Variations in the Dragonfly Microbiome Through Life stages and Its Ability to Harbor Antibiotic Resistant Bacteria

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VARIATIONS IN THE DRAGONFLY MICROBIOME THROUGH LIFESTAGES AND ITS
ABILITY TO HARBOR ANTIBIOTIC RESISTANT BACTERIA

A Thesis
presented in partial fulfillment of requirements
for the degree of Master’s of Science
in the Biology Department
The University of Mississippi

by
Sarah Russell
May 2019
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Abstract
Juvenile dragonflies (nymphs) may possess the ability to pass their microbiome to the adult life stage through metamorphosis. If this is so, the environment in which the nymph develops may have an effect on the adult microbiome. In this study, the gut microbiomes of 13 species of dragonfly were compared across life stages and when collected from environments at different levels of urbanization. The gut of each dragonfly was removed, DNA extracted, and a portion of the bacterial 16S rRNA gene amplified and sequenced. Gut suspensions were also plated on antibiotic amended plates to determine the potential for dragonflies to contain antibiotic resistant bacteria. Gut microbiomes of dragonflies mainly separated by life stage, with nymphs further separating by the environment from which they were collected from. Dragonfly species was not a significant factor in the separation of either nymph or adult microbiomes. The microbiomes of nymphs and adults differed in levels of their dominant bacterial phyla, with Proteobacteria being dominant in adults, while nymphs showed a higher proportion of Acidobacteria and Bacteroidetes compared to adults. Nymphs also contained bacteria phyla that were not present in the adult microbiome. Both life stages contained antibiotic resistant bacteria, with the guts of dragonfly adults having higher counts of resistant bacteria than nymphs. The environment from which the dragonflies were collected had a significant influence on the counts of resistant bacteria for multiple antibiotics, as did dragonfly species. These results suggest that the gut microbiomes of dragonfly nymphs and adults are fundamentally different, and that both life stages have the potential to contain antibiotic resistant bacteria. The local environment influences
both the numbers of these antibiotic resistant bacteria and the composition of the gut microbiome in general.
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CHAPTER I:
EFFECTS OF LIFE STAGE, SITE AND SPECIES ON THE DRAGONFLY GUT MICROBIOME

INTRODUCTION

An animal’s microbiome plays a major role in the health and fitness of the host (Mueller & Sachs, 2015; Lloyd-Price et al., 2016). A healthy microbiome can improve longevity and reproduction, while an altered microbiome can increase the chance of disease and death. As DNA sequencing methods have increased in affordability and ease of use, microbiome analysis has moved outside the focus of human health and into other areas of biology, including entomology (Dillon & Dillon, 2004). The microbiomes of insects have been analyzed for a variety of reasons, including conservation, and pest/disease management (Crotti et al., 2012). From a broader perspective, insects are among the most diverse and abundant animals and they play key roles in ecosystems (Price et al., 2011). Insects occupy a variety of habitats and the insect microbiome may, at least in part, be dependent on their specific environmental location (Yun et al., 2014). The microbiome of an insect can also depend on its specific lifecycle: holometabolous insects complete a full cycle of metamorphosis from egg to larvae to pupae to adult; hemimetabolous insects develop from egg to nymph to adult, skipping the pupae stage (Price et al., 2011). Insects can show gut microbiome profiles that are specific to each life stage,
as has been shown in mosquitoes (Wang et al., 2011). Depending on the particular insect species, different developmental stages can occupy different environments; for example, many insects have aquatic juvenile stages and terrestrial adult forms. The impacts of this on the gut microbiome have rarely been examined.

Dragonflies and damselflies (Order: Odonata) are hemimetabolous but juveniles and adults experience different lifestyles. The juvenile nymph stage can live in water for up to four years, feeding on other aquatic animals and molting as they grow. After the final molt, the dragonfly emerges as a terrestrial adult that can live for up to a year, feeding primarily on smaller flying insects (Glaser, 2007). The few studies that have examined bacterial communities associated with dragonflies have focused on the gut microbiome of adults, and often used culture-based approaches (Schilder & Marden, 2007; Yun et al., 2014; Nair & Agashe, 2016; Deb et al., 2018). Adult dragonfly gut microbiomes may be more diverse than that of other carnivorous insect groups (Deb et al., 2018) and geographic location and season can explain much of the variation in the composition of the adult microbiome (Nair & Agashe, 2016; Deb et al., 2018). However, little is known about how the dragonfly gut microbiome might differ between the nymph and adult life stages, or between dragonfly species.

Habitat degradation is a known disturbance for aquatic insects (Dolný et al., 2012), but whether the degree of human development around an ecosystem influences the aquatic insect microbiome is unknown. Similarly, it is unknown if habitat-driven variation in the microbiome of dragonfly nymphs would be carried into the adult stage, as no study has compared the gut microbiomes of nymphs and adults of the same species. Dragonflies do not go through the non-feeding pupae stage that results in substantial microbiome changes in other insects (Minard et al., 2013), and nymphs and adults are both carnivorous, which may mitigate the effects that diet can have on the gut microbiome (Swei & Kwan, 2017). Thus, it is possible that either the whole or
parts of the nymph gut microbiome could be retained in adult dragonflies. Regardless, the specific habitat occupied by the nymph is likely to be important, and nymphs occupy a wide range of habitats, including burrowing in sediment or hiding in organic matter in both ponds and rivers (Glaser, 2007). Dragonfly nymphs can be found in both disturbed and pristine habitats, and they are typically more tolerant of pollution than other aquatic insect nymphs (Hodkinson & Jackson, 2005).

In this study we compared the gut microbiomes of 13 species of dragonfly nymphs and adults collected at five different sites across north Mississippi and Tennessee, USA. Sites varied in their degree of urbanization, and we hypothesized that dragonfly microbiomes would be more influenced by site than by life stage or dragonfly species. Our findings suggest that all three of these factors play a role in influencing the gut microbiome of dragonflies.

METHODS

Site Selection

Dragonflies were collected from five sites in northern Mississippi and southern Tennessee, USA. Sites were selected based on levels of potential human impact because of the degree of urbanization and use associated with each site. Sites with high levels of urbanization or human use were considered high impact, while sites in more rural locations were considered low impact. The University of Mississippi Field Station (UMFS; 34°25'05.6"N, 89°23'32.3"W) near Oxford, MS, is a 307-hectare site with minimal human disturbance and no urbanization. UMFS was originally a fish hatchery and is now used by the University of Mississippi as an ecological research site. Camp Lake Stephens (CLS; 34°18'40.7"N, 89°28'31.3"W), Oxford, MS, is a 35-hectare camp with minimal urbanization. The site contains buildings used for housing, dining and other camp associated activities. CLS hosts occasional youth events throughout the year, and
weekly youth camps during June and July. The third site was a treatment reservoir at a wastewater treatment plant (TP) in Oxford, MS (34°16'36.7"N, 89°31'01.4"W) that is no longer in use. The site has a history of use as a retention pond and is located next to a busy highway and soy cropland. Two sites were located in Memphis, TN: Beaver Lake (35°08'32.2"N, 89°49'17.3"W) is located in Shelby Farms (SF; a 1821-hectare park with multiple outdoor activities). This site is downstream of horse barns and subject to moderate agricultural impact, as well as heavy use by park visitors. Wolf River Greenway (WRG; 35°07'40.9"N, 89°51'11.1"W) is an urban site downstream of Memphis Baptist Memorial Hospital and collects runoff from major roads and residential areas.

**Dragonfly Collection and Processing**

Nymphs were collected from the five sites between January and April 2018 and adults were collected between May and June 2018. Ten individuals of each life stage were collected from each site, for a total of 50 nymphs and 50 adults. No attempt was made to identify individuals to species during collection. Each individual was netted and placed in a sterile plastic bag then placed on ice for transportation to the lab. The particular aquatic microhabitat that each nymph was collected from was noted and designated as sediment (in which the nymph was buried in the sediment), leaf litter (in which the nymph was found in submerged, decaying leaves), or littoral (in which the nymph was collected from aquatic vegetation along the littoral edge). Adults were collected aerially or when perching so had no microhabitat to record.

Individuals were processed within 24 h of collection. Dragonflies were measured for length and weight, then surface sterilized using 70% ethanol and the gut tract removed. The gut was vortexed in 1 ml sterile saline (0.8% NaCl) at maximum speed for 10 minutes. The mixture
was then centrifuged (10,000 xg, 15 minutes) to form a pellet of tissue and cells. The supernatant
was removed, leaving only the pellet, which was used for DNA extraction.

**DNA extraction and Amplification**

DNA was extracted from the pellet using a DNeasy PowerSoil Kit and protocol (Qiagen,
Germantown, MD). Purified DNA underwent barcoded amplification targeting the V4 region of
the 16S rRNA gene (Kozich et al., 2013; Jackson et al., 2015). Amplification products were
standardized with SequalPrep Normalization Plates (Life Technologies, Grand Island, NY) and
pooled prior to sequencing. The cleaned and pooled samples were sequenced using the Illumina
MiSeq platform at the Molecular and Genomics Core Facility at the University of Mississippi
Medical Center (UMMC). DNA was also used to amplify a portion of the CO1 gene for
dragonfly species identification. Odonate specific primers were based on those developed by
Karthika et al. (2012),

\[ \text{OdoF1}_\text{t15}' \text{TGTAAAACGACGGCCAGTATTCAACHAATCATAARGATATTGG3'} \text{and} \]

\[ \text{OdoR1}_\text{t15}' \text{CAGGAAACAGCTATGACTAAACTTCTGGATGYCCRAARAAYCA3'}. \]

CO1 amplification followed methods developed by Karthika et al. (2012). Amplification products
were purified and sequenced through a commercial provider (Functional Biosciences, Madison,
WI).

**16S rRNA Gene Sequence Analysis**

Raw data files (FASTQ) were processed using the Mothur bioinformatics pipeline
version 1.40.5 following methods recommended by Schloss et al. (2011) and Kozich et al.
(2013). Sequences were aligned to the SILVA database (version 128) and classified according to
the RDP database release 16. Erroneous sequences including chimeras, and mitochondria and
chloroplast sequences were removed. Analyses of alpha and beta diversity used operational taxonomic units (OTUs) defined by 97% sequence similarity and by subsampling (1000 iterations) the number of reads to that in the lowest remaining sample (453 sequences following removal of OTUs defined by just one or two sequence reads). Beta diversity was assessed using the abundance-based Bray Curtis dissimilarity index, which was used to ordinate samples using non-metric multidimensional scaling (NMDS). Analysis of similarity (ANOSIM) was used to determine if dragonflies were influenced by site, microhabitat, life stage or species. Analysis of variance (ANOVA) using R version 3.0.2 was used to determine if species, life stage, site, or habitat had any influence on species richness (alpha diversity) of the gut microbiome.

**CO1 Gene Analysis**

FASTA files from CO1 sequencing were trimmed to retain confirmed bases and compared to those in GenBank (BLAST searches in January 2019) to determine dragonfly species identity. The criteria used to determine species was based on the top three matches received from BLAST results, based on a BLAST “Ident” percentage of 96 or higher.

**RESULTS**

A total of 100 dragonflies were sampled, however, 13 yielded low numbers of 16S rRNA gene sequence reads (see below) and were excluded from further analyses. Of the remaining 87 individuals, CO1 gene sequencing identified them as belonging to 13 species, with eight species being represented by both nymphs and adults (Table 1). Three adults (A15, A23, A34) showed poor CO1 sequencing and were unable to be identified to dragonfly species (Table 1); these were removed from species-focused data analyses but retained for site or life stage analyses.
Table 1: Individual dragonflies collected for gut microbiome analysis from five aquatic sites representing a range from low to high human impact (UMFS, CLS, TP, WRG, SF). Dragonflies were adults caught as they perched on vegetation or nymphs collected from the littoral edge, leaf packs, or burrowed in sediment. Dragonfly species were identified by partial sequencing of the CO1 gene.

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The number of dragonfly species collected at each site varied for each life stage and finding a species as an adult at a given site did not relate to its presence in the nymph life form (Table 1). More species were found as nymphs (13) than adults (eight; Table 1). Camp Lake Stephens, one of the more rural sites, had the highest number of species collected (eight), with *Erythemis simplicicollis* and *Celithemis elisa* being the most common (four individuals of each). The Shelby Farms site and the abandoned Treatment Pond had the fewest dragonfly species (five at each). Across all sites, *E. simplicicollis* was the most collected species (19 individuals) followed by *Libellula luctuosa* and *Pachydiplax longipennis* (15 and 13 individuals, respectively). Only one individual of each of *Tetragoneuria cynosura*, *Erythrodiplax fusca*, and *Ladona deplanata* was collected across all sites (Table 1).

Thirteen dragonflies gave low numbers of 16S rRNA gene sequence reads, and rarefaction curves showed inadequate sequencing depth for these individuals. These individuals were removed from the dataset and the remaining 87 dragonflies all had >500 valid bacterial 16S rRNA gene sequences recovered. These 87 dragonflies yielded a total of 265,953 16S rRNA gene sequences, of which 3,345 were identified as potential chimeras and were removed. Thus, the final microbiome dataset consisted of 262,608 valid bacterial sequences, or a mean of 3,018 sequences per individual (ranging from 503 to 27,053). 83% of these sequences were identified as representing 33 different bacterial phyla, while 17% were unclassified Bacteria. Four phyla (Proteobacteria, Firmicutes, Bacteroidetes, and Acidobacteria) accounted for 73% of all sequences recovered. Both adults and nymphs showed high proportions of Proteobacteria (Fig. 1A, B) but they differed in the occurrence of subphyla (Fig. 1C, D). The gut communities of adults yielded more sequences identified as Gammaproteobacteria, especially those at the two sites within the city of Memphis (Shelby Farms and Wolf River Greenway) for which Gammaproteobacteria accounted for almost all of the Proteobacteria sequences obtained.
The nymph gut microbiome showed more variation among the subphyla that made up the Proteobacteria, and typically had greater percentages of the community comprised of Alphaproteobacteria and Betaproteobacteria than adults (Fig. 1C, D). An exception were nymphs collected from Shelby Farms, for which Gammaproteobacteria accounted for almost all of the Proteobacteria in their microbiome (Fig. 1C). Proportions of other bacterial phyla also varied by dragonfly life stage. Firmicutes was the second most commonly occurring bacterial phylum among adult dragonflies, while for nymphs, Bacteriodetes was the next most commonly detected phylum after Proteobacteria (an exception being nymphs collected from Shelby Farms, which exhibited high percentages of Firmicutes; Fig. 1B). Verrucomicrobia, Planctomycetes, Chloroflexi, Acidobacteria and Actinobacteria all tended to be more prevalent in nymphs compared to adults, and nymphs also tended to have more variation in the major phyla comprising their microbiome (Fig. 1).

When grouped by dragonfly species, there were no consistent patterns in the proportions of specific bacterial phyla in the nymph and adult gut communities (Fig. 2). However, there was variation in the composition of the microbiome within species, largely based on site (Fig. 1). For example, *L. luctuosa* and *Plathemis lydia* nymphs that were acquired from Shelby Farms had higher proportions of Firmicutes in their gut microbiome than individuals of the same species found at other sites (Fig. 2B).

Sequences grouped into 8,656 OTUs based on 97% sequence similarity. 5,571 of these OTUs were represented by just one or two sequence reads, and were removed prior to further analyses of community similarity and diversity, retaining 3,085 OTUs (from a total of now 255,686 sequences). Of these 3,085 OTUs, five OTUs represented 133,901 reads (52% of the dataset). Four of these prominent OTUs were identified as being members of the Gammaproteobacteria, three from order Enterobacteriales (accounting for a combined 60% of
the adults sequences and 21% of nymphs) and one from Aeromonadales (accounting for 5% of adult sequences and 16% of nymph sequences; Table 2). Two of the OTUs identified as Enterobacteriaceae (OTU0001, 0004) represented over 50% (56,258 reads) of the adult sequences, while these OTUs were much scarcer (2.5%) in the nymph dataset (Table 2). Other abundant OTUs were identified as belonging to phylum Firmicutes, represented by orders Lactobacillales and Clostridiales, phylum Chlamydiae, represented by order Chlamydiales, and phylum Fusobacteria, represented by order Fusobacteriales. In total, the nymph sequences grouped into 2,336 OTUs (from a total of 154,725 reads) while adult sequences grouped into 954 OTUs (from a total of 100,961 reads).

In terms of overall bacterial community composition, the dragonfly gut microbiome primarily separated by life stage (Fig. 3A, ANOSIM p<0.001). The microbiome of adults did not separate by sample site (Fig. 3B, ANOSIM p>0.05), whereas the nymph gut microbiome separated by both site and microhabitat (Fig. 3C, D, ANOSIM p<0.001 for each). Species was not a significant factor in determining either the nymph or adult microbiome (Fig. 3 E, F, ANOSIM p > 0.05) although there was some suggestion of a species effect for nymphs but that was not significant (Fig. 3E, ANOSIM p>0.056).

When standardized by subsampling to the same number of sequences, observed species richness (S) of the dragonfly gut microbiome varied across life stage, species and site (ANOVA, p<0.001 for all factors). Interactions between these variables were also significant, with the interaction between site and life stage being the most significant (p<0.0001), followed by the interactions between site and species (p<0.004), and life stage and species (p<0.02). Nymphs had a more species rich gut bacterial community than adults at all sites, although this richness varied between sites (Fig. 4A, B). There was also significant variation in richness based on the nymph’s microhabitat (p<0.0001, Fig. 4C), with the gut microbiomes of nymphs found along the littoral
edge being more species rich than those collected from leaf packs or sediment. Both nymphs and adults had higher levels of richness across the rural sites (University of Mississippi Field Station, Camp Lake Stephens) compared to more urban sites (Shelby Farms, Wolf River Greenway; Fig. 4A, B), and even adults at the abandoned Treatment Pond had richer gut communities that those at Shelby Farms and Wolf River Greenway (Fig. 4B). Dragonfly species varied in the richness of their gut communities for both adults and nymphs (Fig. 4D, E). Adults and nymphs of *L. luctuosa, P. longipennis*, and *L. quadrimaculata* tended to have more species rich gut communities than other species, while *P. lydia* had varying levels of richness. Of the 16 *E. simplicicollis* adults, three showed more species rich gut communities than the other adults, while the nymphs showed overall richer gut communities than the adults. Within species and life stage, *Anax imperator* nymphs showed a strong, significant negative correlation with body length and species richness (*r* = -0.93, *p* = 0.006) and body weight and species richness (*r* = -0.87, *p* = 0.02). *E. simplicicollis* adults showed a significant, positive correlation between weight and species richness (*r* = 0.55, *p* = 0.03). No other specific life stage and species combinations showed significant correlations with body weight or length.

Because some dragonfly species were only sampled as adults or nymphs, there is the potential for effects of species and life stage to be confounded. Thus, we ran a reduced model containing only species for which both nymphs and adults were sampled (71 individuals, eight species). This analyses yielded similar results such that microbiome species richness varied significantly across all variables (site, *p* < 0.0001; life stage, *p* < 0.0001; species, *p* = 0.004) and across all interactions (site x life stage, *p* < 0.0001; site x species, *p* = 0.0003; and species x life stage, *p* = 0.02).
Figure 1: Major Bacteria Phyla of Dragonflies. Major bacterial phyla identified in the gut microbiomes of dragonflies, as separated by life stage (A: adults, B: nymphs). Phyla were identified by partial 16S rRNA gene sequencing and “Other” represents a total of 23 other phyla. Percentages of the major subgroups of Proteobacteria are also shown for adults (C) and nymphs (D). Dragonflies were collected from five different sites in Mississippi and Tennessee, USA, that varied in their degree of human impact (UMFS, CLS, TP, WRG, SF).
Figure 2: Proteobacteria Subphyla of Dragonflies. Major bacterial phyla identified in the gut microbiomes of 13 species of dragonflies, separated by life stage (A: adults, B: nymphs). Phyla were identified by partial 16S rRNA gene sequencing and “Other” represents a total of 23 other phyla. Dragonflies were collected across five sites in Mississippi and Tennessee, USA, and species numbers represent 1: Anax imperator, 2: Anax junius, 3: Celithemis elisa, 4: Celithemis eponina, 5: Erythemis simplicicollis, 6: Erythrodiplax fusca, 7: Ladona deplanata, 8: Libellula luctuosa, 9: Libellula quadrimaculata, 10: Pachydiplax longipennis, 11: Plathemis lydia, 12: Sympetrum corruptum, 13: Tetratoneuria cynosure.
Table 2: Abundant operational taxonomic units (OTUs) of bacteria in the gut microbiomes of 13 species of dragonflies collected from five sites in Mississippi and Tennessee, USA. Dragonflies were collected as both nymphs and adults. Size is the total number of reads per OTU, and % Total reads, % Adult reads, and % Nymph reads is the percentage that given OTU represents in those datasets. One dragonfly adult accounted for 99% of the sequences of Otu0006 found.

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Figure 3: NMDS of Dragonfly Microbiomes. Nonmetric multidimensional scaling ordinations based on Bray Curtis dissimilarity showing patterns in the gut microbiomes of dragonflies based on A: adults (A) and nymphs (N; stress=0.26), B: adults separated by sample sites with different levels of human impact (WRG>SF>TP>CLS>UMFS; stress=0.20), C: nymphs separated by site.
(stress=0.24), D: nymphs separated by microhabitat (stress=0.24), E: nymphs separated by
dragonfly species (stress=0.20), and F: adults separated by species (stress=0.20). The effects of
life stage (nymph vs. adult) was significant, as was the effect of site and microhabitat on nymphs
(ANOSIM, p<0.0001 for each). Species were identified as Ai: *Anax imperator*, Aj: *Anax jenius*,
Ce: *Celithemis elisa*. Cep: *Celithemis eponina*, Es: *Erythemis simplicicollis*, Ef: *Erythrodiplax
fusca*, Ld: *Ladona deplanata*, Ll: *Libellula luctuosa*, Lq: *Libellula quadrimaculata*, Plo:
cynosure* (n=1-23 for each).
Figure 4: Observed Species Richness of Dragonfly Gut Microbiomes. Observed species richness (S) across dragonfly gut microbiomes, as measured in operational taxonomic units detected by subsampling 453 sequences from each sample over 1,000 iterations. S is represented by boxplots with quartiles, including median line, outliers (circles) and whiskers representing the minimum and maximum S for each sample type. Species richness was compared in the context of A: dragonfly nymphs at sites with different levels of human impact (WRG>SF>TP>CLS>UMFS; n=8-10 individuals per site), B: dragonfly adults at the different sites (n=6-10 individuals per site), C: nymphs collected from different microhabitats: leaf litter (n=10), littoral edge (n=27), and sediment (n=10), D: adults separated by dragonfly species, and E: nymphs separated by dragonfly species. Species were identified as: 1: Anax imperator, 2: Anax junius, 3: Celithemis elisa, 4: Celithemis eponina, 5: Erythemis simplicicollis, 6: Erythromela fusca, 7: Ladona deplanata, 8: Libellula luctuosa, 9: Libellula quadrimaculata, 10: Pachydiplax longipennis, 11: Plathemis lydia, 12: Sympetrum corruptum, 13: Tetraneuraria cynosure (n=1-23 for each).
DISCUSSION

Our aim was to compare the gut microbiome of nymph and adult dragonflies, as well as to determine how the microbiome varies between dragonfly species and across habitats with different levels of urbanization and human use. Although the number of studies on insect microbiomes are increasing (Lewis & Lize, 2015), few studies have compared the insect microbiome across life stages. This is the first study investigating the gut microbial communities of dragonfly nymphs compared to those of adults, while also characterizing microbiome variation across habitat and dragonfly species.

Dragonfly microbiomes were dominated by Proteobacteria, which is consistent with results of a similar study on adult dragonflies (Nair & Agashe, 2014), as well as studies on adult butterflies and honey bees (Hamdi et al., 2011; Hammer et al. 2014). The microbiomes of adult dragonflies showed a particularly high proportion of Gammaproteobacteria, similar to a large-scale study characterizing the microbiomes of multiple groups of insects (Jones et al., 2013). Although nymphs showed the presence of Gammaproteobacteria, their microbiome also contained appreciable proportions of Alphaproteobacteria and Betaproteobacteria. There have been few prior studies on the gut microbiomes of insect nymphs, although the nymphs of European firebug showed similar proportions of Alphaproteobacteria and the presence of Betaproteobacteria (Sudakaran et al., 2012). Betaproteobacteria are not typically found as a major component of the insect gut microbiome but have been reported for some insects such as Melolontha hippocastani and Anopheles gambiae larvae (Wang et al., 2011; Arias-Cordero et al., 2012). Other dominant phyla varied across life stage, with adults typically having more Firmicutes and nymphs having higher numbers of Bacteroidetes. These findings are consistent with those of research analyzing the gut microbiomes of beetle life stages (Arias-Cordero et al., 2012; Menchaca et al., 2013), although some studies report higher proportions of Bacteroidetes.
among adult insects (including flies, froghoppers, leafhoppers, moths, and ants) than observed here (Wang et al., 2011; Jones et al., 2013).

Specific OTUs, rather than bacterial phyla, provide more information on the potential roles of members of the gut microbiome. Three of the four most abundant OTUs were members of the Enterobacteriaceae, and this family includes enteric bacteria such as *Escherichia coli* and *Enterobacter sp.* While often indicative of human impacts on aquatic systems, other members of this family include insect pathogens, such as *Photorhabdus luminescens*. Other members of the Enterobacteriaceae are symbionts within insects and contribute a nutritional benefit or provide the insect with a defense against gut colonization by pathogens (Dillon & Dillon, 2004; Moran et al, 2005; Rajagopal, 2008, Duchaud et al, 2011). The family has been found previously in adult dragonflies, with some dragonfly species showing greater proportions of Enterobacteriaceae in their gut microbiome than others (Deb et al, 2018). The roles of members of the Enterobacteriaceae in dragonfly nymphs and adults is unclear but the high prevalence of this family suggests more than passive acquisition from the environment.

Another proportionally abundant OTU, identified as the genus *Aeromonas*, was found in 73% of our dragonflies, although other analyses of the adult dragonfly gut microbiome have found this genus to be less common (Nair & Agashe, 2016). *Aeromonas* includes species that can be symbiotic or pathogenic to insects, and a mutualistic relationship has been found between *Aeromonas* bacteria and aquatic chironomid larvae, with evidence suggesting that *Aeromonas sp.* protect their host from toxic metals (Laviad & Halpern, 2016). Whether dragonflies have such a relationship with a specific *Aeromonas* species is unknown, as this has not been studied.

Sequences identified as *Wolbachia* (phylum Alphaproteobacteria, family Rickettsiaceae) have been previously reported in the gut microbiome of adult dragonflies (Deb et al., 2018) but only 265 sequences (0.10% of the total) were identified as *Wolbachia* in our study, and only from one
adult Pachydiplax longipennis dragonfly from UMFS (making up 49% of the hosts total sequences). *Wolbachia* are insect pathogens that manipulate the host through a variety of methods targeting reproduction, although they may also provide some protection against viral infections (Werren, 1997; Hedges et al, 2008). That members of this genus have been found in other dragonfly species and in other environments, but were scarcely detected here, suggests site- or species-specific patterns in their distribution.

Dragonfly microbiomes differed by life stage, a phenomenon that has been reported for other insects, especially when those life stages occupy different habitats (Wang et al., 2011; Arias-Cordero et al., 2012). Juvenile insects have been found to have higher species richness in their gut microbiomes than adults, which is consistent with our findings. Studies on *M. hippocastani* and *A. gambiae* gut microbiomes found that juvenile forms that were submerged (in either an aquatic environment or in soil) contained a more diverse gut community (Wang et al., 2011; Arias-Cordero et al., 2012). It is possible that insect nymphs or larvae retain higher gut bacterial diversity because they are constantly submerged in a medium that contains a diverse mix of potential bacterial inoculants, although no research has been done to confirm this.

The level of urbanization and land use had a significant influence on the gut microbiome, especially for nymphs, and site has been found to influence the microbiome of other insects, such as honey bees (Yun et al., 2014). The microbiomes of nymphs and adults collected from the Shelby Farms site that receives run off from a horse farm showed the lowest species richness, and microbiome richness was also low for dragonflies collected from site the Wolf River Greenway site downstream from a hospital and subject to road runoff. Other studies have shown shifts in the gut microbial communities of aquatic organisms when they are exposed to high levels of environmental contamination or collected from polluted sites (Hacioglu & Tosunoglu, 2004; Gaulke et al., 2016; Carlson et al., 2017), and disturbance decreased the overall diversity.
of the oyster gut microbiome, primarily through a loss of rare phylotypes (Wegner et al., 2013). These studies and ours suggest that the gut microbiomes of invertebrates in sites that are subjected to higher urbanization are likely to differ from, and be less diverse than, those from rural sites.

At a finer scale, the particular microhabitat from which a nymph was collected also influenced the richness of their gut microbiome. The microbiomes of nymphs collected from sediment had the lowest richness and those collected along the littoral edge had the highest. While habitat has been found to have an impact on the gut microbiome of insects (Yun et al., 2014), this has been previously assessed at geographic scales and there is little to no research on how microhabitat can influence the microbial communities of individuals occupying the same location. The nature of the microhabitat or substratum can affect the ability of dragonfly nymphs to capture prey (Folsom & Collins, 1984) so that microhabitat differences might relate to differences in diet, and diet has been shown to influence the gut microbiome of adult dragonflies (Deb et al., 2018). For some species of dragonfly, different instars of nymph inhabit different microhabitats (Cherrill & Brown, 1992), so that microhabitat differences could also relate to host age, which could in turn influence the composition of the gut microbiome within the general nymph life stage.

Gut microbiomes of dragonflies showed substantial variation within the particular life stage of each species, likely representing the influences of site and microhabitat. However, even within different individuals of a species collected from the same site and habitat there was appreciable variation. Different species of adult dragonflies have been reported as having distinct gut bacterial communities (Nair & Agashe, 2014; Deb et al., 2018), but while species was a significant influencer of gut bacterial richness in this study, no individual species showed a
distinct gut microbial profile. Rather, the local environment as determined by both site use and microhabitat appeared to be a stronger determinant of the microbiome, particularly for nymphs.

Host diet can influence the composition of the gut microbiome of insects (Broderick et al., 2004; Chandler et al., 2011; Yun et al., 2014) and seasonal variation in prey availability can be an important determinant of the microbiome of adult dragonflies (Deb et al., 2018). Similar diet-driven seasonal changes in the gut microbiome have also been reported for mammals (Maurice et al., 2015). We did not assess the effects of seasonality or diet in this study, in part because nymphs and adults had to be sampled in different seasons based on the organism’s life history. However, each life stage was collected within a particular time of year (winter/early spring for nymphs, spring/early summer for adults) so any seasonal affects within a life stage should be minimized. While seasonal patterns in the availability of prey may not have played a role in this study, differences in the availability of prey between sites could have. Habitat degradation from urbanization or heavy use can result in changes in food availability and therefore diet in mammals, which can in turn influence the gut microbiome (Amato et al., 2013). The significant site influence on dragonfly microbiomes could be a product of species loss and thus different prey availability at the more human-impacted sites. The availability of prey or other food is rarely considered when assessing spatial patterns in gut microbiomes between sites or habitats but could be an explanation for geographic variation in microbiome composition, as well as differences between particular microhabitats.

This study is one of few to show how life stage is a major driver of the gut microbiome of insects, and we found that the type of land use exerts a strong influence on the microbiome of dragonfly nymphs and less of an effect on adults. Aerial adults likely travel over broader ranges than nymphs, potentially limiting the effects of site, and nymphs are also continually exposed to a local bacterial community in the water they inhabit. Unexpectedly, species was not as dramatic
a factor in influencing the gut microbiome of dragonflies, but this may be a limitation of the experimental design, which was more focused on elucidating life stage or site-related differences. The finding that dragonfly nymphs and adults have significantly different microbiomes brings up an interesting question as to what really is the microbiome of an organism that has substantially different life stages. For mammals there is a tendency to view the adult as having the mature microbiome (Yatsunenko et al., 2012), but in insects such as dragonflies the adult form may be relatively short-lived compared to living up to four years as a nymph (Glaser, 2007). While they may be the same species, nymphs and adults are essentially different holobionts. They inhabit entirely different environments and, as shown here, have fundamental differences in their gut microbiome and how it is influenced by habitat variability.
CHAPTER II: DRAGONFLY NYMPHS AND ADULTS AS RESERVOIRS FOR ANTIBIOTIC RESISTANT BACTERIA

INTRODUCTION

Increased use of antibiotics has led to higher levels of antibiotic contamination entering aquatic ecosystems, and, in turn, increased the load of antibiotic resistant bacteria in animals that live in or frequent these environments (Pathak & Gopal, 2005; Manaia et al., 2016). Antibiotics select for resistance genes in bacteria by inhibiting the growth of susceptible bacteria and allowing resistant strains to be more competitive (Bírošová et al., 2014). Many of these resistance genes are plasmid-borne and can be transferred between bacterial species, allowing resistance to become more prevalent in a bacterial community (Sørensen et al., 2005). Although most bacteria in aquatic environments are non-pathogenic to humans or other mammals, there is the potential for plasmids that these bacteria carry to spread to pathogens (Alexander et al., 2015).

Antibiotics can enter aquatic ecosystems from various sources including urban pollution and agricultural runoff (McManus et al., 2002; Krummer & Henninger, 2003). Antibiotics in waste water are not fully removed by treatment plants, resulting in low to moderate levels of contamination in the effluent (Watkinson et al, 2007). Once in an aquatic system, antibiotics can influence the microbial communities in water, sediment and even the microbiomes of aquatic organisms (Park & Choi, 2008; Li et al, 2012). Antibiotics can be taken up by aquatic invertebrates and alter the composition of their gut bacterial communities (Basu et al., 2010;
Meredith-Williams et al., 2012; Pennington et al., 2015). Of particular interest are the aquatic juvenile forms of various insects that subsequently develop into terrestrial and migratory adults (Daly, 1998). If resistant bacteria present in aquatic juveniles are retained following transformation into the adult, then such organisms present a potential mechanism for the spread of antibiotic resistance over large geographic scales. However, little research has been done to identify if antibiotic driven selection on bacterial communities in juvenile insects leads to increased numbers of antibiotic resistant bacteria in adults.

One group of insects that is particularly amenable to study this phenomenon are dragonflies (Odonata, infraorder Anisoptera). Dragonfly juveniles, or nymphs, are aquatic and live in water for up to four years (Glaser, 2007). Nymphs inhabit both ponds and rivers, typically in free floating vegetation or buried in sediment (Glaser, 2007). Because nymphs are in constant contact with water or sediment for long periods of time, they could be particularly susceptible to increased presence of antibiotic resistant bacteria when antibiotic contamination is present. Dragonflies are hemimetabolous and do not go through the non-feeding pupa phase that has been shown to substantially change the microbiome of other insects (Wang et al, 2011). Thus, adult dragonflies may retain portions of the nymph bacterial community, suggesting a chance for the persistence of resistant bacteria from juvenile to adult. Adult dragonflies are migratory, sometimes flying over continental scales (Wikelski et al., 2006), so there is potential for dragonflies to disperse bacteria, including antibiotic resistant bacteria, over large areas. Dragonflies are also important in food webs, with both nymphs and adults preying on other insects, as well as being preyed upon by fish, birds and other dragonflies (Knight et al., 2005; Roberts, 2012). Because of these trophic interactions, resistant bacteria in dragonflies could be passed on to other organisms. While the spread of antibiotic resistant bacteria from prey to predator has not been investigated, birds, for example, can acquire pathogenic bacteria from their
food sources (Reed et al, 2003). Few studies have examined bacterial communities associated with dragonflies, none of which considered antibiotic resistance among those bacteria and all of which focused solely on the gut microbiota of adults (Yun et al., 2014; Nair & Agashe, 2016; Deb et al., 2018). To determine if dragonflies harbor antibiotic resistant bacteria, 13 species of dragonflies, both nymphs and adults, were collected from five sites in North Mississippi, and Memphis, Tennessee, USA that vary in levels of human impact and potential antibiotic contamination. Colony counts of gut bacteria resistant to the antibiotics tetracycline, vancomycin, amoxicillin, kanamycin, and cefazolin were determined for a total of 100 individuals.

METHODS

Site Selection

Sites were chosen for their potential levels of historic antibiotic contamination based on the extent of human impact in the form of urbanization and land use. Areas that had high levels of urbanization and/or human use were considered to have higher potential for antibiotic contamination. Areas considered to have less potential for antibiotic contamination were those found in rural settings with minimal use. The University of Mississippi Field Station (UMFS) in Oxford, MS, is a rural 307 hectare site with essentially no human disturbance or impact (34°25'05.6"N, 89°23'32.3"W). UMFS is used by the University of Mississippi as a research station but was once used as a fish hatchery. Camp Lake Stephens (CLS), Oxford, MS, is a 35 hectare camp with minimal urbanization and likely low levels of antibiotic contamination (34°18'40.7"N, 89°28'31.3"W). This site hosts a variety of activities throughout the year but sees most of its traffic during the summer months when it holds weekly summer camps during June and July. The third site is an old water treatment reservoir at a wastewater treatment plant (TP)
that was used until 2015 to treat wastewater but is no longer in use in Oxford, MS (34°16'36.7"N, 89°31'01.4"W). The retention pond once held contaminated water but was drained and allowed to fill with rainwater but no measures were taken to decontaminate the area. The site still experiences human impact from its location next to a highway and farmland. Two sites are located in the city of Memphi, TN, and have more potential for human impacts associated with urban areas. Wolf River Greenway (WRG) is downstream of Memphis Baptist Memorial Hospital and collects run off from major roads and residential areas (35°07'40.9"N, 89°51'11.1"W). Shelby Farms (SF) is a 1821 hectare park used for outdoor activities use and containing multiple animal areas (horses, American bison, wildlife). Dragonflies at this site were collected from Beaver Lake (35°08'32.2"N, 89°49'17.3"W), directly downstream of horse barns and subject to potential antibiotic contamination from agriculture.

**Dragonfly Collection and Processing**

Dragonflies were collected and processed using the methods detailed in Chapter I. Ten individuals of each life stage were collected from each site between January and June 2018, for a total of 50 nymphs and 50 adults. Individuals were placed in sterile bags on ice for transportation to the lab, where they were processed within 24 h of collection. Dragonflies were not visually identified to species but were identified by subsequent CO1 gene sequencing (see Chapter I). Dragonflies were surface sterilized using 70% ethanol and the gut tract removed. The gut was vortexed in 1 ml sterile saline (0.8% NaCl) at maximum speed for 10 minutes. Subsamples of the resulting suspension were used to determine counts of antibiotic resistant bacteria.
**Counts of Antibiotic Resistant Bacteria**

Counts of antibiotic resistant bacteria were determined from colony counts on agar plates amended with five different antibiotics, as well as regular agar. Subsamples of the saline suspensions were diluted 1:2 and 1:50 in trypticase soy broth and 0.1ml of each dilution was plated onto trypticase soy agar (TSA) containing no antibiotics, TSA + tetracycline (at 130 µg/ml), TSA + vancomycin (at 105µg/ml), TSA + amoxicillin (at 420 µg/ml), TSA + kanamycin (at 63 µg/ml), and TSA + cefazolin (at 12 µg/ml). Concentrations of each antibiotic were selected to be 3x the minimum inhibitory concentration for susceptible bacteria in order to account for increasing antibiotic resistance among bacteria (Andrews, 2001). These antibiotics are medically relevant, include some of high use (e.g. amoxicillin) and others (e.g. vancomycin) of more restricted use, and have various resistant and medically relevant gut bacteria associated with them (CDC, 2018). Plates were incubated at 37°C for 48 h after which colonies were counted for plates containing between 20-300 colonies. Counts of antibiotic resistant bacteria for each antibiotic were reported as colony forming units (CFUs) per mass of dragonfly.

**Data analysis**

Analysis of variance (ANOVA) was used to determine if there were significant differences in CFUs for each growth medium based on dragonfly species, life stage, or site (sitexlife stagexspecies). Tukey’s HSD was used to examine any specific differences for variables that were significant. To account for covariance among species and life stage, as well as species and site, a reduced model ANOVA (and Tukey’s HSD follow-up as needed) was performed that was limited to species that were found in both life stages and at more than one site. All analyses were conducted using R version 3.0.2.
RESULTS

One hundred dragonflies were used in this study, 50 nymphs and 50 adults, representing 13 different species (Chapter I, Table 1). All dragonflies yielded colonies on unamended media (TSA), while growth on antibiotic-amended media varied. Vancomycin plates consistently gave the most growth, but this was variable, ranging from 163 to 7,500,000 CFUs g\(^{-1}\) dragonfly. High levels of growth (ranging from 269 to 5,350,000 CFUs g\(^{-1}\) dragonfly) were also obtained using cefazolin treated plates and counts on cefazolin and vancomycin amended media were highly correlated with each other (R=0.89), and with counts obtained on TSA (R=0.80-0.83). The fewest colonies were observed on tetracycline plates and only 11 individual dragonflies, eight adults and three nymphs, yielded any colonies that were resistant to tetracycline. These dragonflies came from different sites, but five of the adults were the same dragonfly species, *Erythemis simplicicollis*. 62 individuals yielded colonies on plates treated with amoxicillin, with counts ranging from 147 to 1,136,363 CFUs g\(^{-1}\) dragonfly. Kanamycin resistant bacteria were detected in 36 individual dragonflies and CFUs for kanamycin plates ranged from 333 to 2,142,857 CFUs g\(^{-1}\) dragonfly. Other than the correlations reported above, relationships between numbers of antibiotic resistant bacteria on different media types were low (R<0.25), other than a positive correlation (R=0.50) between counts of amoxicillin- and cefazolin-resistant bacteria.

Prior to statistical analysis, four adults were removed from the dataset because CO1 sequencing did not give a clear species identification. For the remaining 96 individuals, life stage was only a significant predictor for counts of bacteria resistant to kanamycin (p=0.008), with nymphs from Shelby Farms and Camp Lake Stephens having higher numbers of kanamycin-resistant bacteria than adults, while adults had higher numbers at the other sites (Fig. 1). Site was a significant factor influencing the numbers of bacteria that were resistant to cefazolin (p<0.001), vancomycin (p<0.0001) and amoxicillin (p=0.0008). Site was also a significant factor for growth
on TSA (p<0.0001). Dragonflies from Shelby Farms, the site most likely impacted by human activity, had significantly higher counts of antibiotic resistant bacteria than the much less impacted Camp Lake Stephens for every antibiotic tested except tetracycline and kanamycin (both of which showed no significant influence by site; Fig.1). Dragonflies from Shelby Farms also had significantly higher counts of cefazolin (Fig. 1 C) and vancomycin (Fig. 1F) resistant bacteria than those collected from the University of Mississippi Field Station, the other low impact site, as well as higher counts of culturable bacteria on TSA. The two sites most likely to be impacted by human activity (Shelby Farms, Wolf River Greenway) did not significantly differ from each other in terms of growth on any plate type, but both had significantly higher numbers of colonies on TSA, vancomycin, and cefazolin than the Treatment Plant site. Nymphs from Shelby Farms yielded the greatest number of colonies for any site-life stage combination on TSA (Fig. 1A), amoxicillin (Fig. 1B), and kanamycin (Fig. 1D), and dragonfly adults from Wolf River Greenway gave the greatest number of colonies on cefazolin (Fig. 1 C) and vancomycin (Fig. 1F). While it was our least urban site, adult dragonflies from the Field Station, along with those from Wolf River Greenway, yielded the most tetracycline-resistant bacteria (Fig. 1E).

Dragonfly species (Fig. 2) was a significant variable for the numbers of culturable bacteria determined on TSA (p=0.0003), amoxicillin (p=0.028), cefazolin (p=0.0016), kanamycin (p=0.006) and vancomycin (p=0.0003), the latter antibiotic also showed a significant species x life stage interaction (p=0.02). *Sympetrum corruptum* and *E. simplicicollis* yielded the most growth on TSA, while *Anax junius* and *Ladona deplanata* yielded the fewest (Fig. 2A). *Libellula luctosa* gave the highest number of amoxicillin-resistant bacteria (Fig. 2B), but while there was an overall impact of species on counts of amoxicillin resistant bacteria, there were no significant pair-wise species combinations. *S. corruptum* yielded the highest counts of cefazolin resistant bacteria (Fig. 2C), but this was not significantly greater than counts from any other
species following post hoc Tukeys HSD. While species was a significant variable determining counts of kanamycin-resistant bacteria, there were no significantly different pairwise species combinations, although counts from *L. luctosa* were typically the highest (Fig. 2D). On vancomycin amended plates, counts from *E. simplicicollis* and *S. corruptum* were significantly greater than those from *A. junius* (Fig. 2F).

Although species was a significant influence for all media types except tetracycline, not every dragonfly species was represented by both nymph and adult life stages. Thus, we reanalyzed the data using a reduced dataset that consisted of dragonfly species for which both nymphs and adults were collected. This dataset consisted of 46 adults and 37 nymphs, representing eight species. Life stage was still only significant for counts of kanamycin resistant bacteria (*p*=0.01), with nymphs having higher numbers than adults. Site remained a significant influence on overall culturable bacterial counts on TSA (*p*<0.0001), as well as bacteria that were resistant to amoxicillin (*p*=0.002), cefazolin (*p*<0.0001), and vancomycin (*p*<0.0001). Species was still significant for numbers of bacteria obtained on TSA (*p*=0.001), cefazolin-resistant bacteria (*p*=0.001), kanamycin-resistant bacteria (*p*=0.001) and vancomycin-resistant bacteria (*p*=0.0003), but was no longer significant for amoxicillin-resistant bacteria.
Figure 1: Site and Lifestage Antibiotic Growth. Colony forming units (CFUs) of antibiotic resistant bacteria obtained from dragonfly nymphs (N) and adults (A), collected from sites subject to different levels of human impact. CFU counts were determined using: (A) unamended trypticase soy agar (TSA), and TSA amended with either (B) amoxicillin, (C) cefazolin, (D) kanamycin, (E) tetracycline, or (F) vancomycin, at 3x minimum inhibitory concentrations. Sites reflect a gradient of least human-impacted to highest impacted sites (UMFS-CLS-TP-WRG-SF). Bars represent means +/- standard error for 8-10 individuals for each site x life stage combination.
Figure 2: species antibiotic growth. Colony forming units (CFUs) measured in colonies g$^{-1}$ dragonfly separated by species. Plate types include: (A) CFUs on Trypticase Soy Agar (TSA) and TSA amended with either (B) Amoxicillin, (C) Cefazolin, (D) Kanamycin, (E) Tetracycline or (F) Vancomycin at 3x minimum inhibitory concentrations. Species include: Anax imperator (Ai), Anax junius (Aj), Celithemis elisa (Cel), Celithemis eponina (Cep), Erythemis simplicicollis (Es), Erythrodiplax fusca (Ef), Ladona deplanata (Ld), Libellula luctuosa (Ll), Libellula quadrimaculata (Lq), Pachydiplax longipennis (Plo), Plathemis Lydia (Ply), Sympetrum corruptum (Sc) and Tetragonuria cynosure (Tc). Bars represent +/- standard error for 1-23 individuals for each species.
DISCUSSION

This study provides a first look at the ability of dragonflies to harbor antibiotic resistant bacteria. Our aim was to identify if nymph and adult life stages harbored different levels of antibiotic resistant bacteria, and to determine if sites subject to more human impact or urbanization resulted in higher counts of resistant bacteria in aquatic insects. Kanamycin and tetracycline resistant bacteria, as well as bacteria resistant to antibiotics from the same class as others used in our study (cefotaxime, ampicillin), have been found in the feces of nymph and adult damselflies (close relatives of dragonflies) especially for individuals collected from urban environments (Yamaguchi et al., 2018). Damselflies also showed a higher occurrence of multidrug resistant bacteria when obtained from more urban environments, compared with those in more rural or pristine settings. The minimum inhibitory concentration of these bacteria was highest for ampicillin and kanamycin, meaning it required higher volumes of antibiotic to kill bacteria (Yamaguchi et al., 2018). The typical gut bacterial load for those damselflies, as determined on antibiotic-free media, was $10^8$ bacteria per individual, 10-100x higher than the average counts we obtained ($10^6$ for nymphs, $10^5$ for adults). However, those counts were determined from plated fecal matter, as oppose to a gut suspension, so the differences in numbers is not surprising as the fecal matter has a greater concentration of bacteria than a suspended gut solution. Non-biting midge larvae (*Chironomidae*) have also been found to harbor antibiotic-resistant bacteria, and counts determined from chironomid larvae collected from sewage drains had significantly higher levels of antibiotic resistant bacteria than those collected from a non-contaminated pond (Basu et al, 2010). Thus, it’s becoming clear that aquatic insects can harbor antibiotic resistant bacteria and that the numbers of these bacteria may be highly dependent on the particular environment.
For four of the five antibiotics examined in this study, counts of antibiotic resistant bacteria in adults were not significantly different from those in nymphs. Other studies on insects have found significant differences in the loads of resistant bacteria in adult and juvenile life stages, with adults having higher numbers (Wei et al., 2013). We predicted that nymphs would have higher loads of resistant bacteria relative to adults in environments with high levels of urbanization. Nymphs are fully submerged in an aquatic environment and more likely to be exposed to both antibiotics and antibiotic resistant aquatic bacteria. However, adults could acquire antibiotic resistant bacteria from other sources, including their diet. Diet can have a major impact on the microbiome of adult dragonflies (Nair & Agashe, 2016; Deb et al., 2018) and other organisms can acquire antibiotic resistant bacteria from their food (Reed et al., 2003; Ahmad et al., 2011). Adult dragonflies move more widely than nymphs, potentially acquiring bacteria from multiple environments (Glaser, 2007). In this study, however, counts of antibiotic resistant bacteria did not differ between nymphs and adults, while site urbanization and species were much more important than life stage in determining bacterial load.

The highest counts of resistant bacteria were determined for cefazolin and vancomycin, and numbers of bacteria resistant to those antibiotics were also correlated with overall culturable numbers derived from TSA. Vancomycin is a glycopeptide antibiotic that is a Gram positive bacteria inhibitor, while cefazolin is a cephalosporin antibiotic used to combat Gram positive bacteria and some Gram negative bacteria (Krummer, 2009). As both of these antibiotics primarily target Gram positive bacteria, they may be selecting for similar resistant bacteria. However, the insect gut microbial community is diverse (Dillon & Dillon, 2004), and it is possible that there are many resistant strains. Interestingly, while amoxicillin is another antibiotic that primarily targets Gram positive bacteria, counts of amoxicillin resistant bacteria were much lower and more varied than for either cefazolin or vancomycin.
The species of dragonflies collected in this study showed differences in the numbers of antibiotic resistant bacteria found in their gut. We were able to compare counts in 13 species (including eight that were present as both nymphs and adults at multiple sites), more than other studies that have assessed environment driven changes in the microbial community of insects by focusing on one or two species (Pai et al, 2005; Wei et al, 2013; Pennington et al, 2015). Because species may be cofounded by the variables of site and life stage, a reduced model that only incorporated species sampled of both nymphs and adults and at least two sites still showed significant species effects. *S. corruptum* and *E. simplicicollis* had the highest overall counts on TSA, and also harbored the most bacteria that were resistant to cefazolin and vancomycin. Neither of these species have particularly different behaviors than the other dragonfly species that we collected, so an explanation as to why they contain greater numbers of bacteria is difficult. *E. simplicicollis* can take on larger prey and eat a significantly higher proportion of its body mass compared to *Pachydiplax longipennis* study (May & Baird, 2002), another species sampled in this study, so it may relate to dietary behavior, but a mechanism for this is unclear.

In comparing the counts of antibiotic resistant bacteria found in dragonflies, host species and site were important drivers of bacterial numbers while life stage was not. We did not identify the bacteria obtained in this study, so whether the same antibiotic resistant bacteria are present in adults as nymphs is unknown. Nymph and adult dragonflies do differ in their overall gut microbiome, although this is also influenced by level of human impact in the form of urbanization (see Chapter I). Tracking the microbiome through time, as an individual dragonfly grows and metamorphoses would specifically allow one to check for the passage of antibiotic resistant bacteria from nymph to adult life stage, but this would be difficult in a natural environment, or if using the gut collection methods utilized here. The fecal sampling approach of Yamaguchi et al. (2018) might, however, lend itself to such studies. Regardless, urbanization
clearly had a major influence on the numbers of antibiotic resistant bacteria present in
dragonflies, with dragonflies collected from sites subject to more human impact having more
antibiotic resistant bacteria. Such findings should be a concern for the health of aquatic
ecosystems and suggest that the effects of contamination and pollution can extend to the gut
microbiome of aquatic organisms.
LIST OF REFERENCES
REFERENCES


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Sharma, J.B. Russell, S. *The Effects of Atomic Force Microscopy as an Undergraduate Research Tool*. Georgia State University, AAPT Conference, Atlanta GA. April 2015