Abstract

CRABS & ‘CROBES: THE TRIPARTITE RELATIONSHIP OF A HOST, PARASITE, AND THEIR RESPECTIVE MICROBIOMES

by

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Growing evidence suggests that the associated microbiome of organisms (the holobiont) has been shaping the evolutionary pathways of macroorganisms for thousands of years, and that these tiny symbionts can influence species interactions. Yet, while studies have investigated host-parasites and microbiomes separately, how the two systems interact and influence each other has only begun to be explored. This relationship among hosts, parasites, and microbial communities changes the dynamic of host-parasite evolution from a more traditional co-evolution, to a tripartite evolutionary relationship. My research aimed to resolve questions about the community composition and diversity of microbial symbionts associated with host and parasite separately and when combined (parasitized hosts). My research also developed a methodology that can be used in future studies to manipulate microbiomes, in the pursuit of understanding how the loss or manipulation of the microbiome affects parasitism. Developing the methodology for future microbiome manipulation included determining a method for bacterial inhibition and testing the effects of inhibition on community diversity. For the first part of the research, infected and uninfected crab hosts were sampled from a coastal North Carolina

oyster reef three times over a four-month period. Tissue samples were collected from four biological groups: uninfected crab viscera, infected crab viscera (i.e. host + parasite), the entire adult parasite externa, and parasite larvae. Microbial DNA was extracted from tissue samples and sequenced using the V6-V8 region of the 16S rRNA microbial gene to determine the community composition and diversity of the microbiome for each biological sample and across time. Microbial community analysis revealed that parasite externae and larvae had very similar microbiomes but were significantly different from the microbiomes of the crabs. Microbiomes of infected versus uninfected crabs were also significantly different. Both adult parasite externae and parasite larvae were found to have bacteria including *Pseudoalteromonas* species, which provide natural antimicrobial defenses, while uninfected crabs were mainly comprised of Rhodobacteraceae, commonly associated with photo- and chemoautotrophy. To develop the methodology for the second part of the research, modified Kirby-Bauer disk diffusion technique was used to determine the effects of biological sample, antibiotic type, antibiotic concentration, and time to inhibition of bacteria. Three broad spectrum antibiotics (ampicillin, chloramphenicol, and gentamicin) and their combinations (total of seven antibiotics and combinations) were tested across four concentrations against all four biological groups. Chloramphenicol, at 2mg/mL, was found to be the most effective antibiotic at inhibiting microbial growth across all four biological samples. To determine the effect of chloramphenicol on microbial community composition and diversity, this treatment (or a no treatment control) was applied to live infected and uninfected crabs for 24 hours. After which, microbial DNA was extracted from all four biological samples and will be sent for sequencing, thus the results of bacterial community diversity are pending. Understanding the microbial community composition of a host and parasite, and developing a methodology for manipulating those microbiomes, is an important step to beginning to

understand the microbiome's role in the host-parasite relationship and determining how the tripartite relationship impacts coevolutionary processes.

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Introduction:

The biosphere is filled with millions of species that live in restricted ranges but do not exist in a microcosm. This means that species are interacting with each other constantly, and in a variety of different ways. Interactions can be commonly placed into five groupings (Lidicker 1979), based on how individuals are impacted when they interact. While broad interactions, such as those that occur between predator and prey, tend to be more easily observable, the more cryptic and intimate interactions between host and parasite can be just as important to community interactions (Toft and Karter 1990). These close-knit interactions, referred to as symbioses, occur when two or more species coevolve, and form a longer-term, interactive relationship. Symbioses can take a variety of forms, depending on which organisms are benefiting from the interaction and how dependent an organism is on its symbiont. Once such symbiotic interaction is parasitism, where one organism gains an advantage at the detrimental cost to another (Lidicker 1979, Toft and Karter 1990, Alvarez et al. 1995, Lively 1996, Morran et al. 2011). While the macro-symbiotic relationship between hosts and parasites has been well studied for decades, broader understanding of the microbiome has opened up new interactions between these microbial symbionts and macro-organisms.

The microbiome is the collection of symbiotic bacteria that work in tandem with an organisms' cells to assist in tasks ranging from digestion of material that the host would be otherwise incapable of obtaining nutrients from, to pathogen defense that a host's immune system might be unable to resist (Turnbaugh et al. 2007, Grice and Segre 2011, Koch and Schmid-Hempel 2011, King et al. 2016). In these microbial endosymbiotic relationships, the host

provides protection against biotic and abiotic factors that could disrupt, or even kill, the microbial community, and also provides a stable environment for the microbial population to flourish (Ley et al. 2006, Lau and Lennon 2012, Wong et al. 2015, Dirksen et al. 2016). A stable environment is important to microbial symbionts as they are heavily influenced by changes in temporal, seasonal, and spatial scale (Fierer 2008, Wilhelm et al. 2014, Sun et al. 2017). However despite the relative stability of a host, seasonality can still affect the microbial symbionts of host, due to changes in behaviors or diet in a host, that are associated with changes in seasons (Davenport et al. 2014, Hu et al. 2018). Because of their high variability due to space and time, and to moderate the changes to the microenvironment or to increase the number of hosts, some microbial symbionts directly affect the behavior of their host organism (Ley et al. 2006, Huttenhower 2012, Li et al. 2012).

While extensive literature exists on the community composition of the human microbiome, and several other vertebrate and invertebrate species, the evolutionary implications of hosts and their microbiomes has only recently been explored (Turnbaugh et al. 2007, Grice and Segre 2011, Huttenhower 2012, Li et al. 2012). With the explosion of research into microbial ecology and symbioses, there is more compelling evidence that the interdependence of microbes and their hosts cannot be ignored, and that the interactions these groups share may have far ranging effects on the evolution of a species (McFall-Ngai et al. 2013, Barbearán et al. 2014, Colston and Jackson 2016, Kopac and Klassen 2016, Webster 2017). One of the most compelling cases for microbial influence on hosts is the modification of host behavior to suit a situation that allows the spread of microbes. If populations of host organisms have slightly different microbiomes that modulate a critical behavior, such as mating, variations can lead to hosts being unable to identify each other as the same species, and over the evolutionary course of

hundreds of generations, these groups can differentiate into new species (Vavre and Kremer 2014, Venu et al. 2014, Archie and Tung 2015, Wong 2015). In conjunction with the behavior modification, microbes may also act as a sort of 'evolutionary toolbox' that a host species might use to adapt to novel abiotic or biotic conditions (Montllor et al. 2002, Colston and Jackson 2016). This toolbox may not be something that is consciously used by a host, but rather if a host encounters a stressor, such as a parasite, and it has a microbial symbiont that provides a greater resistance to parasitism, the host and microbe might coevolve to mitigate the stressor and increase fitness (Kim et al. 2012). Microbial symbionts have been shown to have significant effects in populations of *Caenorhabditis elegans*, and their ability to survive diseases. *Staphylococcus aureus* is a pathogenic bacteria that lyses the gastric cell lining in the nematode *C. elegans*, causing 100% mortality. However, when worms were exposed to a less virulent pathogen, *Enterococcus faecalis*, the mortality dropped to <1%, indicating that microbial communities might potentially reduce the virulence of a pathogen when working in concert with a host's natural defenses to increase their own fitness (Ford et al. 2016, King et al. 2016).

With microbes influencing these kinds of processes, it is likely that microbes could play a role in more complex interactions between macroorganisms, such as the coevolutionary arms race between parasites and their hosts. The host-parasite coevolutionary race is when a host constantly evolves traits and defenses to reduce its chances of being parasitized, while at the same time the parasite is evolving to counteract those adaptations (Lively 1996, Morran et al. 2011). If parasites behave in a similar manner to some pathogens, then hosts with weakened microbiomes may become easier potential targets for parasitic infection as there are fewer challenges to overcome, similar to an immunocompromised organism being more susceptible to a pathogen (Ford et al. 2016, King et al. 2016). This changes the coevolutionary arms race

between parasites and hosts from its more traditional two part struggle into a tripartite relationship, with the host, parasite, and the microbiota all evolving in concert to improve their own fitness (Currie et al. 2003, Little et al. 2005, Koch and Schmid-Hempel 2011).

With a tripartite relationship, the same "Red Queen" relationship exists (Van Valen 1973), where organisms are constantly evolving at the same rate as their parasite. In this scenario, it is occurring among the host, the parasite, and each of their respective microbiomes (Toft and Karter 1990, Lively 1996, Morran et al. 2011). For the hosts and parasites, this remains relatively unchanged, with hosts continuing to evolve defenses against parasites, and parasites evolving new adaptations to facilitate infection. But the microbial community is evolving too and at a faster rate than their eukaryotic host, with a host's microbiome changing to provide a competitive edge to the host, so that both host and microbial symbiont can continue their partnership (Toft and Karter 1990, King et al. 2016, Dheilly et al. 2017). This can result in systems where hosts become dependent on their microbial symbionts to perform functions, rather than evolving their own responses to a stressor. For example, in leafcutter ants, the combination of sterile working techniques employed by the ants, paired with a microbial antibiotic defense against the fungal parasite *Escovopsis spp.*, produces a potent combination of ant behavior and microbial secretions that have helped the ant colonies cultivate fungi for millions of years (Currie et al. 2003, Little et al. 2005). Though it is hard to pinpoint exactly what behaviors are induced by microbial communities and which are natural defenses from the host itself, it is clear that the tripartite relationship among microbes, hosts, and parasites is not just feasible, but likely (Currie et al. 2003, Little 2005, Koch and Schmid-Hempel 2011, Clay 2014, Vavre and Kremer 2014, Dheilly et al. 2015, Dheilly et al. 2017, Zeng 2017). This seems more possible when considering that in experiments where microbial communities have been disrupted, the occurrence of

parasitism is at higher levels than in populations where the microbial community is left intact (Dong et al. 2009, Dheilly et al. 2015, Dheilly et al. 2017).A study investigating mosquito (*Anopheles gambiae*) reliance on the microbiome to defend against parasitic malaria (*Plasmodium falciparum*) was detrimentally impacted by the removal of the microbiome (Dong et al. 2009). Mosquitos were found to possess genes that were used to inhibit parasitism, however their effectiveness at inhibiting the malaria parasite was dependent on the presence of their natural microbiome (Dong et al. 2009). This highlights the fact that within the host-parasite tripartite relationship both the host and the hosts' microbiome are developing in concert with each other to defend against a parasite, however the lack of gene activation with the loss of the microbiome also shows the interdependence of the host on microbial symbionts within this tripartite relationship.

In this study, I investigated this tripartite relationship among host, parasite, and their respective microbiomes in a Western Atlantic system, where a native host crab, *Eurypanopeus depressus* (flatback mud crab), is infected by an invasive parasite *Loxothylacus panopaei* (Van Engel et al. 1966, Hines et al. 1997, Kruse and Hare 2007). The host crab, *E. depressus*, is a common mud crab species found primarily in mid to higher salinity estuarine waters in structured environments like oyster reefs (Williams 1984).

The parasite, *L. panopaei*, is a member of the derived superorder Rhizocephala, a group of parasitic barnacles that specialize in the infection of decapod hosts (Van Engel et al. 1966, Walker et al. 1992, Walker 2001). *Loxothylacus panopaei* starts its life cycle when an externa releases a brood of nauplii, which are tiny free swimming larvae (Fig. 1). Because the larvae have a limited amount of reserved resources, they quickly molt into their next stage, the cyprid larvae, which begin to actively seek out a host using chemical cues (Walker et al. 1992, Alvarez

et al. 1995, Høeg 1995, Pasternak et al. 2005). Once a prospective host has been identified, the female cyprid attaches to the gills or body of a molted crab and undergoes another metamorphic molt, becoming a kentrogon, which quickly penetrates the host with a specialized stylet (Høeg 1995, Walker 2001, Glenner et al. 2001). After penetration, the female injects a vermigon larvae into the hemolymph of the host, which the parasite uses to transport to the hepatopancreas where the female barnacle will mature and spend the rest of her life (Høeg 1995, Walker 2001, Glenner et al. 2001). Once the vermigon is situated near the digestive organs of the host, it begins to form a root system that burrows into the soft tissues of the crab and extracts nutrients from the host (Høeg 1995, Walker 2001, Glenner et al. 2001). The barnacle grows over a 20-25 day period into a mature interna, which consists of little more than a small conglomeration of parasite cells attached to the host via the network of rootlets (Høeg 1995, Walker 2001, Glenner et al. 2001). Water is pulsed over the interna to supply oxygen, but otherwise the parasite remains immobile for the rest of its life. Once the interna has matured, the parasite begins to develop a virgin externa which, once fully developed, can then be fertilized by a male to begin the process over (Høeg 1995, Walker 2001, Glenner et al. 2001). In male crabs, the parasitic infection results in changes in pleon shape and modifies crab behavior so that they will protect and brood the parasitic externa, instead of trying to remove it (Walket et al. 1992, Høeg 1995, Walker 2001). *Loxothylacus panopaei* is a warm water parasite, naturally occurring throughout the Gulf of Mexico all the way to Florida (Van Engel et al. 1966, Hines et al. 1997), but with the assistance of anthropogenic forces, it has been able to expand its range up a large portion of the Eastern Seaboard. In the native coevolved range of the parasite, crab infection rate is relatively low, ~10% of the population, or so-to-speak a "crab winning" population (Tepolt et al. 2019). However, the invaded range sees a much higher infection rate, 80-90% but sometimes as high as

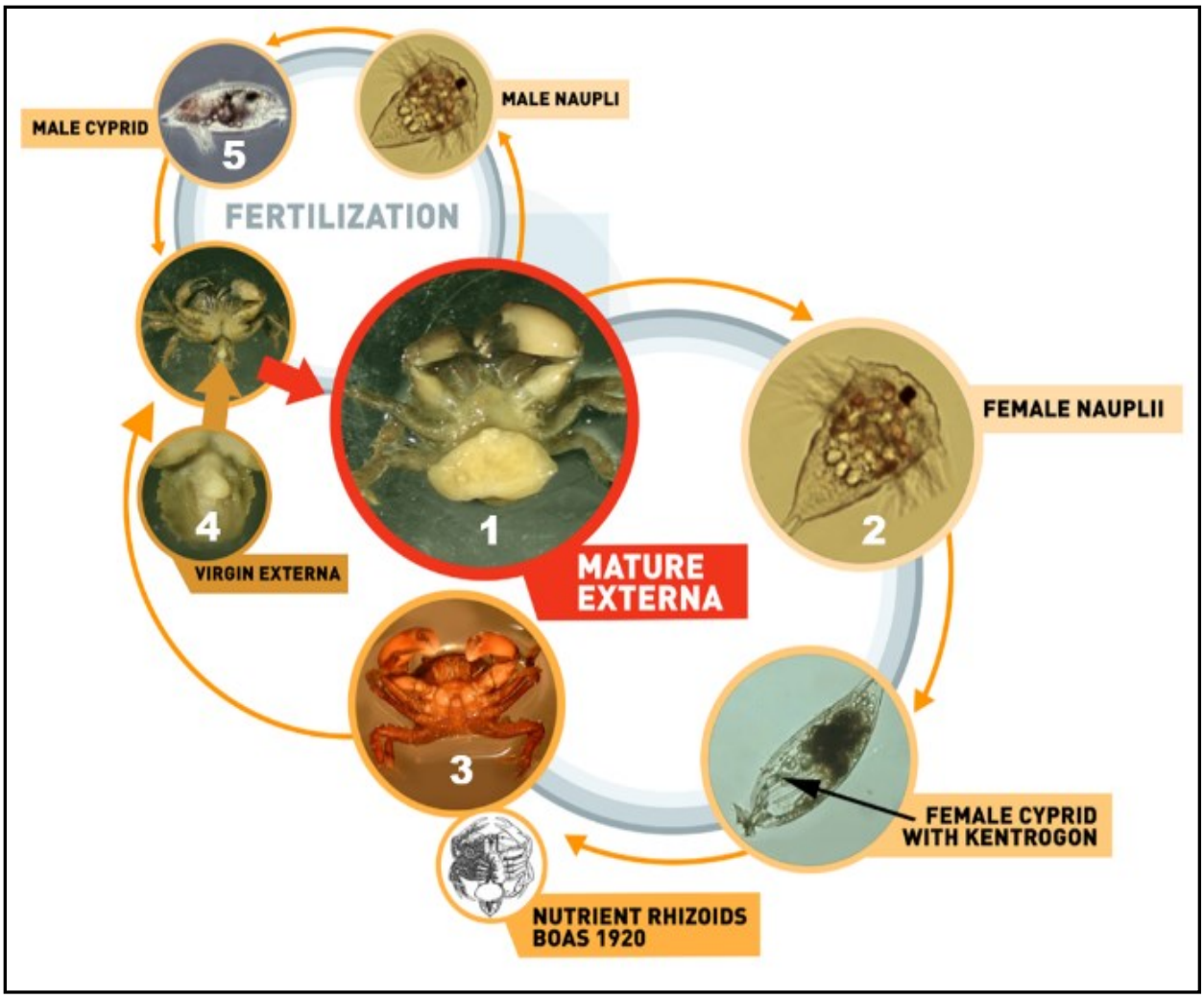

Figure 1: Infection cycle of *Loxothylacus panopaei* with its crab host. (1) In an infected crab, a mature (fertilized) externa of the parasite releases (2) male and female larvae. (3) The latter infects a new crab, extending a series of nutrient rhizoids throughout its body and eventually produces (4) a virgin externa, an unfertilized sac on the crab's abdomen. (5) Once fertilized by the male, (1) the externa becomes mature, beginning the cycle anew **[Diagram: R. Houghton, A. Blakeslee, A. Fowler]**

100% in some samples, indicating a "crab losing" scenario (Van Engel et al. 1966, Hines et al. 1997, Tepolt et al. 2019). Parasites are prevalent in these crab losing regions likely because of their hosts' naiveté (Tepolt et al. 2019). *Loxothylacus panopaei* has coevolved with crabs throughout its natural range in the gulf coast, and both it and its microbial community have had to adapt to continue infecting a host constantly evolving to reduce parasitism. Since the naïve hosts have never had to coevolve, they lack any of the natural defenses that conspecifics have gained over thousands of years of evolution, which is likely why infection is more prevalent in the invaded range (Tepolt et al. 2019).

My study focused on identifying microbial community composition and diversity of microbes in uninfected crabs, infected crabs, and the rhizocephalan parasite (externa of adult parasite and parasite larvae). Uninfected crabs and parasite larvae were expected to have significant differences in microbial community composition. This is because the parasite larvae are free-living and unassociated with the crab host (Fig. 1) at the larval stage, thus I expected that microbial community composition and diversity would be distinct between these two biological samples. Parasite larvae and parasite externae were expected to have the most similar microbiomes. This is because the externae is a parasite structure, so it was expected to contain mostly parasite microbial communities. Infected crabs were expected to differ from both uninfected crabs and free-living parasites. This was expected because of the widely distributed rootlet system of the parasite throughout the host, which should contain parasite microbial symbionts, and the tissues of the crab which contain crab microbial symbionts. The result of these mixed microbial communities was hypothesized to result in unique microbiomes in infected crabs. My study further addresses the potential effect of season on community composition and diversity. Because microbial communities are impacted by seasonal changes, I

hypothesized that microbial communities would change over time (from summer to fall). To test these hypotheses, crabs and parasites were collected three times over a four-month period, and microbial communities were sequenced and analyzed to determine the community composition and diversity of the crab and barnacle microbiomes, and how these communities changed over time.

Methodology:

Crab Collection:

Prior to the start of the experiment, passive crab traps were deployed at a restored oyster reef near Bogue Sound, NC (Fig. 2) which is inhabited by both infected and uninfected *E. depressus* mud crabs. Passive crab traps ("crab condos") consisted of a 19 x 22 x 16 cm milk crate filled with 3.5lbs of autoclaved oyster shell, and covered in mesh (Fig. 3). These traps were staked into place just outside an oyster reef where infected *E. depressus* had been observed before (Walker 1992). Passive traps were left for several weeks before the first sampling trip, to allow crabs to use them as an artificial refuge, and to make crab collection easier (Roche and Torchin 2007, Fowler et al. 2013, Tepolt et al. 2019). In cases where passively collected crab numbers were low, hand collection from the oyster reef was used to supplement collections. To reduce the chance of contaminating the microbiome of crabs, gloves were worn by collectors, and when sorted gloves were wiped with 70% ethanol between handling individuals. Crabs were visually inspected *in situ* to determine infection status (presence of a mature externa) (Fig. 1). Thirty crabs, 15 each of infected and uninfected, were collected. Five additional crabs were to account for crabs that might have died during transportation. An additional 15 infected crabs

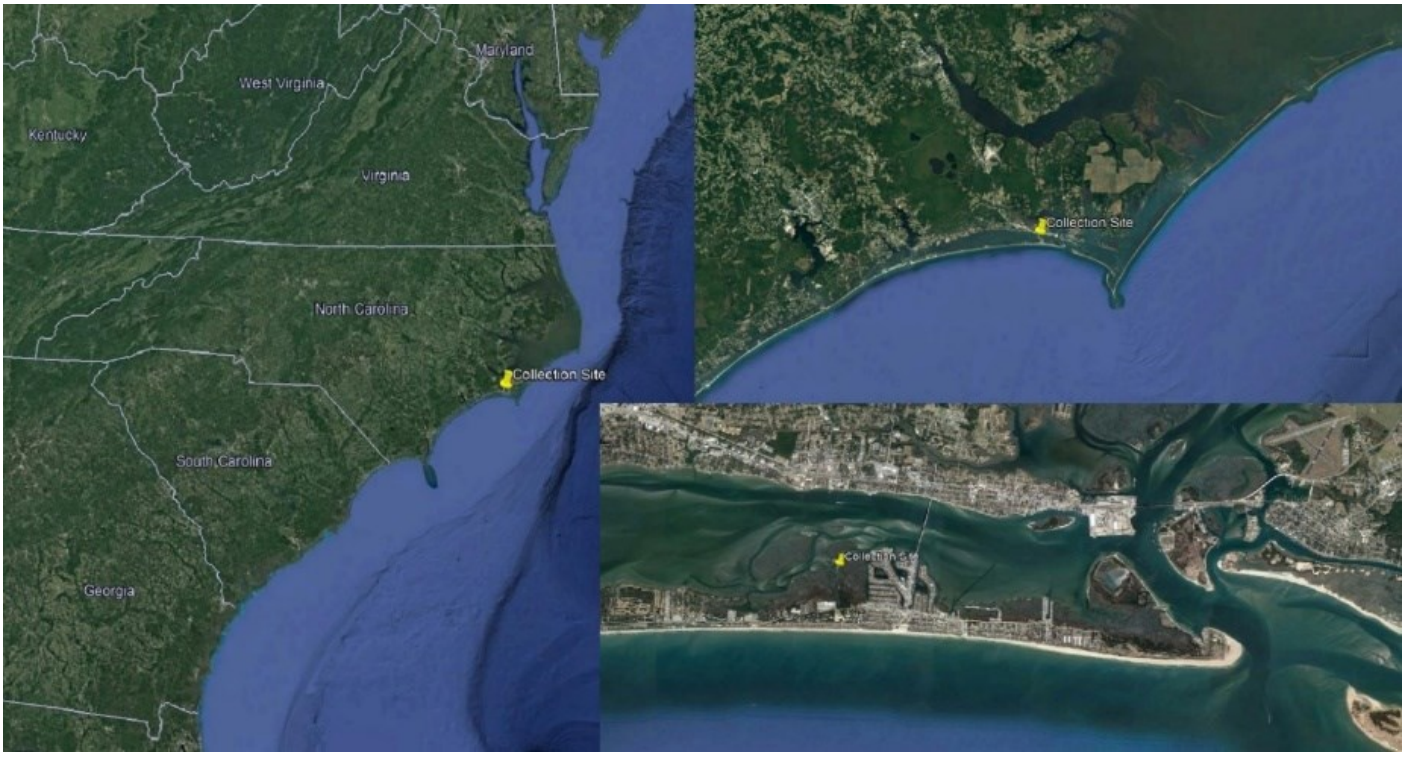

Figure 2: Location of crab collection site at Hoop Pole Creek, NC (34°42’29.12”N, 76°45’6.84”W) **[Figure made with Google Earth Pro]**

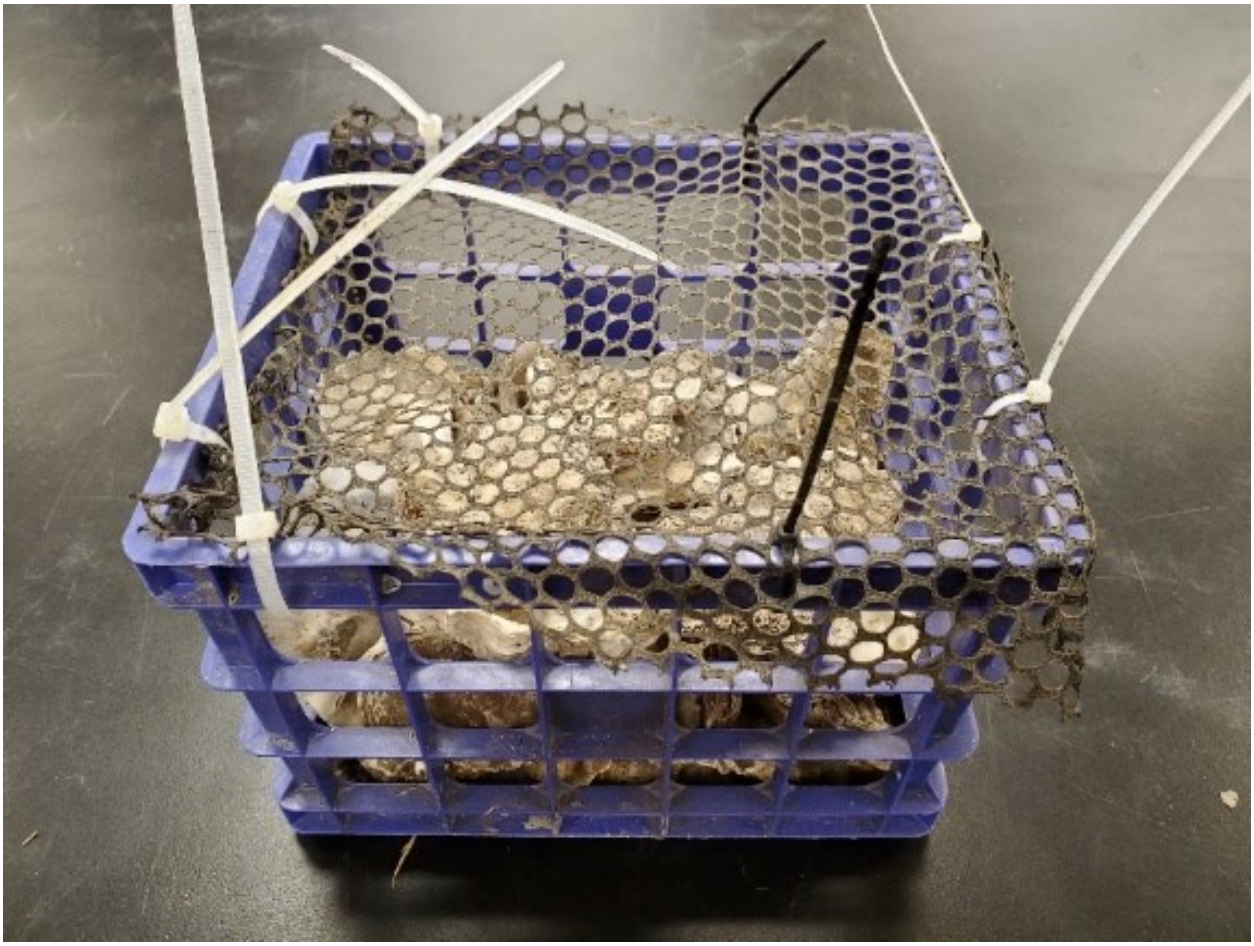

Figure 3: Passive collector “crab condos” that were deployed at the collection site **[Photo taken by KL Swanson]**.

were collected to act as lab brooders so samples of parasite larvae could be collected for microbial analysis. All crabs were rinsed with sterilized sea water, reducing the possibility of transportation of foreign microbial communities from nearby sediment and oyster shells. Once crabs were washed, they were placed into individual compartments of an 18-compartment 26 x 15 x 4 cm plastic jewelry case to prevent any possible cross microbial contamination among individuals. Crabs were transported back to the ECU campus in coolers and all experimental crabs, with the exception of the predetermined brooders, were rinsed with sterilized sea water and then immediately frozen. Crabs were dissected within 12hrs of being collected. Brooder crabs were stored in a new sterilized jewelry case once returned to the lab, with 30mL of sterilized 20‰ water. To reduce the chance of microbial contamination of larvae samples water was changed daily in brooding crabs and wells were checked daily for any signs of larval broods. Collections were repeated three times (20th July, 14th September, 26th October 2019) to test the seasonal effects on the microbiome, and 45 crabs (15 infected and uninfected, and an additional 15 designated lab brooders) were collected at each time point.

Crab Dissections:

Dissections were performed such that all the soft tissues inside the crab, including gonads, hepatopancreas, heart, and gills, could be extracted. Because of the distributed nature of the adult barnacle rootlets when it is parasitizing a crab, the best method of extracting as much usable microbial DNA as possible, based on unpublished pilot studies that I performed, was to remove all visceral materials. During dissection, uninfected crab viscera were visually examined to ensure that no parasite rootlets were present, as the externae in parasitized crabs can be removed with the parasite still alive, making infected crabs appear uninfected from external

inspection. Because only visual examination was used, it is possible that may have been in early stages of infection and counted as uninfected. Dissections were performed using a sterilized razor blade to cut the crab's carapace between the telson and the top of the carapace, and sterilized fine nose tweezers were used to separate the upper and lower carapace. Next, a different pair of sterilized tweezers was used to collect the visceral mass of the crab from within the upper and lower carapace. All tools and work stations were sterilized with 70% ethanol solution between dissections. All visceral mass per crab was collected into 1.5mL Eppendorf tubes and mixed with 250µL of PBS (phosphate buffer solution). Infected crabs also had their externa(e) removed using a third set of sterilized tweezers, which was deposited in a separate Eppendorf tube and also submerged in 250µL of PBS, resulting in two samples from each infected crab, one viscera and one externa, and one viscera sample from each uninfected crab. For parasite larvae, brooder crabs were observed daily. For any crabs that released a brood of nauplii larvae, multiple live larvae (>100 individuals) were collected via positive phototaxis using a 250µL pipette. Five samples were collected from every brood, then all samples from the same crab were centrifuged to pellet the larvae. This pellet, in addition to 50µL of the water, were added together to produce one sample with a higher concentration of larvae to increase the amount of microbial DNA.

A total of 98 samples were collected over a four-month period from three distinct time periods. Each time period had 10 infected viscera, 10 externae, and 10 uninfected viscera, and additionally September had 6 larvae samples, and October had 2 larvae samples (Table 1). Larval sampling was highly variable as there was no way of inducing the release of larvae, so samples could only be collected if females were brooding and releasing larvae. Since females generally

Table 1: Number of usable samples in each sampling trip and biological group. Ten of each group besides larvae were sampled, but for some samples, there was not enough DNA amplification for microbial community analysis. Sample size refers to the number of samples that had enough DNA for amplicon sequencing. Sampling occurred on 20th July, 14th September, 26th October 2019.

Sample Group	**Sample Size**	**Air Temp (℃)**	**Water Temp (℃)**	**Salinity (ppt)**
July Externae	10	36	32	15
July Infected Viscera	9			
July Uninfected Viscera	7			
September Larva	6	31	28	15
September Externae	10			
September Infected Viscera	7			
September Uninfected Viscera	6			
October Externae	10	26	22	16
October Infected Viscera	10			
October Larva	2			
October Uninfected Viscera	5			

release larvae every 5-6 days (Walker 2001), larvae were only collected within the first week of being captured, to retain as much of the natural microbiome as possible.

Microbial Extraction:

Samples were subjected to a rigorous DNA extraction process, designed to separate as much microbial DNA as possible from the crab and parasite tissues. Samples were first placed in a sonication water bath for 10 minutes. Samples were then sonicated at 35 Khz to lyse the host eukaryotic cells and release more bacterial cells to increase the potentially accessible microbial DNA from each sample. After sonication, samples were extracted using the Qiagen DNeasy PowerSoil Pro kit (Hilden, Germany). Initially, samples were extracted with the DNeasy PowerSoil kit; however, quantification numbers obtained from the amplified samples were insufficient to be sequenced. The inclusion of Qiagen's inhibitor removal technology seemed to be critical to obtaining samples that could be sequenced, and so the PowerSoil Pro kit was chosen for its inclusion of this technology. Although designed for extracting microbial DNA from soil samples, the PowerSoil kit was also ideal for separating microbially symbionts from the tissues of the crabs and parasite larvae. Once microbial DNA had been extracted, samples were amplified using the V6-V8 region of the 16S rRNA gene. Although the V4 region of the gene is commonly used to amplify and identify microbial DNA, the V4 region in bacterial DNA has a corresponding region in the Eukaryotic 18S rRNA gene, and thus a section of the gene that did not have a corresponding Eukaryotic region had to be selected. DNA extracts from positive amplified samples that also contained more than 2ng/µL of DNA were shipped to Dalhousie University for bacterial V6-V8 amplicon sequencing.

Statistical Analyses:

Microbial sequences were analyzed using open source Mothur v1.44.1 and a published pipeline (Kozich et al. 2013). These analyses produced taxonomic level data from phylum to species of microbial associates for each of the biological sample types. To examine community richness across broader and more specific taxonomic classifications, phyla, class, and genus level community richness data were included as response variables in statistical analyses. Normality was assessed via community richness, using the lm function from base RStudio v1.2.5033, and goodness-of-fit tests did not show significant deviation from normality. Biological group, time, temperature, and salinity were all included as fixed effects in initial linear models. In preliminary runs, neither salinity nor temperature had a significant effect on taxonomic diversity and were removed from subsequent models. Sampling time, biological sample, and the interaction of time and sample were therefore included in linear models at the phyla, class, and genus levels to determine their effects on community richness.

Visualizations of sampling time, biological sample, and the interaction of time and biological samples were performed using non-metric multi-dimensional scaling models (nMDS) at the three taxa levels on bacterial diversity. nMDS models were run using the vegan v2.5-4 package from taxa diversity data obtained from Mothur, and models were plotted in ggplot v3.3.1.

Taxa level plots compared the relative abundance of bacterial communities of each taxonomic group in samples, thereby indicating the bacterial community diversity of samples. To create taxa level plots of microbial community diversity, taxfiles and sharedfiles generated in Mothur were imported to RStudio. Phyloseq v1.16.2 and dplyr v0.7.8 were used to import and interpret data (Haverkamp 2018,

https://norwegianveterinaryinstitute.github.io/BioinfTraining/phyloseq_tutorial.html). Phylum level plots were sorted by taxonomic diversity with a >2% cutoff to filter groups that had low community representation, and all phyla that were below the 2% community representation of the total 16S rRNA gene sequences were pooled as 'Other' in order to reach a sum of 100% relative abundance. This was performed for each sample. When possible, genera level plots used a >5% cutoff for community diversity; however, some samples had too high a diversity making visualization difficult. For these groups, a cutoff of >7.5% or 9% was used to filter out the numerous less abundant taxa, and to make community analysis visualization clearer. As with phyla level taxonomic plots, groups of bacteria that were below the 5%, 7.5%, or 9% taxonomic diversity cutoff were combined into an 'Other' category so that relative abundance summed to 100% relative abundance.

Results and Discussion:

Influence of the Interaction of Biological Sample and Time on Microbial Community Richness and Composition:

Interactions between time and biological sample type were found to significantly influence microbial community richness for all three taxonomic groupings (phyla $t=6.528$, class $t=6.309$, genera $t=7.563$, $p<0.0001$) (Fig. 4) suggesting that microbial communities associated with the different biological samples are influenced by seasonal changes (e.g. time). The biological group that experienced the greatest change in community richness, at all three taxonomic levels, due to seasonality was the uninfected crab viscera (Fig. 4, Table 2). At the genera level, uninfected crabs had significantly lower taxa richness than parasite externae

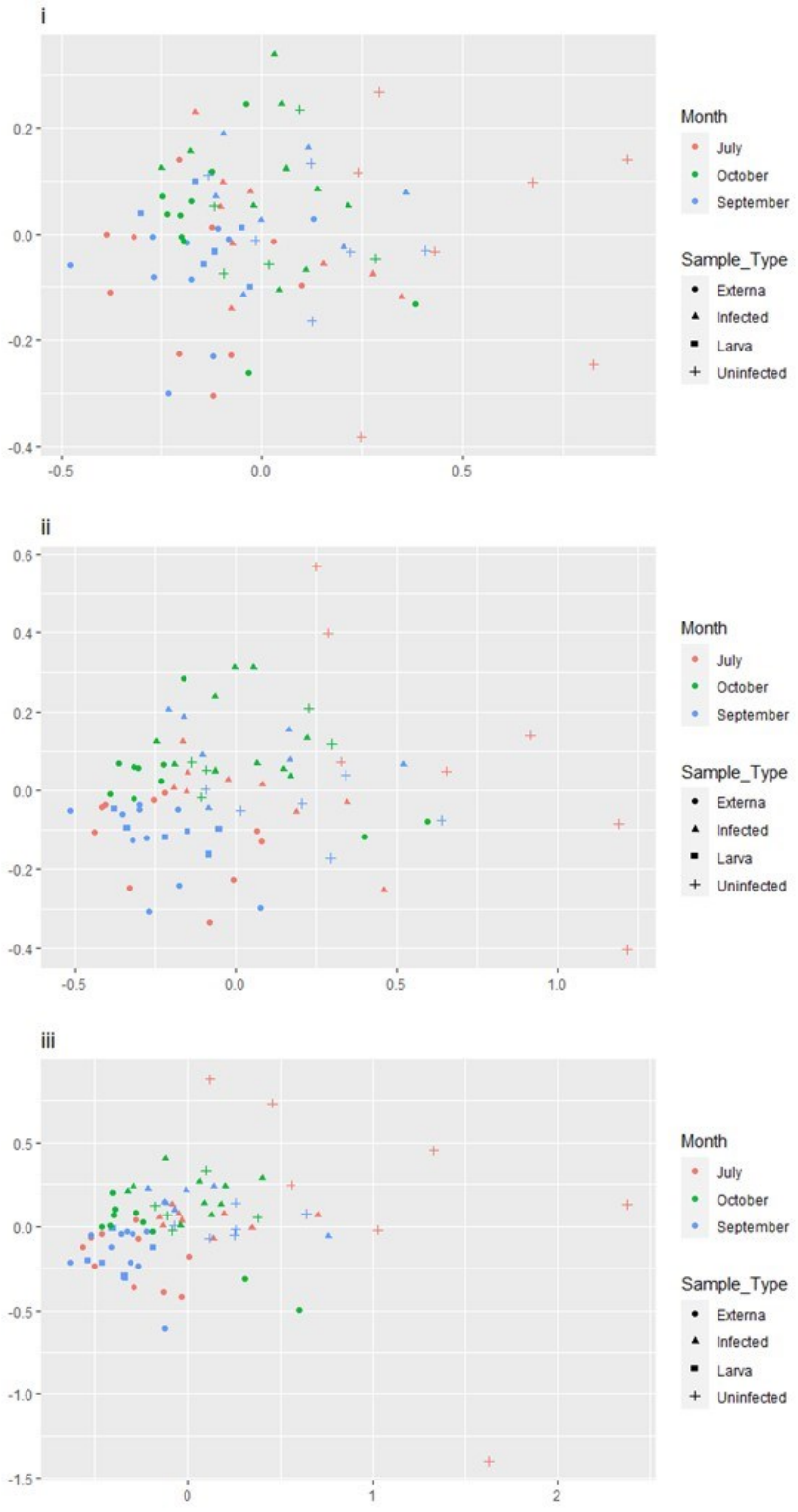

Figure 4 i) phylum level, ii) class level, iii) genus level. NMDS plots of microbial community diversity of all samples comparing the interaction of biological sample and sampling time at. Colors signify month and shapes signify biological groups.

le of statistical analysis of taxa richness, comparing interactions of sampling time and biological

Taxa richness was compared at three taxonomic levels (phylum, class, genus) for all samples.

	Phylum			Class			Genus		
	estimate	t value	p value	estimate	t value	p value	estimate	t value	p value
(Intercept)	26.6	22.087	<0.001	12.5	15.232	<0.001	371	13.988	<0.001
October	-0.5	-0.294	0.7699	3.2	0.551	0.5831	15	0.4	0.6904
September	0.7	0.411	0.6823	3.6	0.62	0.537	11.8	0.315	0.7540
Infected Viscera	-3.4889	-1.994	0.05	-11.278	-1.724	0.0891	-97.333	-2.526	0.0138
Larvae	-1.3	-0.661	0.5107	-4.267	-0.0637	0.5289	12.7	0.293	0.7702
Uninfected Viscera	-11.1714	-5.952	<0.001	-34.5	-5.395	<0.001	-240.714	-5.824	<0.001
October:Infected Viscera	0.7889	0.323	0.7476	-3.222	-0.387	0.6997	-0.2667	-0.005	0.996
September:Infected Viscera	-1.6683	-0.65	0.5177	-4.822	-0.552	0.583	-0.6095	-0.011	0.9914
October:Uninfected Viscera	8.6714	3.09	0.0028	21	2.197	0.0313	160.7143	2.601	0.0113
September:Uninfected Viscera	4.5387	1.669	0.0994	10.067	1.087	0.2808	81.2476	1.357	0.179

samples in July samples (t=-5.824, p<0.001); however, in September and October sampling taxonomic the genera richness increased, bringing it closer to the parasite externae samples (t=1.357, p=0.179; t=2.601, p=0.0113 respectively). A similar trend was observed in infected crab viscera, with infected crab genera being significantly different in July samples (t=-2.526, p=0.0138); and greater genera diversity in the September and October sampling periods (t=-0.011, p=0.9914; t=-0.005, p=0.996 respectively). Externae remained consistent among the three sampling periods with only slight fluctuations in genera taxa richness in September and October samples (t=0.315, 0.754; t=0.4, p=0.6904). The higher seasonal variation in uninfected crabs, and the less extreme changes in infected crabs suggests that uninfected crab microbiomes may be more impacted by seasonal changes, while the parasite seems to have a stabilizing effect on the taxa richness in parasitized crabs (Table 2). Because only two time periods were sampled (summer and fall), it is unknown if this trend would continue to fluctuate throughout the year.

Influence of Time on Microbial Community Richness and Composition:

Sampling time did not have significant impacts on taxa richness at the phylum and class taxonomic levels (F=1.526, p=0.2237; F=1.77, p=0.177, respectively), but showed near significance at the genus level (F=2.532, p=0.0859). Taxa richness at the genus level increased from the summer July sampling to the fall September sampling, which resulted in a near significant shift in community richness (t=1.925, p=0.0578). The later October samples were slightly more diverse than the earlier September samples resulting in a significant increase of taxa richness between the July and October sampling times (t=1.994, p=0.0496). Because larvae were only sampled in September and October time periods (Table 1), it may be that parasite larvae were driving some of the increased taxa richness in fall samples. To compensate for this, a

second analysis at the genus level was performed with larvae removed, to determine if time had significant impacts on community richness in the uninfected crabs, infected crabs, and parasite externae. Overall the, model with larvae removed was still not significant, even at the genus level ($F=1.904$, $p=0.1565$). Between months, there was no significant change in genus taxa richness between July and September ($t=1.247$, $p=0.2165$), and there was a near significance between July and October sampling periods at the genus level ($t=1.915$, $p=0.0595$). Comparing the analysis with larvae and the analysis without larvae, the same trend appears, with taxa richness increasing throughout the end of summer and into the fall sampling periods. October had the greatest community richness at the genus level, with September as an intermediary, suggesting that taxa richness increases from the summer months towards the fall (Table 2). A similar trend was observed in genus level nMDS plots, in which July community diversity was visually disparate from September and October sampling periods, which were clustered together more tightly (Fig. 5). These results were contrary to other work in deer and humans that showed that an organisms' microbial richness decreases in the colder months (Davenport et al. 2014, Hu et al. 2018). It could be that crabs and parasites are more seasonally impacted than deer and humans, possibly due to the lack of homeothermy, and thus their microbiomes mirror the environmental bacterial community shifts more closely. Although time was only near significant, the observable changes in taxa richness and diversity, combined with literature, suggest that a longer study time with more sample points would show time and seasonality to be a significant driver of bacterial richness and diversity (Ghiglione and Murray 2011).

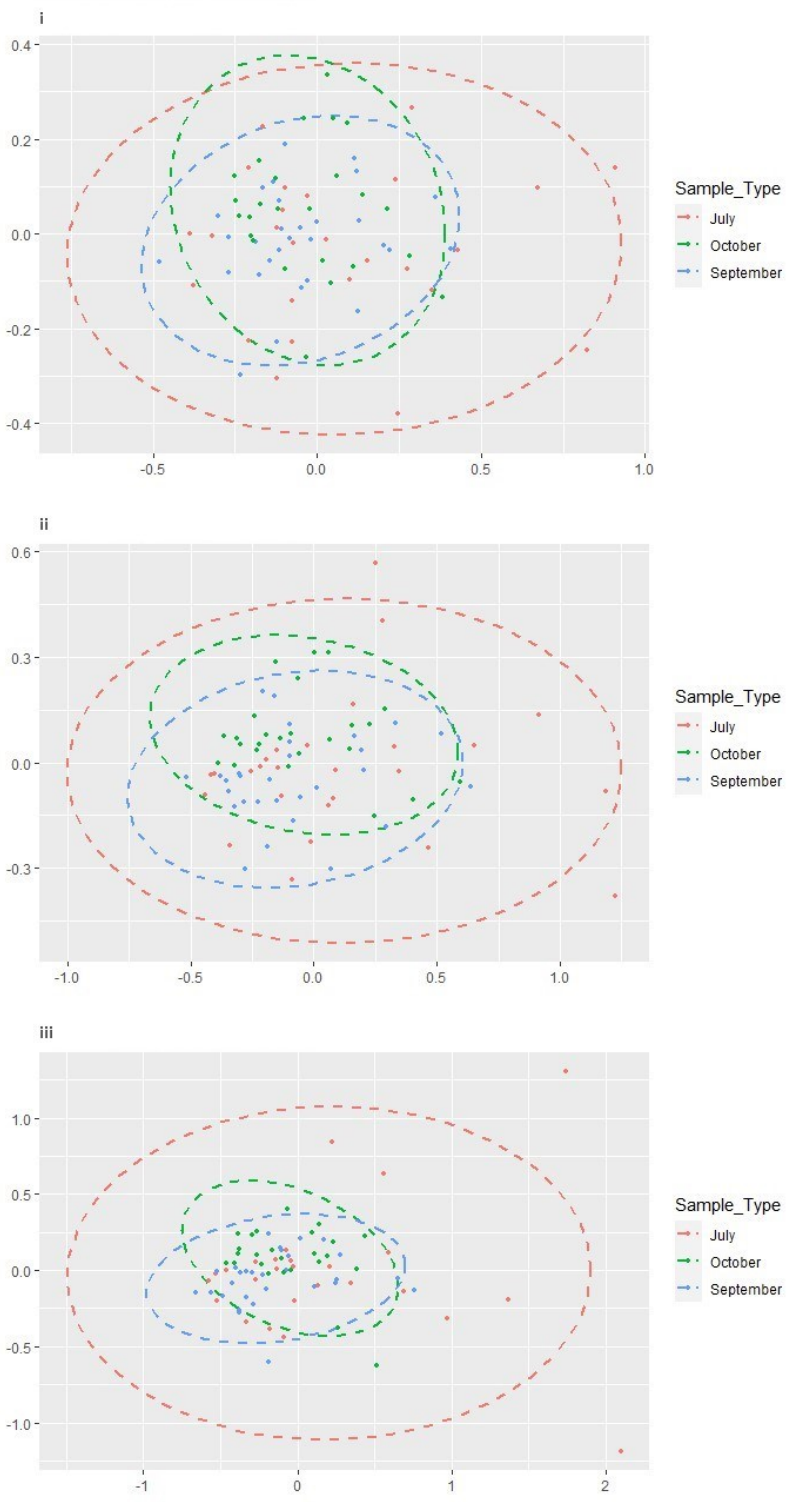

Figure 5 i) phylum level, ii) class level, iii) genus level. NMDS plots of microbial community diversity of all samples compared by sampling month at. Colors signify month. Dashed circles represent the confidence of the mean of the time groups.

Influence of Biological Sample on Microbial Community Richness and Composition:

Taxa richness was found to be significantly impacted by the type of biological tissues that bacterial communities came from at all three taxonomic levels (phylum; $F=12.58$, $p<0.0001$, class; $F=14.39$, $p<0.0001$, genus; $F=16.87$, $p<0.0001$). Yet, in pairwise comparisons, not all biological groups were significantly different from each other. Parasite externae and parasite larvae were very similar in taxa richness ($t=0.067$, $p=0.947$), with larvae having only around two genera more than the parasite externae, which had around 380 genera. The similarity in taxa richness of the parasite externae and parasite larvae is mirrored in the community diversity found in the nMDS plot (Fig. 6iii) where parasite larvae are nested entirely within parasite externae at the genus level. This supports the hypothesis that, because parasite externae and parasite larvae are two different tissue types of the same organism, they would contain similar microbiomes. Infected crab viscera samples were significantly different from parasite externae ($t=-4.161$, $p<0.0001$) containing almost 100 fewer genera then the parasite externae's 380. This difference paled in comparison to the uninfected crabs, which were also significantly different ($t=-6.507$, $p<0.0001$), and only contained around 130 genera, less than half of the parasite externae's genus level taxa richness of 380. This showed a clear trend with parasite samples having the highest taxa richness and uninfected crabs having the lowest, while infected crabs had an intermediate taxa richness. These results also supported the hypotheses that uninfected crabs and parasite samples would be most different from each other, while infected crabs would have intermediate taxa richness due to mixing of parasite and crab microbiomes. This was also visualized in nMDS plots which showed that infected crab's viscera partially overlapped with the parasite externae, and the majority of the uninfected crab viscera samples (Fig. 6iii).

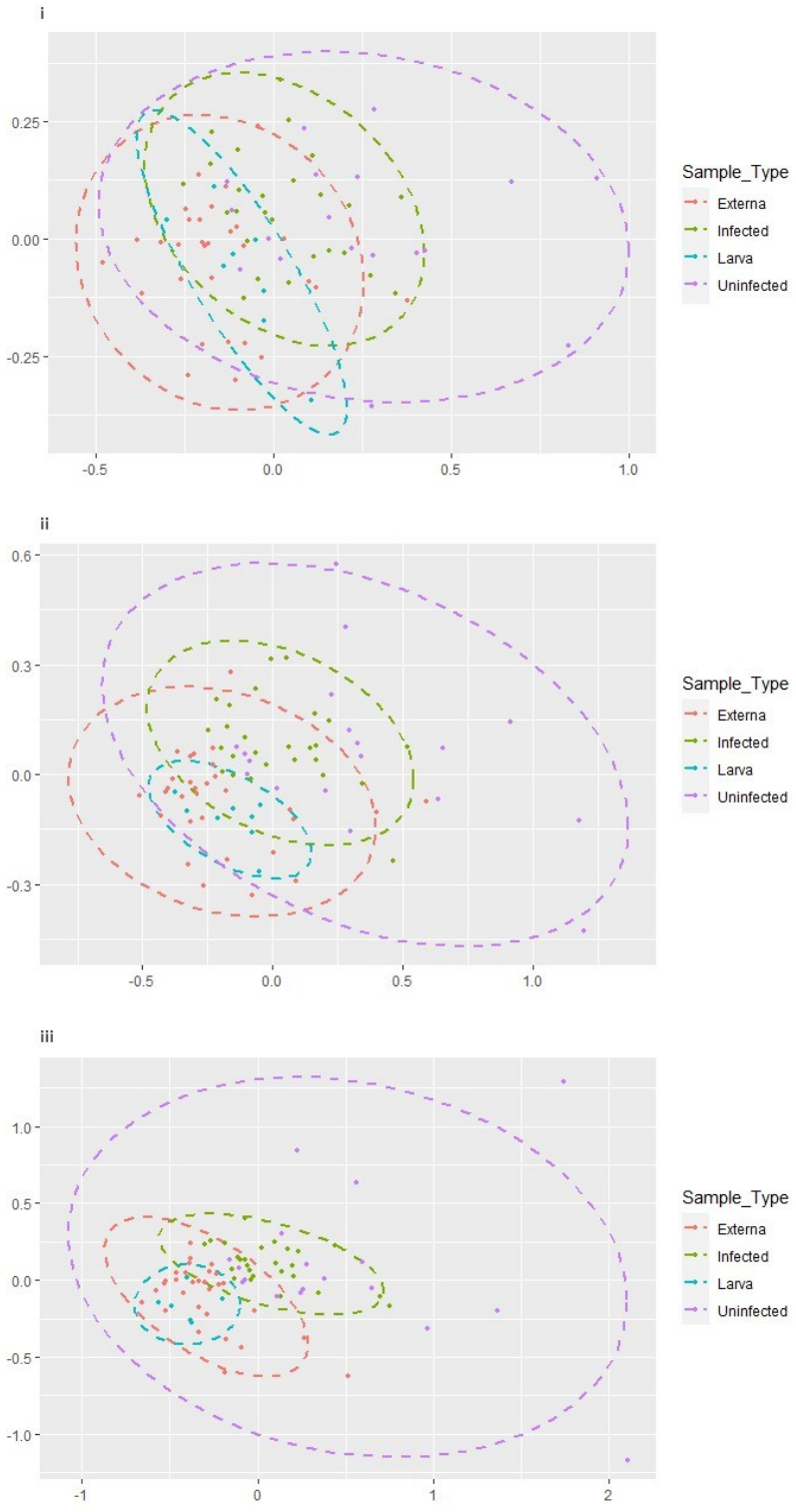

Figure 6 i) phylum level, ii) class level, iii) genus level. NMDS plots of microbial community diversity of all samples compared by biological type at Colors signify month. Dashed circles represent the confidence of the mean of the biological groups.

***Community Composition and Diversity of Biological Groups**:*

All Samples:

Proteobacteria and Bacteroidota were the two most abundant bacterial phyla, and both groups were found in high proportions in the four biological groups sampled (uninfected crabs, infected crabs, parasite externae, parasite larvae) (Fig. 7i. 8i. 9i). Proteobacteria has been shown to consist of a high proportion of marine seawater samples, with communities consisting of ~25-28% Proteobacteria in the Southeastern United States Atlantic coast (González and Moran 1997, Wagner-Döbler and Biebl 2006). In this study, they comprised ≥50% Proteobacteria in all samples (Fig. 7i. 8i. 9i). Bacteroidota are also common bacteria in sediment and sea water communities, and they also have strong symbiotic ties with the gut microbiota of many animals, where they can be some of the most abundant community members (Thomas et al. 2011). The high proportions of Bacteroidota in all biological groups in my study are likely due to their close association with the gut microbiomes of macro-organisms, although some rare taxa may have been obtained from the environment.

Uninfected Crabs:

The family of bacteria, Rhodobacteraceae, were consistently the most prominent group of Proteobacteria in uninfected crabs, comprising 50-75% of almost all Proteobacteria diversity (Fig. 7ii). This trend continued from July to September, and although October uninfected crab samples still contained high amounts of Rhodobacteraceae, they were in lower relative abundancies (Fig. 8ii, 9ii). Rhodobacteraceae are a fairly common family of bacteria that are associated with chemo- and photoautotrophs found in marine sediments (Pohlner et al. 2019). Because of the vast underrepresentation of Rhodobacteraceae (Pohlner et al. 2019), it was

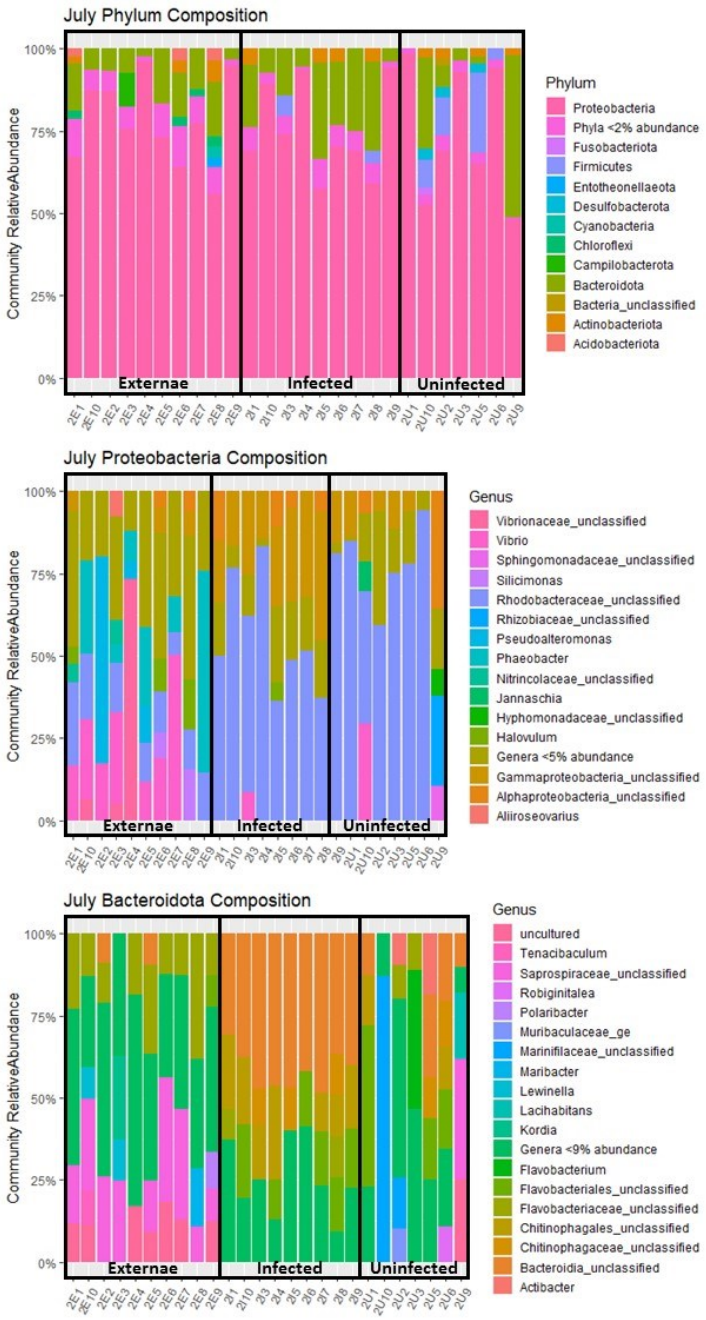

Figure 7 (top) phyla level taxa plots with >2% relative abundance cutoff, (middle) Proteobactera genera taxa plots with >5% relative abundance cutoff, (bottom) Bacteroidoia genera taxa plots with >9% relative abundance cutoff. Community diversity taxa plots for the July sampling period. Biological groups are boxed together to highlight community differences between individuals. The first number in labels refers to the sampling period (2 = July), the letter refers to sample type (E is externa, I is infected viscera, U is uninfected viscera, and the second number refers to replicate id (e.g. 2E1 is July externa sample 1, 2I7 is July infected viscera sample 7).

difficult to determine which, if any, of these may have been actual symbionts of the uninfected crabs. It may also be that, because of their more variable seasonal microbiome (Fig. 5), uninfected crabs are able to incorporate many different bacteria into their microbiome, resulting in more cosmopolitan microbial communities. This incorporation of microbiomes may be correlated with environmental microbial communities; however, because some crab's total microbiome is comprised of up to 10% Rhodobacteracea, it seems unlikely that this large community composition is entirely due to environmental contamination, but rather the sediment may be a source of the uninfected crab's microbiome. Additionally, because the dissection methodology was designed to reduce any outside microbial contamination, it seems more likely that the uninfected crabs are something of 'microbial community generalists' that are able to use wide ranges of communities.

Rhizobiaceae were detected in a few uninfected crabs in July and October samples (Fig. 7ii, 9ii). These bacteria are close symbionts of plants, assisting or hindering plant development (Spaink et al. 1998). Because of this group's close association with plant material (Spaink et al. 1998), in addition to the dissection method of collecting all internal viscera, including digestive organs, it could be that the Rhizobiaceae were detected in uninfected crabs that had consumed algae containing these microbial communities.

Another group unique to uninfected crabs were Marinifilaceae. As with Rhizobiaceae, these bacteria were sporadic, only appearing in some crabs in July and October (Fig. 7ii, 9ii). The Marinifilaceae are also typically associated with sediment microbiota, which may indicate that crabs are able to incorporate abundant taxa from their environments into their microbiomes, resulting in some groups that are facultative microbial community members, as opposed to a more obligate microbial symbiont.

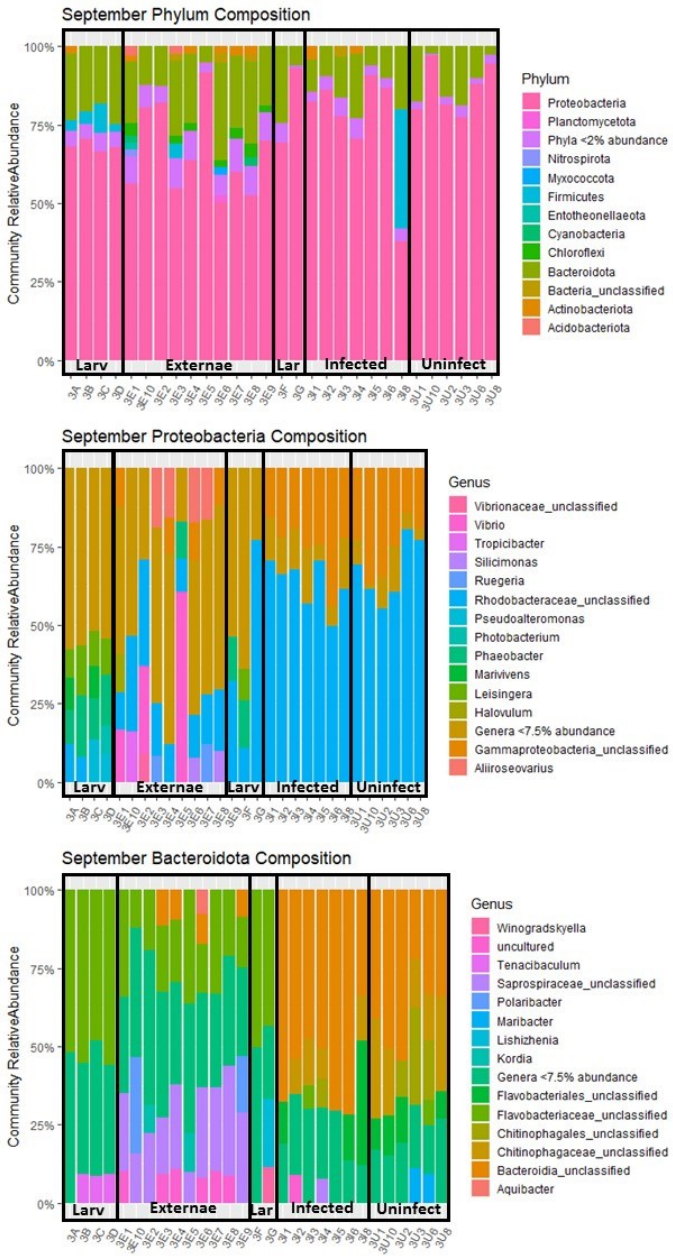

Figure 8 (top) phyla level taxa plots with >2% relative abundance cutoff, (middle) Proteobactera genera taxa plots with >7.5% relative abundance cutoff, (bottom) Bacteroidoia genera taxa plots with >7.5% relative abundance cutoff. Community diversity taxa plots for the September sampling period. Biological groups are boxed together to highlight community differences between individuals. The first number in labels refers to the sampling period (3 = September), the letter refers to sample type (E is externa, I is infected viscera, U is uninfected viscera, and the second number, or letter for larvae samples, refers to replicate id (e.g. 3E1 is September externa sample 1, 3A, B, C, D, F, G are larvae samples).

Infected Crabs:

One of the most abundant groups within the infected crab samples were members of the Chitinophagaceae family. This group has the ability to break down complex molecules, including chitin, the compound the group is named for (Sangkhobol and Skerman 1981). The higher abundance of these chitin-hydrolysing bacteria could be a potentially significant interaction of the crab and parasite microbiomes during infection. One of the side effects of parasitism by some groups of Rhizocephalan barnacles is the inability for hosts to molt and continue growing (Walker 2001). If communities of Chitinophagaceae associated with the barnacle parasite are able to proliferate within the crab host's body, and are able to hydrolyse any new chitin that the host might produce, then host crabs would be unable to produce new layers of chitin, stunting growth of the crab. The increased numbers of Gammaproteobacteria could be a direct result of this Chitophagaceae infection, and an example of the microbiomes of crab and host competing to produce more desirable outcomes for their respective symbiont. If Chitinophagaceae have strong ties with crab parasitism, then their high abundance in samples could be used as a potential indicator for crabs that have no visual infection, due to the parasite not being fully developed.

Uninfected and Infected Crabs:

Unclassified members of Gammaproteobacteria comprised a major portion of the Proteobacteria communities in both uninfected and infected crabs. Gammaproteobacteria contains a variety of bacteria; however, many of these are associated with microbial symbionts that produce natural antibiotics. Because of the unclassified nature of the group, it is challenging to determine their interaction with uninfected and infected crabs; however, research has shown that some marine crabs have defenses against bacterial infection (Noga 2010). If crab immunological defenses against bacteria are amplified by the presence of bacterial symbionts,

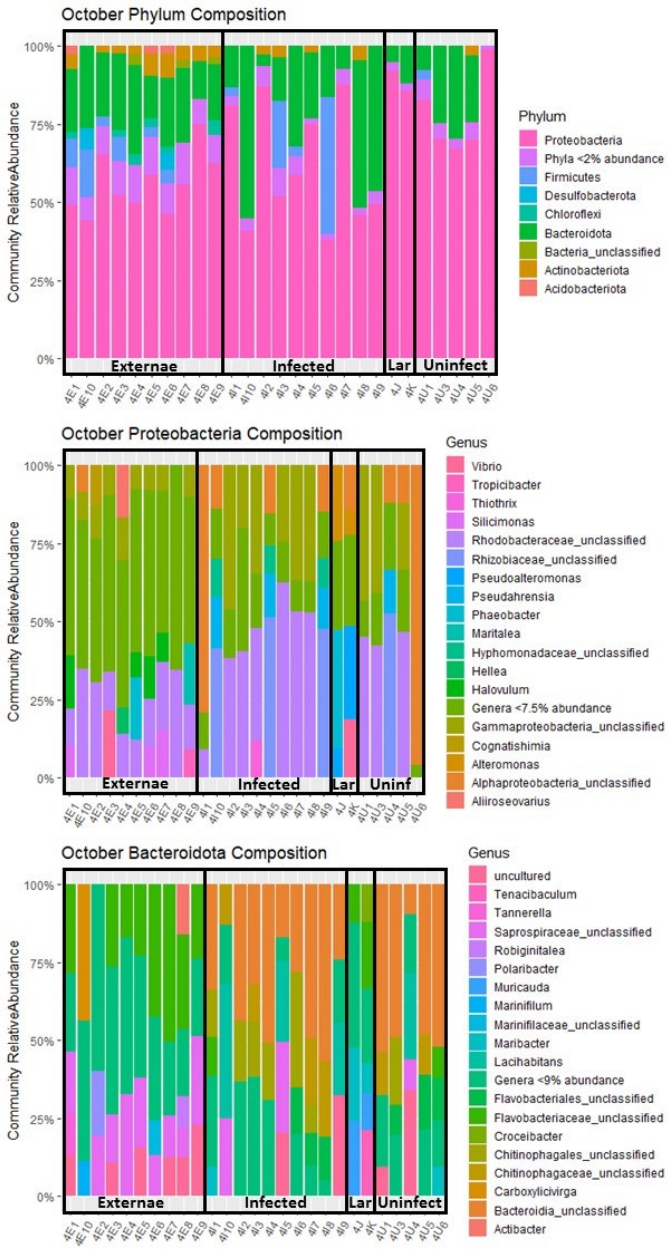

Figure 9 (top) phyla level taxa plots with >2% abundance cutoff, (middle) Proteobacteria genera taxa plots with >7.5% abundance cutoff, (bottom) Bacteroidota genera taxa plots with >9% abundance cutoff. Community diversity taxa plots for the October sampling period. Biological groups are boxed together to highlight community differences between individuals. The first number in labels refers to the sampling period (4 = October), the letter refers to sample type (E is externa, I is infected viscera, U is uninfected viscera, and the second number, or letter for larvae samples, refers to replicate id (e.g. 4E1 is October externa sample 1, 4J and 4K are larvae samples).

then the unclassified Gammaproteobacteria could possibly assist in the protection of crabs from diseases (Dong et al. 2009, Noga 2010). The differences in community composition of Gammaproteobacteria between uninfected and infected crabs lends some support to the hypothesis that these unclassified Gammaproteobacteria may be immunologically symbiotic in nature. Infected crabs had much higher proportions of the unclassified Gammaproteobacteria than uninfected crabs, which could be the crab microbiome's response to the presence of the parasite and the parasitic microbial community.

Unclassified representatives of the order Flavobacteriales were present in uninfected and infected crab samples, although the frequency that they appeared in biological groups varied with the presence of parasitic infection, with only ~50% of uninfected crabs containing Flavobacteriales, and infected crabs containing 100%. Flavobacteriales are a common community member in marine sediments and marine water, comprising of up to 20% of community composition in some systems, but can also be found as symbionts in the gut of macro-organisms (Yang et al. 2017). Although other groups, such as the Marinifilaceae, are thought to have been incorporated into the crab microbiome from abundant environmental communities, the Flavobacteriales likely have some community members that are gut symbionts of the uninfected and infected crabs. Why infected crabs have a higher frequency of Flavobacteriales is unknown; however, the variation of Flavobacteriales, may indicate that both uninfected and infected crabs are able to utilize some available communities to assist in gut functions, as opposed to requiring specific microbial symbionts for all gut functions. Varied gut communities could also be indicative to crab diet, as changes in diet can change prevalence of gut microbial communities (Thomas et al. 2001, Davenport 2014, David et al. 2014).

Externae and Larvae:

A shared group between parasite larvae and parasite externae samples is *Pseudoalteromonas*. This group of bacteria have been well documented as symbionts of marine eukaryotes that produce antibacterial agents, and additionally reduce settlement of fouling plankton on an organism (Holmström and Kjelleberg 2006). This could suggest that the *Pseudoalteromonas* collected from parasite externae and parasite larvae samples also assist in preventing bacterial infection in the parasite. Additionally *Pseudoalteromonas* could provide additional benefits for parasite externae by reducing fouling of the parasite brood sac (Holmström and Kjelleberg 2006).

Parasite Externae:

Parasite externae had several taxa that were unique when compared to uninfected and infected crab samples, and additionally taxa composition seemed much more consistent both between samples, and over seasonal gradients. This more highly conserved microbiome in the parasite externae could be the result of the barnacle being more dependent on specific taxa, and these biological groups may not be able to passively incorporate as much of the microbiome, as hypothesized in the uninfected crab samples. The first of these prevalent groups in parasite externae were unclassified Vibrioaceae, and the members from the genus *Vibrio* (Fig. 7ii, 8ii, 9ii). *Vibrio* bacteria are most commonly found in estuarine systems, and several species are associated with marine organisms, such as oysters (Marhsall et al. 2016). *Vibrio* bacteria are notorious pathogens that occur in the tissues of marine biota, causing diseases in organisms that consume the *Vibrio* contaminated tissues (Marhsall et al. 2016). It may be that the *Vibrio* associated with the parasite externae could act as a deterrent to predators and fouling organisms.

Phaeobacter were another genus prominent throughout seasonal samplings of parasite externae (Fig. 7ii, 8ii, 9ii). *Phaeobacter* has been shown to have a selective, pathogenic relationship with marine algae (Bramucci et al. 2018). (Bramucci et al. 2018). The relationship results from the bacteria killing certain haploid cell types in the tissue it inhabits. It is unclear if the relationship with the parasite externae is similar, and if *Phaeobacter* is present in both internal and external portions of the externae, there may be some kind of evolutionary tradeoff that exists between potential protection from predation and the production of parasite offspring.

Externae also had communities of *Tenacibaculum* throughout the seasons (Fig. 7iii, 8iii, 9iii). *Tenaciaculum* are known for infecting the kidneys of marine organisms, especially fish, and inducing necrosis in the infected tissues, usually killing the host (Avendaño-Herrera et al. 2006). While these *Tenaciaculum* are likely pathogenic, their interaction with the host and parasite are unknown. They could be a pathogen of the parasite itself, or they could be a pathogen of the crab.

The last group unique to the parasite externae is the family Saprospiraceae, whose members are best known for their abilities to break down complex organic compounds in the environment (Rosenberg et al. 2014) (Fig. 7iii, 8iii, 9iii). As with the Rhodobacteraceae in uninfected crabs, these Saprospiraceae bacteria may be community members that are incorporated into the parasite via passive acquisition of abundant taxa in the environment.

Larvae:

Three unique genera of Flavobacteraceae were sampled from larvae, including *Muricauda*, *Maribacter*, and *Croceibacter*. Flavobacteriaceae were thought to be associated with the microbiome of the host crab's gut microbiome, with some potential for incorporation from

environmental samples. Because great care was taken to limit contamination of designated brooders, it seems unlikely that these are passive environmental communities that are being incorporated into the parasite larvae. Some work has shown that a few species in *Maribacter* have some natural antibiotic resistance, and the presence of these groups might be indicative of groups of bacteria that assist the larvae in overcoming defenses in a potential host and its microbiome (Nedashkovskaya et al. 2010).

Conclusion:

Because of the differences in bacterial richness between infected crabs and uninfected crabs, there seems to be evidence of parasitism changing the microbiome of the crabs. The changes in microbiome due addition of a parasite were most evident at the genus level indicating that microbiome studies should focus on this detailed taxa level analysis (Fig. 6iii). Some of these changes may be due to two organism's microbiomes, the host crab and parasitic barnacle, being combined in each of the infected crab samples. It is also possible that host microbiome was being modified by the parasite, adding to the changes in microbiome. A few examples of host microbiome modification have been studied, and there is evidence that host microbiomes can be pushed towards either higher or lower taxa richness by a parasite (Zaiss and Harris 2015, Kelly 2017). For instance, in experiments with humans and pigs, the addition of parasitic helminths increased the bacterial species richness in stool samples when compared to uninfected individuals (Zaiss and Harris 2015). Some of this change was noted to be the host's own microbiome responding to physiological changes in the host due to the increased mucosal habitat that was formed due to the parasite. It could be that host modification of the microbiome is not entirely due to competition between host and parasite microbiomes, but that anatomical or

physiological changes in the host due to parasitism can also induce changes in bacterial community richness and/or diversity (Zaiss and Harris 2015). Understanding when and how the parasite modifies the host's microbiome could reveal more about the parasitism process, and specifically the roles that microbes play in the process of infection. For example, studies on a freshwater prawn have shown that the microbial community varies drastically between different molt stages of the decapod (Mente 2016). It could be that *L. panopaei* takes advantage of this shift in the microbiome when it infects crabs during the molting stages (Dheilly et al. 2015, Zaiss and Harris 2015, Kelly 2017). All infected crabs that were sampled had already developed their carapace from the molt when they had been parasitized, so there is no baseline in this system for how the microbiomes changes during molts, or if the parasite used these changes in molt microbiomes to its advantage.

The significant differences among biological samples at every taxa level, but especially the genera level (Fig. 6iii) also highlight the uniqueness of each biological group's microbiome and how the microbiomes of the host and parasite interact with each other, and are impacted by time (Fig. 4iii). Differences in community composition, and the symbionts of each biological group, could be used to understand more about the evolutionary pressures that each species is under. For instance, the presence of *Phaobacter* in parasite externae samples suggest that fouling of the externae is detrimental to the parasite, and so it has coevolved with a microbial symbiont to overcome this problem. Community composition of hosts and parasites could also create the framework for understanding how the microbial symbionts of the host and parasite interact with each other in the tripartite relationship. With more sampling times and a longer sampling period, the bacterial communities of the uninfected crabs and infected crabs could even be used as early bio-indicators for parasite infection, especially if groups such as the Chitinophagaceae are an

important community member in the microbiome of infected crabs, while uninfected hosts have lower or absent representatives of these bio-indicator species.

Although crabs were checked for infection *in situ* via presence of externae, and visually inspected during dissection for the presence of rootlets, there was still a potential for early infection crabs, infected crabs where the externa had fallen off, or crabs with a less distributed rootlet system, to be counted as uninfected crabs. This could potentially skew data and make uninfected crabs appear more similar to infected crabs. Additionally, there were potentials for bacterial contamination at several steps in the collection and dissection process, despite precautions. One potential example of this was the dissection process for the parasite externae. Because the externae is fully exposed to the environment, environmental bacteria could have remained embedded on the internal or external surfaces of the externae, despite the washes of sterilized water *in situ* and in the lab prior to dissections.

In sum, there are clear differences in groups of bacteria among biological groups, and in groups where parasites and hosts are interacting. The community composition of each organism suggests that interactions between host and parasite microbial communities are occurring, resulting in significantly different communities in infected and uninfected crabs. The variability of uninfected crabs and, to a lesser degree, infected crabs, also suggests that crabs are able to incorporate a variety of bacteria, resulting in more microbiomes that may not have as strong of association to hosts as the microbiomes in the parasite. When a parasite infects a host, although there is a significant increase in taxa richness within infected crabs, the microbial community also loses some of the diversity between individuals. This trend seems to be a direct result of parasitism, either due to combinations of both host and parasite microbiomes, or through host microbiome modification by the parasite. Although parasite externae and larvae have the most

genera richness of any samples, they also have the most similar communities between samples, indicating higher conservation of microbial communities. Future studies in host and parasite microbial communities could examine the microbial communities utilized in the native range of the parasite, to determine if the trend of microbial community conservation occurs only in the invasive range of the parasite, or if it is a trend that continues in both its native and invasive ranges.

CHAPTER 2: ANTIBIOTIC TREATMENT OF MICROBIAL COMMUNITIES

Introduction:

While many studies examining close-knit species interactions focus on the two primary members of that relationship (e.g., a host and its parasite) (Lidicker 1979, Toft and Karter 1990, Morran et al. 2011), it is clear that species interactions are often much more complicated and likely involve multiple species beyond just the host and parasite (Little et al. 2005, Koch and Schmid-Hempel 2011, Dheilly et al. 2015). For example, recent studies have shown that microbial symbionts can be highly influential to macroorganism species interactions, potentially shaping, protecting, or even inhibiting those relationships (Barbearán 2014, Clay 2014, Dirksen 2016). Yet to understand the role that these microbiota have on species interactions, a methodology for disrupting the microbiome must be developed, such that species interactions can be compared with conspecifics that possess an intact microbiome (Currie et al. 2003, Kennedy et al. 2018). Developing a methodology that could be used to manipulate the microbiome is a critical step in studying and understanding the microbiome on the tripartite relationship between host, parasite, and microbial symbionts.

One methodology for testing the microbial holobiont's role in a tripartite relationship, such as among a host, parasite, and their respective microbiomes, would be to lab rear microbially-sterile hosts and parasites and test infection prevalence by crossing groups of sterile and non-sterile parasites and hosts. While it is possible to rear organisms that are completely microbe-free, there are several hurdles with this methodology. Mainly, the rearing of germ-free organisms is an expensive and time-consuming process that requires special facilities to maintain microbe-free environments (Forbes and Park 1959, Kennedy et al. 2018). Additionally, because

the extent of crab reliance on the microbiome is unknown, the loss of the microbiome to create a microbe-free crab lineage could have additional impacts on the host-parasite relationship.

Because obtaining microbial sterile crabs and hosts is challenging, different methods are required to effectively disrupt the microbiome without killing the test subjects and still be able to test the infection rates of parasites. Perhaps one of the most mainstream and cost-effective methods of altering the microbiome in test subjects is the use of antibiotics (Gould et al. 1952, Blaser 2016, Kennedy et al. 2018, Zhu and Zhou 2018). Antibiotics are readily controllable by changing dosage and targeting of specific taxa is feasible as an antibiotics' effectiveness against specific bacterial groups is well-studied (Kennedy et al. 2018, Zhu and Zhou 2018). In addition, the use of broad-spectrum antibiotics can be effective at removing large groups of microbial communities, thereby reducing the number of treatments that might need to be exposed to a subject (Robinson and Young 2010, Blaser 2016, Kennedy et al. 2018). If smaller groups of bacteria are to be tested, then replacing broad spectrum antibiotics with more targeted antibiotics is a simple change in methodology to manipulate the microbiome (Robinson and Young 2010, Blaser 2016).

Antibiotic exposure can greatly influence how microbial communities are disrupted, while dosage and frequency of antibiotic delivery can impact the amount of antibiotic resistance microbial communities develop over time (Blaser 2016, Zhu and Zhou 2018). To test this, many researchers use press and pulse experiments to determine the resilience of an ecosystem in response to some kind of stressor or disturbance and the frequency of that disturbance (Ostfeld and Keesing 2000, Braverman and Mamdani 2008, Hamman and McCoy 2018, Jentsch and White 2019). In medical fields, press and pulse experiments test antibiotics used to treat bacterial infections and a bacterial community's response and resistance to antibiotics (Levy and Marshall

2004, Tan et al. 2012). When bacteria are exposed to intense, but brief, concentrations of antibiotics, many bacteria are either killed or become inhibited in their ability to grow and divide due to excessive stress (Kirby and Craig 1981, Roberts et al. 2016). However, when the antibiotic stressor has been flushed from the system, or as bacteria develop antibiotic resistance in response to stress, bacterial communities become active once more. Any bacteria that survived propagate and spread, potentially allowing the spread of antibiotic resistant strains (Roberts et al. 2016, Zhu and Zhou 2018). Bacteria with a greater resistance to antimicrobial antibiotics sometimes respond better to high-dose pulse treatments (Kirby and Craig 1981, Roberts et al. 2016). Yet, the most effective way of eliminating infections is by using a long-term press application of antibiotics to completely eliminate the bacteria through attrition (Roberts et al. 2016, Zhu and Zhou 2018). The downside to this approach is that longer exposure to an antibiotic increases the chances of a microbial community developing antibiotic resistance (Blaser 2016, Zhu and Zhou 2018)

Additionally, a metric was needed to determine the stress the host crabs endured during antibiotic treatment trials. Several studies have shown that when crabs are exposed to a stressor, their righting response slows as they struggle to flip over, resulting in a reliable proxy for determining the stress that a crab is experiencing (Rebach 1974, Warrenchuk and Shirley 2002, Blakeslee et al. 2015). Because the disruption of the microbiome via the use of antibiotics could incur a stress to the crabs, the use of righting response trials provided understanding of that stress on the host.

In my investigation, I aimed to test the influence of microbial disruption on community composition and diversity in infected and uninfected crabs, and how microbial disruption may affect host behavior in terms of righting response. Three broad spectrum antibiotics (ampicillin,

chloramphenicol, and gentamicin) were selected to provide the greatest potential in community disruption as they are all commonly used antibiotics, broad-spectrum, and cost effective (Box 1). Ampicillin weakens the cell wall of gram-positive bacteria and therefore was expected to be most effective against groups like Actinobacteria (NCBI 2019). Chloramphenicol is a very potent antibiotic that blocks the addition of proteins to amino acids and was expected to be effective against a broad range of bacterial colonies (NCBI 2019). Gentamicin interrupts the reading of tRNA and tends to be most effective against gram-negative bacteria such as Bacteroidetes and Proteobacteria (NCBI 2019). Based on prior antibiotic work (Hamamoto et al. 2004), and some preliminary work in the Field lab (unpublished), I hypothesized that chloramphenicol would be the most effective at disrupting a broad range of microbial communities during single antibiotic treatments from four host and parasite biological samples (uninfected crab viscera, infected crab viscera, parasite externae, parasite larvae). This is because

Box 1: Background information on Antibiotics.

Ampicillin (Amp) – This broad-spectrum antibiotic is a derivative of penicillin antibiotics. While it is generally used in pharmacology to fight gram-positive bacteria, it can also be used on gram-negative species. Ampicillin targets the penicillin-binding proteins of the bacterial cell wall, resulting in wall weakening, which leads to cell lysis. Ampicillin is also resistant to several types β-lactam, which is a naturally occurring antibiotic resistance that is produced by a number of gram-positive and negative species of bacteria. This antibiotic is expected to be most effective against Actinobacteria (National Center for Biotechnology Information 2019).

Chloramphenicol (Chl) – Another broad-spectrum antibiotic, chloramphenicol is an incredibly toxic substance to both Prokaryotes and Eukaryotes. Because of its potency, it is generally used against more extreme bacterial infections, like typhoid fever. This antibiotic targets peptidyl transferase, interfering with a bacteria's proteins ability to add amino acids to peptide chains. While chloramphenicol prevents bacterial reproduction and growth, it does not necessarily kill bacteria. Chloramphenicol is expected to be effective against all bacteria types (National Center for Biotechnology Information 2019).

Gentamicin (Gen) – The third broad-spectrum antibiotic being tested is gentamicin, which is generally used against aerobic gram-negative bacteria. While it can be used to target gram-positive bacteria, the concentrations are typically toxic to hosts as well. Gentamicin works by targeting the 30S ribosomal subunit and interrupts the reading of tRNA, resulting in an inability of targeted bacteria to synthesize proteins. This antibiotic is expected to work best against Bacteroidetes, Proteobacteria, and Tenericutes (National Center for Biotechnology Information 2019).

of chloramphenicol's recorded effectiveness against several pathogens that are difficult to remove without potent antibiotics, like typhoid (NCBI 2019) (Box 1). Yet past research has also shown that a cocktail of antibiotics can sometimes compensate for individual antibiotic weaknesses, making them more effective than any one antibiotic (Robinson and Young 2010, Blaser 2016, Kennedy et al. 2018). I therefore hypothesized that a combination of the three antibiotics would induce the greatest disruption in microbial communities. I also hypothesized that the highest concentrations would make the antibiotic combinations the most effective, as there would be more antibiotic molecules to interact with the microbial communities and would also limit the amount of antibacterial resistance the microbial communities would be able to produce (Levy and Marshall 2004, Tan et al. 2012). In righting response trials, I hypothesized that infected crabs would have slower righting response times versus uninfected crabs due to the combined stress of parasitism and antibiotic exposure, as demonstrated in past studies (Blakeslee

PART 2A. ANTIBIOTIC TESTING

Methodology:

Antibiotic Calculations:

Antibiotic concentrations were normalized by antibiotic units (X) so the effectiveness between different antibiotics could be directly compared, as molecular weights and solubility between differ between antibiotics. Although the maximum concentration of chloramphenicol that is soluble in water is 2.5 mg/mL (125X), in combination with other antibiotics, chloramphenicol precipitated out of solution (O'Neil 2013). Because of this, the maximum testable solubility of each antibiotic, was 100X (10 mg/mL ampicillin, 2 mg/mL chloramphenicol, 2 mg/mL gentamicin). For treatments where combinations of antibiotics were used, concentrations remained constant across tests and treatments were not diluted. For example, in the 100X ampicillin and gentamicin (100X amp + gen) treatment, 100X ampicillin (10 mg/mL) and 100X gentamicin (2 mg/mL) were combined. This was done rather than dilute samples, such that they would add up to 100X (e.g., 50X (5mg/mL) amp + 50X (1 mg/mL) gen, for a total of 100X) (Table 3).

Disk diffusions:

A modified Kirby-Bauer disk diffusion technique was used to determine the most effective dosage of antibiotics for the four biological sample types. Disk diffusions are commonly used in antibiotic concentration experiments to determine a bacterial communities' response to different concentration levels of antibiotics (Andrews 2001, Fiebelkorn et al. 2003, Jorgensen and Ferraro 2009). To perform disk diffusions, a single biological sample was added to 500µL of PBS (phosphate buffer solution) after dissection, which was then absorbed with a

Table 3: Table showing concentrations of antibiotics that were used in modified Kirby-Bauer disk diffusion and crab experiments. Amp, Chl, and Gen correspond to ampicillin, chloramphenicol, and gentamicin, respectively. Concentrations in 'X' are normalized concentrations of each specific antibiotic.

	Amp	Chl	Gen	Amp & Chl	Amp & Gen	Chl & Gen	Amp & Chl & Gen
100X	10 mg/mL Amp	2 mg/mL Chl	2 mg/mL Gen	10 mg/mL Amp & 2 mg/mL Chl	10 mg/mL Amp & 2 mg/mL Gen	2 mg/mL Chl & 2 mg/mL Gen	10 mg/mL Amp & 2 mg/mL Chl & 2 mg/mL Gen
75X	7.5 mg/mL Amp	1.5 mg/mL Chl	1.5 mg/mL Gen	7.5 mg/mL Amp & 1.5 mg/mL Chl	7.5 mg/mL Amp & 1.5 mg/mL Gen	1.5 mg/mL Chl & 1.5 mg/mL Gen	7.5 mg/mL Amp & 1.5 mg/mL Chl & 1.5 mg/mL Gen
50X	5 mg/mL Amp	1 mg/mL Chl	1 mg/mL Gen	5 mg/mL Amp & 1 mg/mL Chl	5 mg/mL Amp & 1 mg/mL Gen	1 mg/mL Chl & 1 mg/mL Gen	5 mg/mL Amp & 1 mg/mL Chl & 1 mg/mL Gen
25X	2.5 mg/mL Amp	0.5 mg/mL Chl	0.5 mg/mL Gen	2.5 mg/mL Amp & 0.5 mg/mL Chl	2.5 mg/mL Amp & 0.5 mg/mL Gen	0.5 mg/mL Chl & 0.5 mg/mL Gen	2.5 mg/mL Amp & 1.5 mg/mL Chl & 1.5 mg/mL Gen

sterilized cotton swab and applied over a large agar plate. Plates were poured from a marine agar (BD Difco, Fisher Scientific), as preliminary work showed that the most bacteria were able to grow on marine agar compared to standard nutrient agar plates likely due to their natural marine habitat . Once the material had been spread across the entire surface, eight 6.35 mm diameter sterilized antibiotic sensitivity disks were applied equidistant from each other and ~3-4cm from the outside of each agar plate (Fig. 10). 10µL of each of the seven antibiotic treatments (Table 3) were applied to a randomly assigned disk on each plate, and the eighth disk received 10µL of deionized (DI) water to act as a control. Five replicates from each biological group (uninfected viscera, infected viscera, parasite externae, parasite larvae) were used at each of the four antibiotic normalized concentrations (Table 3) for a total of 80 plates, all of which were stored in a 20°C incubator and monitored for 72 hours. Zones of inhibition, i.e., the radius from the edge of the disk to the edge of clearing where microbial growth began, were measured at 24hr, 48hr, and 72hrs. Only the best microbial inhibitor, defined as the antibiotic treatment had the greatest zone of inhibition, over the longest time, among all biological groups, was chosen for use on live subjects (Gould et al. 1952, Andrews 2001). In addition to complete inhibition, zones needed to last for the entire duration of the timed trials (72hrs), and the selected antibiotics needed to be effective against all four types of biological samples. Inhibition (Fig. 11) was determined by the highest letter ranking as described in Gould et al. (1952), with ‘A’ inhibition meaning that all bacterial growth was completely inhibited. Once bacterial colonies reached the disk but if growth was still patchy, the zone would be measured as a ‘B’ group. Once bacterial growth had formed a lawn, the disk was given an ‘E’ grouping, and zones of inhibition were not measured in ‘E’ groups.

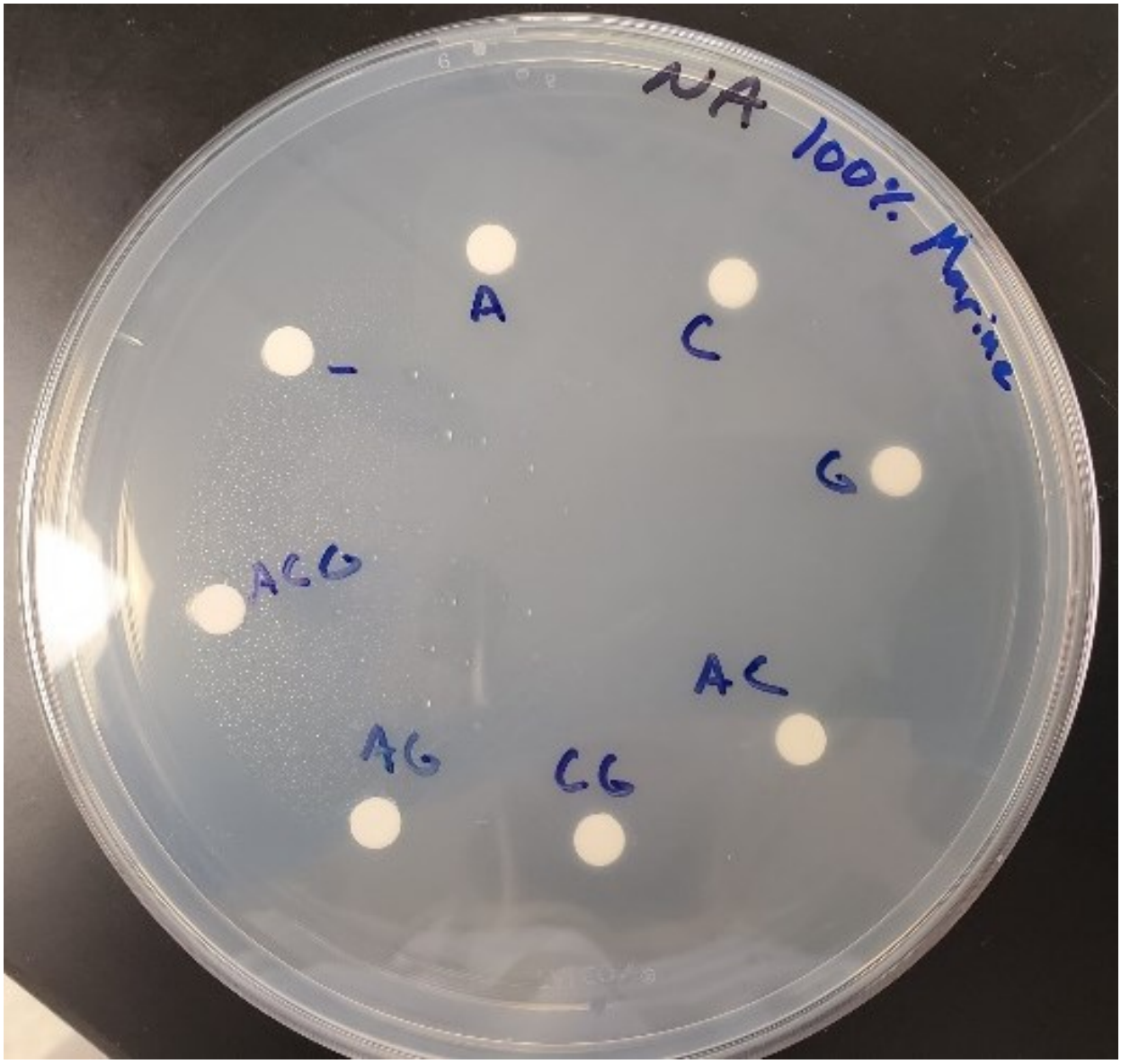

Figure 10: Example disk diffusion plate. A, C, and G correspond to ampicillin, chloramphenicol, and gentamicin respectively and '-' is the control disk. Treatments with combined antibiotics have multiple letters (e.g. ACG is the ampicillin, chloramphenicol, and gentamicin combination treatment). **[Photo taken by KL Swanson]**.

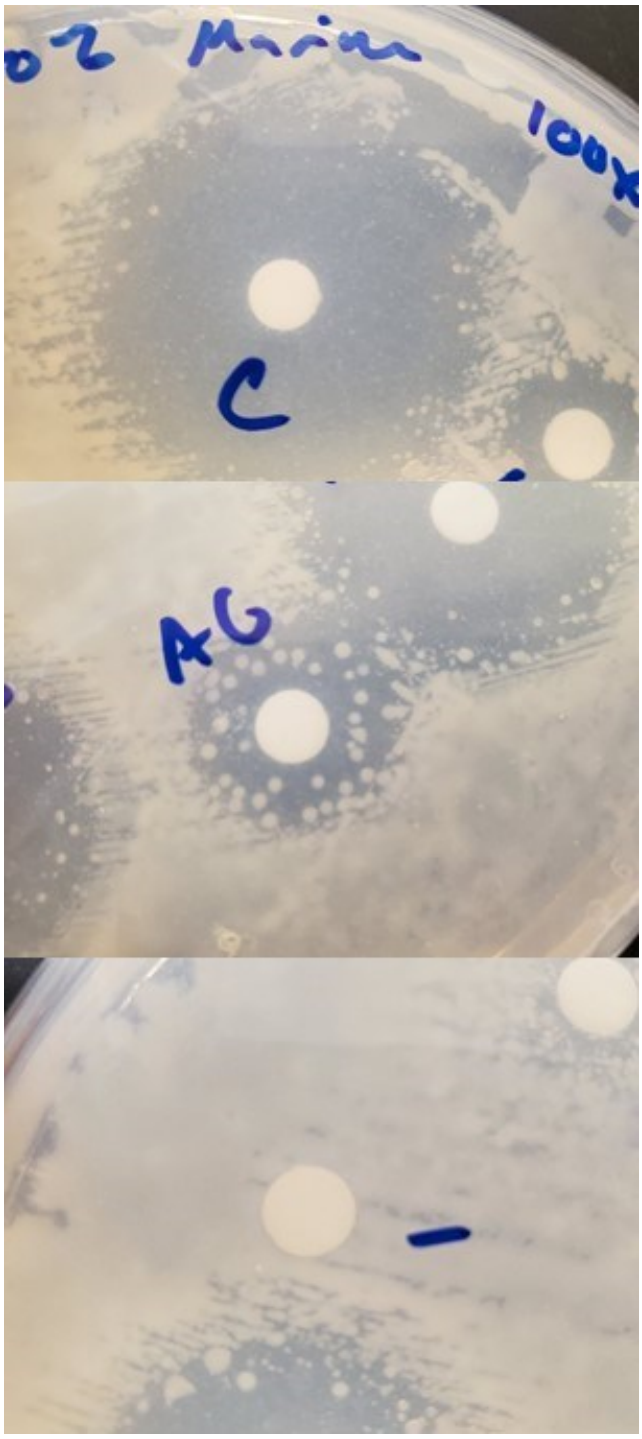

Figure 11: Top) Example of an 'A' inhibition zone surrounding a disk that was treated with chloramphenicol labeled as "C". Center) Example of a 'B' inhibition zone around a disk that was treated with a combination of ampicillin and gentamicin, labeled as "AG". Bottom) Example of an 'E' inhibition zone surrounding a control disk labeled with a "-". **[Photos taken by KL Swanson]**.

Statistical Analyses:

Because of the variable growth on some disk diffusion plates (likely due to differences in original cell densities among biological sample tissues), data were normalized to prevent trends that made it appear that zones of inhibition increased over time due to larger sample size. To compensate for this, the first day that plates were observed to have growth was called 24hrs for that specific plate. To test effects on 'A' inhibition radii, a linear mixed-effects model (lmer) from the package lme4 1.1-23 was run in Rstudio. The inhibition radius response variable was tested against the fixed effects of antibiotic concentrations (100X, 75X, 50X, 25X), biological samples (uninfected viscera, infected viscera, parasite externae, parasite larvae), antibiotic types and combinations (Table 3), and observation time points (24hrs, 48hrs, 72hrs). Additionally, a random effect was added for the response of radii to their plate. Because inhibition radii depended on the time that each plate was measured at, time points were nested within plate replicates. Initial models with interactions between all four fixed effects found only the interaction between antibiotic treatments and concentrations to be significant. Biological sample type and time were included as non-interacting fixed effects in the model analyzing inhibition radii.

Results and Discussion:

Influence of the Interaction of Antibiotic Type and Concentration:

Antibiotic types, and the dose at which they were administrated, was shown to have a significant effect on the amount of bacterial growth that was inhibited ($F=3.7831$, $p<0.0001$). This shows that, in addition to the type of antibiotic that is administered, the dosage had significant impacts on how quickly bacteria will develop antibiotic resistance. This dosage and

treatment dependency was best demonstrated when comparing similar antibiotic groups across different concentration levels. For instance, at 100X normalized concentrations, a combination of ampicillin and chloramphenicol inhibited an average radius of 1.9mm. As concentration of antibiotics decreased the overall effectiveness of antibiotics decreased: 75X amp+chl inhibited 0.8mm; 50X amp+chl inhibited 0.4 mm; and 25X inhibited 0.4 mm. Overall, among all antibiotic combinations and concentrations, 100X chl (2mg/mL) had the highest amount of inhibition over the course of the experiment ($t=11.987$, $p<0.0001$) (Table 4).

Influence of Antibiotic Type:

The types of antibiotics that were administered had significant effects on the amount of inhibition that was observed on the plates across all time periods ($F=80.1774$, $p<0.0001$). Because of the difference in effectiveness of antibiotics against different groups of bacteria, there were expected to be variations in overall inhibition. It was expected that the combination of all three antibiotics would provide the greatest range of bacterial inhibition, with gentamicin being most effective against gram-negative bacteria, ampicillin inhibiting gram-positive bacteria, and chloramphenicol inhibiting both groups. However, contrary to predictions, chloramphenicol by itself was the most effective antibiotic, inhibiting significantly larger radii ($t=12.139$, $p<0.0001$) for longer periods of time (Fig. 12, 13). Possible reasoning for why chloramphenicol and gentamicin performed poorly together is that they have an antagonistic relationship, meaning the presence of one, in this case chloramphenicol, negatively affects the effectiveness of another (White and White 1964, Sande and Overton 1973). This antagonistic relationship between antibiotics is due to the chemical pathways that are inhibited. When a bacterial cell is undergoing protein synthesis, mRNA is transcribed from cellular DNA and is received by bacterial peptidyl

Table 4: Interaction between antibiotic combinations and antibiotic concentrations, and their effect on the inhibition of bacterial growth. Amp is ampicillin, Chl is chloramphenicol, and Gen is gentamicin. Concentration correspond to calculated concentrations in Table 3.

	Estimate	t-value	p-value
(Intercept)	1.655	5.816	1
25X	-0.0463	-0.279	0.7802
50X	0.0112	0.08	0.9361
75X	-0.0061	-0.05	0.96
Amp+Chl	0.8545	8.863	<0.0001
Amp+Chl+Gen	0.6915	7.158	<0.001
Amp+Gen	-0.121	-1.179	0.2387
Chl	1.154	11.987	<0.0001
Chl+Gen	0.5593	5.773	<0.0001
Gen	-0.3024	-2.962	0.003
Uninfected Viscera	-0.088	-1.467	0.1436
Infected Viscera	-0.1966	-3.331	0.001
Parasite Larvae	-0.2451	-3.823	0.0001
Time 48	-0.5292	-1.386	1
Time 72	-0.8495	-2.22	1
25X:Amp+Chl	-0.6708	-3.619	0.0003
50X:Amp+Chl	-0.3662	-2.31	0.021
75X:Amp+Chl	-0.2256	-1.633	0.1028
25X:Amp+Chl+Gen	-0.6699	-3.578	0.0003
50X:Amp+Chl+Gen	-0.2653	-1.647	0.0998
75X:Amp+Chl+Gen	-0.3201	-2.298	0.0217
25X:Amp+Gen	-0.0256	-0.128	0.8984
50X:Amp+Gen	0.0955	0.536	0.5918
75X:Amp+Gen	0.0298	0.204	0.8382
25X:Chl	-0.8576	-4.595	<0.0001
50X:Chl	-0.3354	-2.113	0.0348
75X:Chl	-0.4339	-3.117	0.0018
25X:Chl+Gen	-0.9091	-4.761	<0.0001
50X:Chl+Gen	-0.4073	-2.505	0.0123
75X:Chl+Gen	-0.3294	-2.365	0.0182
25X:Gen	-0.0385	-0.129	0.8974
50X:Gen	-0.4263	-2.132	0.0332
75X:Gen	-0.3215	-2.13	0.0333

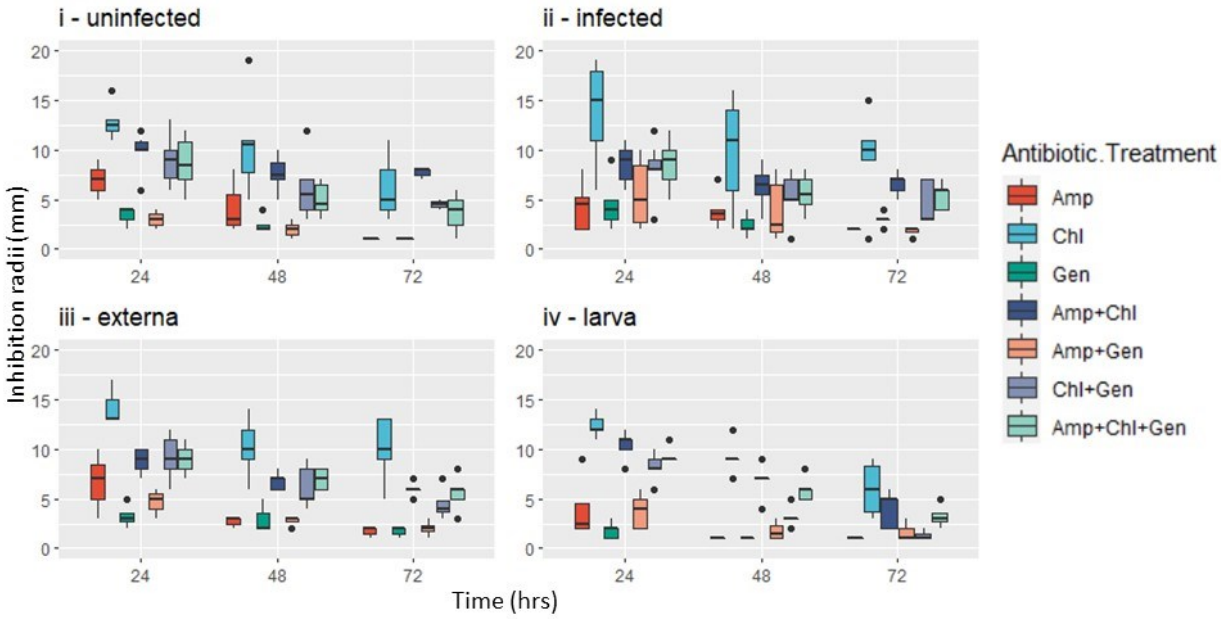

Figure 12: i) uninfected crab viscera, 22) infected crab viscera, iii) parasite externae, iv) parasite larvae. Inhibition radii of 'A' zones grouped by antibiotics at 100X concentrations broken down by biological samples. Antibiotics are compare over the 72 hour observation period. Amp is ampicillin, Chl is chloramphenicol, and Gen is gentamicin. Inhibition is measured by inhibition radius in millimeters of 'A' inhibited zones.

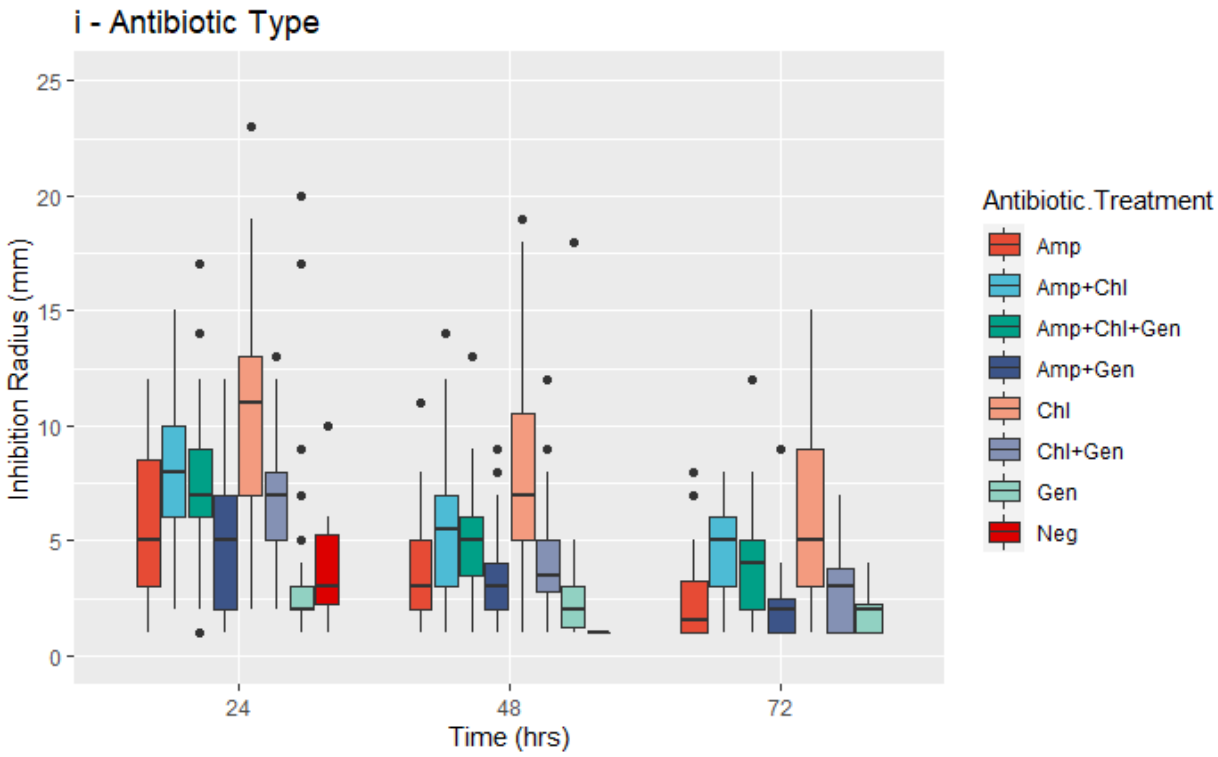

Figure 13: Inhibition radii of 'A' zones grouped by antibiotics at 100X concentrations and all biological samples. Antibiotic treatments are compared over the 72 hour observation period. Amp is ampicillin, Chl is chloramphenicol, and Gen is gentamicin. Inhibition is measured by inhibition radius in millimeters of 'A' inhibited zones.

transferase where the mRNA strand is read with the 30s subunit protein, and the transferase forms peptidyl bonds between amino acids and produces a strand of tRNA corresponding to the mRNA strand. However, chloramphenicol blocks the peptidyl transferase, interfering with the cell's ability to read mRNA and add amino acids to peptidyl chains (White and White 1964, Sande and Overton 1973). This directly impacts the function of gentamicin, which prevents the 30s RSU from reading the mRNA strand, thereby preventing translation and the formation of proteins. But if peptidyl transferase is being inhibited, then everything downstream from that step-in protein synthesis, including the reading of mRNA strands, will be blocked, making gentamicin redundant and decreasing its overall effectiveness (White and White 1964, Sande and Overton 1973). If this were the entirety of gentamicin's shortcoming when combined with chloramphenicol, then inhibition would be expected to be higher in the gentamicin treatments then what was observed in the gen+chl treatments. The fact that gentamicin was consistently the lowest performing antibiotic suggests that gentamicin and chloramphenicol's antagonistic relationship is likely not the only reason for lowered inhibition in treatments where the antibiotics are combined, and that there must be another reason that gentamicin performs so poorly in inhibiting the growth of the crab and parasite microbiomes.

Influence of Concentration:

Concentration also had a significant effect on the inhibition of bacterial groups within biological samples and antibiotic types ($F=16.0399$, $p<0.0001$). On average lower concentrations had decreased inhibition radii (Table 4), although the 50X concentration had a slightly larger zones of inhibition than 100X treatments over the 72 hour study period ($t=0.08$ $p=0.9361$) (Fig. 14). Most of this increased

inhibition at the 50X concentration level seems to come from the amp+gen combination

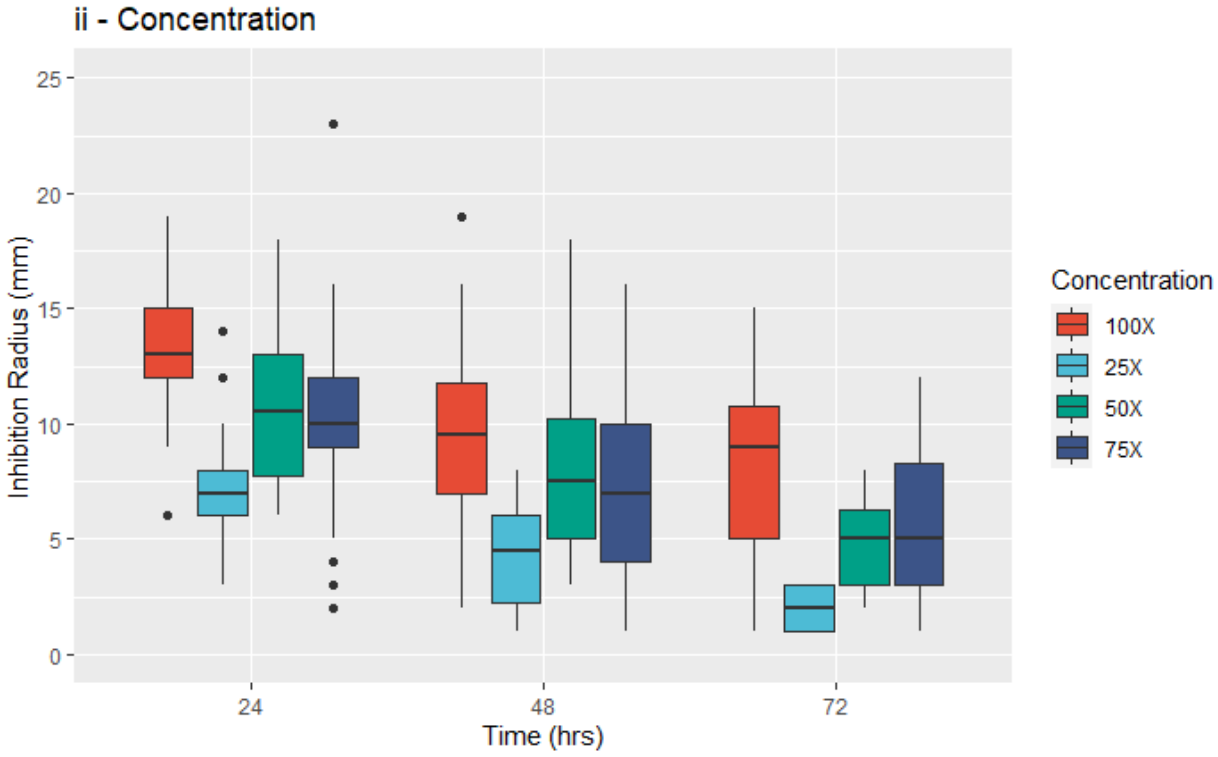

Figure 14: Inhibition radii of 'A' zones of the top performing antibiotic: chloramphenicol. Inhibition radii are grouped by concentration level and all biological samples. Antibiotic treatments are compared over the 72 hour observation period. Inhibition is measured by inhibition radius in millimeters of 'A' inhibited zones.

treatments (Table 4). At 100X, the amp+gen treatments had smaller measured zones of inhibition (t=-1.179, p=0.2387) than the 50X treatments (t=0.536, p=0.5918) which had larger zones of inhibition. This could be a result of the gentamicin, which seemed to inhibit bacteria slightly better at lower concentration levels. At 100X concentration, gentamicin had much smaller inhibition zones (t=-2.962, p=0.003), and inhibition zones size increased through moderate treatments at 50X (t=-2.132, p=0.0332), and had the highest inhibition at 25X concentration (t=-0.129, p=0.8974).

Influence of Biological Sample:

While interactions among biological samples were small, there was still a significant effect of biological sample on inhibition radii (F=6.216, p=0.0004), with some sample types seeming to be more resistant to bacterial inhibition (Fig. 14). Bacterial communities were shown to be quite different among different biological samples in Chapter 1 (Fig. 7, 8, 9), and these changes in community composition are likely the main drivers for why different biological samples experienced different amounts of inhibition. The interesting result from the influence of biological sample, is that the parasite externae and uninfected crab viscera, two groups that were shown to be significantly different from each other in experiment 1, had the most similar amounts of inhibition. This could be due to bacterial communities in different biological samples being susceptible to the same types of antibiotics, despite being comprised of different species. Interestingly, the two biological groups that had the most similar taxa communities, had the most dissimilar amount of inhibition that occurred, with larvae having smaller inhibition radii than the parasite externae (t=-2.731, p=0.0068) (Fig. 15). Since parasite larvae and externae were most

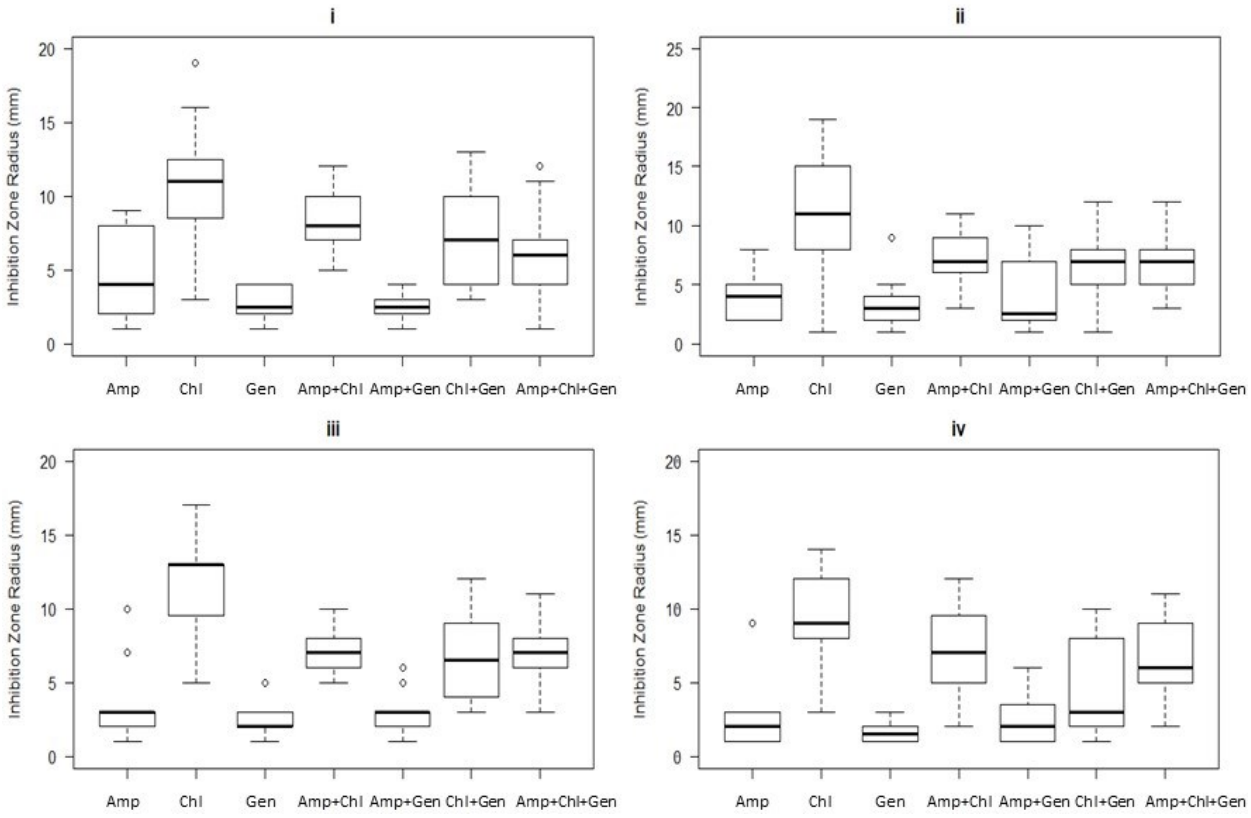

Figure 15: i) uninfected crab viscra, ii) infected crab viscera, iii) parasite externae, iv) parasite larvae. Inhibition of all antibiotics at 100X concentration split by biological sample. Amp is ampicillin, Chl is chloramphenicol, and Gen is gentamicin. Inhibition is measured by inhibition radius in millimeters of 'A' inhibited zones. All inhibition radii are compared at 24 hrs.

similar to each other, in terms of taxa richness and community composition, these were the groups that were expected to have similar amounts of inhibition. These differences in bacterial inhibition between samples that should have behaved similarly could have come from the media that antibiotic tests were performed on. Marine agar was used to grow the most microorganisms due to the marine environment the biological samples came from, and it is possible that some groups of bacteria grew in different proportions than what would normally be in the crab tissues. It could also be that some groups of bacteria unique to parasite larvae, such as the *Maribacter*, could be responsible for the increase in antibiotic resistance in the larvae samples. Literature has shown that some species within *Maribacter* have natural antibiotic resistance, and it could be groups of bacteria like these, with associations with only the parasite larvae, that are increasing community antibiotic resistance via horizontal gene transfer between bacterial groups.

Influence of Time:

The amount of time that bacteria were expose to antibiotic treatments had significant impacts on the amount of inhibition that occurred ($F=2.5162$, $p<0.0001$). This trend was observed across all biological samples, antibiotic treatments, and concentration levels (Fig. 12, 13, 14, 15), and resulted in smaller inhibition zones at later time points. Initial measurements of inhibition radii at 24hrs had significantly larger inhibition radii ($t=49.557$, $p<0.0001$), than the intermediate sized 48hr time period ($t=-7.932$, $p<0.0001$), or the smallest 72hr inhibition radii ($t=-11.515$, $p<0.0001$). The significant differences in inhibition radius sizes between all three treatments shows that bacterial antibiotic resistance was gained throughout the experiment, which was why shorter pulse dosages of antibiotics was expected to result in less antibiotic resistance gained by crabs and parasite microbiomes. The fact that this trend was consistent

across all biological sample types, antibiotic concentration levels, and antibiotic combinations, suggests that future experiments performed with antibiotic disruption of microbial communities will be impacted by the microbiome's ability to develop antibiotic resistance.

PART 2B. COMMUNITY DISRUPTION AND HOST BEHAVIOR

Methods:

Antibiotic Dosing and Community Diversity:

To test the effects of antibiotic exposure on host stress response, crabs were exposed to the most effective antibiotic dosage (100X, 2 mg/mL chl). Crabs were exposed to the antibiotics either for a 24 hour period (pulse) or a 72 hour period (press). However, following preliminary trials, the press experiment had to be abandoned as it led to excessively high mortality (91%) in infected crabs exposed to the antibiotic. As a result, only the 24 hour pulse experiment is described below. Crabs were subjected to righting response trials before and after the 24 hour experiment (described below). After testing righting response, crabs were given a 24 hour resting period before the start of the dosage trial to ensure that any stress induced mortality was due to the antibiotics and not the righting response trials.

Sterilized jewelry boxes were randomly assigned based on treatment (antibiotic or no antibiotic = control) and infection status (infected or uninfected). A total of 19 crabs were used in each of the uninfected control, the uninfected antibiotic, and the infected control groups, and 18 crabs were used in the infected antibiotic group (one died prior to the start of the experiment). A treatment stock was made with 1.14L of 20‰ sterilized sea water and 2.28g of chl to create a 100X (2 mg/mL) stock solution. The solution was extracted using a motorized pipette, and 30mL of the antibiotic stock was added to each of the appropriate wells within the jewelry cases, while the control wells received 30mL of 20‰ sterilized sea water. After all wells had been filled,

crabs were transferred from holding cases to experimental cases, which were randomly stored in an incubator for 24hr at 20°C and a 16:8 light:dark cycle. After 24 hours, cases were removed from the incubator and placed at 0°C for 30 minutes. Crabs were dissected as described in Chapter 1. The crab visceral mass was removed and stored in Eppendorf tubes with 250µL of PBS (phosphate buffered solution), and externa(e) from infected crabs were removed and stored in a separate tube with 250µL of PBS. Crabs that died during the experiment were not included in downstream genetic analyses because the microbiome of organisms begins to change as soon as the host has died (Hauther et al. 2015). A total of five crabs died in the infected chl treatment group from an initial population of 18 (27% mortality), and one crab died in the uninfected chl treatment group from an initial population of 19 (5% mortality). DNA extractions followed the same protocol as described in Chapter 1. PCR and sequencing are in progress, and thus the results of antibiotic disruption on community composition and diversity are forthcoming and not included in this chapter.

***Righting Response Trials*:**

All 69 living crabs (19 uninfected control, 19 infected control, 18 uninfected treatment, 13 infected treatment) were tested for righting response trials pre- and post-treatment. Each crab was individually placed on the middle of a large flat cutting board and subjected to three repeated flip trials, and the time it took for a crab to right itself was recorded. Occasionally, it was noted that crabs would flip most of the way until they were resting right on the joining of their carapace and abdomen, and sometimes would remain there for the rest of the time trial. If a crab performed this type of partial flipping behavior, the partial flip time was recorded, and a second time was taken if they completed righting themselves within the time limit. If a crab did

not right itself within 120 seconds, that trial was ended. This was repeated three times, concurrently to get an average righting response for each individual crab. Once a crab had finished its righting response trials, it was placed in a new jewelry box with 30mL of 20‰ sterilized sea water, and the cutting board was wiped down with 70% ethanol solution between each individual to reduce the chance of cross contamination.

Statistical Analyses:

For crab righting response, data was skewed to the right in a poisson distribution, and a generalized linear model was used to analyze the results using the glm function in base RStudio. Interactions between treatment and infection were analyzed in the glm, and righting response times were visualized in the base Rstudio boxplot function with the righting response times as a function of antibiotic and infection groups.

Analysis of disrupted microbial communities will be performed using the methodology from Chapter 1. Mothur v1.44.1 will be used along with the previous pipeline (Kozich et al. 2013) for interpretation of sequenced communities. Community composition between antibiotic treated crabs and control crabs will be compared to determine antibiotic effectiveness at disrupting the microbiome. These communities will be plotted in RStudio v1.2.5033 in nMDS plots using the vegan v2.5-4 package and ggplot v3.3.1, and community taxa plots will be made using Phyloseq v1.16.2 and dplyr v0.7.8. Phyla plots will again be sorted by taxa >2% while genera will use the >5% cutoff, unless high numbers of rare taxa exist, in which cast the >7.5% cutoff will be used.

Results and Discussion:

Righting Response Trials:

Prior to treatment, there was no statistical difference in righting response between infected and uninfected crabs ($p=0.8252$). This was surprising, given that *L. panopaei* has been shown to have a significant detrimental effect on its host, altering behavior and enhancing mortality (Alvarez et al. 1995). Unlike some parasite species that require host death to progress to the next stage in their life cycle (Trail 1980), *L. panopaei* has a direct lifecycle, and thus if the host dies, the parasite also dies. Thus, behavioral modification that too greatly enhances host mortality would not be adaptive to the parasite. Therefore, the righting response trials likely suggest that crabs that survived the infection process can compensate for the parasite and continue to perform defensive and predator avoidance behaviors.

Interactions between infection and treatments was shown to significantly impact the righting response time of crabs ($df=68$, $t=-2.437$ $p=0.0176$), with infected crabs taking a longer time to right themselves then uninfected crabs that were also treated (Fig. 16). There was also a difference in mortality in treatment crabs; five (27%) infected treatment crabs died during the experiment, while only one (5%) uninfected treatment crab died. For both infected and uninfected crabs, the addition of 100X (2mg/mL) chloramphenicol increased righting time by over six times the initial righting times ($t=0.889$, $p<0.0001$). This supported the hypothesis that the addition of antibiotics would increase the righting response time, although the response time was much longer in the uninfected crabs than was anticipated. Infected control crabs were not significantly different from uninfected control crabs in righting response at the end of the experiment ($t=-0.506$, $p=0.615$).

The increased righting response time suggests that treatment groups were being affected

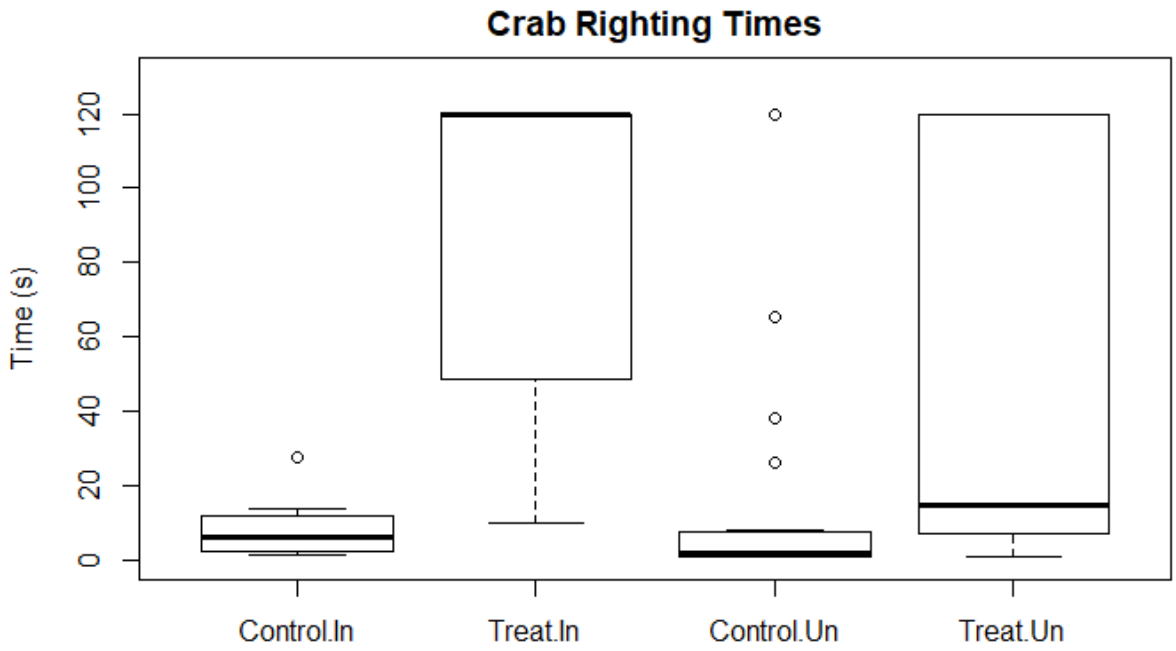

Figure 16: Boxplots showing crab righting response times after the 24hr experimental period. In (infected) and Un (uninfected) indicate if crabs were infected with the parasite *Loxothylacus panopaei*. Treatment groups refer to if crabs were dosed with chloramphenicol (treat) or not (control). All trials were stopped at 2 minutes so the upper limit of the righting response times is 120 seconds.

by the addition of antibiotics, although if this was due to loss of microbiome or a physiological response from the crabs remains unknown. The observed increased stress in the infected crabs versus the uninfected crabs also suggests that while the control infected crabs were able to right themselves at nearly the same rate as control uninfected crabs, the additional stressor of the antibiotics had a significantly greater strain on the infected treatment crabs, when compared to their uninfected counterparts. This type of response has been observed in a few systems. For instance, the parasitized moth larvae of *Lymantria dispar* were unable to handle environmental stressors that non-parasitized con-specifics were able to withstand (Bischof 1995). In the stickleback *Gasterosteus aculeatus*, parasitized individuals died much faster than their non-parasitized counterparts under food stress (Pascoe and Mattey 1977).

Community Diversity:

To date, all DNA has been extracted from the treated or untreated crabs. Following methodology from Chapter 1, I will perform PCR using the same primers and protocols and will send these samples for sequencing. Sequencing results are forthcoming.

Conclusion and Future Directions:

The main purpose of Chapter 2 was to establish a methodology that can be used in future studies to disrupt the microbiome of the crab and parasite to test the microbiome's role in the tripartite relationship. Because much of the evolutionary impacts of the microbiome on the host-parasite relationship are still unknown, developing a methodology that can be used to begin manipulating the microbiomes is a much needed step towards understanding the tripartite relationship. Concentration and antibiotic type both showed significant impacts on the amount of

microbial community that was able to be disrupted over a short period. The broad spectrum chloramphenicol was also shown to be an effective inhibitor of the microbiomes of both parasitized and unparasitized crabs. Because of chloramphenicol's effectiveness against bacteria that are notoriously resistant to weaker antibiotics, this suggests that more potent antibiotics are required to inhibit the microbiome of this host and parasite. This could simply be due to the fact that the antibiotics that were tested were not specifically designed to eliminate the microbiomes that are native to the crabs and parasites. This could be compounded by a number of factors, such as the marine environment of the microbiomes, however without further testing it is difficult to say why gentamicin and ampicillin did not inhibit as much of the microbiome as chloramphenicol. Further refinement of antibiotics and dosages will likely be required, as different biological samples have been shown to response differently to different antibiotics, and some communities might be resistant to the broad spectrum antibiotics that were tested in this experiment. Analysis of treated crab microbiomes from the dosage trials will assist in determining the efficacy of chloramphenicol at disrupting the microbiomes of the crabs and parasites. Future work using the methodology developed could be critical in unraveling the tripartite relationship in the host-parasite-microbiome system, and uncovering how the microbiome impacts this complex relationship between two organisms.

REFERENCES

Alvarez F, et al. 1995. The effects of parasitism by the barnacle *Loxothylacus panopaei* (Gissler) (Cirripedia: Rhizocephala) on growth and survival of the host crab *Rhithropanopeus harrisii* (Gould) (Brachyura: Xanthidae). Journal of Experimental Marine Biology and Ecology 192:221-232.

Andrews JM. 2001. BSAC standardized disc susceptibility testing method. Journal of Antimicrobial Chemotherapy 48(Supplemental):43-57.

Archie EA and J Tung. 2015. Social behavior and the microbiome. Current Opinion in Behavioral Sciences 6:28-34.

Avendaño-Herrera R, et al. 2006. Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: A review. Diseases of Aquatic Organisms 71(3):255-266.

Barbearán A, et al. 2014. The microbial contribution to macroecology. Frontiers in Microbiology 5:203.

Bischof C. 1995. Effects of heavy metal stress on carbohydrate and lipid concentration in the haemolymph and total body tissue of parasitized *Lymantria dispar* L. larvae (Lepidoptera). Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology, and Endocrinology 112(1):87-92.

Blakeslee AM, et al. 2015. Assessing the effects of trematode infection on invasive green crabs in eastern North America. PloS one 10(6):e0128674.

Blaser MJ. 2016. Antibiotic use and its consequences for the normal microbiome. Science 352(6285):544-545.

Bramucci AR, et al. 2018. The bacterial symbiont *Phaeobacter inhibens* shapes the life history of its algal host *Emiliania huxleyi*. Frontiers in Marine Science 5(188):doi:10.3389/fmars.2018.00188.

Braverman E and R Mamdani. 2008. Continuous versus pulse harvesting for population models inconstant and variable environment. Journal of Mathematical Biology 57:413-434.

Brown BE. 1997. Coral bleaching: Causes and consequences. Coral Reefs 16(S1):S129-S138.

Clay K. 2014. Defensive symbiosis: A microbial perspective. Functional Ecology 28(2):293-298.

Colston TJ and CR Jackson. 2016. Microbiome evolution along divergent branches of the vertebrate tree of life: What is known and unknown. Molecular Ecology 25(16):3776-3800.

Currie CR, et al. 2003. Experimental evidence of a tripartite mutualism: Bacteria protect ant fungus gardens from specialized parasites. Oikos 101(1):91-102.

Davenport ER, et al. 2014. Seasonal variation in human gut microbiome composition. PLoS One 9(3):e90731.

David LA, et al. 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature 505:559-563.

Dheilly A, et al. 2010. Antibiofilm activity of the marine bacterium *Pseudoalteromonas sp*. Strain 3J6. Applied and Environmental Microbiology 76(11):3452-3461.

Dheilly NM, et al. 2015. Biological warfare: Microorganisms as drivers of host–parasite interactions. Infection, Genetics and Evolution 34:251-259.

Dheilly NM, et al. 2017. Parasite microbiome project: Systematic investigation of microbiome dynamics within and across parasite-host interactions. mSystems 2(4): https://doi.org/10.1128/mSystems.00050-17.

Dirksen P, et al. 2016. The native microbiome of the nematode *Caenorhabditis elegans*: Gateway to a new host-microbiome model. BMC Biology 14(38): https://doi.org/10.1186/s12915-016-0258-1.

Dong Y, et al. 2009. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLOS Pathogens 5(5): https://doi.org/10.1371/journal.ppat.1000423.

Fiebelkorn KR, et al. 2003. Practical disk diffusion method for detection of inducible clindamycin resistance in *Staphylococcus aureus* and coagulase-negative Staphylococci. Journal of Clinical Microbiology 41(10):4740-4744.

Fierer N. 2008. Microbial Biogeography: Patterns in microbial diversity across space and time. Accessing Uncultivated Microorganisms. Chapter 6:95-115. In Zengler, Karsten (ed).
Forbes M and JT Park. 1959. Growth of germ-free and conventional chicks: Effect of diet, dietary penicillin and bacterial environment. The Journal of Nutrition 67(1):69-84.
Ford SA and KC King. 2016. Harnessing the power of defensive microbes: Evolutionary implications in nature and disease control. PLOS Pathogens 12(4): https://doi.org/10.1371/journal.ppat.1005465.
Ford SA, et al. 2016. Microbe-mediated host defense drives the evolution of reduced pathogen virulence. Nature Communications 7(13430).
Fowler AE, et al. 2013. The North American mud crab *Rithropanopeus harrisii* (Gould, 1841) in newly colonized Northern Baltic Sea: Distribution and ecology. Aquatic Invasions 8(1):89-96.
Ghiglione JF and AE Murray. 2011. Pronounced summer to winter differences and higher wintertime richness in costal Antarctic marine bacterioplankton. Environmental Microbiology 14(3):617-629.
Glenner H. 2001. Cypris metamorphosis, injection and earliest internal development of the Rhizocephalan *Loxothylacus panopaei* (Gissler). Crustacea: Cirripedia: Rhizocephala: Sacculinidae. Journal of Morphology 249:43-75.
González JM and MA Moran. 1997. Numerical dominance of a group of marine bacteria in the alpha-subclass of the class Proteobacteria in coastal seawater. Applied and Environmental Microbiology 63(11):4237-4242.
Gould JC, et al. 1952. The determination of bacterial sensitivity to antibiotics. Edinburgh Medical Journal 59(4):178-199.
Grice EA and JA Segre. 2011. The skin microbiome. Nature Reviews Microbiology 9:244-253.
Hines AH, et al. 1997. Introduced and native populations of a marine parasitic castrator: Variation in prevalence of the Rhizocephalan *Loxothylacus panopaei* in Xanthid crabs. Bulletin of Marine Science 61(2):197-214.
Hamaoto H, et al. 2004. Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. Antimicrobial Agents and Chemotherapy 48(3):774-779.
Hamman EA and MW McCoy. 2018. Top-down pulses reduce prey population sizes and persistence. Scientific Reports 8:9346.
Hauther KA, et al. 2015. Estimating time since death from postmortem human gut microbial communities. Journal of Forensic Sciences 60(5):1234-1240.
Holmström C and S Kjelleberg. 2006. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microbiology Ecology 30(4):285-293.
Høeg JT. 1995. The biology and life cycle of the Rhizocephala (Cirripedia). Journal of the Marine Biological Association of the United Kingdom 75:517-550.
Hu X, et al. 2018. High-throughput analysis reveals seasonal variation of the gut microbiota composition within forest musk deer (*Moschus berezovskii*). Frontiers in Microbiology 9:1674.
Huttenhower C, et al. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486:207-214.
Jentsch A and P White. 2019. A theory of pulse dynamics and disturbance in ecology. Ecology 100(7): doi: 10.1002/ecy.02734
Ji X, et al. 2012. Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China. Journal of Hazardous Materials 235-236: 178-185.
Jorgensen JH and MJ Ferraro. 2009. Antimicrobial susceptibility testing: A review of general principles and contemporary practices. Medical Microbiology 49:1749-1755.
Kelly PH, et al. 2017. The gut microbiome of the vector *Lutzomyia longipalpis*is essential for survival of *Leishmania infantim*. mBio 8(1):10.1128/mBio.01121-16.

Kennedy EA, et al. 2018. Mouse microbiota models: Comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. Frontiers in Physiology 9:1534
King KC, et al. 2016. Rapid evolution of microbe-mediated protection against pathogens in a worm host. Multidisciplinary Journal of Microbial Ecology 10:1915-1924.
Kim Y, et al. 2012. Enhancement of plant drought tolerance by microbes. In: Aroca R. (eds) Plant Response to Drought Stress, Ch15:383-415. Springer, Berlin, Heidelberg.
Kirby WMM and WA Craig. 1981. Theory and applications of pulse dosing: A summary of the symposium. Reviews of Infectious Diseases 3(1):1-3.
Koch H and P Schmid-Hempel. 2011. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proceedings of the National Academy of Sciences 108(48):19288-19292.
Kopac SM and JL Klassen. 2016. Can they make it on their own? Hosts, microbes, and the holobiont niche. Frontiers in Microbiology 7:1647.
Kozich JJ, et al. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and Environmental Microbiology 79(17):5112-5120.
Kruse I and MP Hare. 2007. Genetic diversity and expanding nonindigenous range of the Rhizocephalan *Loxothylacus panopaei* parasitizing mud crabs in the Western North Atlantic. Journal of Parasitology 93(3):575–582.
Lau JA and JT Lennon. 2012. Rapid responses of soil microorganisms improve plant fitness in novel environments. Proceedings on the National Academy of Sciences 109(35):14058-14062.
Levy SB and B Marshall 2004. Antibacterial resistance worldwide: Causes, challenges and responses. Nature Medicine 10:S122-129.
Ley RE, et al. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124(4):837-848.
Li S, et al. 2012. The intestinal microbial diversity in mud crab (*Scylla paramamosain*) as determined by PCR-DGGE and clone library analysis. Journal of Applied Microbiology 113(6):1341-1351.
Lidicker WZ. 1979. A clarification of interactions in ecological systems. BioScience 29(8):475-477.
Little AEF, et al. 2005. Defending against parasites: Fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens**.** Biology Letters 2(1): https://doi.org/10.1098/rsbl.2005.0371.
Lively CM. 1996. Host-parasite coevolution and sex. BioScience 46(2):107-114
Marshall DL, et al. 2016. Insects as sustainable food ingredients: Production, processing, and food applications. Academic Press. 223-253.
McFall-Ngai M, et al. 2013. Animals in a bacterial world, a new imperative for the life sciences. Proceedings of the National Academy of Sciences 110:3229-3236.
Mente E, et al. 2016. Gut microbial communities associated with the molting stages of the giant freshwater prawn *Macrobrachium rosen bergii*. Aquaculture 463(1):181-188.
Montllor CB, et al. 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecological Entomology 27(2):189-195.
Morran LT, et al. 2011. Running with the Red Queen: Host-parasite coevolution selects for biparental sex. Science 333(6039):216-218.
National Center for Biotechnology Information. PubChem Database. Ampicillin, CID=6249, https://pubchem.ncbi.nlm.nih.gov/compound/Ampicillin. (Accessed 2019).
National Center for Biotechnology Information. PubChem Database. Chloramphenicol, CID=5959, https://pubchem.ncbi.nlm.nih.gov/compound/Chloramphenicol. (Accessed 2019).
National Center for Biotechnology Information. PubChem Database. Gentamicin, CID=3467, https://pubchem.ncbi.nlm.nih.gov/compound/Gentamicin. (Accessed 2019).
Nedashkovskaya OI, et al. 2010. *Maribacter stanieri* Sp. Nov., a marine bacterium of the family Flavobacteraceae. International Journal of Systematic and Evolutionary Microbiology 60(pt. 1):214-218.

Noga EJ, et al. 2010. Primary structure and cellular localization of callinectin, an antimicrobial peptide from blue crabs. Developmental and Comparative Immunology 35:409-415.
O'Neil M (ed.). 2013. The Merck index – An encyclopedia of chemicals, drugs, and biologicals. Cambridge, UK: Royal Society of Chemistry p. 367.
Ostfeld RS and F Keesing. 2000. Pulsed resources and community dynamics of consumers in terrestrial ecosystems. Trends in Ecology & Evolution 15(6):232-237.
Pascoe D and D Mattey. 1977. Dietary stress in parasitized and non-parasitized *Gasterosteus aculeatus* L. Zeitschrift für Parasitenkunde 51:179-186.
Pasternak Z, et al. 2005. The morphology of the chemosensory aesthetasc-like setae used during settlement of cypris larvae in the parasitic barnacle *Sacculina carcini* (Cirripedia: Rhizocephala). Marine Biology 146:1005-1013.
Pohlner M, et al. 2019. The majority of active Rhodobacteraceae in marine sediments belong to uncultured genera: A molecular approach to link their distribution to environmental conditions. Frontiers in Microbiology 10:10.3389/fmicb.2019.00659
Rebach S. 1974. Burying behavior in relation to substrate and temperature in the hermit crab, *Pagurus longicarpus*. Ecology 55:195-198.
Roberts JA, et al. 2016. Continuous versus intermittent β-lactam infusion in severe sepsis: A meta-analysis of individual patient data from randomized trials. American Journal of Respiratoru and Critical Care Medicine 194(6):681-691.
Robinson CJ and VB Young. 2010. Antibiotic administration alters the community structure of the gastrointestinal microbiota. Gut Microbes 1(4):279-284.
Roche DG and ME Torchin. 2007. Established populations of the North American Harris mud crab, *Rhithropanopeus harrisii* (Gould 1841) (Crustacea: Brachyura: Xanthidae) in the Panamá Canal. Aquatic Invasions 2(3):155-161.
Rosenberg E, et al. 2014. The Prokaryotes: Other major lineages of bacteria and the archaea. 10.1007/978-3-642-38954-2.
Sande MA and JW Overton. 1973. In vivo antagonism between gentamicin and chloramphenicol in neutropenic mice. The Journal of Infectious Diseases 128(2): 247-250.
Sangkhobol V and VBD Skerman. 1981. *Chitinophaga*, a new genus of chitinolytic myxobacteria. International Journal of Systematic Bacteriology 31(3):285-293.
Spaink HP, et al. 1998. The Rhizobiaceae: Molecular biology off mosel plant-associated bacteria. Springer Science.
Sun W, et al. 2017. Seasonality affects the diversity and composition of bacteriplankton communities in Dongjian river, a drinking water source of Hong Kong. Frontiers in Microbiology 31:https://doi.org/10.3389/fmicb.2017.01644.
Tan C, et al. 2012. The inoculum effect and band-pass bacterial response to periodic antibiotic treatment. Molecular Systems Biology 8(1):617.
Tepolt CK, et al. 2019. Recent introductions reveal differential susceptibility to parasitism across an evolutionary mosaic. Evolutionary Applications 13(3):545-558.
Thomas F, et al. 2011. Environmental and gut Bacteroidetes: The food connection. Frontiers in Microbiology 2(93):doi:10.3389/fmicb.2011.00093.
Trail DRS. 1980. Behavioral interactions between parasites and hosts: Host suicide and the evolution of complex life cycles. The American Naturalist 116(1):77-91.
Toft CA and AJ Karter. 1990. Parasite-host coevolution. Trends in Ecology & Evolution 5(10):326-329.
Turnbaugh PJ, et al. 2007. The human microbiome project. Nature 449:804-810.
Vavre F and N Kremer. 2014. Microbial impacts on insect evolutionary diversification: From patterns to mechanisms. Current Opinion in Insect Science 4:29-34.
Venu I, et al. 2014. Social attraction mediated by fruit flies' microbiome. Journal of Experimental Biology 217:1346-1352.
Van Valen L. 1973. Body size and numbers of plants and animals. Evolution 27(1):27-35.

Wagner-Döbler I and H Biebl. 2006. Environmental biology of the marine *Roseobacter* lineage. Annual Review of Microbiology 60:255-280.
Walker G, et al. 1992. Aspects of the life-cycle of *Loxothylacus panopaei* (Gissler), a sacculinid parasite of the mud crab *Rhithropanopeus harrisii* (Gould): A laboratory study. Journal of Experimental Marine Biology and Ecology 157:181-193.
Walker G. 2001. Introduction to the Rhizocephala (Crustacea: Cirripedia). Journal of Morphology 249:1-8.
Warrenchuk JJ and TC Shirley. 2002. Effects of windchill on the snow crab (*Chionoecetes opilio*). Crabs in Cold Water Regions: Biology, Management, and Eonomics 19:81-96.
Webster NS. 2017. Conceptual and methodological advances for holobiont research. Environmental Microbiology Reports 9(1):30-32.
White JR and HL White. 1964. Streptomycinoid antibiotics: Synergism by puromycin. Science 146(3645): 772-774
Wilhelm SW, et al. 2014. Seasonal changes in microbial community structure and activity imply winter production is linked to summer hypoxia in a large lake. Microbial Ecology 87(2):475-485.
Williams AB. 1984. Shrimps, lobsters, and crabs of the Atlantic coast of the eastern United States, Maine to Florida. Smithsonian Institution Press. Washington, D.C. 1984.
Wong CAN, et al. 2015. Behavioral microbiomes: A multi-dimensional approach to microbial influence on behavior. Frontiers in Microbiology 6: https://doi.org/10.3389/fmicb.2015.013959.
Yang J, et al. 2017. c. Genomae Announc 5(5):e01551-16.
Zaiss MM and NL Harris. 2015. Interactions between the intestinal microbiome and helminth parasites. Parasite Immunology 38(1):5-11.
Zeng Q, et al. 2017. Models of microbiome evolution incorporating host and microbial selection. Microbiome 5(127): https://doi.org/10.1186/s40168-017-0343-x.
Zhu L and Q Zhou. 2018. Optimal infusion rate in antimicrobial therapy: Explosion of evidence in the last five years. Infection and Drug Resistance 11:1105-1117.