

Multi-scale ecological filters shape the crayfish microbiome

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Abstract A general and practical understanding of the processes that drive microbiome assembly and structure are paramount to understanding organismal biology, health, and evolution. In this study of stream-dwelling crayfish, we conceptualized colonization of microbial symbionts as a series of ecological filters that operate at the environment, host, and host microsite levels, and identified key ecological processes at each level. A survey of *Cambarus sciotensis* in western Virginia, USA, showed that the local environment and host microsites interact to create complex patterns of microbial diversity and composition. An in situ experiment confirmed a prevailing

effect of host microsite on microbial composition, and also showed that an ectosymbiotic worm (Annelida; Branchiobdellida) which feeds on biofilms and other symbionts had significant effects on microbial composition of the host carapace, but not gills. Bacterial communities of the carapace were taxonomically rich and even, and correlated with microbial communities of the ambient environment. Conversely, communities on gills were less diverse and dominated by two taxa with potential functional significance: Comamonadaceae and Chitinophagaceae. The bacterial communities of the gills appear to be tightly coupled to host biology, and those of the carapace are mostly determined by environmental context. Our work provides the first characterization of the crayfish microbiome and shows how multi-scale and experimental studies of symbiont community assembly provide valuable insights into how the animal microbiome is structured under conditions of natural complexity. Furthermore, this study demonstrates that metazoan symbiont taxa, i.e., the branchiobdellidans, can alter microbiome assembly and structure.

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1 Introduction

Recent advances in our understanding of the intimate and diverse interactions between plants, animals, and their microbial symbionts have blurred the lines that define individual organisms and fundamentally changed the way we think about ecology and evolution (Gilbert et al. 2012). Animals and plants are colonized by diverse communities of symbionts that can significantly influence the survival, growth and reproduction of their hosts, and in turn symbionts affect the impact their hosts have on their environments (Brown et al. 2002, 2012;

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Palmer et al. 2010). Given these strong direct effects of symbionts on their hosts and potential indirect effects on their host's communities and ecosystems, our understanding of populations, communities and ecosystems should be greatly enhanced if we have greater knowledge of which symbionts are associated with various hosts and how these symbionts may influence integration of hosts into various communities and ecosystems.

While there is an extensive empirical and conceptual literature on the associations between many metazoan symbionts and their hosts, we have only recently begun to accumulate comparable data and concepts for microbial symbiont communities. Observational studies utilizing new sequencing technologies have highlighted patterns of microbial symbiont diversity and composition across a range of contexts from specific host body parts to geographic regions (e.g. Costello et al. 2009; Dominguez-Bello et al. 2010; Kuczynski et al. 2010; Huttenhower et al. 2012; Shafquat et al. 2014). Increasingly, frameworks that embrace multi-scale processes of community structure are being used to explain the typically complex patterns of symbiont diversity (e.g. Dethlefsen et al. 2007; Pedersen and Fenton 2007; Graham 2008; Mihaljevic 2012). Though promising, these multi-scale frameworks are in need of empirical evaluation, and particularly experimental evaluation.

Although animal microbiome research has been largely focused on humans and other terrestrial vertebrates, essential relationships with microbial symbionts are also ubiquitous among aquatic animals (Scheuring and Yu 2012; Clay 2014). Skin bacteria serve as protective agents against amphibian pathogens (Harris et al. 2009; Loudon et al. 2014). Microbial symbionts unlock novel food resources, and competitively exclude potentially harmful pathogens for many aquatic invertebrates, including sponges, corals, and arthropods (Scheuring and Yu 2012; Peerakietkhajorn et al. 2015). Given the ubiquity of important microbial associations in aquatic animals, important and yet undiscovered relationships between crayfish and microbial symbionts seem likely and may have far-reaching consequences. Crayfish have a worldwide distribution in freshwaters, serve as keystone species and ecosystem engineers (e.g. Creed 1994; Statzner et al. 2000, Statzner et al. 2003; Usio and Townsend 2002, 2004; Creed and Reed 2004), and include many endangered, as well as invasive nuisance species (Taylor et al. 2007; Helms et al. 2013; Owen et al. 2015). Crayfish are also one of the most frequently used model organisms in organismal biology (Holdich and Crandall 2002). Part of the interest in crayfish research stems from their amenability to field and laboratory studies, including experimental studies of symbiotic associations (Skelton et al. 2013). Despite the wealth of interest and intense study of crayfish, we are unaware of previous investigations focused on the development of the crayfish microbiome.

In this study, we conceptualized the bacterial microbiome of stream inhabiting crayfish as the result of a series of nested filters operating at scales from the geographic region to microsites on the hosts' bodies (Fig. 1). At the coarsest scale, physical and biological characteristics of a local environment filter out a subset of all bacterial taxa that could potentially colonize the habitat from the regional species pool (Fig. 1a). This subset of the regional pool represents the environmental pool of potential microbial symbionts for hosts living in a given local habitat. Microbiome community membership may again be filtered at the interface between host and environment because only a subset of environmental microorganisms are able to colonize and thrive on any individual host (Fig. 1b). The specific characteristics of different parts of the host's body, here called "microsites", may further filter colonizing microbial symbionts at the microsite level (Fig. 1c). For example, the gills of crayfish are the site of gas exchange and ammonia excretion. Conversely, the carapace is thicker and far less permeable. Thus, the chemical environments of these two microsites are likely quite different and likely to host very different microbial communities.

We implemented field surveys and an in-situ experiment to identify the processes that filter colonizing microbial communities at each level in our framework. We examined the strength of environmental filtering on microbial symbiont communities by comparing environmental and symbiotic bacterial communities in four watersheds. To assess the relative strength of environmental filtering at the level of host microsite versus host habitat, we compared microbial communities of the gills and carapace across all four watersheds. Finally, we conducted a field experiment with manipulated co-infection treatments of *Cambarincola ingens* Hoffman 1963 (Annelida: Branchiobdellida), an obligate ectosymbiont

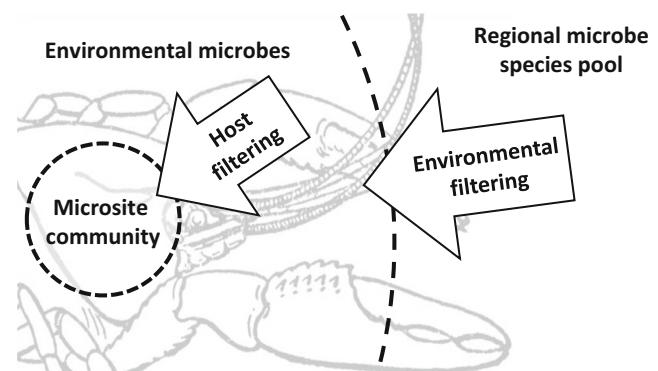


Fig. 1 Conceptual diagram showing multi-level filtering of microbial symbiont community during symbiont community assembly. Potential symbionts from the regional species pool are filtered by habitat characteristics such as water chemistry and pH at the host habitat level, by host characteristics at the host-habitat interface, and by symbiont interactions and habitat characteristics at the host microsite level. Thus the community structure observed at any microsite within the host is the product of a series of nested ecological filters that operate at scales from geographic to cellular

that feeds on crayfish biofilms and other co-occurring symbionts, to explore the effects of metazoan symbionts on the diversity and composition of the crayfish microbiome. Our results demonstrate the necessity of a complete multi-level framework to understand patterns of microbial symbiont diversity and to identify key interactions that may influence microbiome assembly and structure.

2 Materials and methods

Field survey We examined the crayfish microbiome at 4 sampling sites within the New River drainage near Blacksburg, Virginia, USA; Sinking Creek ($37^{\circ}18'9.34''$ N, $80^{\circ}29'6.9''$ W), Big Stoney Creek ($37^{\circ}24'53.33''$ N, $80^{\circ}34'53.58''$ W), Tom's Creek ($37^{\circ}14'23.82''$ N, $80^{\circ}27'30.55''$ W), and Spruce Run Creek ($37^{\circ}15'54.43''$ N, $80^{\circ}35'52.67''$ W). Six adult crayfish (*Cambarus sciotensis* Rhoades, 1944) of similar size (35 mm mean carapace length [CL], ± 5 mm SD) from each site were sampled. Terminal restriction fragment length polymorphism (TRFLP) analysis of the 16S rRNA gene was used to estimate bacterial diversity and compositional variation (Thies 2007), in addition to 3 replicate samples of benthic substrate biofilms from each site. TRFLP is a largely automated process suited for high sample throughput and is useful for tracking changes in microbial community structure at coarse taxonomic scales over time and space (Schütte et al. 2008). All sampling sites were within the contiguous range of *C. sciotensis* (Hobbs et al. 1967). Sites were chosen based on accessibility and to capture a range of benthic substrata types, including limestone (Spruce Run Creek and Sinking Creek), shale (Big Stoney Creek), and sand (Toms Creek). Sampling of benthic substrata consisted of swabbing a 1 cm^2 upward facing surface of randomly selected cobbles from the streambed.

Cleaner symbiont experiment We conducted a field experiment to assess the influence of metazoan ectosymbionts on the crayfish microbiome. Twenty crayfish enclosures (“cages”) were installed in the South Fork of the New River near the campus of Appalachian State University in Boone, N.C. in June 2012. Cages consisted two layers of hardware cloth mesh (12 mm) on the front and back to permit unimpeded flow of stream water, while isolating individual crayfish for recapture and preventing transmission of ectosymbionts to caged crayfish from external crayfish (cages described in Brown et al. 2012; Skelton et al. 2013). Approximately 40 L of mixed substrate collected immediately downstream was added to each cage. Cages were arrayed according to a randomized block design, with 5 blocks perpendicular to stream flow to control for upstream/downstream effects of cage placement (Fig. 2). Each block contained four cages, wherein a single crayfish received one of four treatments: no worms (all

branchiobdellidans removed), 6 worms, 12 worms, or immediate harvesting for assessment of initial microbial communities at the experiment’s outset. Our 6 worm treatment represents typical densities of *C. ingens* for *Cambarus* of the size used in our experiment (30–35 mm CL; Brown and Creed 2004; Brown et al. 2012; Skelton et al. 2016). Our 12 worm treatment represents a higher than typical density, but is within the observed range of densities for our site.

Cambarus chasmodactylus James, 1966 of carapace length (30–35 mm) and bearing all appendages were collected from tributaries of the New River. All branchiobdellidan worms were removed in the laboratory via manual removal by forceps, followed by 5 min submersion in a 0.5 M MgCl₂ solution (Brown et al. 2002). Crayfish were then kept in the experimental cages for 13 days to allow reconditioning of gill and carapace surfaces with native microorganisms. Then, we began the experiment by transplanting large worms (*C. ingens*) collected from local tributaries onto crayfish at treatment levels. Midway through the 43 d experiment, crayfish were physically examined and worm treatments adjusted by reapplying missing worms as necessary to achieve initial treatment levels. Debris was removed from the exterior of cages every other day throughout this period to prevent accumulation of sediment or altered flow in/around cages.

Microbial sampling protocols For both the survey and field experiment, sampling of microorganisms was performed in-field using flame-sterilized equipment. Carapace communities were sampled by wiping one randomly selected lateral half of the carapace with a sterile swab for 10 s and immediately preserving the swab head in a microcentrifuge tube preloaded with a sucrose lysis buffer (pH = 9) (Mitchell and Takacs-Vesbach 2008). Similar techniques were used to sample a 1 cm^2 area of cobbles comprising the benthic substrate of our field survey. For gill samples, the carapace was removed and the rear-most gill tuft from one lateral side (randomly chosen) was clipped and stored in a tube of buffer. All samples were kept on ice and moved to -80°C storage within hours. All remaining crayfish carcasses were preserved in 70% ethanol for determination of final worm densities.

DNA extraction was performed using a conservative technique optimized for potentially low-biomass and low diversity environmental samples (Geyer et al. 2013). Briefly, DNA was extracted using a cetyltrimethylammonium bromide (CTAB) procedure that involves a mixture of 1% CTAB, 10% sodium dodecyl sulfate, phenol/chloroform/isoamyl alcohol (pH = 7.5), lysozyme (0.2 $\mu\text{g}/\mu\text{L}$), and proteinase K (20 $\mu\text{g}/\mu\text{L}$) with either a swab head or gill tissue. Extracted DNA was resuspended in Tris buffer (pH = 8.0) and quantified via spectrophotometry (NanoDrop 2000; Thermo Scientific, Wilmington, DE, USA). The average recovery of DNA from carapace swabs was ~ 50 ng DNA/ μL and ~ 500 –1000 ng

Fig. 2 In-stream enclosures arranged in a randomized block design field experiment with 4 treatment levels and 5 blocks perpendicular to streamflow. Double walls of wire mesh prevent contact between experimental and wild crayfish, and prevent transmission of worms into cages, while allowing natural stream flow and colonization of crayfish prey



DNA/ μ L per gill filament (a majority of which was assumed to be crayfish DNA).

TRFLP analysis PCR amplification of extracted DNA took place in triplicate (25 μ L reaction volume) using a standard 2 μ L of diluted template, 5 units/ μ L of Taq Hot Start Polymerase (Promega Corporation, Madison, WI, USA), and the universal bacterial primers 8F (5'-AGAG TTTGATCMTGGCTCAG-3') and 519R (5'-ACCG CGGCTGCTGGCAC-3'), the forward primer labeled with a 5' 6-FAM fluorophore (Integrated DNA Technologies, Coralville, IA, USA). Amplification was optimized for concentrations of MgCl₂ (2.5 mM per reaction), BSA (1 μ L/reaction), annealing temperature (53 °C), and final extension time (5 min). Amplification replicates were pooled and cleaned using a QuickClean II PCR Extraction Kit (GenScript, Piscataway, NJ, USA). Amplifications were digested with HaeIII (New England BioLabs, Ipswich, MA, USA) in triplicate (20 μ L reaction volume) for 3 h at 37 °C following manufacturer's suggested protocols. Digestion replicates were then pooled and cleaned using GenScript extraction kits. Fragment separation/quantification took place in quadruplicate with an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) and fragments binned using the GeneMarker software AFLP protocol. Resulting sample profiles were standardized using the procedures outlined in Dunbar (2001) to produce both a consensus profile among replicates and final normalization of all sample profiles by total sample fluorescence.

Next-generation sequencing Illumina MiSEQ amplicon sequencing was used to examine the diversity of bacterial communities. Approximately 150 ng of 48 DNA extracts were

provided to the Virginia Bioinformatics Institute at Virginia Tech for 150 bp paired end sequencing of the V4-V6 region of the 16S rRNA bacterial gene. The result was ~500 k reads per sample with an average length of 253 bp after stitching of paired reads. PANDAseq was used to merge forward and reverse reads, correct errors in the region of overlap, and reject any reads that failed to overlap sufficiently (Masella et al. 2012). Taxonomy was assigned to all unique sequences using the UCLUST method and GreenGenes reference database (13_8 release) (DeSantis et al. 2006) in QIIME (1.7.0) (Caporaso et al. 2010). USEARCH was used to cluster all dereplicated reads into OTUs at the 97% similarity level and filter chimeras (Edgar 2010). The UPARSE pipeline was followed for all data denoising (Edgar 2013). A total of 10,489 OTUs were successfully annotated and formed the basis of subsequent multivariate analyses. To reduce the influence of spurious OTUs, we aggregated OTUs at the finest taxonomic level and removed all OTUs that comprised less than 0.1% of the total dataset prior to analysis. We also removed OTUs identified as chloroplasts by BLAST search. Sequences for all bacterial OTUs were deposited in GenBank (accession numbers KY250848 - KY260576).

Statistical methods To assess bacterial diversity observed on carapaces and gills, we calculated Simpson diversity for each sample from normalized TRFLP fragment peaks using the *diversity()* function of the vegan package for R v2.0–10 (Oksanen et al. 2016). Simpson diversity indices were converted to effective numbers of taxa following Jost (2006). This conversion approximates the total number of equally abundant taxa in a community and makes comparisons among communities more interpretable and intuitive than raw diversity indices (Jost 2006). We used linear regression to correlate

the observed diversity of crayfish samples with bacterial diversity of the local substrate and to compare diversity observed on gills to that of carapaces. We first calculated the average diversity of all substrate samples from each sampling locality, and then used average substrate diversity as a continuous predictor and crayfish microsite (gill versus carapace) as a categorical factor, with an interaction term. We also used linear regression to assess the effects of sample type (gills versus carapaces) and branchiobdellidan treatment level with an interaction term in our symbiont experiment. In this case, diversity was calculated as described above using the number of reads assigned to each OTU after sequence binning.

We examined the main and interactive effects of host microsite (gills versus carapaces) and sampling site on the TRFLP fragment composition (a proxy for bacterial community composition) using permutations multivariate analysis of variance (PERMANOVA; Anderson 2001) implemented using the *adonis()* function in the R package Vegan v2.0–10 (Oksanen et al. 2016), with a Bray-Curtis dissimilarity matrix and 10,000 permutations. Compositional effects were visualized by non-metric multidimensional scaling (NMDS) using the *metaMDS()* function in vegan v2.0–10. The same statistical methods were used to assess the effects of branchiobdellidan treatment levels on the bacterial composition of gills and carapaces in the symbiont experiment. We also used Mantel tests to assess the strength of multivariate correlations between average within-site TRFLP profiles from substrate samples, to those of gills and carapaces across all 4 sites. This was done by taking the average peak height for each fragment length from each sample type (gills, carapaces, and cobbles) within each site to create 3 site by fragment length matrices; 1 matrix each for cobbles, gills and carapaces. We then used 2 Mantel correlations to correlate gills and cobble, and carapaces and cobbles across sites (*mantel()* function; vegan 2.0–10; Oksanen et al. 2016).

3 Results

Field survey DNA fingerprinting (TRFLP) results recovered an average of 37.9 (min = 21, max = 47) and 20.3 (min = 8, max = 36) OTUs from carapace and gill microsites, respectively, suggesting greater overall phylum-level richness of carapace biofilms (Fig. 3). Cobbles sampled from the stream bed had an average of 30.2 taxa (min = 13, max = 50). There was significant variation in microbial diversity among host microsites and among sampling locations (Fig. 3), and marginally significant, interactive effects of sampling location and host microsite on symbiotic microbial diversity (Table 1). Variation among watershed in the microbial diversity inferred from TRFLP analysis of the carapace was correlated with the microbial diversity of local substrate, however among-site

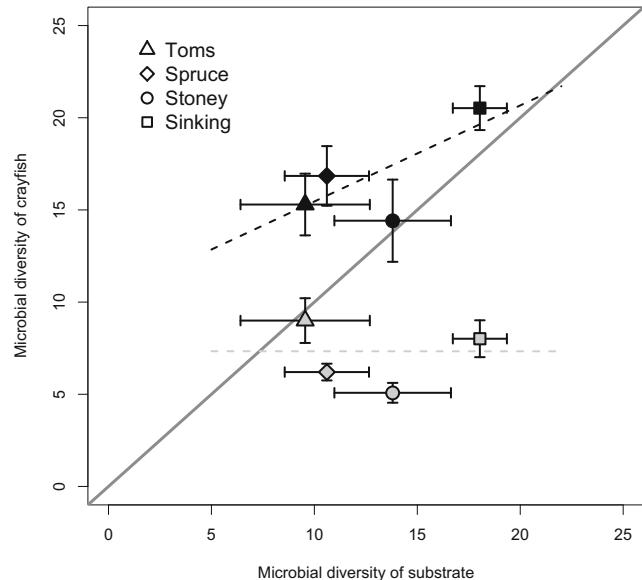


Fig. 3 Field survey results depicting the relationship between microbial diversity originating from crayfish (gills [grey symbols]; carapace [black symbols]) and surrounding environment (substrate) from four sampling sites, ± 1 SE. Diversity is shown as the effective number of equally abundant unique fragment lengths, derived from Simpson's Index of relativated TRFLP data. Grey reference shows 1:1 relationship. Diversity of carapace was typically higher than, and positively correlated with substrate diversity. Gill diversity was typically lower than, and uncorrelated with substrate diversity

variation in the diversity of the gills was not related to substrate diversity (Fig. 3).

Host microsite accounted for most of the compositional variation inferred from TRFLP analysis among samples (PERMANOVA, $F_{1,33} = 23.86$, $p < 0.001$, $R^2 = 0.33$). Additionally, there was significant among-site variation in microbial composition (PERMANOVA, $F_{3,33} = 2.68$, $p = 0.003$, $R^2 = 0.11$), and interactive effects of sampling site and microsite on composition (PERMANOVA, $F_{3,33} = 2.54$, $p = 0.003$, $R^2 = 0.10$). NMDS ordination recovered 2 convergent solutions after 12 tries and a final stress of 0.12. The bacterial composition inferred from TRFLP analysis of the carapace biofilm was largely indistinguishable from that of local substrate biofilms, whereas the composition of the gills was distinct from the substrate (Fig. 4). Across watersheds,

Table 1 Linear regression for effects of average substrate diversity and crayfish microsite on bacterial diversity. The overall model was highly significant ($F_{3,37} = 30.21$, $p < 0.001$). Bold font p -values indicate significance for $\alpha = 0.05$

Coefficients	Estimate	t statistic	<i>p</i> value
Intercept	10.25	3.685	< 0.001
Sample type (Gills)	-2.91	-0.718	0.477
Mean substrate diversity	0.521	2.507	0.017
Sample type \times substrate diversity	-0.521	0.300	0.090

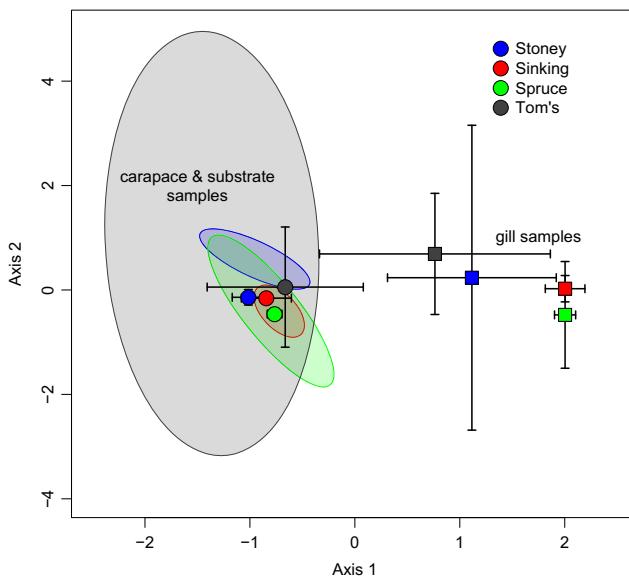


Fig. 4 Non-metric multidimensional scaling (NMDS) of field survey results showing relationships among the bacterial biofilm community of gills (squares), carapaces (circles) and substrate (shaded ellipses) from four locations; red = “Sinking Creek”, blue = “Big Stoney”, green = “Spruce run”, grey = “Toms Creek”. Symbols represent centroid (mean) of each group. Ellipses represent 95% confidence envelope for centroid of substrate samples. Community composition of the carapaces was less variable, but within the range of local substrate. Gill composition was variable at some sites, but always distinct from substrate and carapaces

there was a significant correlation between the bacterial composition of the benthic substrate and the carapace microbiome (Mantel $r = 0.812, p = 0.034$), and a marginally significant correlation between substrate composition and the composition of the gills biofilm (Mantel $r = 0.527, p = 0.089$).

Cleaner symbiont experiment Examination of all crayfish at the middle and conclusion of the experiment confirmed that our methodology prevented colonization of worms on crayfish in the 0 worm treatment. Previous studies using these cages have had similar success (Brown et al. 2012; Skelton et al. 2013). There was some mortality of adult worms, as well as worm reproduction during the experiment that led to variation in worm density among crayfish within each worm treatment level. After the experiment, the high density treatment had an average of 4.6 (± 0.75 SE) adult worms and 16.8 (± 3.01 SE) juvenile worms (21.4 total). The medium density treatment had an average of 3.0 (± 0.58 SE) adult worms and 10.0 (± 4.58 SE) juvenile worms (13.0 total). Thus the high density treatment remained high compared to the medium density treatment, with an average of 1.64X more worms than in the medium density treatment.

Similar to the TRFLP results from the field survey, Illumina sequencing of the experimental crayfish biofilms confirmed that the bacterial communities of carapaces were much more diverse (mean adjusted Simpson’s index = 35.2) than those of the gills (mean = 4.75). There were no significant effects of branchiobdellidan treatments on the microbial diversity of the

carapace or the gills (Table 2). Again, similar to the results of our field survey which were inferred from TRFLP analysis, Illumina sequencing of microbial composition of crayfish samples showed that the carapace was distinct from that of the gills. Bacterial communities of the carapaces contained many evenly abundant taxa, whereas the gills contained many fewer taxa and were dominated by two bacterial families; Comamonadacea and Chitinophagacea (Fig. 5). Comamonadacea had the highest relative abundance of all taxa recovered from gill samples, comprising an average of 41.0% ($\pm 3.9\%$ SE) of reads in gill samples. Second was Chitinophagacea which comprised an average of 15.5% ($\pm 2.4\%$ SE) of reads from gill samples. Conversely, these taxa comprised an average of 4.4% ($\pm 0.007\%$ SE) and 0.4% ($\pm 0.003\%$ SE) of reads from carapace samples respectively. Illumina sequencing showed that branchiobdellidan treatments had no detectable effects on the microbial composition of the crayfish gills (Fig. 6 left panel). In contrast, there was a significant effect of worm treatment on the bacterial composition of the carapaces in which crayfish exposed to high symbiont densities were distinct from controls and medium density treatments (Fig. 6 right panel). There was no significant difference in multivariate dispersion among treatments ($F_{2,12} = 0.169, p = 0.847$).

4 Discussion

Although some microbial symbionts are transmitted from parent to offspring, or from host to host, most are obtained from environmental sources (Bright and Bulgheresi 2010; Walke et al. 2014). In this study, we examined ecological filters that operate at multiple levels as the crayfish microbiome is assembled from environmental sources. We found significant sources of variation at each level, from geographic sampling location, to interactions among metazoan and microbial symbionts within host microsites. By far, the strongest influences over microbiome community composition were observed at the microsite level, where colonizing bacterial symbionts interact with host tissues and other symbionts.

Host habitat level Our field survey showed that local environment had variable influence on the microbial communities

Table 2 Linear regression for effects of branchiobdellidan worm treatment and crayfish microsite type on bacterial diversity. The overall model was highly significant ($F_{3, 28} = 79.56, p < 0.001$). Bold font p -values indicate significance for $\alpha = 0.05$

Coefficients	Estimate	t statistic	<i>p</i> value
Intercept	35.51	18.033	< 0.001
Worm treatment	0.182	0.249	0.470
Sample type (Gills)	-29.53	-9.629	< 0.001
Sample type \times worm treatment	-0.252	-0.655	0.518

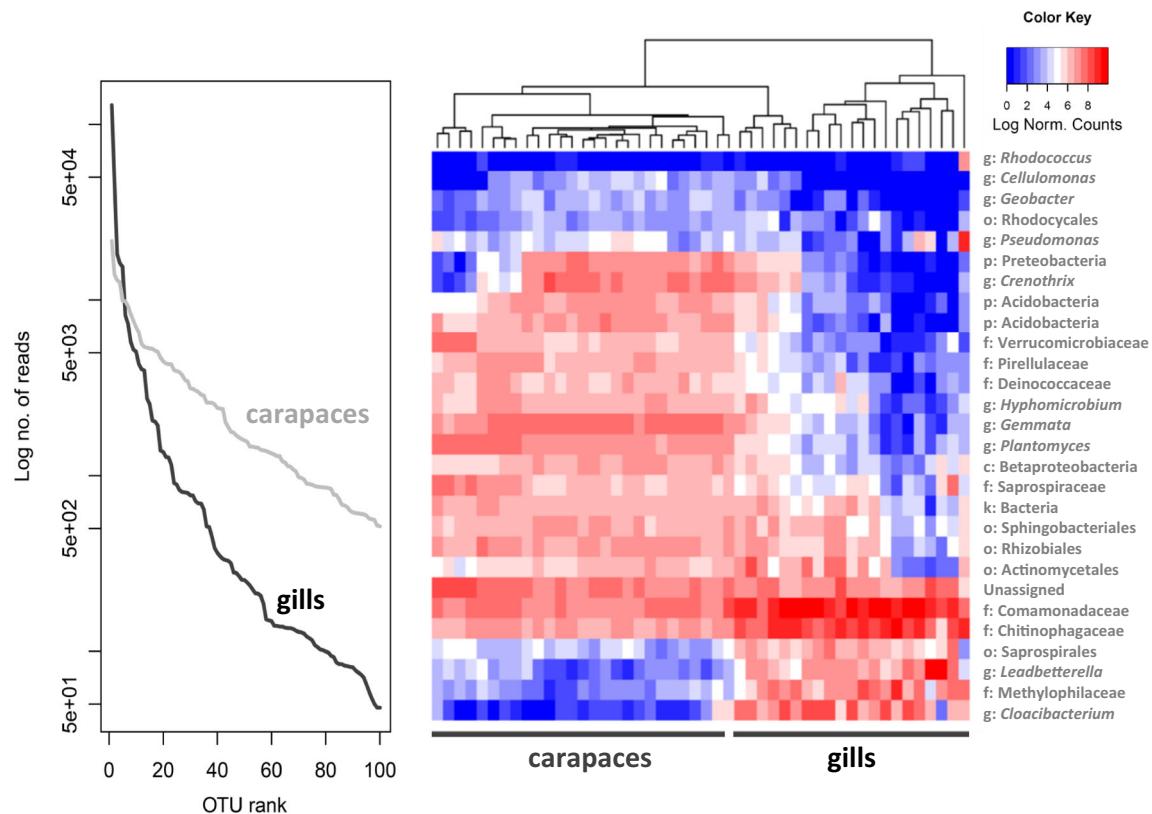


Fig. 5 Comparison of microbial communities of the crayfish carapace and gills. (left) Rank abundance curves for the 100 most frequently observed taxa showed that carapace communities were more taxonomically even than those of the gills, and gills were largely dominated by few taxa. (right) Heat map of showing the log counts of reads for 30 most frequently observed taxa

revealed major compositional differences between gills and carapaces. Columns are arranged by similarity using hierarchical clustering. The gills were dominated by two taxa, Comamonadacea and Chitinophagaceae. Letters before taxa indicate finest taxonomic rank resolved from bioinformatics pipeline (e.g., g = genus, f = family, o = order)

of each host microsite. Microbial composition of the gills and carapaces varied among watersheds, and were correlated with the composition observed from the local substrata, indicating

that many taxa sampled from crayfish are probably opportunistic environmental bacteria. This result is similar to those of other studies of aquatic animal microbiomes (Walke et al.

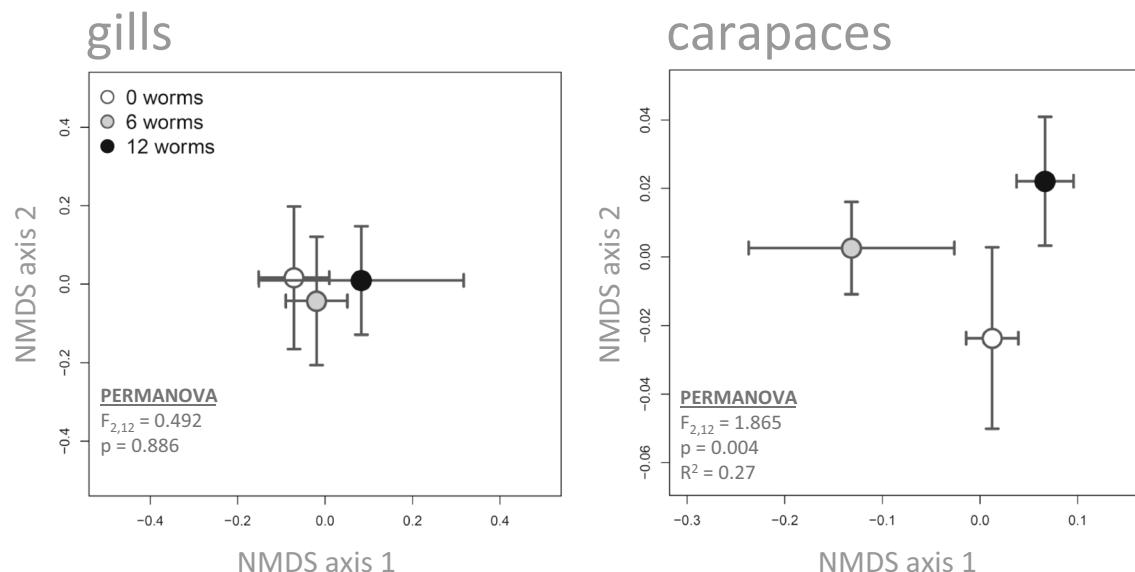


Fig. 6 Non-metric multidimensional scaling ordination showing significant effects of brachiobdellid treatment on carapace (right; stress = 0.14;) but not gill (left; stress = 0.08) microbial communities

2014). Our results also indicate that processes acting at a finer level limit gill community diversity, but not carapace diversity. The microbial richness of the carapace was similar to, and positively correlated with the richness of environmental samples, indicating that richness of the carapace is determined by richness of the environment. However, environmental richness and gill richness were not correlated, and gills consistently had many fewer taxa than environmental samples. Thus a process that operates at the microsite level, such as host immunity, restricts successful colonization of most environmental taxa on the gills (discussed below).

We suspect that the observed variation in microbial communities among watersheds may be attributed to local physiochemical characteristics of each watershed. It is well-known that global patterns in soil and stream bacteria composition and diversity are shaped by pH gradients at both world-wide and local spatial scales (Fierer et al. 2007; Lauber et al. 2009; Rousk et al. 2010). Although we did not assess physiochemical characteristics of our sampling sites in this study, pH seems to be a likely underlying environmental driver of among site variation based on the results of work from another temperate watershed in the eastern United States (Fierer et al. 2007).

Host-habitat interface As microbial symbionts are acquired from the surrounding environment, membership of the microbiome may again be filtered at the interface of host and environment. It is not likely that all microorganisms present in an environment are suited for symbioses with an animal host, and variation among hosts (species and individuals) may select for different subsets of the environmental pool (McKenzie et al. 2012; Kueneman et al. 2014; Walke et al. 2014). Ecological and host-mediated differences among microsites on, or in, the host body may select for a more specialized subset of the global pool. For example, microbial communities of human skin vary widely among parts of the human body (Costello et al. 2009; Grice and Segre 2011). Therefore, the communities of bacteria found in association with a host microsite may represent a restricted subset of the global pool of potential colonists. Carapace diversity was similar to bacterial samples taken from local substrates. Conversely, microbial communities of the gills were shown by TRFLP analysis of survey data to be less diverse than local substrate and carapaces at all sampling sites, and again found to be less diverse than carapace biofilms by direct sequencing of experimental animals. Thus the gills appear to impose a strong filter on microbial symbiont communities, but similar filtering is weak or non-existent on the less physiologically active carapace.

Microsite processes Crayfish gills are a vital interface between the crayfishes' internal physiology and the external environment. The gills exchange respiratory gases with the environment, are sites of nitrogenous waste excretion, and

regulate ion exchange. Consequently, the gills are composed of thin, un-sclerotized epithelia. Conversely, the carapace is composed of heavily sclerotized and calcified cuticle, and is approximately 300 times less permeable than the gills (Pequeux 1995). Both microsites are accessible to microbial colonization from environmental sources. Although the gills are contained within a partially closed gill chamber, water is routinely moved across the gills to maintain a respiratory current. This water contains environmental sediments that serve as sources of microbes that could colonize the gills, and accumulations of sediments and microbial biofilms on the gills are a major challenge to crayfish physiology (Bauer 1998; Rosewarne et al. 2014).

Because of the physiological challenges imposed by microbial colonization of the gills, we predicted that the microbial communities the gills would be limited by host defenses to few specialized taxa. Our results supported our predictions. In contrast to the carapace, crayfish gills were found to have less diverse microbial communities that were dominated by two taxa. While carapaces were characterized by many evenly distributed taxa, the gills were dominated by Comamonadacea and Chitinophagacea. Reduced bacterial diversity, and constrained microbial membership of the crayfish gills may be at least in part attributable to host immunity and the production of antimicrobial chemicals. Previous work has shown that the hemolymph of some crayfish species can inhibit the growth of some bacterial taxa in culture and may reduce bacterial accumulations on crayfish gills (Farrell et al. 2014).

Microbiome studies are increasingly focused on non-random microbiome community assembly, with a particular focus on beneficial microbial taxa (e.g. Bäckhed et al. 2005; Dethlefsen et al. 2006, 2007; Huttenhower et al. 2012; Scheuring and Yu 2012; Shafquat et al. 2014). Most beneficial microbes provide either nutritional advantages or defense against invading pathogens (Bäckhed et al. 2005; Huttenhower et al. 2012). Based on recent theoretical developments (Scheuring and Yu 2012), we suspect that microbial resources released through the thin and permeable cuticle of the gills fuels the competitive dominance of Comamonadacea and Chitinophagacea, and that one or both taxa may produce antimicrobial compounds to maintain dominance and protect the host from invading pathogenic taxa. The gill cuticle itself could be a resource for Chitonphagacea, as several strains within this family are known to degrade chitin (Rosenberg 2014). Similar relationships between animals and protective microbes are common in nature and have been described in many marine invertebrates such as sponges and corals (Scheuring and Yu 2012; Clay 2014). Given the ubiquity of protective microbes on marine animals it is likely that similar associations may occur in freshwater habitats.

Evidence from other freshwater animals further indicate that Comamonadacea and Chitinophagacea may serve a significant function in crayfish biology. Recent experimental work has

demonstrated a positive effect of bacterial symbionts on the population growth rates of a small freshwater crustacean, *Daphnia* (Peerakietkhajorn et al. 2015). Similar to our study, beneficial microbial communities were largely composed of Betaproteobacteria belonging to the Comamonadacea; a finding congruent to previous investigations of the bacterial symbionts of *Daphnia* (Qi et al. 2009; Freese and Schink 2011). Moreover, Comamonadacea and Chitinophagacea are often dominant members of the skin microbiomes of aquatic amphibians (Harris et al. 2009; McKenzie et al. 2012; Kueneman et al. 2014; Walke et al. 2015), yet the processes that led to their ubiquity and dominance in aquatic animal microbiomes, and their potential functional significance are not resolved.

Because our field survey used DNA fingerprinting, the identity of the dominant taxa on the gills of *C. sciotensis* could not be determined. Because the sequencing study and the field survey were conducted on different *Cambarus* species, it is possible that the gills of *C. sciotensis* and *C. chasmodactylus* are dominated by different microbial taxa. Host specificity in the microbial symbionts of crayfish gills remains an unexplored and potentially fruitful avenue for future research. Nonetheless, the observations and methodologies presented here clearly indicate that patterns of microsite diversity are consistent among both *Cambarus* species. Specifically, microbial communities of the carapace have higher taxon richness and evenness than the gills.

The study of symbioses continues to expand beyond concepts based on pairwise species interactions and is embracing the realistic complexity of symbiosis. Recent synthetic work has highlighted the importance of direct and indirect interactions among symbionts during symbiont community assembly (Graham 2008; Skelton et al. 2015, 2016; Thomas et al. 2016). However, perspectives that transcend microbe-microbe or microbe-host interactions are not typically considered. We have shown that metazoan symbionts exert influence over microbial symbiont communities at the microsite level. Crayfish growth and survivorship can be increased by hosting branchiobdellidan worms (reviewed in Skelton et al. 2013). Several lines of evidence suggest that the positive effect of worms on their host is mediated by interactions with microbial communities that develop on the gills. *Cambarincola ingens* often enter the gill chamber and consume organic matter that accumulates therein, in addition to grazing on the exoskeleton biofilm (Brown et al. 2002, 2012). Accumulations of organic matter, particularly bacterial flocs, present a major challenge to crayfish in their natural benthic environment (Bauer 1998; Rosewarne et al. 2014). Thus researchers have hypothesized that *C. ingens* increase the growth and survival of their hosts by consuming harmful organic matter, including bacteria flocs, from the gill epithelia (Brown et al. 2002, 2012). Support for this hypothesis has come from multiple studies demonstrating context-dependent outcomes in the

interactions between crayfish and their worms (Lee et al. 2009; Brown et al. 2012; Thomas et al. 2013, 2016; Skelton et al. 2014).

In this study, branchiobdellidans had a significant effect on the composition of carapace microbial communities, but no detectable effect on microbial composition of the gills. Bacterial richness was also unaffected on both gills and carapaces. The methods used in this study could only detect changes in bacterial composition and not bacterial biomass, so it remains possible that branchiobdellidans reduce bacterial biomass on the gills without specific effects on the composition of bacterial communities. Because the gills were found in this study to be dominated by a few taxa and perhaps influenced directly or indirectly by the host, any effects of worms on gill bacterial communities is more likely to be due to reductions in microbial biomass and not changes in microbial taxonomic composition. In contrast to the gills, microbial communities of the carapace appear to be more variable, and composed of abundant opportunistic colonizers whose relative abundances are influenced by local factors such as environment and branchiobdellidan presence. The effect of branchiobdellidans could be either from direct grazing or indirect effects of grazing via resource alteration. Whatever the cause, the disparity between worm effects on gills and carapaces suggests that gill microbial communities are less susceptible to alteration from local biological factors, and that the symbiosis between crayfish and the bacterial communities of their gills is more tightly coupled to host biology than environmental context.

5 Conclusions

Our goal for this study was to examine the effects of ecological processes on patterns of microbial symbiont communities at multiple scales, from watersheds to host microsites. We provided the first sequenced-based characterization of the crayfish microbiome and identified taxa with potential functional significance to crayfish biology. The crayfish microbiome was influenced by local environment, host microsite, and interactions with metazoan symbionts. Predominant ecological processes that dictated membership of the crayfish microbiome operated at the finest spatial scales, where microorganisms interact with each other and with host tissues. The majority of variation in microbial composition and diversity was explained by host microsite, a result that was consistent across DNA fingerprint analyses and high-throughput amplicon sequencing. The microbial communities of the gills were found to be less responsive than carapaces to the influences of environment and interactions with metazoan symbionts, suggesting a stronger influence of host control over microbial colonization of gills. Gills were dominated by two bacterial families identified in other studies as symbionts of other aquatic animals, suggesting

that these organisms may represent important specialized aquatic animal symbionts.

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