activity and this result confirms that essentially all activity measured resulted from dissolved enzymes. Most enzymes adsorb strongly to particles and the majority of enzyme activity in marine sediments (Meyer-Reil 1991) is expected to be particle-associated. However, in the gut of a detritivore, dissolved enzymes released from microbial cells are presumably more valuable to the host than cell-attached microbial

enzymes. Products of dissolved enzymes are more likely to be lost to the producing cell (Vetter et al. 1998) and, consequently, greater microbial loss may translate into greater host gain, in the form of a greater likelihood of product absorption by the host.

Enzyme activity trends and bacterial abundances: stomach and hepatopancreas

In the stomach, enzyme activities and bacterial abundances were consistently greater in regular treatments than in antibiotic treatments, however, activities generally did not correlate significantly or positively with bacterial abundances. If bacterially-produced extracellular enzymes were responsible for a substantial fraction of the enzyme deficit observed in intermolt antibiotic treatments (Fig. 5.3; typical IA activities were approximately half that of IR activities in the stomach), we would expect bacterial abundances to correlate with enzyme activities, for regular treatments. Two enzymes, lipase and esterase, had activities that correlated positively with bacterial abundances and lipase was also significantly greater in intermolt regular crabs than in intermolt antibiotic crabs (Table 5.4).

The lack of a positive correlation in the stomach between enzyme activity and bacterial abundance for the other enzymes may result if: 1) bacterial enzyme release does not scale directly with bacterial numbers, 2) stomach bacteria do not contribute extracellular enzymes, or 3) the host's enzymes were produced in such quantities as to overwhelm any bacterial contribution. In general bacterial abundances are thought to correlate with extracellular enzyme release, as indicated by stable cell-specific extracellular enzyme activities measured in various environments (Huston and Deming 2002; Lehman and O'connell 2002). Yet numerous environmental factors may influence total enzyme production and cellular release (Chróst 1991). The absence of a positive correlation between certain enzyme activities and bacterial abundances has been reported previously in the stomach of another marine crustacean, the northern krill species,

Meganyctiphaunes norvegica (Donachie et al. 1995), however in the Donachie et al. experiment the krill were not fed and lack of a correlation was attributed to nutrient limitations. Bacterial abundances may not parallel total enzyme activity, in both *U. pugnax* and *M. norvegica*, if these are commensal bacteria that simply utilize the concentrated organic matter in the stomach and do not contribute to the pool of extracellular enzymes. In particular, stomach bacteria attached to pyloric finglets in *U. pugnax* (Chapter 3, this thesis) are unlikely to profit from releasing their own extracellular enzymes because they are in a region of active fluid circulation, in which released enzymes would be quickly removed from the site of release. These bacteria may be able to survive on the host's digestive products or they may produce cell-attached extracellular enzymes, which we did not measure in this study. While the lack of extracellular enzyme release may explain the absence of any positive correlations between activity and bacterial abundance in the stomach, this explanation does not account for the observed activity reduction in antibiotic treatments.

Two alternative explanations may account for the reduced enzyme activities detected in the stomach and hindguts of crabs from antibiotic treatments. Firstly, antibiotics exposure may have had a negative impact on the hosts, impairing their enzyme production or secretion into the gut. Hepatopancreas enzyme activities were reduced in antibiotic treatments (IR vs IA, PR vs PA), but generally not significantly. Although other studies have applied these antibiotics to invertebrates in a similar manner and observed no toxic side-effects (Erasmus et al. 1997; Zimmer and Bartholme 2003; Zimmer et al. 2001), we cannot exclude this possibility. Enzyme activity measured in the hepatopancreas is the most appropriate estimator of host enzyme production. All enzymes produced by decapod crustaceans are thought to be generated here and passed into the stomach and midgut lumen (Brunet et al. 1994; Icely and Nott 1992). The majority of active enzymes are expected to be retained within the stomach and midgut, possibly via gut fluid reflux (Jumars 1993; Mayer et al.

1997). Yet some host-derived enzymes are likely passed with gut contents into the hindgut. Therefore any negative impact on hepatopancreas enzyme production may be detected throughout the gut.

A second, alternative, explanation for reduced activities in antibiotic treatments depends on whether hepatopancreas-associated bacteria are important for enzyme production. It is possible that these bacteria release extracellular enzymes and contribute to total enzyme activities observed in the other gut sections. If these hepatopancreas bacteria were inactivated by the antibiotic application, this effect may reduce enzyme secretion and activities in other gut sections, as compared with regular activities. Although we cannot reject this explanation, bacterial count data do not support this theory. Bacterial abundances in the hepatopancreas were not significantly different between regular and antibiotic treatments (Fig. 5.6). It is unclear if these bacterial counts represent transient (food-associated) bacteria ingested and passed into the hepatopancreas, or a stable, resident population (see Chapter 3, this thesis). Either transients or residents may contribute to overall enzyme activity (Harris 1993). The transient bacteria may be lysed during digestion and consequently release active enzymes, while resident bacteria may release extracellular enzymes intended for their own gain, but effectively increasing the host's enzyme activity. Because we did not observe a consistent reduction in bacterial abundance with antibiotic application, and because enzyme activities were not significantly correlated with bacterial abundances, we expect that bacteria detected in the hepatopancreas are transient bacteria. Still, this bacterial population and its role, if any, in enzyme production warrant further study.

Enzyme activity trends and bacterial abundances: midgut

There was not a consistent treatment effect in midgut activities across molt stages. Furthermore, we did not detect a significant reduction in midgut bacterial abundances with antibiotic treatment in either intermolt or postmolt

crabs. The overall abundance of resident bacteria in the midgut, for regular treatments, was substantially less than the resident populations found in the stomach and hindguts (Fig. 5.6, also see Chapter 3, this thesis). Therefore the total population size of the midgut bacterial population may be insufficient to contribute measurably to extracellular enzyme activity. However, a more likely reason that bacterial numbers were not significantly reduced with antibiotics and no consistent enzyme contribution was detected may be a function of the type of bacteria present along the midgut. As observed in SEM images (Chapter 3, this thesis), bacteria appear scattered along the midgut, suggesting that they are opportunistic transient bacteria, also known as epimural transients (Harris 1993), rather than a stable, persistent population. These bacteria may have slipped by the peritrophic membrane or may have been transported anterior-ward from the hindgut. Abundances of epimural transients may not be consistently reduced with antibiotics, as we found, if midgut populations are re-seeded from surviving transient bacteria. Furthermore, transient bacteria are likely to specialize in adhesion to passing organic matter and may not release large quantities of free extracellular enzymes.

In postmolt crabs we observed reduced midgut activities in antibiotic treatments. Midgut bacterial abundances, however, were not reduced in these crabs and correlations between bacterial numbers and activities were not significant. We suspect that elevated activities may have been due to the host's endogenous production, because hepatopancreas-derived enzymes are secreted into the stomach and midgut. Therefore, if greater quantities of enzymes were released in postmolt regular crabs, compared with postmolt antibiotic crabs, we would expect to observe elevated activities in the midguts of postmolt regular crabs. In particular, chitinase and protease activities were greatest in postmolt regular individuals, as measured in the hepatopancreas, stomach, midgut and hindgut.

Enzyme activity trends and bacterial abundances: hindgut

In the hindgut, the significant reduction of enzyme activities and bacterial abundances in antibiotic treatments, and the positive correlation between activities and bacterial abundances all indicate a bacterial contribution of enzymes. Enzyme activities detected in the hindgut may be produced by resident or transient microbes or they can be transferred from the midgut, but no host enzymes are secreted directly into this section (Dall and Moriarty 1983). Our results, from intermolt crabs, suggest that bacterial enzymes may contribute to overall dissolved enzyme activity in the hindgut, especially for chitinase, glucosidase and protease. An alternative possibility, that these elevated hindgut activities were simply an artifact of up-stream activity passed into the hindgut, is not substantiated for intermolt crabs because midgut activities did not show the same activity pattern as observed in the hindgut.

Significant correlations between activity and bacterial abundances may have only been commonly detected in the hindgut due, in part, to host enzyme conservation and the consequential reduction in enzyme activities. The observed decline in hindgut activity, compared with the other gut sections, has been reported in other invertebrate detritivores (Mayer et al. 1997). In order to reduce enzyme loss to the environment, the host may reabsorb enzymes in the posterior midgut and/or may control fluid transport along the gut (Jumars 1993). Digestion in detritivores has been compared with laundry processing, in which contents are 'rinsed' before egestion (Mayer et al. 2001). In crustaceans, the combination of reverse peristalsis and anal uptake of water (Fox 1952) may facilitate conservation of host-derived enzymes, as well as transport of bacterial extracellular enzymes and products to the absorptive midgut.

Estimated bacterial enzyme contribution: stomach and hindgut

To assess the potential importance of bacterial contributions to total enzyme activities, we estimated total possible bacterial enzyme production based

on literature values (Lehman and O'Connell 2002). These authors calculated protease and glucosidase activities of attached bacteria and normalized to a per-cell basis. Using our estimates of total bacterial abundance and Lehman and O'Connell's (2002) per-cell enzyme activities, we estimated total bacterial enzyme activities in both the stomach and hindgut and compared these values with our measurements of total enzyme actitvities for intermolt regular crabs. In the hindgut, total estimated bacterial production was 4.2 times greater than measured protease activity and 44.7 times greater than measured glucosidase activity. Measured activities may be lower than these estimates if many of the bacterial enzymes in the hindgut are attached to substrates, and therefore not detected. Alternatively, not all hindgut bacteria may produce glucosidase or protease. Specifically, if hindgut bacteria are involved in a metabolic consortium, some bacteria may specialize in particular enzyme production.

In the stomach, total measured enzyme production was greater than estimated bacterial production, likely due to host enzyme production. Estimated bacterial production may account for 5% of total protease activity and 45% of total glucosidase activity, in intermolt regular crabs. Therefore, for glucosidase, reduced activity measured in antibiotic treatments may be due to loss of bacterial production.

Enzyme roles: protease and chitinase in the hindgut

Our results suggest that hindgut bacteria may be contributing extracellular protease, glucosidase and chitinase enzymes to overall hindgut activity. Although we would expect the host crab to have extracted most of the proteinaceous material from the diet by the time this material was passed into the hindgut, resident bacteria may specialize in degrading residual protein in the gut contents or, more likely, remnants of the peritrophic membrane. The peritrophic membrane is a protein, chitin and glucosaminoglycan-rich membrane (Tellam et al. 1999; Terra 2001) formed in the anterior midgut that envelops the food bolus

as it passes through this section (Bignell 1984). It is thought to function in protecting the midgut from bacterial colonization, and abraded in the hindgut by cuticular projections and muscular contractions (Bignell 1984). In *U. pugnax*, we have observed indications of a peritrophic membrane along the midgut (Chapter 3, this thesis), however this membrane is not apparent in the hindgut or in fecal pellets (pers. obs.). Therefore we propose that this membrane is shredded in the hindgut by the host's projections and that resident bacteria release extracellular proteases and chitinases to degrade this material. If the host does benefit from any of these bacterial products, this process would be an efficient means of 'recycling' in which the host may recoup some of its energetic investment in the protein- and chitin-rich peritrophic membrane.

If resident bacteria are producing substantial quantities of chitinase, they may also be degrading the chitinous hindgut cuticle. Chitinase activity has been detected in bacterial cultures established from other crustacean hindgut communities (Harris 1992; Pinn et al. 1997). Bignell (1984) suggested that hindgut-associated bacteria may degrade the hindgut cuticle, producing 'thin-spots' that may increase permeability and facilitate nutrient uptake by the host. We did observe a dimpled surface along the anterior hindgut lining (Chapter 3, this thesis), which may result from bacterial activities. Further research using high-resolution transmission electron micrographs of hindgut cross sections may indicate if the hindgut cuticle is thinned, in particular around bacterial attachment sites.

Enzyme roles: cellulose

Although we did not detect any cellulase activity along the digestive tract of *U. pugnax*, we suspect that our method was inappropriate for this system. In order to conclude that no host- or microbially-produced cellulase activity exists along the digestive tract of *U. pugnax*, other methods should be tested. In

particular, a reducing sugar assay has been successfully used in other crustaceans to detect cellulase activity (Xue et al. 1999).

Postmolt enzyme roles: protease and chitinase

Protease and chitinase activities were greatest in postmolt regular treatments for the hepatopancreas, stomach and hindgut (Fig. 5.3). However, these activities may have been due to endogenous production, rather than bacterial contribution. Digestive enzymes are affected by the molting process (Vega-Villasante et al. 1999) and these two enzymes, in particular, may have an important role in host molting. Chitinase and protease enzymes have been connected with release of the molt (Lustigman et al. 1996; Vega-Villasante et al. 1999), and may be elevated throughout the crab tissue.

Enzyme activity trends and Eccrinales abundances: stomach and hindgut

Because molting removes all Eccrinales from the gut, any eccrinid contribution of enzymes should have been apparent in a comparison of postmolt and intermolt activities. Specifically, the difference in activities between intermolt and postmolt regular treatments should be due to the absence of Eccrinales and possible reduction in bacterial abundances. However, a substantial reduction in postmolt regular treatments was observed only for esterase (stomach) and glucosidase (stomach and hindgut) activities. We expect that these results were complicated by effects of molt stage on host enzyme production. Protease and chitinase activities in the hepatopancreas were greatest for postmolt regular crabs. If these endogenously produced enzymes contributed to activities throughout the gut, any potential association between Eccrinales biomass and enzyme activity would have been obscured.

In the stomach, no enzyme activities were significantly, or even highly positively, correlated with *Enteromyces callianassae* length. The low stomach colonization rate of measured intermolt crabs may have prevented us from detecting any potential relationship. Colonization was more frequent and

extensive in the hindgut, where we measured a positive, significant correlation between *Enterobryus* sp. length and esterase activity and a positive, non-significant correlation with protease activity (Table 5.9).

Little is known about the physiology of Eccrinales protists and therefore we have a limited basis of information to generate predictions about which types of enzymes Eccrinales may produce. However two lines of evidence support the proposition that Eccrinales may produce and release extracellular protease. Firstly, (Kimura et al. 2002) found that callianassinid shrimp, *Nihonotrypaea harmandi*, colonized by Eccrinales released greater quantities of amino acids from ingested sediment than uncolonized shrimp. Secondly, Eccrinales are related to the parasitic protist, *Perkinsus marinus*, which have been reported to release a number of extracellular proteins including highly potent proteases (La Peyre et al. 1995). This parasite relies on extracellular proteases to degrade host matrix proteins, allowing it to propagate within host tissue. However, if *Enterobryus* sp. releases extracellular proteases into the gut lumen, this would explain the positive correlation between Eccrinales length and protease activity in the hindgut. *Enterobryus* sp. proteases may also target peritrophic membrane proteins, as postulated for hindgut bacterial enzymes. Further research, focusing on either culturing and/or molecular approaches to detecting enzyme-encoding genes, is needed to resolve if these protists are producing extracellular enzymes.

Sediment activities compared with gut activities

Enzyme activities in sediment were typically greater in antibiotic treatments than in regular treatments. We suspect the antibiotics may have lysed sediment-associated cells and released dissolved enzymes, thereby increasing sediment enzyme activities. Although crabs in both treatments (antibiotic and regular) were exposed to regular sediment before measuring enzyme activities, pre-conditioning crabs on the higher activity sediment may have altered host enzyme production. The sediment activity differences were

small compared with those measured along the digestive tract, and even if sediment-associated enzymes are concentrated in the gut, this effect was not expected to change our findings.

Enzyme activities in sediments were considerably less than activities measured along the gut, for all enzymes and treatments. Hindgut enzyme activities were expected to be greater than sediment activities for multiple reasons. Firstly, although the crabs may reduce the amount of fluid-associated enzymes passed from midgut to hindgut, this process is likely to be inefficient and some enzymes would pass into the hindgut along with the gut contents. Secondly, fiddler crabs are highly selective detritivores (Miller 1961) and are adept at selecting for the most organic-rich fraction of the sediment. Therefore fiddler crabs are also concentrating sediment-associated enzymes upon ingestion. These sediment-associated enzymes may still be present in the hindgut contents. Finally, any contribution from resident gut microbiota along the hindgut would also increase hindgut enzyme activities.

We speculate that if substantial amounts of bacterial enzymes, or other products, are passed into the environment with the fecal pellets, this effect may factor into salt-marsh geochemistry. Although hindgut activities were greater than sediments, we do not know if activities in fecal pellets are also elevated in comparison with sediment activities. Our measurements of hindgut activities combined all hindgut contents, obscuring any possible gradients in enzyme activity. For multiple deposit-feeding species, Mayer et al. (1997) found that the posterior-most hindgut had lower enzyme activities than the anterior hindgut. If gut content 'rinsing' efficiently retains most enzyme activity, the egested material should have relatively low enzyme activities. Mayer et al. (2001) reported that fecal material from deposit-feeding polychaete *Arenicola marina* had equivalent or lower enzyme activity compared with sediments. To assess if *U. pugnax* and/or its gut microbes 'export' digestive enzymes into the environment, further research on enzyme activities in fecal pellets and along the hindgut is needed.

Antibiotics treatments

Bacterial abundances, as measured by direct counts, were significantly reduced in the stomach and the hindgut, in crabs exposed to antibiotic treatments. As a group, the antibiotics used in this study kill or inactivate both gram-negative and gram-positive bacteria. Due to their synergistic effects, these three antibiotics are an especially potent combination that reduce bacterial cell counts more efficiently than if any were used alone (Brown et al. 1989). Penicillin and ampicillin disrupt the formation of cross-linkages in the bacterial cell wall, producing leaky cell walls that allow streptomycin to penetrate the cell. Streptomycin inhibits protein synthesis by binding to ribosomes and should terminate all enzyme production.

Still, abundances of gut-associated bacteria were reduced only by approximately half or less in multiple sections. Due to various factors, we did not expect to eliminate all bacteria from the gut. Observed reductions in bacterial abundance were consistent with those measured in our method-development trials (results not shown). Some bacteria may have developed resistance to the suite of antibiotics applied (Andersson 2003). Alternatively, bacteria may have been inactivated, but not lysed, and therefore still enumerated. Finally, archaeal cells were mostly likely unaffected by these antibiotics. Penicillin and ampicillin would not impact Archaea because they target the bacterial cell wall, which is unique from the archaeal cell wall (Madigan et al. 2003).

Our measured reductions of associated bacteria for *U. pugnax* was comparable to those reported for other aquatic invertebrates, after antibiotic exposure. As measured by direct counts, bacterial abundances in the stomach of the krill species, *Meganyctiphanes norvegica* were reduced by an order of magnitude after 24 h of antibiotic exposure (Donachie et al. 1995). Yet, for the same comparison, colony-forming units were reduced by four orders of magnitude. For the freshwater detritivorous isopod, *Asellus aquaticus*, bacterial

application reduced gut-associated bacteria to a third of their untreated abundances (Zimmer and Bartholme 2003).

Impact on host

A contribution of enzymes from stomach-associated bacteria would be of greatest value to the host because of the large amount of absorptive gut region 'downstream' of the stomach (Plante et al. 1990). Specifically, released enzymes or products could be absorbed in the hepatopancreas or along the midgut. Although our results indicate that enzyme activities and bacterial abundances are reduced in the stomach, we cannot exclude the possibility that this activity pattern results from hepatopancreatic contribution. It is possible that stomach microbes are commensals, utilizing some fraction of digestive products without contributing substantially to the pool of dissolved enzymes, yet without inflicting harm on the host.

If the host can absorb the resultant products, hindgut bacterial or eccrinid enzymes may allow for nutrient recovery from otherwise 'lost' material. However, it is possible that these enzymes benefit only the hindgut microbes and that any products are released with fecal material, rather than reclaimed by the host. Further research is needed to address whether the host can benefit from microbial enzymes. In particular, if a distinguishing quality existed to discriminate endogenous from bacterial enzymes (i.e. K_m or adsorption measure), this characteristic could be used to differentiate between total dissolved bacterial contribution and total host production.

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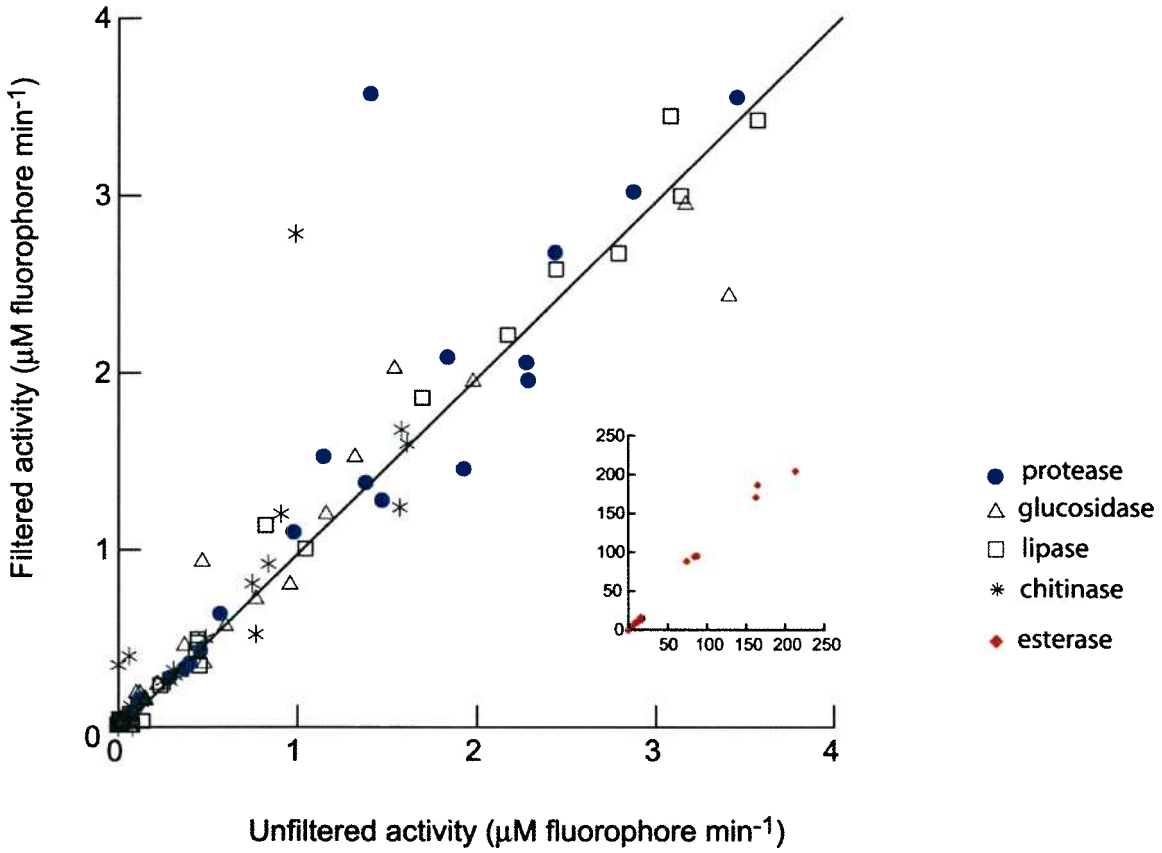
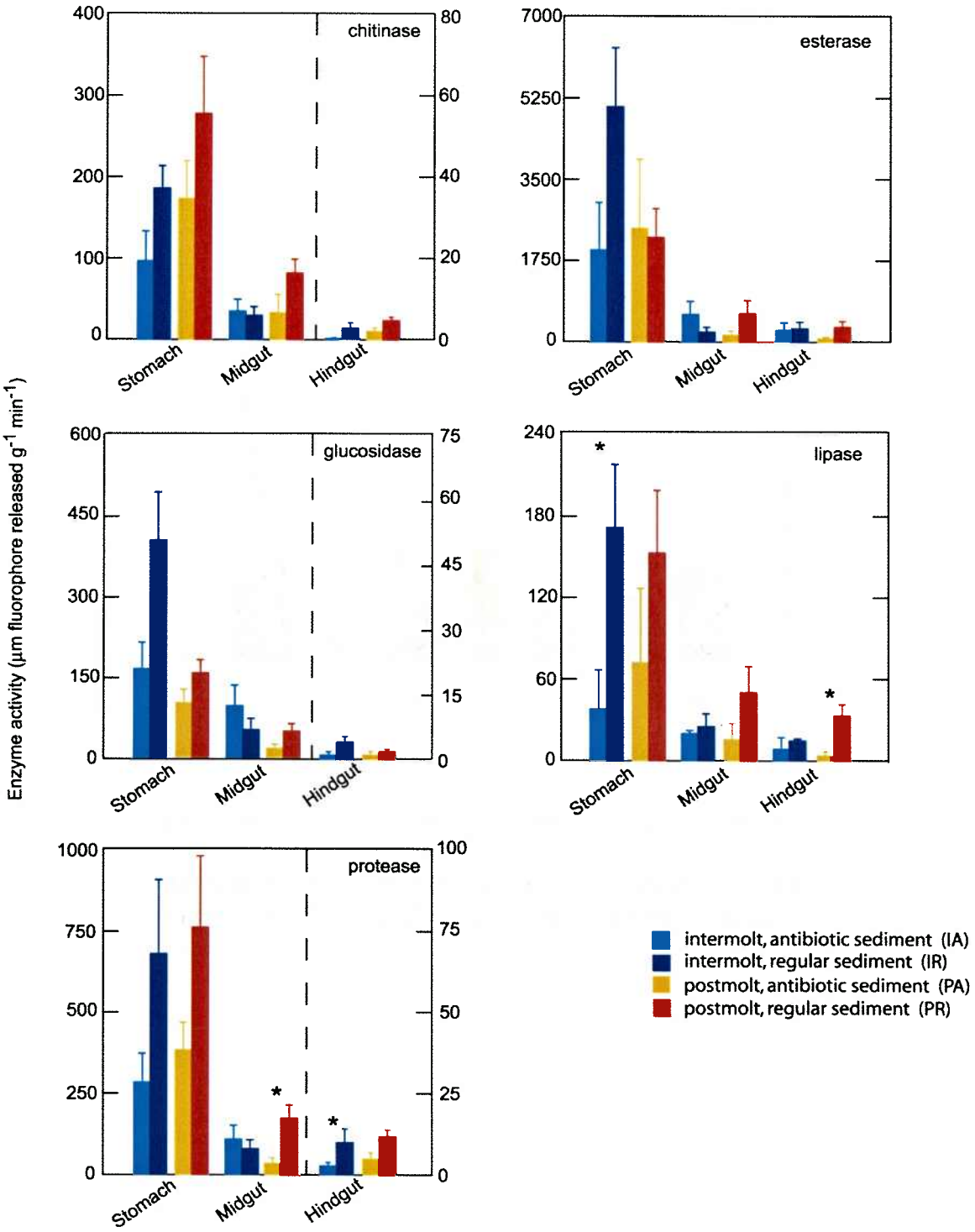


Figure 5.2 Comparison of paired filtered and unfiltered activities for all enzymes, including all treatments and sections. Esterase activities are shown on inset.

Figure 5.3 Enzyme activities among each gut section and treatment (mean + SE). Significant pairwise differences ($P < 0.05$) between IA and IR or PA and PR pairs are denoted with an asterisk (*). Note that hindgut activities correspond with the second Y-axis for chitinase, glucosidase and protease.


chitinase
esterase
glucosidase
lipase
protease
Stomach
Midgut
Hindgut
Enzyme activity (µm fluorophore released g-1 min-1)
intermolt, antibiotic sediment (IA)
intermolt, regular sediment (IR)
postmolt, antibiotic sediment (PA)
postmolt, regular sediment (PR)

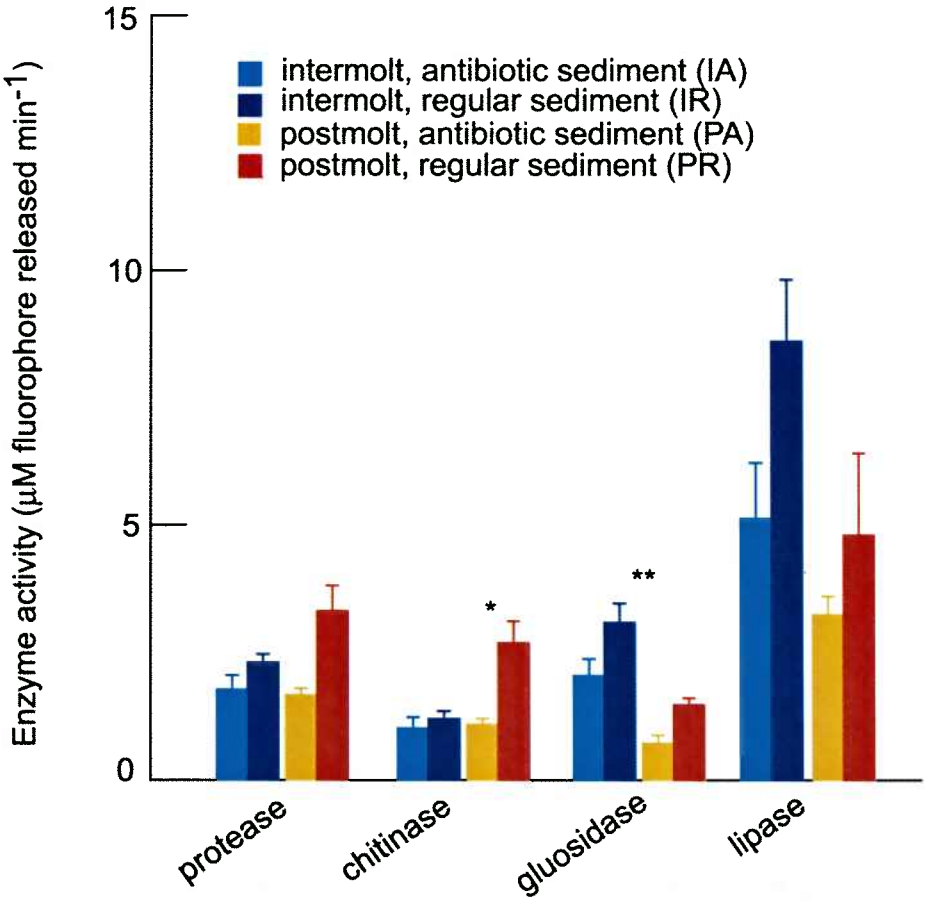


Figure 5.4 Hepatopancreas enzyme activitites among treatments and by enzyme (mean + SE). Any significant pairwise differences ($P < 0.05$) between IA and IR or PA and PR pairs are denoted with an asterisk (*). Any significant pairwise differences between IR and PA are denoted with a double asterisk (**).

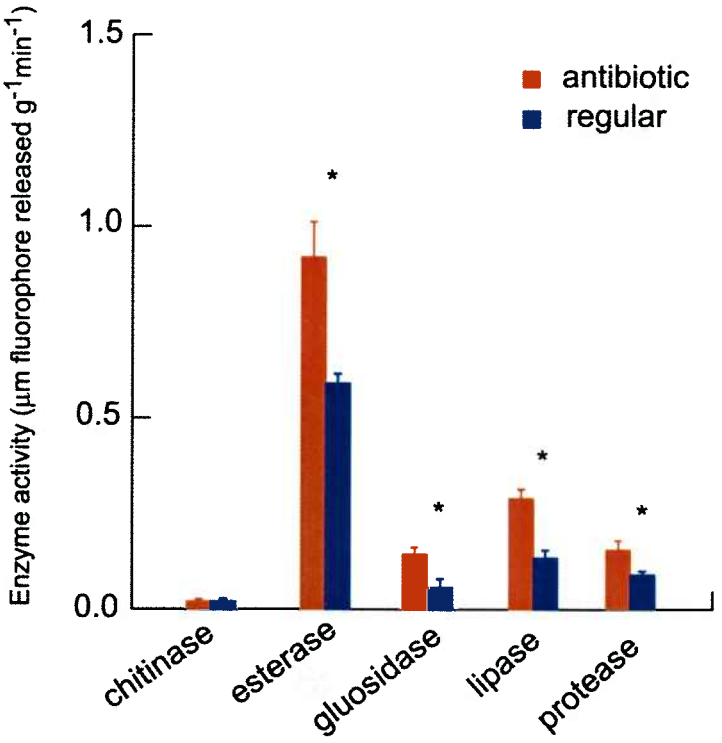


Figure 5.5 Activities of all enzymes in antibiotics-dosed and regular sediments (n = 4; mean + SE). Any significant pairwise differences ($P < 0.05$) between treatments are denoted with an asterisk (*).

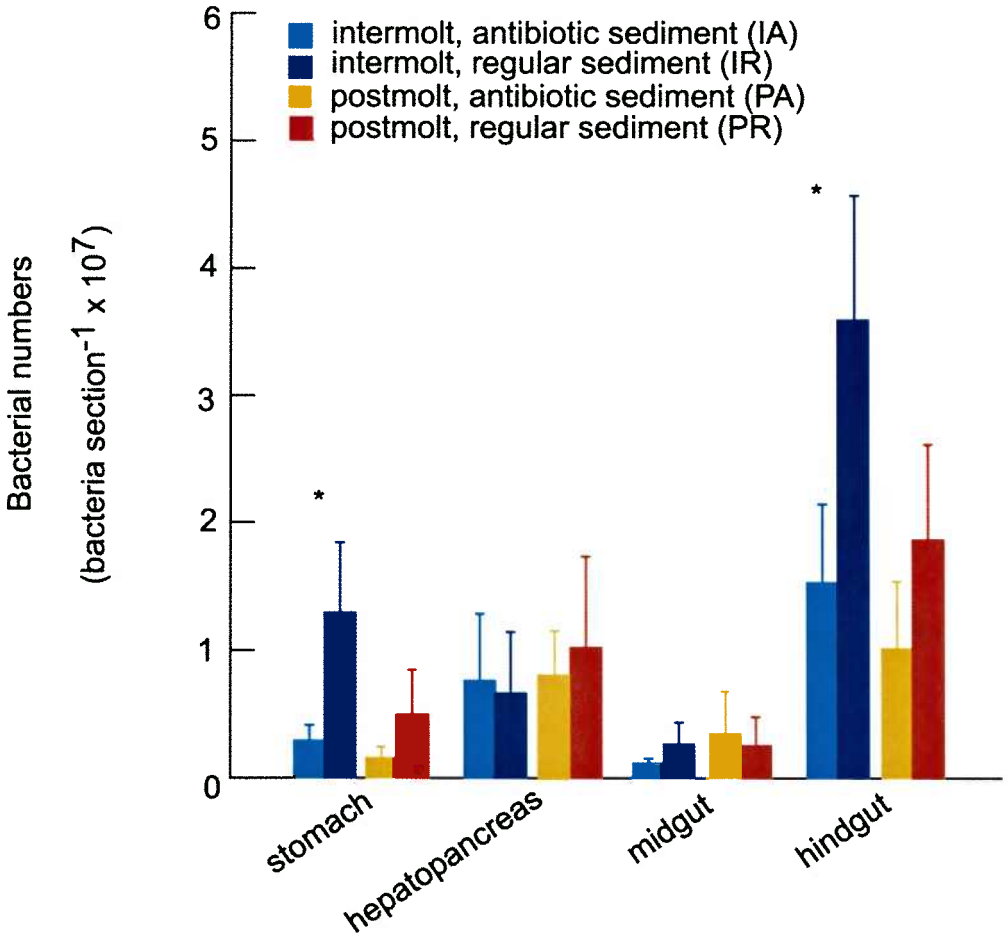


Figure 5.6 Bacterial numbers in each gut section for all four treatments (n = 4-8; mean + SD). Any significant pairwise differences ($P < 0.05$) between IA and IR or PA and PR pairs are denoted with an asterisk (*).

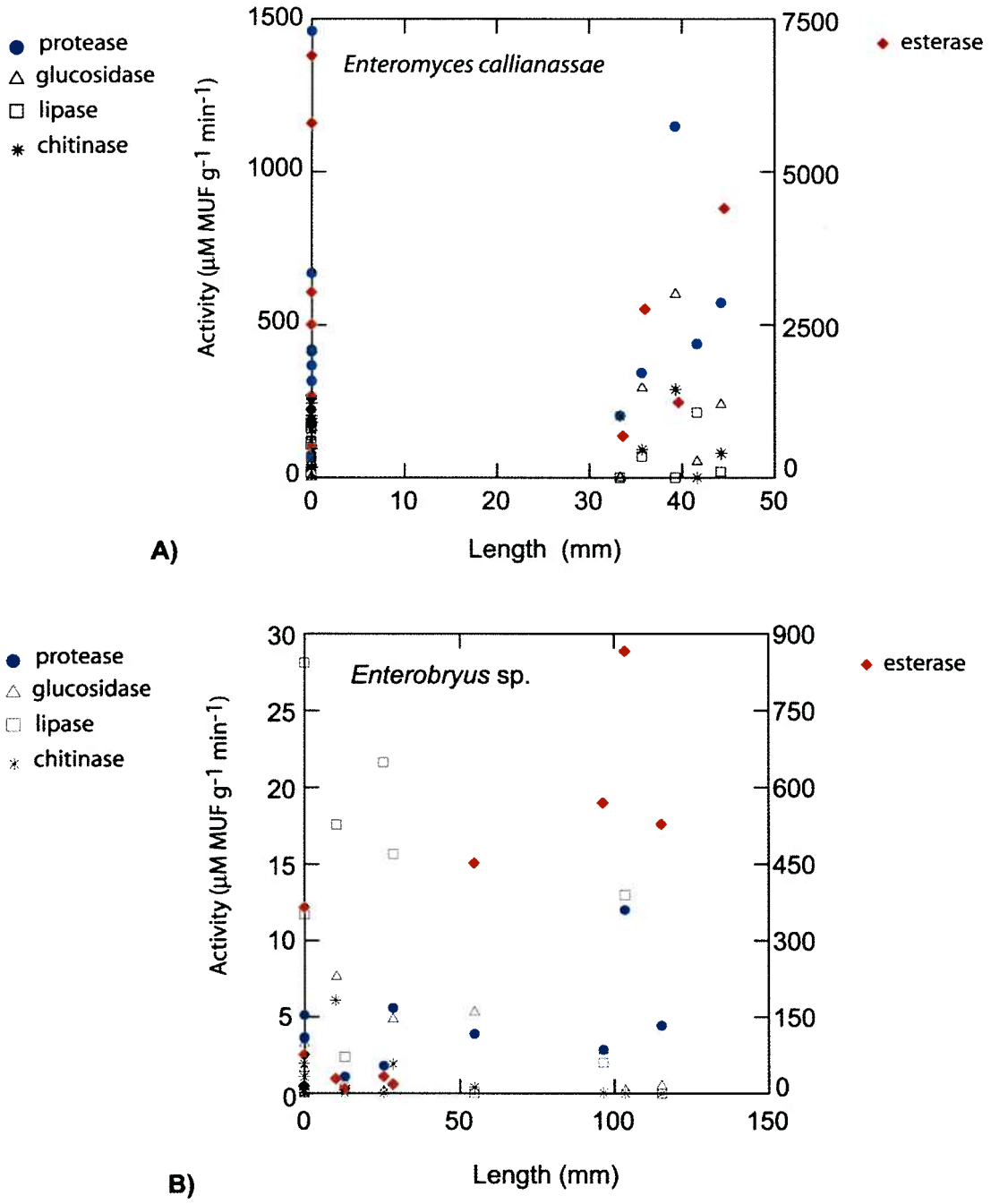


Figure 5.7 Eccrinales length and enzyme activity. A) *Enteromyces callianassae* (stomach) and B) *Enterobryus* sp. (hindgut). Esterase activities correspond with second Y-axes. Filled symbols designate enzymes with positive correlation coefficients for *Enterobryus* sp. correlation.

Chapter 6: Conclusions

General summary

The overarching goal of this thesis was to characterize the ecology and genetic diversity of resident gut microbes in order to advance our understanding of their interactions with their host, the marsh fiddler crab, *Uca pugnax*. The results of this thesis also provide a basis for further studies addressing how these gut populations may influence marsh microbial ecology and biogeochemistry. The greatest abundances of both bacteria and protists were documented in the stomach and hindgut. For these sections, I have described morphologies, measured abundances and characterized the genetic diversity (bacteria) of resident microbes. Both bacterial and protozoan symbionts appear to be consistent features of these sections. Furthermore, bacterial diversity patterns appear comparable among individuals and, likely, over time. Community composition, however, differs between stomach and hindgut populations. Functionally, resident bacteria, particularly in the hindgut, may contribute to total activities of certain enzymes in the gut of their host. The significance of this finding in terms of the host physiology is still to be determined.

One unique aspect of this thesis that will contribute to future studies of invertebrate-microbial interactions was our effort to characterize all communities of resident microbes in *Uca pugnax* and our attempt to isolate enzyme contributions from each. This thesis not only characterized the existence of resident, symbiotic microbial communities, but also studied these populations within the ecological context of their host.

I. Chapter 2: Eccrinales

This thesis is the first report of the two protists, *Enteromyces callianassae* and *Enterobryus* sp. together in the fiddler crab, *Uca pugnax*. These attached symbionts are a common feature of the crab digestive tract: *E. callianassae* was present in > 50% of intermolt crabs and *Enterobryus* sp. was present in > 90%. Both Eccrinales symbionts were found to attach exclusively to restricted regions

of the gut, suggesting that this association is specific and that each species has adapted to particular gut sections. Furthermore, each form of *Enterobryus* sp. colonizes particular sub-sections of the hindgut. The short, bushy form of *Enterobryus* sp. is found only in the posterior-most hindgut and the long, spiraling form is found only in the anterior hindgut.

One clear result of this study was that determination of host molt stage is critical for comparing Eccrinales abundances among individuals and/or among species with accuracy. Other researchers have compared total Eccrinales lengths among different species without reporting molt stage (Hibbits 1978; Mattson 1988). Yet, if any useful patterns between extent of colonization and salient host characteristics are to be discovered, molt stage of the host needs to be considered. Additionally, we propose that our direct method of measuring Eccrinales length offers an improvement over a previously published method, in which total length was estimated with an intersection method (Mattson 1988).

Lastly, our results corroborate the established precedent of Eccrinales species colonizing only detritivorous, algivorous, and/or omnivorous hosts and not inhabiting carnivores (Lichtwardt 1986; Mattson 1988). We predict that, in *Uca pugnax*, the two Eccrinales species may play a role in processing detritus. Finding that *E. callianassae* groups with the Mesomycetozoan protists, rather than with Fungi, may assist in constraining theories on the nature of interactions with crustacean hosts.

II. Chapter 3: Bacterial microbiota: morphologies, attachment sites, and abundances

We measured the greatest abundances of bacteria in chitin-lined gut sections of *Uca pugnax*, the stomach and hindgut. Specifically, SEM images indicated dense clusters of bacteria on the pyloric fingerlets in the crab stomach and along the length of the hindgut. Dense aggregations of attached bacteria in the pyloric stomach have not been reported previously in crustaceans (Harris 1992; Pinn et al. 1997). We postulate that these bacteria are taking advantage of a particular location in the stomach (pyloric fingerlets) in which they are exposed

to both digestive fluid flow and concentrated substrates. It is possible that the host utilizes released bacterial products or is able to ingest some of these bacteria directly. Two morphologies of bacteria colonized specific regions along the hindgut: curved rods along the anterior hindgut and mats of rods in the posterior hindgut. We suggest that greater densities of bacteria (per mm^2 surface area) exist in the posterior hindgut due to enhanced cuticle permeability, which may allow for increased fluid and nutrient flux, thereby alleviating diffusion limitations for attached cells. Relatively greater permeability in the posterior hindgut, compared with the anterior hindgut, would facilitate passive nutrient (small molecules) and water uptake by the host by generating a sufficient concentration gradient, in the right direction.

Transient bacteria were approximately twice as abundant as resident bacteria in the crab stomach and hindgut. Resident bacteria, however, are consistently present among individuals, are more likely to be active and, therefore are expected to play a greater role in host physiology.

III. Chapter 4: Bacterial diversity and seasonality

Bacterial diversity patterns appear to be consistent among individual hosts, based on the fingerprinting technique, DGGE. Furthermore, unique bacterial communities appear to associate with specific gut sections, as indicated by distinctive stomach and hindgut DGGE patterns. Although some changes in diversity patterns were observed between summer and winter, resident communities were not entirely different. Hindgut bacterial densities, however, did vary significantly with season. Based on 16S rRNA gene clone libraries, the hindgut is more diverse than the stomach. One of the most interesting results from the clone libraries was detecting 16S rRNA sequences with high sequence similarity to clones from the hindgut of another crustacean, *Neotrypaea californiensis*. This finding suggests that detritivorous crustacean hindguts may provide an ecological niche for certain bacterial phylotypes. Furthermore, many of the identified clones were most closely related to other symbionts and gut-associated bacteria, indicating that there may be functional similarities in the

nature of these host-microbiota interactions. We propose that these bacterial associates are not a haphazard mixture of opportunistic bacteria from the transient community, but rather are a specific assemblage that may play an important role in host physiology.

IV. Chapter 5: Microbial contributions to digestive enzyme activity

The main goal of this research was to determine if the presence of resident microbes correlated with dissolved extracellular enzyme activity. We found that antibiotic treatments reduced both total enzyme activity and total bacterial abundances in the stomach and hindgut. Stomach bacterial and Eccrinales abundances did not correlate significantly with enzyme activities. We propose that most stomach microbes have a commensal relationship with their hosts, and that they utilize the abundant concentration of organic matter without contributing substantially to the pool of dissolved enzymes. Hindgut bacterial abundance correlations with all enzymes, except esterase, were positive, albeit not significant. We suggest that these bacteria may be degrading the remnants of the chitin and protein-rich peritrophic membrane. These enzymes or bacterial products may benefit their host if they are transferred anterior-ward by fluid flow. We also found that, using the described methodology, essentially all measured activity was derived from dissolved extracellular enzymes, rather than from particle-associated or even cell-associated enzymes.

ITEMIZED CONCLUSIONS

1) Bacteria and protists are regular features in the stomach and hindgut of *Uca pugnax*. Bacterial abundances change with season (hindgut) and protozoan abundances (lengths) vary with molt stage. Still these microbes are not irregular associates, but rather characteristic gut symbionts of *U. pugnax*.

2) Specific morphologies of bacteria and certain Eccrinales morpho-species are found in defined regions of the gut. Although these bacterial

morphologies do not reveal any species-specific information, we can infer some functional basis of conserved morphologies (i.e. that some aspect of the posterior hindgut lining allows for attachment and growth of length-wise attached rod-shaped bacteria).

3) Bacterial diversity in the crab stomach and hindgut appears to be mostly conserved among individuals, according to DGGE patterns. Furthermore these diversity patterns are relatively consistent across seasons. Our results suggest that these microbes are not occasional hitchhikers, randomly latching onto the gut surfaces, but rather that some more specific process influences attachment and growth.

4) The stomach microbial community is less diverse than the hindgut microbial community, as assessed by both DGGE patterns and 16S rRNA clone libraries.

5) Particular bacterial phylotypes, with low sequence similarity to any cultured bacteria, may be shared among crustacean detritivores.

6) Hindgut bacteria may release extracellular enzymes, thereby increasing the overall activities measured in the hindgut. These enzymes and/or their products may be transported anterior-ward by fluid movement to be absorbed in the midgut or hepatopancreas. Alternatively, the host may benefit if small molecular products are absorbed across the hindgut cuticle.

FUTURE RESEARCH DIRECTIONS

Eccrinales

Our sequencing results of *E. callianassae* from *U. pugnax* highlight a gap in the current knowledge basis concerning the possibility of cryptic species of Eccrinales among different hosts. Comparisons of complete 18S rRNA gene sequences of *E. callianassae* from numerous crustacean hosts would resolve this uncertainty.

Very little detail is known about the physiology and diversity of the Eccrinales, as underscored by their recent re-classification as protists, rather than fungi (Cafaro 2003). Cloning and sequencing the hindgut *Enterobryus* sp. would help determine if this organism belongs with other eccrinids in the Phylum Protista and assess if the two morphologies observed represent a single species, or multiple species.

We have speculated that these Eccrinales species may contribute protease or esterase to the gut lumen, however, little is known about the physiology of these organisms. If these species could be successfully cultured, further studies on cultured specimens would assist in constraining their possible range of metabolisms. Alternatively, a molecular study to search for unique enzyme encoding genes, such as cellulase, as described by (Byrne et al. 1999) would offer insights into possible enzyme contributions of these Eccrinales.

Bacteria

We have found that diversity patterns appear consistent among individual crabs, however, we have not resolved the distribution of phylotypes represented by these diversity patterns, either numerically or in terms of physical locations. Are many phylotypes present in equal abundances? Is one phylotype numerically dominant? And how do these phylotypes correspond with the observed morphologies? A study in which phylotype-specific fluorescent probes are applied to the fiddler crab gut lining, via fluorescent *in situ* hybridization (FISH) (Delong 1993), would elucidate both the distribution of bacterial diversity and highlight the locations of specific phylotypes along the gut. In particular, these

results would indicate if the dense mats of bacteria in the posterior hindgut are a monoculture, or if they comprise a diverse assemblage. If the latter is found, this community may form a consortium in which bacterial enzymes and/or products are shared among phylotypes. 'Enzyme-sharing' may also occur in sections with clonal populations.

Hepatopancreas-associated bacteria need to be studied carefully and categorized as either transient or resident bacteria, or possibly a combination of both. Further research on the presence and role of hepatopancreatic bacteria is needed to clarify any possible functions of these bacteria in host physiology.

The presence and possible roles of Archaea were not addressed by this thesis. Archaea are present in gut communities of termites (Brauman et al. 2001; Tokura et al. 2000), in the rumen of terrestrial herbivores (Mackie 2002; Tajima et al. 2001), and in salt-marsh sediments (Munson et al. 1997). Thus, Archaea may be present and active in the gut of *Uca pugnax*, as well as other detritivorous crustaceans. A similar approach to that described in Chapter 4, but with archaeal-specific primers, would resolve whether Archaea are present, and the extent of archaeal diversity in the digestive tract of *U. pugnax*.

Functional role of microbial community

Although it appears that some enzyme activities correlate with bacterial or Eccrinales abundance in the hindgut, this research has not definitively resolved if the host may benefit from these enzymes. A study in which radiolabeled enzyme products or fermentation products were injected into the hindgut lumen and later measured to determine if they were incorporated into the crab tissue would indicate if these products benefit the host.

We observed indications that portions of the gut may experience periodic anoxia, suggesting that these sections may support fermentative metabolisms. Fermentation products such as short chain fatty acids (SCFAs) are a potential energy source for the host crab and can be absorbed without active transport, even across the hindgut cuticle (Hogan et al. 1985; Maddrell and Gardiner 1980). Characterizing oxygen concentrations along the gut with microelectrodes and

measuring SCFA concentrations would indicate if the fiddler crab gut supports substantial fermentative activities.

BROAD IMPACT OF RESULTS

Conclusions drawn from this thesis will contribute to future studies of microbial-invertebrate interactions as well as to studies of the diversity and composition of specific gut communities. In particular, this research will provide a basis for further studies on the distribution, ecology, genetic identity, and function of the poorly-understood Eccrinales protists.

Although not tested directly in this thesis, the presence of distinct and stable microbial communities in the fiddler crab gut may influence salt-marsh microbiology and geochemistry. If resident microbes are shed from the gut lining onto egested material, this input may affect microbial diversity of the marsh sediment. Although the overall sediment diversity pattern (DGGE) differed from typical stomach and hindgut patterns, certain DGGE bands appeared to be shared among these communities. These bands may represent shared phylotypes, and if bacteria of these phylotypes are cast off onto fecal pellets, this process may augment environmental population abundances. Also, resident bacteria may contribute enzymes to the gut lumen. If these bacterial enzymes, or other activities, affect the geochemistry of the gut contents and crab fecal material, this effect may factor into salt-marsh geochemistry. These hypotheses are highly speculative, but offer some possibilities for the influence of resident gut microbes on salt-marsh ecology.

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