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Microbial ecology of the salmon necrobiome: evidence salmon carrion decomposition influences aquatic and terrestrial insect microbiomes

Jennifer L. Pechal[1]* and M. Eric Benbow[1,2]
Departments of [1]Entomology and [2]Osteopathic Medical Specialties, Michigan State University, 243 Natural Science Building, East Lansing, MI 48824, USA.

Summary

Carrion decomposition is driven by complex relationships that affect necrobiome community (i.e. all organisms and their genes associated with a dead animal) interactions, such as insect species arrival time to carrion and microbial succession. Little is understood about how microbial communities interact with invertebrates at the aquatic–terrestrial habitat interface. The first objective of the study was to characterize internal microbial communities using high-throughput sequencing of 16S rRNA gene amplicons for aquatic insects (three mayfly species) in streams with salmon carcasses compared with those in streams without salmon carcasses. The second objective was to assess the epinecrotic microbial communities of decomposing salmon carcasses (*Oncorhynchus keta*) compared with those of terrestrial necrophagous insects (*Calliphora terraenovae* larvae and adults) associated with the carcasses. There was a significant difference in the internal microbiomes of mayflies collected in salmon carcass-bearing streams and in non-carcass streams, while the developmental stage of blow flies was the governing factor in structuring necrophagous insect internal microbiota. Furthermore, the necrophagous internal microbiome was influenced by the resource on which the larvae developed, and changes in the adult microbiome varied temporally. Overall, these carrion subsidy-driven networks respond to resource pulses with bottom-up effects on consumer microbial structure, as revealed by shifting communities over space and time.

Received 13 October, 2015; revised 11 December, 2015; accepted 11 December, 2015. *For correspondence. E-mail pechalje@msu.edu; Tel. 517 355 6514; Fax 517-432-7061.

Introduction

Resource subsidies, such as vertebrate carrion, are recognized to cross ecosystem boundaries and influence the structure of ecological networks, especially in systems where animals migrate to reproduce and then die (Polis and Hurd, 1996; Polis and Strong, 1996). Ephemeral carrion resource subsidies are known to influence the structure and function of local communities in both terrestrial and aquatic ecosystems (Cederholm *et al.*, 1999; Chaloner and Wipfli, 2002; Chaloner *et al.*, 2002; Minakawa *et al.*, 2002; Claeson *et al.*, 2006; Hocking and Reimchen, 2006; Hocking *et al.*, 2009) through interactions of the necrobiome. The necrobiome consists of groups of interconnected and interacting organisms, such as bacteria, fungi, invertebrates and vertebrate scavengers, which are associated with decomposing organic material (Benbow *et al.*, 2013). The creation of ephemeral carrion resource subsidies is particularly profound in settings with relatively predictable and large-scale animal die-offs, such as salmon-bearing streams of Alaska. The resulting post-spawning carcasses along with their associated decomposer/consumer communities are a model system to test potential bottom-up biodiversity effects that may drive ecosystem functions (i.e. carrion decomposition) (Carter *et al.*, 2007; Barton *et al.*, 2013a,b). Salmon carcasses move nutrients and energy into watershed ecosystems that are acquired through a lifetime of resource acquisition throughout enormous expanses of the Pacific Ocean. Little is understood about the microbial ecology of salmon migration process and how it may influence consumer ecology and microbial metacommunity dynamics in their natural streams.

Carrion systems in general have been understudied, so to better appreciate the biotic interactions of microbial taxa during vertebrate decomposition, it is necessary to place this community within the larger interaction network of carrion-associated organisms and their genes (e.g. necrobiome) (Benbow *et al.*, 2013). Decomposing heterotrophic biomass, such as these salmon carcasses, represents a unique and nearly global ephemeral resource (Benbow *et al.*, 2015a,b). The diversity, complexity and interactions among microbes and insects during carrion decomposition remain to be understudied

areas of science (Pechal *et al*., 2014a). The microbial communities of the necrobiome are thought to fill an important functional role in the decomposition process (Pechal *et al*., 2013; 2014b), as well as facilitate the attraction of necrophagous insects (Ma *et al*., 2012) and scavengers (Beasley *et al*., 2015). Microbes were initially only considered nutrient recyclers within an ecosystem (Lindeman, 1942). Janzen (1977), however, suspected that these microorganisms competed with other macroorganism consumers for ephemeral resources by producing toxic compounds during decomposition, which resulted in a less "appealing" resource to the higher trophic-level consumers. For instance, scavenging rates by crustaceans on fish carrion in estuaries have been reported to decrease from 89% in the absence of microbes to 66% when microbial communities proliferated undisturbed (microbe laden), which produced metabolites that acted as chemical defence against higher trophic-level consumers (Burkepile *et al*., 2006).

Common eukaryotic consumers of the necrobiome are necrophagous insects, such as blow flies (Diptera: Calliphoridae) and beetles (Coleoptera). Blow flies are spatially and temporally distributed throughout North America (Whitworth, 2006) and are considered the cornerstone of decomposition consumers in terrestrial habitats (Merritt and Jong, 2015). These primary carrion colonizers lay eggs on carcasses that hatch into larvae that feed on the tissue, and are efficient biomass converters (Tullis and Goff, 1987; Byrd and Castner, 2001; Benbow *et al*., 2015a). These necrophagous insects also have alternative important ecosystem functions through pollination of forest vegetation (Lisi and Schindler, 2011), serving as prey for a variety of animals in watersheds (Hocking and O'Regan, 2015) and harbouring their own unique microbial diversity (Wei *et al*., 2014; Singh *et al*., 2015). Better understanding of how decomposing salmon carcass microbial communities influence the internal microbiome of blow flies is important because both the adults and the larvae have the potential to disperse salmon carrion microbial communities into the environment, which may have larger microbial metacommunity effects at the ecosystem scale. In addition to these direct consumers of salmon carrion, there are aquatic insect communities indirectly affected by the salmon decomposition process. There are immature stages of aquatic insects that feed directly on submerged carcasses (e.g. the stonefly *Sweltza* sp.; Chaloner and Wipfli, 2002; Chaloner *et al*., 2002) or indirect consumers, such as Heptageniidae mayflies (Ephemeroptera), that graze on biofilms or accumulated fine particulate organic matter sloughed from carcasses during decomposition and receive nutrients and microbes. Therefore, we seek to better understand the influence of salmon carcasses on stream insect communities by characterizing the internal microbiota of widespread aquatic insects.

Despite the documented importance of microbial communities in many ecosystem functions (Finlay *et al*., 1997; Torsvik and Ovreas, 2002; Hattenschwiler *et al*., 2005), there has yet to be a study focused on the microbial communities of the necrobiome as part of the aquatic–terrestrial interface. This cross-boundary habitat allowed us to test for increased micro- and macroorganism interactions from organisms that may have otherwise been isolated to their respective habitats. To explore the effect of salmon decomposition on direct and indirect consumers at the aquatic–terrestrial interface, we analysed the microbiome (16S rRNA gene region amplicon) from: (i) aquatic insects collected from streams with and without salmon carcasses, (ii) the internal microbiome of blow fly larvae and the salmon carcass they were utilizing, (iii) the internal microbiome of blow fly larvae compared with that of adults and (iv) the microbiome of temporally separated blow fly adults. We predicted that the insects would have their own unique microbiome that was supplemented by a salmon carrion-derived microbiome. Specifically, we wanted to answer the following questions: Do aquatic insects collected in salmon carcass-bearing streams harbour different internal microbiota compared with insects collected in streams without carcasses? Do blow fly larvae that directly fed on carcasses only obtain microbes from their food resource? Do blow fly adults that came into direct contact with this ephemeral resource subsidy acquire any carcass-specific microbes? Our results demonstrate that carrion resource subsidy-driven networks respond to resource pulses with bottom-up effects on the internal microbiome community structure in both aquatic and terrestrial insects. Characterizing the microbial taxa within these carrion–stream–riparian networks allowed us to identify key microbial taxa that may be influencing the decomposition process, necrobiome community assembly and microbial dispersal between the coupled habitats. Here we illustrate how ephemeral resource pulses have a potentially important effect on overall ecosystem function and food web dynamics by influencing the integration of resource subsidy microbial communities into aquatic and terrestrial food web primary consumers.

Results and discussion

Insect community composition

A total of 615 individual aquatic invertebrates from five orders (Ephemeroptera, Plecoptera, Trichoptera, Diptera and Acari) were collected from non-salmon carcass-bearing stream (NoSAL) and salmon carcass-bearing stream (SAL) (Table S1). We focused specifically on the nymphs from three insect taxa within the flat-headed mayfly family (Ephemeroptera: Heptageniidae) due to their spatial and temporal prevalence among sampling

sites (Table S2): *Anepeorus rusticus*, *Cinygmula* sp. and *Epeorus* sp. Additionally, all Calliphoridae larvae (third instar) collected from salmon carcasses were identified as *Calliphora terraenovae* (Table S2); the carcasses from which the larvae were collected were also swabbed for epinecrotic microbial community characterization, which will be discussed in a later section. A total of 264 Calliphoridae adults (identified to 10 taxa) were collected near decomposing salmon carcasses (Table S1). The most spatial and temporal abundant species was identified as *Calliphora terraenovae* (Table S2), and adults were used for further characterization of internal microbiomes. There were two temporally separated cohorts (10 days apart) of *C. terraenovae* adults collected, which will be referred to henceforth as adults_I and adults_II. Due to weather and time constraints, we were unable to sample the adults on the same day as the larval samples were collected.

Aquatic insect microbial community composition

In all, 1 020 421 16S rRNA gene amplicon sequences were generated from 26 individual mayflies (Table S3) with sequences characterized to 1726 operational taxonomic units (OTUs). Of the 26 mayflies, 11 individuals were collected in NoSAL streams (Table S3) and 15 individuals were collected in SAL streams (Table S3). There were five phyla that most represented the microbial community composition (> 90% relative abundance: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Tenericutes) of mayflies across streams; the most common phyla among all individuals were α-, β-, and γ-Proteobacteria and Bacteroidetes (Fig. 1; Table S4).

The phyletic community composition difference between stream types indicated that where the carrion resource decomposed influenced the internal microbiome of the stream insects. In the predatory *A. rusticus* collected from SAL streams, the relative abundance of Proteobacteria was reduced by 53.8% compared with those collected from NoSAL streams. Whereas, in the scraper/gatherer species, *Cinygmula* sp. and *Epeorus* sp., collected from SAL streams, the relative abundance of Proteobacteria was increased by 40.3% and 43.9%, respectively, over those from NoSAL streams. The relative abundance of Tenericutes increased across all taxa in SAL streams: *A. rusticus* had the greatest increase at 92.2%, whereas *Cinygmula* sp. and *Epeorus* sp. increased 81.0% and 65.3% respectively. Additionally, there were nine OTUs detected in greater than 80% of all mayfly individuals: Comamonadaceae and *Sediminibacterium* spp. were the only OTUs detected among all individuals regardless of species or stream type (Table S4), providing candidate core microbiome taxa among the mayflies; Bradyrhizobiaceae was the only OTU detected with a higher frequency among nymphs from NoSAL versus SAL sites (100 versus 87%); and Renibacterium and Acinetobacter were detected with a higher frequency among mayfly nymphs from SAL streams at 73 versus 100% and 73 versus 93% (NoSAL versus SAL) respectively. Further, there were distinct differences in the mean relative abundance of specific microbial OTUs detected between mayfly taxa collected in the different stream types (Table S4).

The phyla detected in the internal microbiome of the mayflies were composed of communities from a wide scope of common environmental bacteria. *Anepeorus rusticus* is a predator, and thus their food source or naturally occurring microbiota may be influencing the microbial community composition, as has been previously documented in terrestrial ecosystems for other insect species (Yang and Gratton, 2014). *Cinygmula* sp. and *Epeorus* sp., however, are scraper/gathers and their primary diet is composed of algal and detrital biomass: *Cinygmula* sp. feed on autochthonous resources, such as epiphyton (i.e. stream biofilms), whereas *Epeorus* sp. are more generalists capable of using autochthonous and allochthonous (e.g. detritus) resources. Therefore, the differences in the microbial community composition among taxa suggest that the diet preference of the individual taxon governs at least some of the internal microbiome structure. The increase in Tenericutes detected in insects in salmon carcass-bearing streams may also be the result of several well-known salmonoid fish pathogens (e.g. *Mycoplasma*) (Ringø *et al.*, 1995; Holben *et al.*, 2002; Nayak, 2010) entering the water column through fish excrement, shedding of the mucosal lining, or during the carcass decomposition and tissue-sloughing process. These salmonoid-derived bacteria may also be incorporated into environmental biofilms, which are directly (scraper/gathers) or indirectly (predator) consumed by mayflies. The increase in frequency of *Renibacterium* and *Acinetobacter* in SAL streams provides further evidence that these potential salmonoid pathogens (e.g., bacterial kidney disease) are influencing the insect microbial community structure. The only OTU that was detected in higher frequency in NoSAL streams was Bradyrhizobiaceae, a taxonomic group that is commonly associated with plants, soil, aquatic sediments and biofilms, and animal hosts; members within the family are key in nitrogen fixation (Marcondes de Souza *et al.*, 2014; Vila-Costa *et al.*, 2014).

Measurements of α-diversity (richness, diversity and evenness) were not significantly different between stream types, among taxa, or between pair-wise comparisons of taxa collected in the different stream types. Phylogenetic (UniFrac) weighted measures of β-diversity were used to compare microbial community assemblages using all OTUs within all individuals across all sample locations and taxa. There was no pronounced effect of mayfly

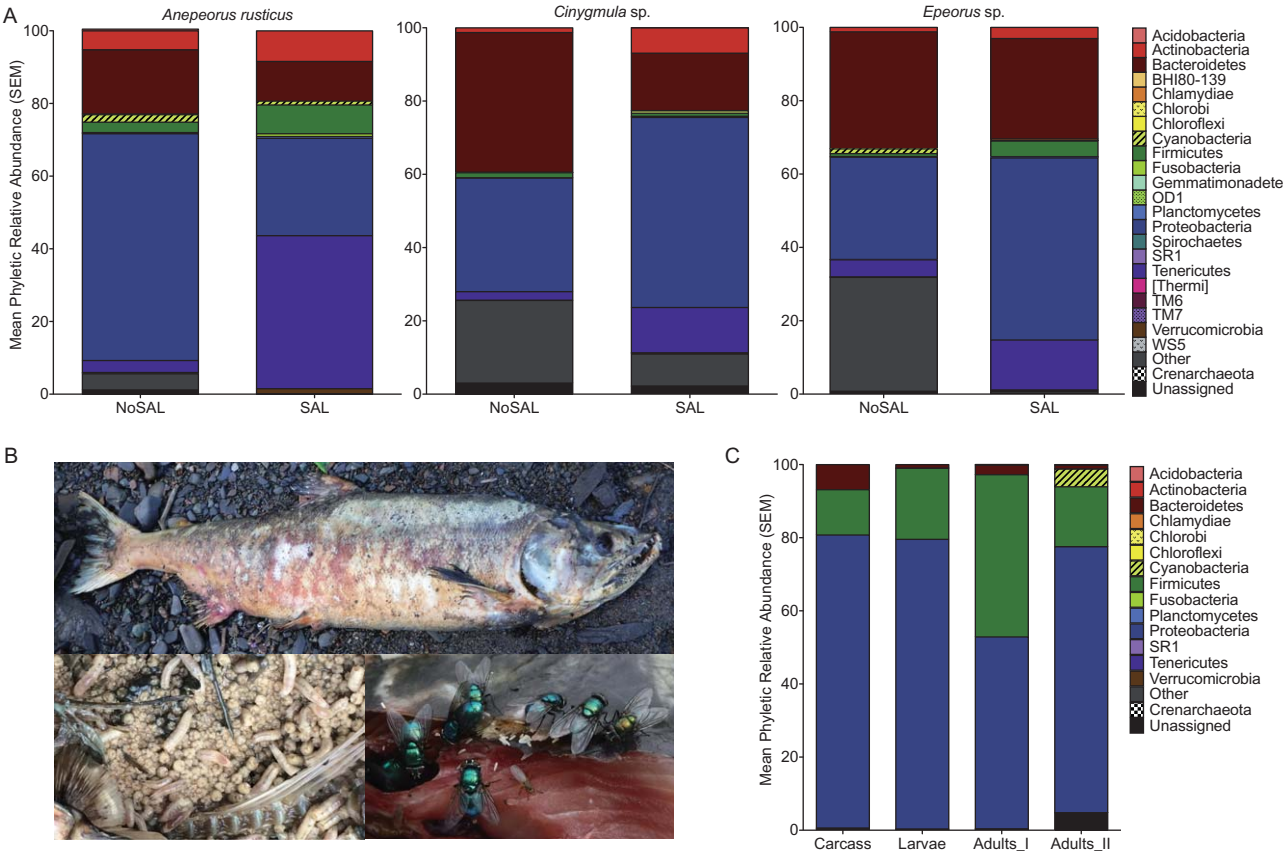

Fig. 1. Microbial community diversity across organisms of the necrobiome.
A. The community structure determined by the mean (±SEM) phyletic relative abundance of mayflies from two functional feeding guilds [*Anepeorus rusticus* (predator) and *Cinygmula* sp. and *Epeorus* sp. (scraper/gather)] that were collected from streams that had naturally occurring salmon carcasses (SAL) or no salmon carcass present in the streams (NoSAL).
B. There were three primary sample types within the carrion network that were analysed for shifts in microbial community structure: the epinecrotic microbial community of naturally occurring decomposing post-spawning salmon carcasses (*Oncorhynchus keta*) – top panel; individual *Calliphora terraenovae* larvae collected from masses occurring on the carcasses – bottom left panel; and individual female *C. terraenovae* adults that use carrion for nutrient acquisition and as an oviposition site – bottom right panel.
C. The community structure of the carrion resource and necrophagous insects during two developmental stages (larvae and adults) as determined by the mean (±SEM) phyletic relative abundance. Community structure differences were further identified between two cohorts of adults that were temporally separated by 10 days between collection events.

species for structuring the internal microbial communities [permutational multivariate analysis of variance (PERMANOVA): $P = 0.770$; Table 1; Fig. S1], but the stream type was nearly a statistically significant effect on the microbial community composition (PERMANOVA: $P = 0.076$; Table 1; Fig. S1). It is possible that the results comparing microbial communities among each taxon are due to low statistical power resulting from the available sample size. Further, bipartite networks were used to visualize the associations between the microbiome OTUs and their respective stream type (Fig. 2). Of the 1726 total OTUs, 32.9% were shared among all mayflies regardless of stream type, whereas communities detected in NoSAL and in SAL streams were composed of 23.6% and 43.5% unique OTUs respectively (Fig. 2). Upon further investigation of microbial community structure of each taxon,

Table 1. Microbial community differences among mayfly nymphs in salmon carcass-bearing and non-salmon carcass-bearing streams.

Factor	d.f.	*SS*	MS	*F*	R^2	*P*
Stream type	1	0.098	0.098	2.061	0.081	0.076
Taxon	2	0.060	0.030	0.632	0.049	0.770
Stream type × taxon	2	0.109	0.054	1.140	0.089	0.301
Residuals	20	0.955	0.047		0.781	
Total	25	1.22			1.000	

This table shows the PERMANOVA results testing microbial community structure based on the weighted phylogenetic distance (UniFrac) matrix for β-diversity among three heptagenid mayfly taxa (taxon) collected in salmon carcass-bearing and non-salmon carcass-bearing streams (stream type) including the interaction with significant results ($P < 0.05$) indicated by an asterisk. SS = sum of squares; MS = mean sum of squares.

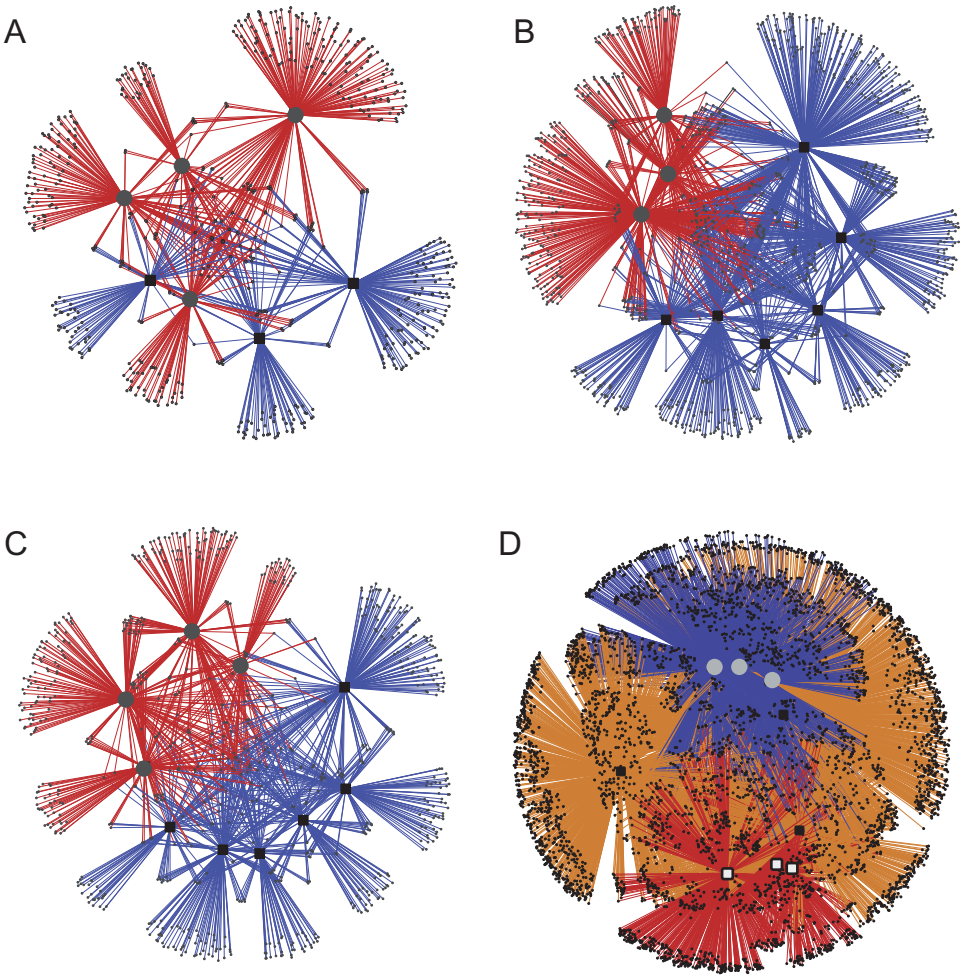

Fig. 2. The relationship of microbial associations among insect (aquatic and terrestrial) communities directly or indirectly influenced by salmon carrion as visualized with bipartite networks. Samples clustered based on their shared OTUs (small grey circles), with those sharing more OTUs being more similar. There were differences in community composition of the three Heptageniidae mayflies [*Anepeorus rustic* (A), *Cinygmula* sp. (B) and *Epeorus* sp. (C)] based on their habitat being salmon carcass-bearing streams (blue vectors, black squares) or non-salmon carcass-bearing (red vectors, grey circles) streams. (D) Internal microbial communities of *Calliphora terraenovae* varied based on stage of development. The larvae (blue vectors; grey circles) were more similar to a second cohort of adults collected 10 days after (red vectors; black squares) an initial cohort of adults (red vectors; white squares) was collected near streams with salmon carcasses.

there was a 48.5% and 23.9% reduction in total OTUs in *A. rusticus* compared with those in *Cinygmula* sp. And in *Epeorus* sp., respectively, which indicates that the predators are accumulating fewer OTUs compared with the mayfly taxa consuming biofilm communities. Additionally, within each taxon, there was distinct grouping based on stream type where individual nymphs sharing more OTUs clustered together depending on carcass presence (Fig. 2). These data provided further evidence that the consumption of available food resources may be an important factor in structuring the internal microbiome of mayflies, but the food resource may be indirectly (e.g. secretions altering biofilm development) or directly (e.g. physical disruption of biofilms; Tiegs *et al.*, 2008) influenced by the presence of salmon carcasses within a stream.

Necrobiome microbial community

There was a total of 811 982 16S rRNA gene amplicon sequences generated from the members of the terrestrial microbial necrobiome: 145 194 sequences were detected from the salmon carcasses (Table S5); 207 009 sequences were from the *C. terraenovae* larvae (Table S5); and 179 220 sequences were from the *C. terraenovae* adults_I (Table S5), whereas the *C. terraenovae* adults_II had 280 559 sequences (Table S5). Three phyla were composed of > 90% relative abundance of the microbial community composition across all individuals: Bacteroidetes, Firmicutes and Proteobacteria (Fig. 1). There were 20 OTUs detected among all sample types (Fig. S3).

The epinecrotic microbial community of salmon carcasses had higher Bacteroidetes-relative abundance in

the *C. terraenovae* larvae (85.7%) when compared with adults (62.5%). The four OTUs detected among all carcasses were: *Sediminibacterium* sp., *Bacteroides* sp., *Dysgonomonas* and *Myroides odoratimimus*. *Sediminibacterium* sp. was also a common OTU detected in the mayflies and has been documented in several freshwater ecosystems (Qu and Yuan, 2008; Kang *et al*., 2014). Therefore, the habitat is likely important for finding this genus among all carcasses because it has also been detected in the gut of trout (Ingerslev *et al*., 2014). Members of *Bacteroides* are well-known pathogens that contaminates important in food safety and have been isolated in cases of salmonoid gill disease (Bowman and Nowak, 2004). *Myroides odoratimimus* has been detected as a pathogen in several fish species including salmonoids (Vancanneyt *et al*., 1996; Loch and Faisal, 2015). *Dysgonomonas* has not only been detected in the gut of rainbow trout (Kim *et al*., 2007) but also has been isolated from Calliphoridae species (Singh *et al*., 2015), which indicates that this OTU may be a naturally occurring member of the microbiota of the carcass or may be introduced by necrophagous insect microbiomes (flies and beetles) frequently associated with carrion decomposition. Furthermore, the most predominant OTUs detected within Firmicutes (Planococcaceae, *Clostridium* and *Erysipelothrix*) and Proteobacteria (Enterobacteriaceae, *Acinetobacter* and Xanthomonadaceae) are all bacteria that have been previously described in association with vertebrate carrion (Metcalf *et al*., 2015) and necrophagous insects (Zheng *et al*., 2013; Kaltenpoth and Steiger, 2014; Singh *et al*., 2015). These results suggest that the microbial communities of the epinecrotic microbiome are either directly from salmon that were infected while alive or through post-mortem dispersal of the microbial communities among carcasses either through spatial cross-contamination among carcasses, or through the transmission of microbiota (e.g. mechanical transmission via tarsi, vomitus or defecation) from necrophagous insects attracted to the carrion resource (Sukontason *et al*., 1999; Talley *et al*., 2009).

Matched *C. terraenovae* larval samples were collected from carcasses to identify shared microbial communities between the larvae and their food resource. There was an increase in relative abundance of Firmicutes (36.6%) compared with the carcass epinecrotic microbiome with the community primarily dominated by *Vagococcus*, *Clostridium* and the candidate family Tissierellaceae. While the relative abundance of Proteobacteria between the epinecrotic and larval samples were equal (*c.* 80%), the composition of predominate OTUs was Enterobacteriaceae, including *Providencia*, and Xanthomonadaceae, including *Ignatzschineria*. Both *Providencia* and *Ignatzschineria* have been detected throughout the life stages of Calliphoridae (Singh *et al*., 2015). To visually assess the relationship of microbial communities between carcasses and larvae, a phylogenetic tree was produced and uploaded into the Interactive Tree of Life (iTOL) (Letunic and Bork, 2007; 2011). This phylogenetic tree reveals differences in the microbial relationships by the grouping of colours based on the sample collection type (i.e. ratio of detection in larvae compared with that in carcass) (Fig. 3A). Further, there were individual clades with distinct microbial communities (phyletic-level taxonomic resolution; Fig. 3B). In some instances, these single clades of microbial communities correspond to a single sample type (larvae or carcass), thus demonstrating distinct phylogenetically relatedness of the microbial communities between the blow fly larvae and the carcasses that they feed upon. There are additional patterns demonstrating larvae with their own unique microbial community not directly related to their diet, which was confirmed with bipartite networks (Fig. S2). Among replicate carcasses, only 22.4–26.2% of the OTUs were shared between the larvae and their diet (carcass) microbiome. These results suggest that the internal microbiome of larval necrophagous insects is at least partially independent of what has been considered their primary food resource (Byrd and Castner, 2001).

As with the larvae, the most predominate phyla among adult *C. terraenovae* were Firmicutes and Proteobacteria (Fig. 1), with the Firmicutes being most represented by Lactobacillales and *Clostridium*, whereas Proteobacteria was primarily represented by Pseudomonadacea. There was, however, a temporal shift in the microbial communities between the cohorts of adults with an increase in Firmicutes (59.3%) in adults_I compared with those in adults_II, which resulted from an increase in Lactobacillales, but the OTU richness within Firmicutes decreased from 940 (adults_II) to 414 (adults_I). Changes in the Firmicutes-relative abundance and composition between cohorts may result from the life history of the females (e.g. gravid status), diet or interactions with other microbiota members in the gut. Further, bipartite networks of the life history stages of *C. terraenovae* showed that of 4697 OTUs detected in the larvae and adults, 29.0% were shared, 5.1% were unique to the larvae and 65.9% were unique to adults. (Fig. 2; Table S5). These data suggest that the larval microbiome may be affected by the lack of heterogeneity in their diet – specifically, the limited dispersal range during the larval stages results in less exposure to various available resources in the surrounding ecosystem, such as flowers, vertebrate carrion and dung – or that the larvae have internal mechanisms, such as antimicrobial peptides found in other Calliphoridae larvae (van der Plas *et al*., 2007; 2010; Čeřovský *et al*., 2010) that are regulating the microbiota composition.

Additionally, there were distinct microbial communities between cohorts of adults (Fig. 2) with 11.2% of OTUs

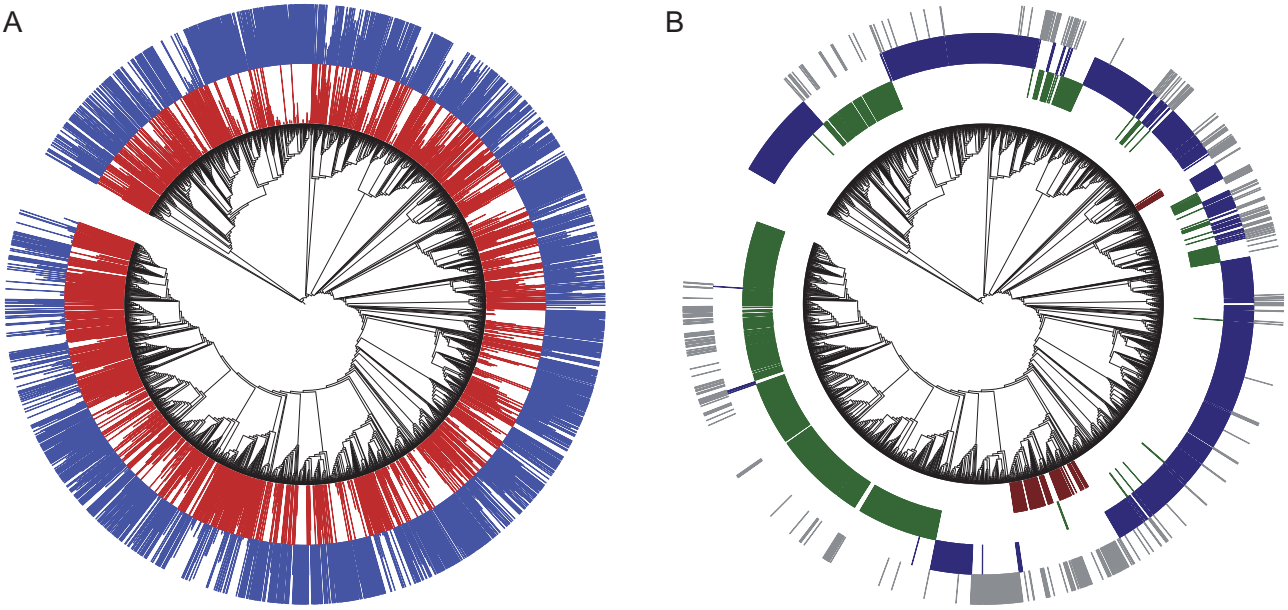

Fig. 3. The phylogenetic relationships of microbial sequences detected in matched carcass and *Calliphora terraenovae* larvae pairs. The phylogenetic tree was generated using QIIME and visualized with iTOL (Letunic and Bork, 2007; 2011).
A. Colours in the outer rings are the mean proportion of abundances for the OTUs in microbial samples from larvae (red) collected on decomposing salmon carcasses (blue).
B. The same phylogenetic tree was used to visualize the microbial community composition with the colours in the outer rings identifying the three predominate phyla detected from innermost to outermost coloured circle: *Bacteroidetes* (dark red), *Firmicutes* (green), *Proteobacteria* (dark blue), remaining phyla (gray).

shared between the adults collected 10 days apart. However, the second cohort of adults were more similar, based on shared OTUs, to the larvae (21.4%) compared with the first cohort of adults (4.5%) (Fig. 2), which indicates that the adult life history, diet or carcass density may have influenced the internal microbiomes of the adults. Carcass density in the study stream increased from the first to second adult collection period due to salmon run timing (J. L. Pechal and M. E. Benbow, unpubl. obs.).

There was not a significant difference in α-diversity (richness, diversity and evenness) among sampling types (carcass, larvae, adults_I, adults_II). There was, however, a pronounced effect of sample type on the microbial community assembly determined by weighted phylogenetic (UniFrac) measures of β-diversity (PERMANOVA: $P = 0.038$; Table 2; Fig. S1). Closely clustered nodes between salmon epinecrotic communities and larvae indicate similar microbial communities shared between the developing *C. terraenovae* larvae and their resource. Additionally, all *C. terraenovae* adults were grouped together with little overlap with larvae or carcass samples providing further evidence of developmental stage partitioning for *C. terraenovae* based on their microbiota.

Conclusions

In this study, we present the microbial community composition of aquatic insects (*Anepeorus rusticus*, *Cinygmula* sp. and *Epeorus* sp.), *Calliphora terraenovae* (larvae and adults) and salmon carcasses that are a part of the salmon necrobiome. These data provide evidence that the microbial ecology of this important ecosystem subsidy is complex with salmon carcass effects on the insect microbiome for both aquatic insects and carrion flies. The salmon necrobiome offers a unique model for testing new hypotheses relevant to ecological networks, community assembly and other aspects of ecological theory. Specifically, we have evidence to suggest that microbial communities can be differentially identified from: (i) aquatic insects collected from salmon carcass-bearing and non-salmon carcass-bearing streams, (ii) the internal community of blow fly larvae from the decomposing salmon

Table 2. Microbial community differences among carrion and Calliphoridae (larvae and adults).

Factor	d.f.	*SS*	MS	*F*	R^2	*P*
Sample type	3	0.300	0.100	3.054	0.534	0.038*
Residuals	8	0.262	0.0328		0.466	
Total	11	0.562			1.000	

This table shows PERMANOVA results testing microbial community structure based on the weighted phylogenetic distance (UniFrac) matrix for β-diversity among the epinecrotic microbial communities from salmon carcasses, *Calliphora terraenovae* larvae and two cohorts of *C. terraenovae* adults collected 10 days apart (sample type) with significant results ($P < 0.05$) indicated by an asterisk. SS = sum of squares; MS = mean sum of squares.

carcass that they develop on and use as food resource, (iii) the internal community of a blow fly larvae from the internal community of a blow fly adult and (iv) differences and similarities between temporally separated adult blow flies. These data are foundational to quantitatively describing subsidy-driven network responses to resource pulses defined by carrion. Ultimately, these carrion networks have direct importance to the decomposition of carcass biomass and indirectly to ecosystem functions, such as riparian plant pollination (Lisi and Schindler, 2011). We are, however, only beginning to understand the role of microbial interactions in the complex and connected marine-derived nutrient systems. Further monitoring and deeper investigations of the microbial communities associated with these insects and their habitat may be necessary to determine the functional role of the microbes during and after salmon runs. Overall, the ability to detect microbial community shifts in the underexplored carrion network and carrion subsidy cross-ecosystem offers insight into how the microbiome of these vertebrate resource pulses are modifying the diet (biofilms) of aquatic insect consumers, and have direct influence on the necrophagous insects and microbial metacommunity dynamics at the aquatic–terrestrial habitat interface.

Experimental procedures

Sample collections and DNA isolation

Aquatic insects were surveyed from streams without salmon carcasses, henceforth referred to as non-salmon carcass-bearing streams (NoSAL), and three salmon carcass-bearing streams (SAL) located near Juneau, AK, in 2013. The streams were a minimum of 7.3 km apart. Sampling consisted of hand collecting aquatic insect nymphs (i.e. instars) from naturally occurring substrate (e.g. stones) in riffle habitats of each stream; all samples were immediately stored in 96–100% molecular grade ethanol for DNA analyses. All aquatic nymphs were identified to the lowest taxonomic level using keys for aquatic insects of North America (Merritt *et al*., 2008). A total of 615 individual aquatic invertebrates from five orders were collected, but we focused on the taxa with the highest abundance among sampling dates and sites (Tables S1 and S2) for characterizing the internal microbiome: *Anepeorus rusticus*, *Cinygmula* sp. and *Epeorus* sp. Using the existing identification keys for Alaska, it was not possible to identify the individual species for *Cinygmula* and *Epeorus*, but all nymphs were morphologically consistent resulting in a single species identification for each genera.

Decomposing post-spawning salmon carcasses (*Oncorhynchus keta*) naturally deposited on the stream bank (gravel bar) were characterized and assessed by dipteran larvae mass activity; those carcasses ($n = 8$) with third instar masses were sampled for matched larvae collections (hand collections of individuals) and epinecrotic microbial communities of the carcass. The epinecrotic microbial communities consisted of microbe communities located on the carcass surface, whether it was the skin/scales or mucous membrane of the opercular cavity (Pechal *et al*., 2014b), and were aseptically collected using sterile cotton swabs. The cotton swabs were immediately stored in 96–100% molecular grade ethanol. Blow fly adults associated with carcasses were surveyed from Sheep Creek, Juneau, AK, on two dates in 2013 collected 10 days apart (Table S2). Adults attracted to carcasses on the stream bank were collected using aerial sweep nets and immediately stored in 96–100% molecular grade ethanol; these carcasses were independent of those carcasses used for larval and epinecrotic microbial community collections, as it was not possible to collect enough adult blow flies from individual carcasses. All Calliphoridae were identified to the lowest taxonomic level using keys for Calliphoridae adults north of Mexico (Whitworth, 2006) and Calliphoridae larvae (Hall, 1948; Kano and Sato, 1951). A total of 264 Calliphoridae adults were collected, and we focused on the most spatially and temporally abundant taxon and sex for characterization of the internal microbiome (Tables S1 and S2).

16S rRNA gene amplicon sequencing and analysis

DNA extraction was performed on individual ephemeropteran nymphs, individual adult female *Calliphora terraenovae*, and three *C. terraenovae* larvae pooled prior to extraction using the Qiagen DNeasy blood & tissue kit (Valencia, CA, USA) with a modified manufacturer's protocol: an addition of 15 mg ml^{-1} of lysozyme was added during the lysis step for each DNA extraction reaction. All insect samples were surface decontaminated before extractions using a 10% hypochlorite wash followed by a triple rinse in sterile water (deionized), as previously described (Ridley *et al*., 2012). DNA quantification was performed using the Quant-iT dsDNA HS Assay kit and a Qubit 2.0 (Grand Island, NY, USA). All DNA preparations were stored at –20°C. Illumina MiSeq 16S library construction (2×250 bp paired-end reads) and sequencing was performed by the Michigan State University Genomics Core Facility using a modified version of the protocol adapted for the Illumina MiSeq, as described by Caporaso and colleagues (2011a). The variable region 4 of the 16S rRNA gene was amplified with region-specific primers, 515F/806R (5′-GTGCCAGCMGCCGCGG-3′, 5′-TACN VGGGTATCTAATCC-3′) according to previously described methods (Claesson *et al*., 2010; Caporaso *et al*., 2011b; 2012). The raw fastq files barcoded Illumina

16S rRNA gene amplicon paired-end reads were assembled, quality-filtered, demultiplexed and analysed using the default settings in QIIME version 1.8.0 (Caporaso *et al*., 2010a). Briefly, the quality-filtering parameters, as suggested by Bokulich and colleagues (2013), were as follows: reads were discarded if they had a quality score of < Q20, contained ambiguous base calls or barcode/primer errors, and/or were reads with < 75% (of total read length) consecutive high-quality base calls. Chimeric reads were also removed using the default settings in QIIME (Haas *et al*., 2011). After quality control, the remaining high-quality sequences were binned into OTUs at a 97% sequence similarity cut-off using UCLUST (Edgar, 2010). The resulting assembled sequence reads were classified into OTUs on the basis of sequence similarity. The highest-quality sequences from each OTU cluster were taxonomically assigned using the RDP classifier (Wang *et al*., 2007) and identified using BLAST against reference sequences from the May 2013 Greengenes 97% reference data set (http://greengenes.secondgenome.com) (DeSantis *et al*., 2006; McDonald *et al*., 2012; Werner *et al*., 2012). The representative sequences of all OTUs were then aligned to the Greengenes reference alignment using PyNAST (Caporaso *et al*., 2010b). We removed singleton OTUs and any remaining low abundance OTUs making up < 0.0005% of reads in the total data set, as recommended for Illumina-generated data (Bokulich *et al*., 2013). Not all samples returned the same number of sequences, and samples with fewer than 1000 sequences were not used in subsequent analyses. Sequence files for all samples used in this study have been deposited in the Sequence Read Archive at the EMBL European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena/): PRJEB9371, PRJEB9779 and PRJEB9780.

Statistical analysis

To examine the associations of microbial communities among samples, estimates of α-diversity were based on OTU matrices and included observed richness, Shannon *H'* diversity and Simpsons evenness as calculated in QIIME. Variation in community composition was visualized using a phylogeny based β-diversity (UniFrac distance) weighted metrics with principal coordinates analysis, and statistically evaluated with PERMANOVA using the adonis function with 999 permutations in the vegan 2.2–1 library in the R statistical package (Anderson, 2001; R Development Core Team, 2010). PERMANOVA was used to test for grouping differences because of its ability to test for interaction effects, adaptation to heterogeneous data and better sensitivity to variation in data sets, as discussed by Anderson and Walsh (2013). All statistical tests performed in this study were considered significant at $P < 0.05$.

Bipartite networks were generated using the sample resource (insects or carcasses) as source nodes and the OTUs as target nodes, with edges (that is, lines connecting nodes) corresponding to positive associations of particular OTUs with the specific sample resource or resource combinations. Bipartite networks were generated using the edge-weighted spring-embedded layout algorithm with edges weighted according to the association strength. Networks were generated using QIIME 1.8.0 (make_otu_network.py) and visualized using default settings in CYTOSCAPE 3.1.1.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Principal coordinates analysis (PCoA) ordinations for patterns of OTUs groupings based on phylogenetic β-diversity (UniFrac) weighted metrics. (A) In mayflies, the taxon was the more important factor for structuring the internal microbial community (*Anepeorus rusticus* – circle, *Cinygmula* sp. – square, *Epeorus* sp. – triangle), while there were trends suggesting the habitat (salmon bearing – solid symbol, non-salmon bearing – shaded symbol) influenced the microbial community structure (Table 1). (B) In the terrestrial system, the microbial communities were significantly grouped based on the sample type: carcass, *Calliphora terraenovae* larvae or adults (Table 2). The epinecrotic microbial communities from salmon carcasses (black triangle), however, were more similar to the *C. terraenovae* larvae (open triangle) developing on their respective carcasses while the temporally separated cohorts of *C. terraenovae* adults grouped similarly to each other (adults_I – open circle, adults_II – open square).
Fig. S2. Bipartite network analysis visualization of blow fly larvae developing on replicate carcasses. Epinecrotic microbial communities were collected from a single decomposing salmon carcass (green vectors) to compare with surface decontaminated *Calliphora terraenovae* larvae (blue vectors) developing on the same carcass. Three separate pairings (A, B, C) were analyzed. Samples (black boxes) cluster based on their shared OTUs (grey circles), with those sharing more OTUs being closer. (A) A total of 704 OTUs were detected after removal of singleton OTUs: there were 171 shared OTUs (24%) between the larvae and carcass, while 159 OTUs (23%) were unique to the carcass, and 373 (53%) were unique to larvae. (B) A total of 2128 OTUs were detected after removal of singleton OTUs: there were 558 shared OTUs (26%) between the larvae and carcass, while 1082 OTUs (51%) were unique to the carcass, and 488 (23%) were unique to larvae. (C) A total of 1058 OTUs were detected after removal of singleton OTUs: there were 237 shared OTUs (22%) between the larvae and carcass, while 456 OTUs (43%) were unique to the carcass, and 365 (34%) were unique to larvae.
Fig. S3. The microbial community structure of the necrobiome determined by mean (±SEM) OTU-relative abundances. The OTUs were selected based on 100% frequency of detection among all salmon carcasses and Calliphoridae (larvae and adults) samples. The taxonomic information provided on the y-axis is the most specific classification for each characterized OTU, which may include class (c_), family (f_), genus (g_) or species (s_) level identifications. *Dysgonomonas* and Moraxellaceae demonstrated an increased relative abundance in adult cohorts (I and II) compared with carcass or larvae (*Calliphora terraenovae*) samples. While the relative abundance of Xanthomonadaceae detected increased in carcass and larvae compared with *C. terraenovae* adults
Table S1. Taxonomic identification and raw counts of aquatic insects and terrestrial flies (Diptera) collected.
Table S2. Five locations (abbreviated name) were sampled near Juneau, AK in 2013; these streams were classified as either salmon carcass (SAL) or non-salmon carcass bearing (NoSAL). Insect sampling for larvae and/or adults occurred over a 10-day period with aquatic and/or terrestrial specimens collected at individual streams; not all insect taxa were collected among all of the streams. Additionally, salmon carcasses were swabbed for their microbial communities at a single location (Sheep Creek).
Table S3. Summary of 16s RNA gene amplicon reads for aquatic insects in salmon carcass-bearing streams (SAL) compared with those in non-salmon carcass-bearing streams (NoSAL).
Table S4. Microbial community abundance of the top OTUs detected in > 80% of mayfly samples with all OTUs being within Bacteria; OTUs detected across all samples ($n = 26$) are indicated with an asterisk at the beginning of the OTU identifier. The change in relative abundance in salmon carcass-bearing streams (SAL) compared with that in non-salmon carcass-bearing streams (NoSAL) was also calculated for each OTU.
Table S5. Summary of 16s RNA gene amplicon reads for salmon carcasses and Calliphoridae (larvae and adults).