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Effects of Pharmaceuticals in Reclaimed Water on Insects in Different Feeding Guilds

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Environmental Toxicology

by

Marcus John Pennington

September 2017

Dissertation Committee:
Dr. John Trumble, Chairperson
Dr. Jay Gan
Dr. Quinn McFrederick

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The Dissertation of Marcus John Pennington is approved:

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Committee Chairperson

University of California, Riverside

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Dedications

To my husband,

To my family,

And

In loving memory of,

Maria Magdalena Pennington

And

Robert Marshall Collier

ABSTRACT OF THE DISSERTATION

Effects of Pharmaceuticals in Reclaimed Water
on Insects in Different Feeding Guilds

by

Marcus John Pennington

Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, September 2017
Professor John T. Trumble, Chairperson

Many countries are utilizing reclaimed wastewater for agriculture because drought, rising temperatures, and expanding human populations are increasing water demands. Unfortunately, wastewater often contains biologically-active, pseudopersistant pharmaceuticals, even after treatment. Runoff from farms and output from wastewater treatment plants also contributes high concentrations of pharmaceuticals to the environment. However, there is a lack of information on how these pharmaceuticals could affect insects living in these environments, the insects' microbial communities, and possible pest management applications. The overall goal of this dissertation is to form a base groundwork to assess the effects of common pharmaceuticals on important insect pests in different feeding guilds. The findings of this research demonstrate a wide range of effects from insect to insect with some unexpected effects.

Culex quinquefasciatus larvae reared in water contaminated with certain pharmecuticals were found to have increased developmental time, susceptibility to

Bacillus thuringiensis subsp. *Israelensis*, and altered microbial communities. Within control treated *C. quinquefasciatus*, the predominant families of bacterial symbionts change with each larval instar despite consistent diets and rearing conditions. This trend was not observed the antibiotic or the mixture treatments. Interestingly, the mixture treatments had greater richness and evenness compared to antibiotic alone treatments, possibly due to the other contaminants facilitating growth of different bacteria.

Female *Megaselia scalaris* flies showed no oviposition preference for treated or untreated diets. Larvae exposed to certain pharmaceuticals in artificial diets showed increased mortality, signs of slowed development, especially in females, and altered sex ratio. There was an overall effect of treatment on the flies' microbial communities; notably, caffeine fed insects displayed higher microbial variability.

Trichoplusia ni showed increased developmental time and mortality when reared on artificial diets containing antibiotics, hormones, or a mixture of contaminants. Mortality was also increased when *T. ni* were reared on tomatoes grown hydroponically with the same concentrations of antibiotics. Microbial communities of *T. ni* changed substantially between developmental stages and when exposed to CECs in their diets.

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Chapter 1: Introduction

With climate change and increasing population density affecting the availability of typical fresh water supplies, it is important to look for new sources of water for agricultural irrigation. In arid California, over 750,000 acres of vegetables were harvested for fresh market in 2012 [1]. Many of these vegetables are not drought tolerant plants and require frequent (often daily) watering. As droughts become more frequent and water more scarce, there is a strong demand to use reclaimed and reused wastewater for irrigation purposes. Problematically, most wastewater increasingly contains pharmaceuticals and personal care products (PPCPs), mostly due to increased prescription rates with a growing population [2]. Currently, we do not know the effects most of these PPCPs have on crops or if they can bioaccumulate. The fear of contamination, primarily in raw vegetables, by bioaccumulating of PPCPs has caused hesitance with respect to reclaimed water use in irrigation. Bioaccumulation and deposition of these PPCPs by sprinkler watering systems could also represent problems for Integrated Pest Management (IPM) systems.

Most PPCPs originate from discharge from manufacturing companies, livestock farms, unaltered pharmaceuticals excreted from humans and livestock, and hospitals. Manufacturing and human-derived excretions go to wastewater treatment plants, which are only capable of removing a portion of all contaminants [3]. On many farms, manure from livestock treated with both growth hormones and veterinary antibiotics is used for fertilization without first being treated [4]. The continuous introduction and accumulation

of anthropogenic contaminants has ultimately led to their presence in biologically active concentrations in the environment, including surface waters such as lakes and streams.

PPCPs of major importance include: acetaminophen, caffeine; the steroidal hormones, 17α- ethynylestradiol, 17β- estradiol, estrone, 19-norethindrone; and the antibiotics lincomycin, oxytetracycline, and ciprofloxacin [5–7]. However, concentrations found in surface water are often much lower than concentrations present in wastewater effluents and field runoff, which can be as much as 5-100 times more concentrated [6,8]. Several pharmaceuticals (mainly anti-inflammatory, psycho-corrective drugs, estrogenic hormones and antibiotics) have been reported to cause physiological effects in a diversity of organisms ranging from the bottom to the top of the food web [9–13]. For instance, the anti-inflammatory drug diclofenac was found to delay reproductive maturity in a freshwater crustacean, *Daphnia magna*, during a multigenerational study that used an environmentally relevant concentration of 0.36 μg/L [14]. Antidepressants have been shown to reduce predator avoidance behaviors in fathead minnows, which has the potential to negatively impact population density [15]. Estrogenic compounds were reported to disrupt mating behavior and secondary sexual characteristics in both fish and frogs [16–18]. Similarly, permatogenesis and oocyte production were greatly hindered in alligators collected from a lake containing elevated xenoestrogen concentrations [19]. Chronic exposure to antibiotics has been found to cause significant decreases in stream detritivore biota [20] and in other cases, increased antibiotic resistant gene expression in many microbiota, potentially leading to the development of resistant bacteria and pathogens [21]. Contamination from PPCPs also has the potential to negatively impact plants. In

Arabidopsis, antibiotics (tetracyclines) were shown to absorb through the roots and also to chelate calcium ions, thereby effecting gene expression and growth [22]. Tetracyclines have also been shown to increase glutathione S-transferase activity in maze, but not in pinto beans [23], suggesting variability in plants' susceptibility and/or ability to metabolize PPCPs. Wu and colleagues (2012) determined the concentration of nineteen PPCPs in six different vegetable crops grown hydroponically. The PPCPs were chosen to cover a wide range of chemical classes and the six crops (celery, tomato, carrot, broccoli, bell pepper, and spinach) cover the various types of vegetables that are eaten raw or with very little processing. These plants were grown in "spiked" nutrient solutions to remove the matrix effect of soil, which could accumulate certain contaminants, thereby slowly increasing concentrations of PPCPs. Wu et al. (2012) showed all nineteen contaminants can be absorbed depending on the plant and plant tissue in varying degrees. The highest concentrated PPCPs found in the tested plants were for the psycho-corrective drugs, carbamazepine (28.7 ± 1.8 ng/g) and fluoxetine (34.0 ± 10.4 ng/g). However, there is no published study to date that has investigated the ability of plants to absorb and disperse antibiotics or hormones in a mixture, nor has there been any exploration into the impact these contaminants may have on critical insect-plant interactions or on insects' endosymbionts that can influence nutrient acquisition, growth, and survival.

Piercing insects, such as aphids, feed by sucking the phloem or xylem contents from plants, while many insects, such as the cabbage looper (*Trichoplusia ni*), consume whole leaves. Chewing insects consume a variety of plant tissues which may sequester stored chemicals such as lipophilic and hydrophobic compounds. The effects of these

instilled PPCPs could have physiological, multigenerational effects, and effect parasitoids' ability to control insects.

Many insects rely heavily on endosymbionts to live and grow. These endosymbionts subsist on nutrients provided by the host and in turn, they often make available nutrients the host is unable to produce or gain from its diet. This can be done by directly producing the nutrient (for example essential amino acids or steroids) or by breaking down plant products such as cellulose. Consequently, insects feeding on a contaminated diet that hinders endosymbionts would suffer from malnutrition resulting from decreased essential nutrient availability, leading to slow growth and development. Thus, insects feeding on contaminated plants could suffer from a decreased growth rate. Reduced rates of growth can lead to potentially greater exposure to pesticides, an increased period of exposure to parasites or predators during susceptible stages [25] an inhibition of defenses against endoparasitoids [26], or increased exposure to induced plant defensive chemicals [27]. Any of these effects could result in substantial changes in insect population development that affect insect control thresholds currently used to manage insect populations.

Insects rely heavily on endogenous and external chemical signals for development, predator avoidance, mating, and oviposition preference [28,29]. The endocrine system of insects produces several chemicals, which bare striking resemblance to those of vertebrates, including humans. Some estrogenic- and androgenic-like substances have already been isolated in moths, *Bombyx mori* and *Manduca sexta*, beetles *Tenebrio molitor*, and flies, *Sarcpohaga bullata* [30]. Exposure of insects to these chemically similar

compounds is likely to disrupt normal functioning and development. The birth control agent, 17 α- ethynylestradiol (EE2), mimics 17β- estradiol and binds antagonistically to the estrogen receptors in mammals to produce its effects. EE2 has been shown to causes changes in mouthpart structure, development, oxidative-stress enzyme concentrations, and emergence of the non-biting midge, *Chironomus riparius* [11,31,32]. Chemicals that do not resemble these structures, but bind to the receptors can also cause effects. In *C. riparius*, it was shown that Bisphenol A, a xenoestrogen and estrogen receptor antagonist, can bind and modulate the ecdysone receptor gene as it does for estrogen receptors in vertebrates, inducing an increase in ecdysone receptors [33]. Bisphenol A has been shown to decrease vitellogenin (the egg yolk protein) in male C. riparius and at higher concentrations, reduces female vitellogenesis [34]. Bioaccumulation of these chemicals has also been shown to increase in a dose dependent manner in members of the genus *Chironomus* [35].

Terrestrial insects must search to find food sources and places suitable for reproduction, which leads to terrestrial insects expending energy to search and choose areas with the best resources possible; however insects are not always able to discern the best places to feed/ forage and reproduce. Argentine ants do not avoid to the contaminant selenium in their diet, which can cause toxic effects from chronic ingestion [36]. Contamination of pork liver with varying concentrations of female contraceptives, in a laboratory setting, effects up to three generations of the blow fly *Chryrysoma megacephala.* Effects of reduced and less developed ovarioles were not seen in the parent generation, facilitating the notion that contraceptive hormones and other chemicals'

effects may be passed on to offspring but show no effect in the parent generation [37]. On the other hand, some PPCPs can hinder the parent generation, which would decrease the production of viable and reproductive progeny. For example, the female mealworm beetles, *Tenebrio molitor*, chose males treated with higher doses of atrazine, an estrogenic herbicide, more than males treated with lower doses. This would suggest that males can "lie" about how healthy they are by producing pheromones that entice females more [38]. The same type of beetle, when injected with antibiotics, displayed an inhibited molting process and after ecdysis often displayed some pupal features [39]. In tomato moths, (*Lacanobia oleracea)*, when either male or female moths were treated with testosterone, the pair produced not only smaller egg clutches but also less viable eggs. In all of these studies, the treatments were delivered in a way that removed any matrix effect of the chemical to the insect. The effects of PPCPs, matrices, plant defensive compounds, and insect-crop interactions are at the forefront of current research needs due to the increased demand for reclaimed wastewater.

The goal of this dissertation is to generate novel insight into the effects PPCPs have on the ecology of insects across tropic levels, including insect microbial communities. My objectives include determining the effects of PPCPs on medically and economically relevant insects; determining the effects of PPCPs on insects' microbial communities; and to determine if feeding style affects insects' susceptibility to PPCPs.

Chapter 2

Pharmaceuticals and personal care products alter the holobiome and development of a medically important mosquito

Abstract

The increasing demand for fresh water has forced many countries to use reclaimed wastewater for agricultural purposes. This water contains pharmaceuticals and personal care products (PPCPs) that remain biologically active following passage through wastewater treatment plants. Run-off from farms and contaminated water from treatment facilities exposes aquatic ecosystems to PPCPs. This study examined the effects of PPCPs on a lower trophic organism. *Culex quinquefasciatus* larvae were reared in water contaminated with environmentally relevant concentrations of common PPCPs. Acetaminophen alone and a mixture of contaminants were found to increase developmental time of larvae. Susceptibility to Bti increased in larvae exposed to antibiotics, acetaminophen, or a mixture of PPCPs. Antibiotics, hormones, and the mixture altered the mosquito bacterial microbiome. Overall, the results indicate that at environmentally relevant concentrations, PPCPs in reclaimed water can have biologically important effects on an ecologically and medically important lower trophic level insect.

Introduction

Use of pharmaceuticals and personal-care products (PPCPs) has been increasing over the past 30 years, doubling or tripling in the past 12 years [40,41]. Common PPCPs include, but are not limited to acetaminophen, mental stimulants, and treatments for heartburn, allergies, and bacterial infections. Since these products are excreted from the human body with little or no changes to the chemical structure [42], they frequently show up in wastewater treatment plants.

Common wastewater treatment facilities are not equipped to remove all PPCPs and therefore discharge a wide range of concentrations into surface waters [43–45]. Untreated water from treatment plants can be released into the environment during powerful storms, when the overflow drains directly into nearby aquatic systems [46]. A study by Kolpin et al. [5] found over 90 PPCPs in U.S. watersheds. In other countries, the concentrations of PPCPs in wastewater treatment plant effluents are even higher [6,47]. In response to an increasing demand for fresh water, it has been proposed to use reclaimed wastewater for agricultural purposes [24]. Unfortunately, PPCPs tend to be relatively long-lived and mobile in both the soil and water [4,48,49]. Therefore, runoff from agricultural fields irrigated with reclaimed water, or fertilized with reclaimed biosolids, is likely to contain increased concentrations of PPCPs.

There is minimal information available regarding the effects of PPCPs on insects in aquatic environments. However, some research has described PPCP effects on bacterial communities in aquatic environments and on aquatic plants. Kołodziejska et al. [50] demonstrated that antibiotic contaminates commonly released from fisheries reduced

growth of green algae (*Scenedesmus vacuolatus*) and duckweed (*Lemna minor*), as well as viability of the crustacean *Daphnia magna*. Rosi-Marshall et al. [51] showed the stimulant, caffeine, significantly reduced respiration rates of stream biofilms, but had no effect on their bacterial composition. They also showed that an antihistamine, diphenhydramine, caused both a reduced respiration rate and a significant change in the bacterial community of the biofilms. These compounds were not designed or intended to affect bacterial communities. Thus, these variable impacts make predicting how chemicals will affect unintended target organisms difficult.

PPCPs usually cause toxicity because they have similar chemical structures to an organism's natural signaling compounds, resulting in expression or blockage of the organism's receptors. For example, mammalian hormones, commonly used in birth control and hormone therapy, have been linked to endocrine disruption in reptiles, birds, and some arthropods [9–11,18,32,52,53]. They also have been shown to alter primary and secondary sexual characteristics such as gonads and mating and courtships behaviors [9,10,52].

Arthropods rely on various hormones for much of their coloration, production of eggs, mating behaviors, and immature development [53–55]. While many insect hormones do not specifically match mammalian hormones, the structure of mammalian sex hormones and ecdysone (the insect molting hormone) are similar. In crustaceans, mammalian sex hormones have been shown to cause infertility, increase molting events, and inhibit chitobiase. Chitobiase plays a role in digestion of the cuticle during a molt [56–58]. Ecdysone is an insect hormone important for initiating the molting process when

larvae are transitioning between instars, and is structurally similar to estrone. Bisphenol A (BPA), a xenoestrogen, has the ability to bind and express not only the estrogen-binding proteins in mammals, but also to the ecdysone-binding protein in *Chironomus riparius* [33]. Exposure of the non-biting midge, *C. riparius*, to estrogenic compounds has been shown to cause mouth deformities, decreased fecundity, and increased developmental time if administered over multiple generations [11,32,53].

Culex quinquefasciatus (southern house mosquitoes) are aquatic arthropods in their larval instars, and terrestrial as adults. Eggs are laid in rafts on the water's surface and the larvae hatch directly into the water. From their first to fourth instar, most mosquito larvae feed on detritus and thereby recycle nutrients back into the environment [59]. Once they reach the fourth instar, larvae cease feeding and prepare to molt into a non-feeding pupal stage. Mosquitoes, like many insects, rely on endosymbionts (microorganisms which provide nutrients or other beneficial functions) to grow and develop [28,60–63]. For example, bacteria in the genus *Buchnera* are commonly endosymbionts of aphids and provide the aphid with essential amino acids [64]. Similarly, bacterial symbionts in the genus *Asaia* have been shown to be crucial in the development of the mosquito, *Anopheles stephensi* [63]. Not surprisingly, mosquitoes treated with antibiotics to eliminate bacteria took significantly longer to develop than untreated control larvae. However mosquitoes "rescued" by a subsequent introduction of the bacteria following antibiotic exposure showed no difference in development [63].

Currently, there is little information available on the effects of PPCPs at environmentally relevant concentrations on aquatic invertebrates, on the effects of PPCPs

on the holobiome of insects, or on the effects of PPCPs on efficacy of *Bacillus thuringiensis* subsp. *israelensis* (Bti), a bacterial insecticide commonly used to control larvae of mosquitoes. Joint exposure to a pollutant and a microbial larvicide can be used to detect sublethal physiological stress [65]. Further, since 1997, 1000s of hectares of floodwater mosquito breeding sites have been treated with Bti [66]. Thus Bti is likely to coexist with PPCPs in aqueous environments. Therefore, we used a series of bioassays to evaluate the effects of PPCPs on development and mortality of the mosquito, *Culex quinquefasciatus*, which is a lower trophic level arthropod found worldwide and native to the Southern U.S. [67]. This species is a vector of encephalitides including West Nile Virus and the nematode, *Wuchereria bancrofti,* which causes filariasis in the tropics and subtropics [68]. Thus, any potential effects would have interesting implications from both ecological and medical perspectives.

Materials and Methods

Chemicals

To determine the effect of PPCPs on *C. quinquefasciatus* a series of representative compounds were chosen based on Kolpin et al. [5] and Mutiyar and Mittal [6]; acetaminophen, caffeine, three antibiotics, and four estrogenic steroidal hormones. Acetaminophen (10 µg/L), caffeine (6 µg/L), estrone (0.112 µg/L), 19-norethindrone (0.872 µg/L), 17β- estradiol (.2 µg/L), 17α- ethynylestradiol (.831 µg/L), lincomycin (0.73 µg/L), and oxytetracycline (72.9 µg/L) concentrations were chosen based on the

maximum concentrations measured by Kolpin et al. [5]. Ciprofloxacin (31,000 µg/L) concentration was chosen from the maximum effluent discharge reported by Mutiyar and Mittal [6]. Six treatments were examined: acetaminophen, caffeine, an antibiotic mixture (lincomycin, oxytetracycline, and ciprofloxacin), hormones (estrone, 19-norethindrone, 17β- estradiol, and 17α- ethynylestradiol), a mixture of all chemicals, and a control, consisting of only Crystal Geyser® Natural Alpine Spring Water (CGSW) (C G Roxane, Olancha, CA).

The chemicals used were purchased as follows: acetaminophen with a purity of ≥ 90%; (MP Biomedicals, LLC, Santa Ana, CA); caffeine at laboratory grade purity (Fisher Scientific, Hanover Park, IL); lincomycin, oxytetracycline, and ciprofloxacin with purities of ≥98% (Alfa Aesar, Ward Hill, MA); estrone, 19-norethindrone, 17β- estradiol, and 17α- ethynylestradiol at ≥98% purity (Sigma-Aldrich, St. Louis, MO); and *Bacillus thuringiensis* subsp. *israelensis* with 37.4% active ingredients of fermentation solids and solubles (Gnatrol® WDG Biological Larvicide, Valent® Professional Products, Walnut Creek, CA). Hydrochloric acid was obtained from Fisher Scientific at 12.1 molar concentrate. Sodium hydroxide was acquired from Sigma-Aldrich (St. Louis, MO) as anhydrous pellets. Stock solutions were prepared by adding powdered chemicals to deionized water. Approximately 5 mL 80% ethanol was added to 250 mL steroidal hormone solutions to facilitate dissolution. Hydrochloric acid (1 M) was added to antibiotic chemical solutions to facilitate dissolution and pH was adjusted using NaOH (1 M) to a pH of 4.00. In all experiments, preparations and concentrations were identical.

Insect rearing

Culex quinquefasciatus mosquito egg rafts were obtained from a parental colony maintained at the University of California, Riverside using the procedures described by Wirth et al. [69]. Rafts were maintained in shallow porcelain pans (30 X 20 X 5 cm) containing 3 L water or water and one of the PPCP treatments. Following emergence, larvae were kept in an incubator (model 818: Precision Scientific Inc., Buffalo, NY) at 28°C, approximately 70% RH, and a light: dark cycle of 16:8.

Second instars were transferred individually by disposable pipette to a bioassay container consisting of a 29.57 mL plastic cup with clear plastic lid containing 15 mL of CGSW. Each mosquito larva was given 67.0 µL of diet on day 1 and 33.5 µL of diet every other day thereafter. Diet was prepared as in Sorensen et al. [70]; briefly a 3:1 (wt/wt) mixture of ground mouse chow: brewers yeast was rehydrated by 50 mL of CGSW for 4 g of dry mixture. Bioassay containers were treated with stock solutions to ensure environmentally relevant concentrations of PPCPs and/or the correct concentration of Bti before larvae were transferred. Volume was checked periodically throughout the experiments with no noticeable difference. This methodology was used for all experiments.

Determination of a chronic LC_{50}

To determine the chronic LC_{50} (dose required to prevent 50% of the population from reaching the adult stage) of Bti for *C. quinquefasciatus*, mosquito larvae were treated with one of seven concentrations (3.075, 6.15 12.3, 24.6, 50, 100, or 200 ng

Bti/mL) and an untreated control. Initially, doses of Bti were chosen by dividing a 24-h acute LC_{50} (123.0 ng/mL), determined by Mogren et al. [65], by ten. Based on the resulting mortalities, an eight-dose concentration range was developed to determine the chronic LC_{50} covering the time span from second instar through adult eclosion. Second instars were transferred from pans to bioassay cups and given an 8-h acclimation period. If a larva died before the end of the acclimation period, it was replaced. Sixty individuals were used for each replicate, with three replicates per treatment. This replication was used throughout all experiments. After treatment, larvae were maintained in incubators as previously described. Larvae were monitored daily until all larvae died or eclosed. The resulting chronic LC_{50} (10.20 ng/ mL) of Bti on *C. quinquefasciatus* was then used as a standard Bti concentration in all subsequent treatments with Bti and/or PPCPs and Bti.

Growth and development

In order to determine the effect of PPCPs on growth and development of *C. quinquefasciatus,* larvae were reared in CGSW treated with each of the five PPCP treatments, or an untreated control. Mosquito larvae were reared and transferred to their respective bioassay containers as stated previously. Larvae were monitored daily for growth, developmental stage, mortality, and number of days to pupation.

Susceptibility assay

To discern the effects PPCPs on susceptibility to Bti, larvae were treated with the PPCPs as before and an additional trial was conducted with both PPCPs and chronic LC_{50}

of Bti (10.20 ng/mL). Individuals were monitored daily for growth, development, and mortality.

Effects of PPCPs on the mosquito as a holobiont

The endosymbiont microbial community of mosquitoes reared under the various PPCP regimes was sequenced and quantified. Mosquitoes were reared in pans as described previously. Three subsets of ten mosquitoes were collected when mosquitoes reached the second, third, and fourth instar. Mosquitoes were then twice washed with 95% ethanol to remove any external microorganisms. After washing, larvae were transferred to a sterile 2 mL microcentrifuge tube with 95% ethanol and frozen at -60 ± 3°C in an ultra cold freezer (Forma Scientific, Inc. Marietta, OH) until DNA extraction. DNA was extracted using a Qiagen DNeasy® Blood and Tissue Kit following the manufacturers protocols with the following amendments. Mosquitoes were crushed by micropestles in a sterile 2 mL microcentrifuge tube and 20 µL of Buffer ATL. After thorough pulverization, 160 µL of the Buffer ATL and the 20 µL of proteinase K was added. Nucleic acid concentration was quantified using a Nanodrop ND- 2000c Spectrophotometer (Cole-Palmer, Vernon Hills, IL).

A commercial sequencing facility (Molecular Research LP MR DNA, Shallowater, TX) performed Roche 454 bacteria barcoded amplicon pyrosequencing (bTEFAP®). The bTEFAP® procedure used the primer set: 27Fmod (GRGTTTGATCMTGGCTCAG) and 519Rmodbio (GTNTTACNGCGGCKGCTG) in a single-step 30 cycle PCR using HotStarTaq Plus Master Mix (Qiagen, Valencia, CA).

PCR was performed using the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s; 53°C for 40 s and 72°C for 1 min; finally an elongation at 72°C for 5 min. After PCR, all amplicons were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA). Samples were sequenced with Roche 454 FLX titanium instruments and reagents following the manufacturer's guidelines.

Statistical analyses

Statistical analyses were performed in R (the R Foundation for Statistical Computing, version 3.1.1). The chronic LC_{50} was calculated using logistic regression, and a logit link function. Lethal concentrations at 95% confidence intervals were calculated using the dose.p function of the MASS package [71]. Growth and development were examined using a generalized linear model with a Poisson probability distribution. Individual treatments were examined using linear contrasts with the untreated control. In susceptibility assays, overall significance in mortality was determined using ANOVA; individual significances were determined using a binomial generalized linear model. Bacterial community data from bTEFAP® was examined with respect to instar and treatment using "permutational MANOVA" (PERMANOVA). PERMANOVA is analogous to MANOVA but is robust to non-normality that is commonly associated with count data [72,73]. Microbial community data from pyrosequencing was further examined with principal component analysis (PCA) performed in the FactoMineR package [74].

Results

Chronic Bti LC_{50}

The percentage of *C. quinquefasciatus* treated with increasing levels of Bti demonstrated a dose dependent response curve and fit a binomial logistic regression line ($y=1.704\ln(x)-3.957$; $R^2= 0.90$). The chronic LC_{50} was determined to be 10.20 ng/mL (95% fiducial limits: 8.81-11.81 ng/mL).

Growth and development

Both acetaminophen and the mixture of PPCPs had significant effects on the developmental time of *C. quinquefasciatus*. Those individuals treated with acetaminophen alone and the mixture of PPCPs required significantly more time on average to pupate ($X^2=19.64$, df=6, $p<0.05$) than the control treatments, but were not significantly different than each other ($p>0.05$). The majority of control mosquitoes pupated on days 5 and 6 while the majority of those in the acetaminophen and the PPCP mixture pupated on days 6 and 7 (Figure 2.1). When treated with Bti and the PPCP(s), trends were similar. However, compared to the non-Bti-treated mosquitoes, all Bti treatments took on average an extra 1-2 days to pupate ($X^2=43.63$, df=1, $p<0.05$) (Figure 2.2).

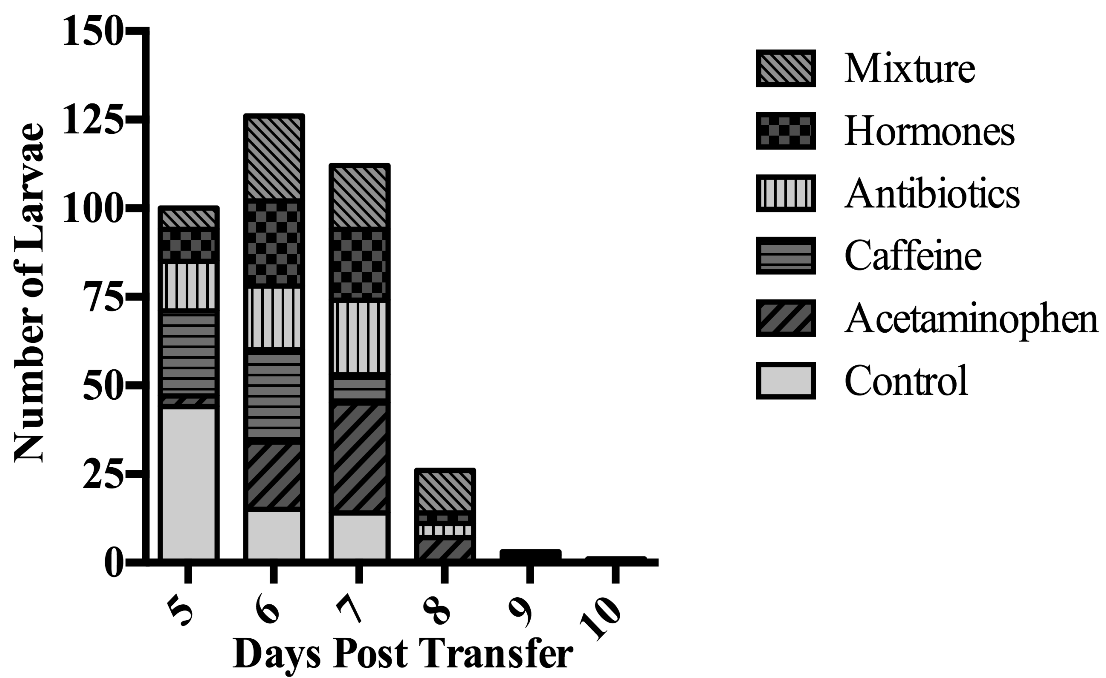


Figure 2.1: Number of mosquito larvae that pupated for all treatments by day

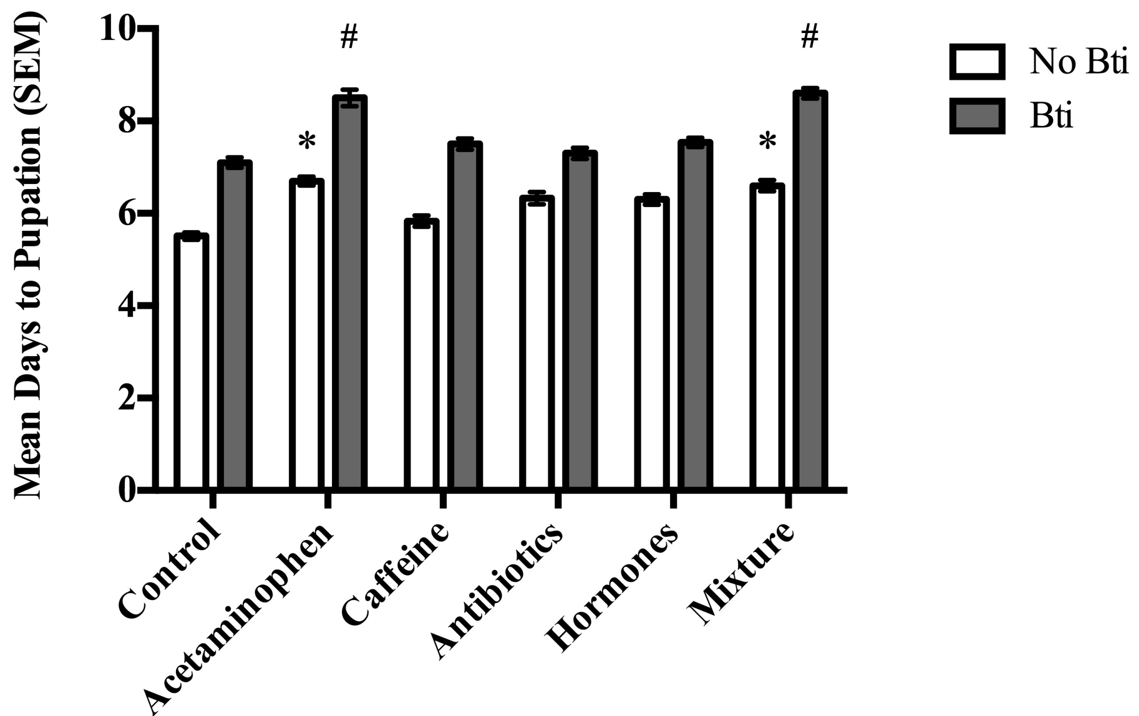


Figure 2.2: Average days to pupation from egg hatch of PCPP treated, untreated, and Bti and control mosquitoes.

Susceptibility to Bacillus thuringiensis *subsp*. israelensis

Overall mortality varied among treatments (X^2= 58.28; df=6; $p<0.01$). Bti-treated mosquitoes exposed to acetaminophen alone, antibiotics alone, and the PPCP mixture were more susceptible to Bti than larvae only exposed to Bti ($p< 0.05$). Mortality of untreated larvae (control) was minimal (3.33%). The mortality of mosquitoes exposed only to Bti was 45%, which was within the 95% confidence limits of the LC_{50} as determined in experiment 1 (Figure 2.3).

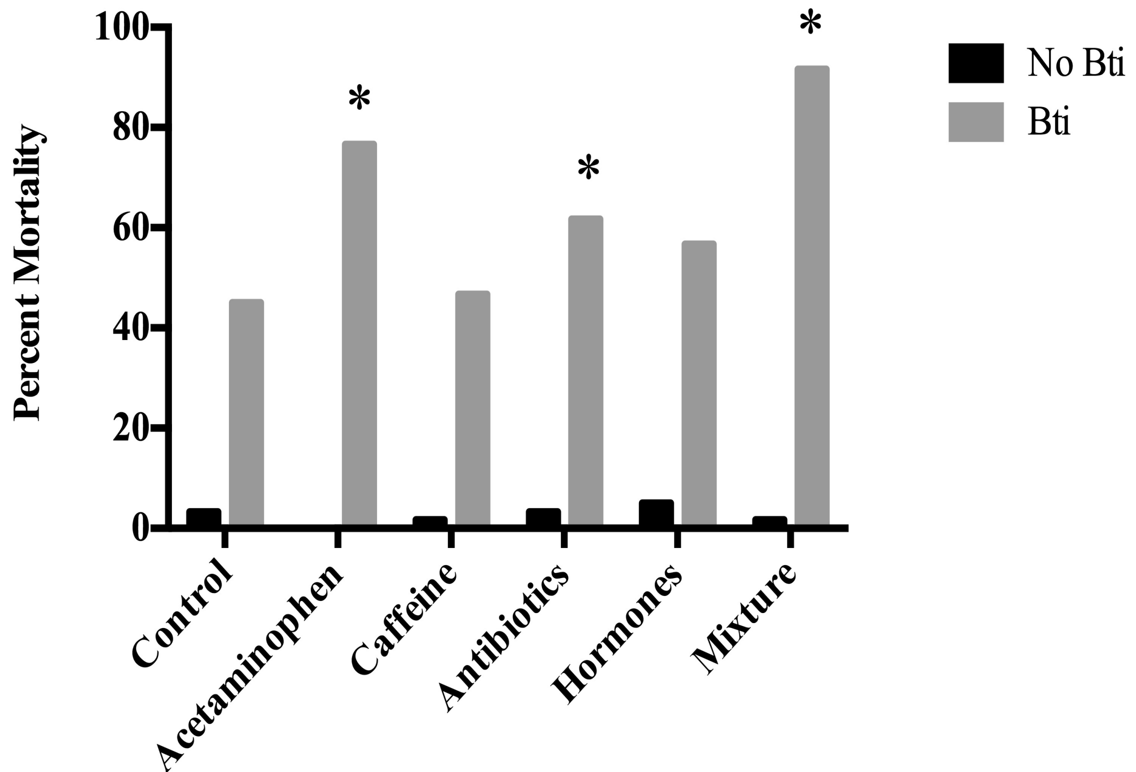


Figure 2.3: Percent mortality of untreated larvae and larvae exposed to the LC_{50} of Bti and PPCPs. *: denotes significant difference from larvae exposed to Bti only ($p<0.05$)

Effects of PPCPs on the mosquito as a holobiome

The microbiome of the mosquito was significantly different among the PPCP treatments (PERMANOVA: F= 6.37; df= 5,48; p<0.0001), between instars (PERMANOVA: F=18.20; df=1,52; p<0.0001), and there was a significant interaction of PPCP treatment and instar (PERMANOVA: F=3.19; df=5,48;p<0.0001). PCA was performed to determine groupings relative to instar. The variable 'instar' loaded onto the first principal component, which explained 28.5% of variation. We tested for differences in the microbiome with respect to instar by examining only the mosquitoes reared in the

untreated controls. Overall there was a significant difference among the instars (PERMANOVA: $F=10.33$; $df=2,6$; $p<0.001$), with third and fourth instars loading together, separately from second instars. Therefore we tested for a difference in third and fourth instars, which were not significantly different (PERMANOVA: $F=1.91$; $df=1,4$; $p=0.22$). Consequently, subsequent analyses were based on combined third and fourth instars.

When third and fourth instar larvae were considered together there was a significant effect of treatment (PERMANOVA: $F=10.80$; $df=5,30$; $p<0.0001$). In contrast to findings when controls only were considered, in which third and fourth instars do not differ, there was a significant difference between third and fourth instars when all treatments were included in the analysis (PERMANOVA: $F=16.79$; $df=1,34$; $p<0.001$). This was likely due to the interaction between treatments and instar (PERMANOVA: $F=2.54$; $df=5,30$; $p<0.001$).

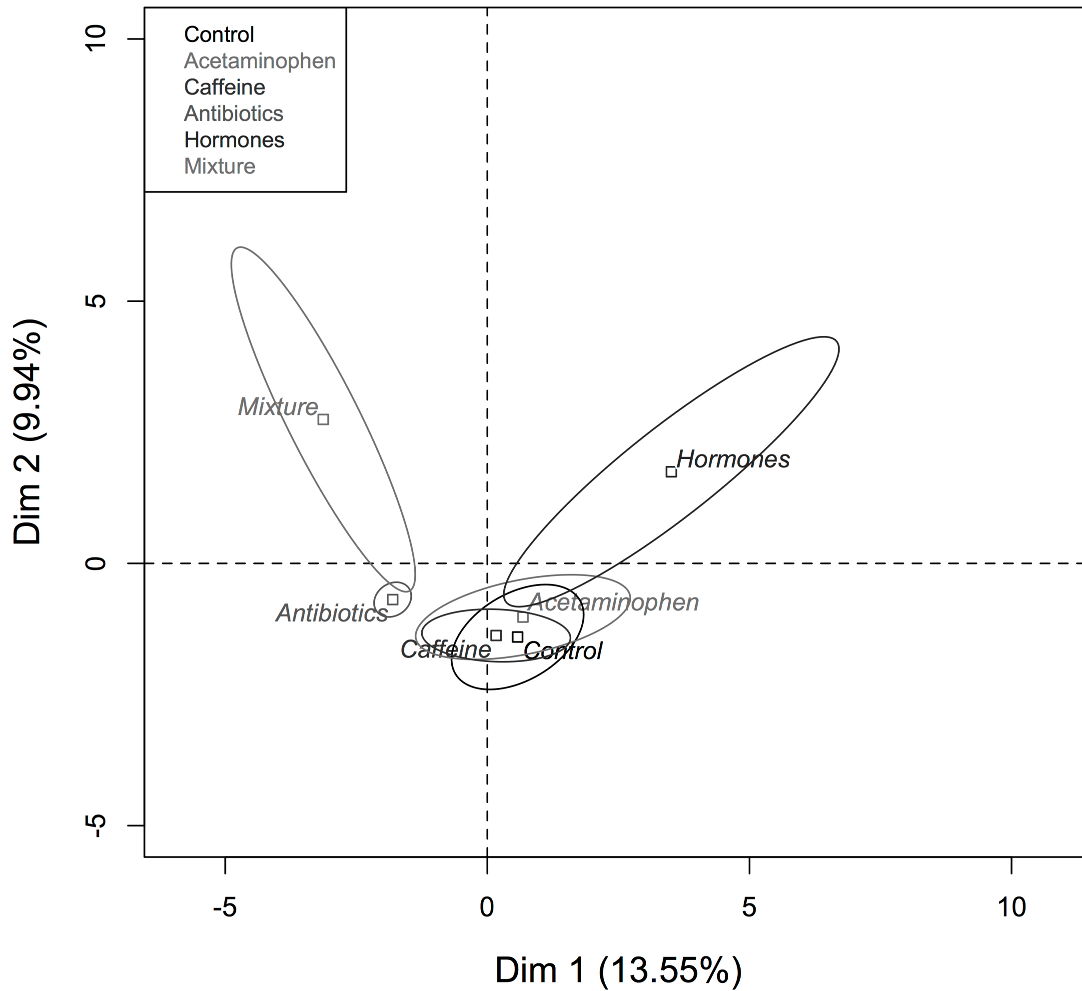


Figure 2.4: Coordination of PPCP treatments and control as determined by the first and second principal component dimensions

When the microbiome of third and fourth instar larvae was examined with PCA, there were 17 dimensions with an Eigenvalue greater than one. However, the first two dimensions explained 23% of the total variation (Figure 2.4). When examined across the first and second principal components (Figure 2.4), bacterial communities treated with

acetaminophen and caffeine and the controls cluster together, suggesting they are similar. The microbiomes of the mosquitoes in the mixture or antibiotic only treatments are similar to each other. The hormone treated mosquitoes are distinct from all other treatment groups.

There were 30 bacterial families with non-zero contributions to one of the first two principal components. Twenty of these families account for at least 96% of the PPCP-treated mosquitoes' bacterial community and cluster in three distinct groups (Figure 2.5). Eight bacterial families each contribute greater than 0.01% to the overall bacterial community in all treatment groups (Table 2.1). The family *Enterobacteriaceae* is mostly described by the first dimension and is associated with the control, acetaminophen, and caffeine treatments (Figures 2.4 and 2.5; Table 2.1). *Rickettsiaceae* is the most abundant bacterial family in all of the treatment groups except for the hormone-treated mosquitoes, where it is second most abundant. *Wolbachia pipientis* accounts for >99% of this family (Table 2.1 and 2.2). *Microbacteriaceae* is the most represented bacterial family in the treatment groups (Table 2.2) and, like *Rickettsiaceae*, has > 9% abundance in all treatment groups (Table 2.1). However, the *Microbacteriaceae* species vary among treatments, but *Rickettsiaceae* bacteria are consistently represented by *Wolbachia pipientis* (Table 2.2).

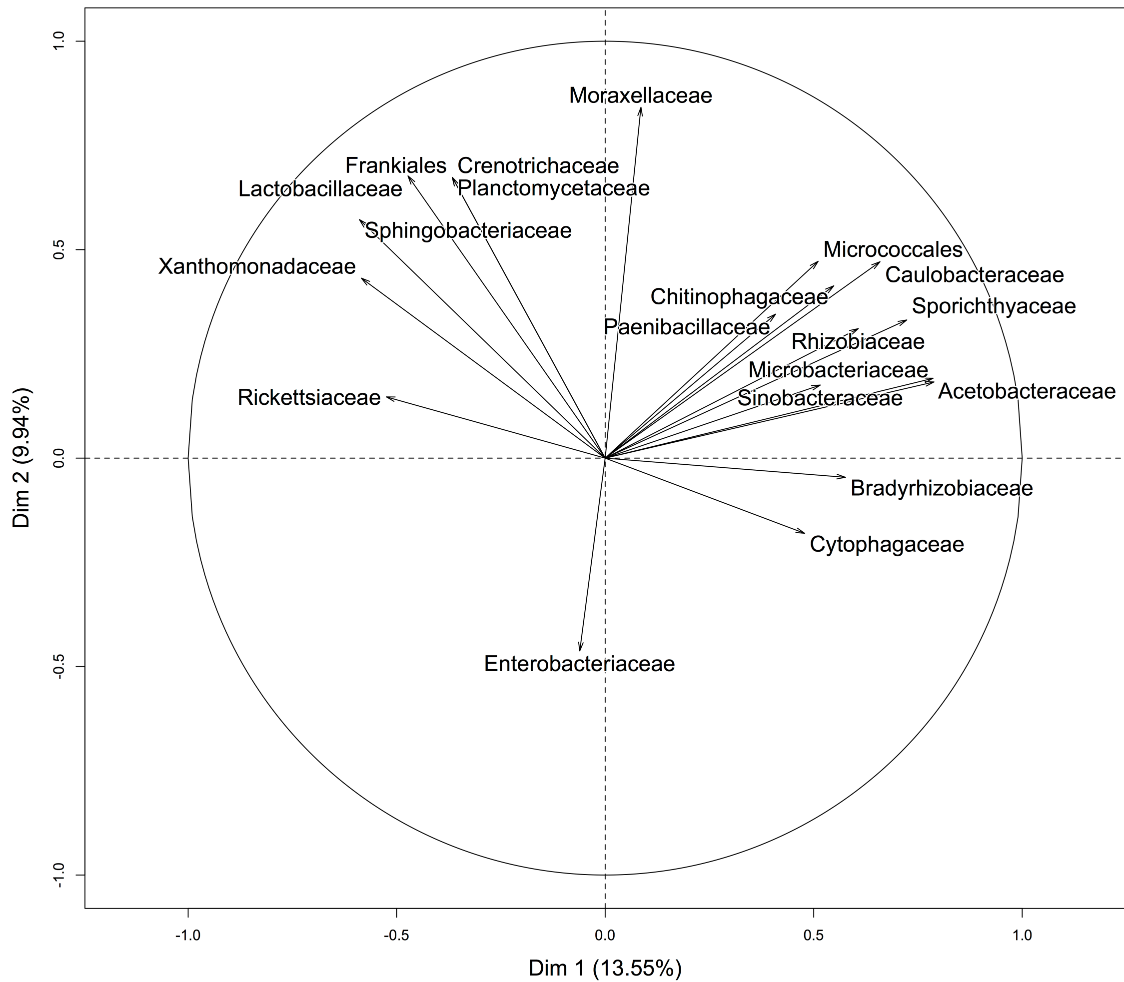


Figure 2.5: PCA factor graph displaying the top twenty families with their associated component

While the eight families presented in Table 2.1 account for at least 96% of the bacterial community, the total counts are reduced by 66% in the antibiotic treatment and reduced by 33% the mixture treatments. Thus, while *Wolbachia pipientis* has a relatively similar number of counts in all distinct treatments, this species accounts for 86.7% of all bacteria in the antibiotic-treated and 69.1% of bacteria in the mosquitoes exposed to mixtures of PPCP.

Table 2.1: Mean percentages, location, and purported function of bacterial families that contribute at least 0.01 percent to the overall mosquito holobiont

Family	Location of Bacteria	Purported Function	Percentage in Control	Percentage in Acetaminophen	Percentage in Caffeine	Percentage in Antibiotics	Percentage in Hormones	Percentage in Mixture
Acetobacteraceae[75,76]	Gut, Whole mosquito,	Possible plant symbiont, Nitrogen fixators, Secondary symbionts of insects dependent on sugar-based diets	0.5	0.6	0.1	> 0.1	1.3	> 0.1
Bradyrhizobiaceae[77,78]	Whole tick	Nitrogen fixation	2.5	5.4	1.4	0	2.5	0.6
Enterobacteriaceae[75,76,79–81]	Whole mosquito, moth midgut, Huhu beetle gut, ant gut, Whole Aphid, Mosquito gut	Insects symbionts both primary and secondary	32.8	27.7	36.8	>0.1	>0.1	0.2
Microbacteriaceae[76,82,83]	Whole mosquito, Mosquito gut, Stem-borer gut	Cellulase activity	24.6	34.5	20.3	2.6	58.2	9.9
Rickettsiaceae[84,85]	Various Tissues and Organs	Reproductive Parasite	33.8	28.9	38.2	86.7	29.5	69.1
Sinobacteraceae[86,87]	Roots area	Possible xenobiotic metabolic function	2.0	1.3	1.5	0.9	5.5	>0.1
Sphingobacteriaceae[82,86]	Whole mosquito, Root area	Heparinase activity	> 0.1	0.3	> 0.1	5.9	> 0.1	15.5
Xanthomonadaceae[75,76]	Gut, whole mosquito	Nitrate/-ite reduction, Chemoorganotrophism, Cellobiose metabolism	0.1	0.1	0.1	3.5	0.1	2.7
		Sum of Percentages	0.1	98.8	0.10.1	99.6	0.1	18.2
		Total Counts	29140	29937	27234	9829	27142	19579

Table 2.2: Most abundant bacterial species by family in four PCPP treatments as they correlate to PCs

Family	Control	Mean Counts	Percentage of Counts
Rickettsiaceae	*Wolbachia pipientis*	4923[*]	33.8
Enterobacteriaceae	*Yersinia mollaretii*	3387[+]	23.3
Microbacteriaceae	*Microbacterium laevaniformans*	3206[#]	22.0
Sphingobacteriaceae	*Pedobacter spp.*	1.2	> 0.1
Xanthomonadaceae	*Stenotrophomonas maltophilia*	8.2	0.1
Microbacteriaceae	*Microbacterium testaceum*	0	0
Microbacteriaceae	*Klugiella xanthotipulae*	154.5	1.1
	Antibiotic		
Rickettsiaceae	*Wolbachia pipientis*	4261[*]	86.7
Enterobacteriaceae	*Yersinia mollaretii*	0	0
Microbacteriaceae	*Microbacterium laevaniformans*	0.3	> 0.1
Sphingobacteriaceae	*Pedobacter spp.*	291.0[+]	5.9
Xanthomonadaceae	*Stenotrophomonas maltophilia*	174.3[#]	3.5
Microbacteriaceae	*Microbacterium testaceum*	3.7	> 0.1
Microbacteriaceae	*Klugiella xanthotipulae*	0	0
	Hormone		
Rickettsiaceae	*Wolbachia pipientis*	4007[+]	29.5
Enterobacteriaceae	*Yersinia mollaretii*	0.3	> 0.1
Microbacteriaceae	*Microbacterium laevaniformans*	5288[*]	39.0
Sphingobacteriaceae	*Pedobacter spp.*	1514	> 0.1
Xanthomonadaceae	*Stenotrophomonas maltophilia*	7.5	> 0.1
Microbacteriaceae	*Microbacterium testaceum*	0	0
Microbacteriaceae	*Klugiella xanthotipulae*	1162[#]	8.6
	Mixture		
Rickettsiaceae	*Wolbachia pipientis*	6764[*]	69.1
Enterobacteriaceae	*Yersinia mollaretii*	17.2	0.2
Microbacteriaceae	*Microbacterium laevaniformans*	250.8	2.6
Sphingobacteriaceae	*Pedobacter spp.*	6764[+]	15.5
Xanthomonadaceae	*Stenotrophomonas maltophilia*	260. 7	2.7
Microbacteriaceae	*Microbacterium testaceum*	359.3[#]	3.7
Microbacteriaceae	*Klugiella xanthotipulae*	16.2	0.2

*- Denotes most abundant
+- Denotes second most abundant
#- Denotes third most abundant

Discussion

Previous studies reporting LC_{50} values for *C. quinquefasciatus* exposed to *B. thuringiensis* subsp. *israelensis* (Bti) have only reported acute values (24 h). The dose-dependent toxicity of Bti to *C. quinquefasciatus* documented in our study was used to calculate the first chronic LC_{50} of Bti on *C. quinquefasciatus.* This value of 10.20 ng/mL is much lower than previously reported acute LC_{50}s determined in 24 h tests. For example an acute LC_{50} value was reported at 140.0 ng/mL by Mogren et al. [65]. Thus, although acute assays are faster to conduct, and can be compared against previously published reports, they overestimate the amounts of Bti needed to kill 50 percent of mosquito populations that are exposed over their entire life spans. Bechmann [88] noted that in life-table experiments some toxicants, especially pesticides, can drive a population to extinction even at concentrations well below an acute LC_{50}. However, effective mosquito control typically requires suppression of late instar larvae even with only an acute exposure and a relatively high dose of Bti is be needed to achieve that goal.

Mosquitoes treated with PPCPs at environmentally relevant concentrations displayed increased developmental time in the acetaminophen and mixture treatments. As these two treatments were not significantly different, it is possible the effects on mixture treated mosquitoes could be from acetaminophen alone. The majority of these two treatments pupated 1-2 days after the control group. The cause of this delay is difficult to determine and may be due to individual or joint actions of many factors including an effect on the nutrients during rearing, an effect of these specific PPCPs on the mosquito physiology, or some general stress. Stress has been shown to influence insect physiology

and behavior [89,90]. Regardless of the cause, increased developmental time would increase larval exposure to PPCPs and potentially to predation. All mosquitoes treated with Bti exhibited an increase in developmental time of 1-2 days, including those in the acetaminophen and mixture treatments. Since Bti is a larvicide that damages the gut lumen in certain mosquitoes [91], a reduced nutrient absorption could lead to increased developmental time. Longer developmental times caused by PPCPs would increase exposure to contaminants and exposure time to Bti. Increased mortality would almost certainly occur.

Larvae in the acetaminophen, antibiotic, and mixture treatments were more susceptible to Bti than the larvae exposed to Bti alone. While the increased time to exposure described above may have played a role in the increased mortality, the pyrosequencing results indicated significant changes in the bacterial community of the mosquito. If some of the affected bacterial communities, especially those eliminated, have detoxifying abilities for Bti-toxin this could also play a role. However, more research in the area is warranted. Previous studies show the inability of *Anopheles* mosquitoes to fend off the malaria parasite *Plasmodium falciparum* following treatment with the antibiotic gentamycin [92]. Contrary to our findings, Broderick et al. (2006) showed the gypsy moth (*Lymantria dispar*) had a reduced susceptibility to *Bacillus thuringiensis* after treatment with antibiotics, which was removed after reintroducing the *Enterobacter* sp. NAB3. As the mosquitoes in our study retained their *Enterobacteria,* the Bti was still activated and the mosquitoes were still susceptible.

In our study, larvae exposed to hormones contained substantially different bacterial communities as compared to controls, suggesting that at least some hormones likely play a role in altering bacterial communities. Overexpression of ecdysone during development could explain why BPA causes mouth deformities and increased pupation time in *C. riparius* [11]. In mosquitoes (*Aedes aegypti*), the vitellogenin gene is a target for ecdysteroid receptor, which can be modulated by the xenoestrogen BPA [55,94]. This suggests that BPA and other xenoestrogens could have an effect on the production of vitellogenin, the egg yolk protein. This also would lead to altered viability of offspring, which we did not assess.

Acetaminophen and the mixture of PPCPs significantly slowed developmental time of *C. quinquefasciatus*, but antibiotics alone did not. In contrast, Chouaia et al. [63] described delayed larval development of *Anopheles stephensi* after treatment with the antibiotic rifampicin at 120 μg/ mL. The developmental time was rescued by reintroducing bacteria from the genus *Asaia*. In our study, there was relatively little difference in development of immature mosquitoes between the antibiotic treatments and the controls for the *Acetobacteraceae*, to which *Asaia* belongs. It is possible that the antibiotics chosen for this study are not effective in eliminating *Asaia*. Alternatively, the antibiotics in our study were used at doses much lower than the rifampicin tested by Chouaia et al. [63], and might have achieved similar effects if applied at higher doses.

With a combination of antibiotics, hormones and other constituents that occur in PPCP-contaminated reclaimed water, it is difficult to know exactly which chemical is affecting which bacterial family and which bacteria were responsible for the deleterious

effects on developmental time. Notably, the antibiotic treatments had approximately 1/3 of the total number of bacteria relative to the control. Therefore even the approximately 8500 counts of *Rickettsiaceae* (the majority) in the antibiotic treatments are lower in number than the *Enterobacteriaceae* (second highest) found in control treatments. Surprisingly, the substantial loss of bacterial counts (density) in the antibiotic only treatments did not slow development.

Although the antibiotics decreased overall bacterial counts in *C. quinquefasciatus*, *W. pipientis* appeared to be relatively unaffected. *Wolbachia pipientis* is susceptible to doxycycline and rifampin [95]. Of the chemicals we tested, oxytetracycline should have had the most impact (based on structural similarity) on *Wolbachia*. However, because the bacterial counts were relatively similar for *W. pipientis* in all treatments, we suspect either the oxytetracycline was too dilute to have an effect, it allowed a non-susceptible strain of *W. pipientis* to dominate, or it is simply ineffective against *W. pipientis*. However the vast majority of the eight bacterial families were greatly reduced in the antibiotic treatments. Of these families, *Enterobacteriaceae* and *Microbacteriaceae* were the most reduced in both the antibiotic and mixture treatments.

The family *Enterobacteriaceae* is highly associated with insect endosymbionts, such as *Buchnera* in pea aphids [64,96]. *Buchnera* bacteria are known to aid the aphid by supplying essential amino acids lacking from a nutritionally deficient diet. Without these endosymbionts the aphids do not develop properly, but the microorganisms apparently have no other direct biological effect on the aphid [97]. The size of the *Enterobacteriaceae* populations (counts) probably had a minimal effect on larval developmental time, as the

counts in the acetaminophen treatment are relatively similar to the hormone-treated group. Additionally, the hormone treated group has a substantially reduced count of *Enterobacteriaceae* bacteria compared to the control, but showed no significant changes in developmental time. We suspect, therefore, that the acetaminophen is negatively impacting some other biological system in the larvae, but determination will require additional research.

The use of reclaimed water for crop irrigation and the release of water from waste treatment plants and farm waste ponds into surface waters is occurring and likely to escalate as demand for fresh water increases. While, the research reported here suggests that PPCPs contaminating reclaimed water will have potentially useful effects for mosquito control, if the data can be extrapolated to other insect species, PPCPs will also have unintended negative effects on other aquatic insects. Very little is known regarding how these contaminants might biomagnify or change chemically as they move through the food web. The eventual impact on populations is also unknown. Further, combinations of PPCPs may be more important for some insects than individual components. Additional research is needed not only on aquatic insects living in surface waters, but also on uptake by plants and associated herbivores in terrestrial environments.

Chapter 3

Culex quinquefasciatus larval microbiomes vary with instar and exposure to common wastewater contaminants

Abstract

Like many insects, mosquitoes, rely on endosymbionts to grow and develop. These can be acquired from the environment. We used next generation 454 pyrosequencing to discern the whole-body microbiome of the mosquito species *Culex quinquefasciatus* in various larval stadia and following exposure to common pharmaceutical and personal care products (PPCPs) found in wastewater. PPCP treatments included environmentally-relevant concentrations; 1) a combination of common antibiotics, 2) a combination of mammalian hormones, 3) a mixture of the antibiotic and hormone treatments plus acetaminophen and caffeine and, 4) an untreated control. Within control groups, the predominant families of bacterial symbionts change with each larval instar despite consistent diets and rearing conditions. This trend was also seen in hormone treatments but not in the antibiotic or the mixture treatments. Richness and evenness were reduced in both antibiotic and mixture treatments, suggesting that antibiotics remove certain bacteria or inhibit them from increasing to proportions seen in the control treatment. Interestingly, the mixture treatments had greater richness and evenness compared to antibiotic alone treatments, possibly due to the other contaminants facilitating growth of different bacteria. These findings illuminate the complexity of the microbiome of *C. quinquefasciatus* and may have implications for more effective control strategies.

Introduction

Endosymbionts, bacterial species known to grow, develop, and vertically transmit in an organisms' cells, usually for mutualistic symbiosis, are essential to the growth, development, and fecundity of many insect species [28,61,82,84,98]. Many aphid species, such as *Acyrthosiphon pisum, Megoura viciae*, and *Myzus persicae*, rely heavily on endosymbionts to survive on the unbalanced diet of phloem sap. Their bacteriocyte endosymbiont, *Buchnera spp*, provides essential amino acids [64]. Without *Buchnera*, aphids demonstrate reduced growth rates and development and produce few or no offspring [64,97]. However, some endosymbionts in the genus *Wolbachia* are known to manipulate insects for their own benefit and can also lead to increased vector competency for transmission of human diseases such as West Nile Virus [60,85]. *Wolbachia* species are common vertically transmitted endosymbionts in many mosquito species and typically infect reproductive tissue [99]. There are many species of *Wolbachia* that influence a variety of insect behaviours and life-history traits. Some endosymbionts can also act defensively by killing parasitoid eggs after they are laid in the host [60,99,100]. Because endosymbionts play major roles in insect development, they have been proposed for use in insect control [101].

Due to the importance of endosymbionts for development in many insect species, some species vertically transfer endosymbionts (from parent to offspring). For example, Estes et al. [102] describes the microbiome of dung beetles' (*Onthophagus taurus*) brood balls, which are used to nourish their offspring until they are adults. When the microbiota in the beetle offspring and their female parent were compared in sterilized dung and soil,

they had proportionally identical 16S rRNA sequences from their microbiome. Interestingly, over their life stages, the proportions of the families of bacteria in the beetles' microbiome changed. The predominant family of the first three instars varied by individual. However, from the pupal stage onwards, *Enterobacteriaceae* was the most common family in the dung beetle. Bees acquire important bacteria through social interaction and also from the environment [103]. Without some of these bacteria, it is thought that honey bees could become more susceptible to outside diseases and increase incidents of colony collapse [104]. More studies are needed to fully understand the importance of microbiota in mosquitoes as they have been linked to increased transmission of pathogens from mosquitoes to humans [85,105].

Mosquitoes are common disease vectors, which spend their juvenile stages in aquatic environments [66]. Bacteria from the water, both symbiotic and free-living, have been shown to influence the microbiome of mosquitoes, suggesting that some of their possible endosymbionts are collected from the environment [106]. Consequently, if the environment is altered, and some of these necessary bacteria are reduced/eliminated, there could be detrimental effects on the development of mosquito larvae. Such a removal effect may occur as the result of antibiotic runoff or other pollution, and/or environmental changes. For example, Rosi-Marshall et al. [51] showed that common pharmaceuticals in streams will alter the respiration and diversity of biofilms. Pennington et al. [107] reported differences in whole-body microbiomes and increased developmental times for *Culex quinquefasiatus* (Say) treated with various pharmaceuticals and personal care products (PPCPs).

Chemicals intended for human use often occur in aquatic environments and/or enter water supplies through water treatment plant overflow or from use of reclaimed water in water scarce areas [5,43,46,108–110]. These chemicals can then affect bacterial communities in the water and the associated aquatic insect community. Presence of these contaminants can alter effectiveness of *Bacillus thuringiensis* subsp. *israelensis* (Bti) a pesticide commonly applied for mosquito control [107]. However, little is known about how such contaminants will influence the microbiome of such insects. Similarly, there is a lack of data determining if mosquito bacterial communities vary during the course of larval development; all available studies we are aware of examine only late instar larvae or mixed lower instars and species [82,111], and information regarding mosquitoes' endosymbionts and their function is very limited or non-existent outside of *Wolbachia*. Therefore, we describe the differences in the bacterial communities of the mosquito *C. quinquefasciatus* in multiple distinct larval stages, as well as when these mosquitoes are reared in environments contaminated with ecologically relevant concentrations of PPCPs that commonly occur in combinations in order to provide a baseline for more in-depth studies.

Materials and Methods

Insect rearing

Culex quinquefasciatus mosquito egg rafts from colonies continuously reared at the University of California, Riverside, CA were maintained using the methods of Wirth et al. [69]. Egg rafts were held in shallow porcelain pans (30 x 20 x 5 cm) containing 3 L

Table 3.1: PPCP treatment group components and rates

Contaminant	Rate (µg/L)	Reference
Antibiotic		
Oxytetracycline	72.90	5
Lincomycin	0.730	5
Ciprofloxacin	31,000	6
Hormone		
17α-Ethynylestradiol	0.831	5
17β- Estradiol	0.200	5
19- Norethindrone	0.872	5
Estrone	0.112	5
Mixture		
Acetaminophen	10.00	5
Caffeine	6.000	5
Antibiotics	As above	
Hormones	As above	

Geyser® Natural Alpine Spring Water (C G Roxane, Olancha, CA) or spring water and one of three PPCP treatments (described below). Egg rafts and larvae were maintained in an incubator (model 818: Precision Scientific Inc., Buffalo, NY) at 28°C, approximately 70% RH, and a light: dark cycle of 16:8. Mosquitoes were fed 2 mL of a mixture of 4 g brewers yeast and ground mouse chow (1:3 wt: wt) rehydrated in 50 mL water every 3 d. PPCP concentrations (Table 3.1) were used as in Pennington et al. [107], and based on concentrations found by Kolpin et al. [5] and Mutiyar and Mittal [6]. The treatment groups examined were: a control consisting of only water, an antibiotic treatment of lincomycin, oxytetracycline, and ciprofloxacin (Alfa Aesar, Ward Hill, MA; purity ≥98%), a hormone treatment of estrone, 19-norethindrone, 17β- estradiol, and 17α- ethynylestradiol (Sigma-Aldrich, St. Louis, MO; purity ≥98%), and a mixture of all PPCPs plus acetaminophen (MP Biomedicals, LLC, Santa Ana, CA; purity ≥ 90%) and caffeine (Fisher Scientific, Hanover Park, IL; laboratory grade purity). Despite Pennington et al. [107] demonstrating

that acetaminophen and caffeine do not alter the microbiome, these were included in the mixture treatment because they can and do co-occur in wastewater with the other contaminants and we did not want to assume there were no possible joint effects. The extended data set showing acetaminophen and caffeine has been presented as Supplementary Figure S1. Hydrochloric acid (Fisher Scientific; 12.1 M) and sodium hydroxide pellets (Sigma-Aldrich, St. Louis, MO) were used to prepare 1 M stock solutions for adjusting final pH in rearing pans to 7± 0.5.

Extraction of bacterial DNA

In preparation for sequencing, three mosquitoes from each PPCP treatment were collected as second, third and fourth instars, as first instars were too small to collect without damage, and twice washed with 95% ethanol to remove any external microorganisms. Larvae were then transferred to individual sterile 2 mL microcentrifuge tubes with 95% ethanol and stored at -60 ± 3°C in an ultra cold freezer (Forma Scientific, Inc. Marietta, OH) until DNA extraction. DNA was extracted using a Qiagen DNeasy® Blood and Tissue Kit following the manufacturers protocols amended as in Pennington et al. [107]. In addition to mosquitoes, samples of water and water plus diet were also extracted using identical protocols, with the additional step of centrifugation at 2900 rpm in an IEC HN-SII tabletop centrifuge for 1 h to create a pellet. Upon extraction, nucleic acid concentration was quantified using a Nanodrop ND- 2000c Spectrophotometer (Cole-Palmer, Vernon Hills, IL), to confirm enough genetic material for sequencing. This process revealed no DNA in water or water and diet samples when no mosquitoes were

present and thus they were not subjected to further analysis because any bacteria would have originated from the mosquitoes, their egg-rafts, or from the air after the water had been altered by the mosquitoes' various biological processes.

Roche 454 bacteria barcoded amplicon pyrosequencing was performed by a commercial sequencing facility (Molecular Research LP MR DNA, Shallowater, TX). The procedure used the forward primer 27Fmod (GRGTTTGATCMTGGCTCAG) and the reverse primer 519Rmodbio (GTNTTACNGCGGCKGCTG) in a single-step 30 cycle PCR using HotStarTaq Plus Master Mix (Qiagen, Valencia, CA). PCR was performed using the following cycle conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s; 53°C for 40 s, 72°C for 1 min; and a final elongation at 72°C for 5 min. After PCR, all amplicons were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA). Samples were then sequenced with Roche 454 FLX titanium instruments and reagents following the manufacturer's guidelines.

Sequence analysis pipeline

Raw bacterial DNA sequences were analysed using a MacQIIME (version: 1.8.0-20140103) based pipeline [112]. Barcodes and primers were trimmed using the split_libraries.py script with default settings. After barcode trimming, data was denoised and re-inflated using the default denoise_wrapper.py script settings and the default setting in the inflate_denoiser_output.py script. The maximum, minimum, and mean number of sequences across all samples was 22128, 3102, and 10099.11 respectively, with the

average length of the reads being 403.84 base pairs. Operational taxonomic units (OTUs) were chosen by the default 97% identity threshold, which roughly correlates to species [113], via the UCLUST method as implemented in the pick_otus.py script [114]. Representative OTUs were chosen using the pick_rep_set.py script and default settings. The Greengenes reference database clustered at 97% identity was used to assign taxonomy using the assign_taxonomy.py script [115]. OTUs were counted and summarized using the make_otu_table.py and summarize_taxa.py scripts respectively. OTUs were aligned using the align_seqs.py and filter_alignment.py scripts, and used to build a phylogenetic tree (make_phylogeny.py). There were 658 distinct OTUs at the species level with 58 distinct families; 15 OTUs failed to match any contained within the database and could not be assigned taxonomically. Fifteen families were chosen by their proportionality being greater than or equal to 1% in at least one sample. The cut-off was chosen at 1% as this was assumed to be the minimum to influence larval development. For alpha diversity, multiple rarefactions were performed using the multiple_rarefaction.py script with the lowest rarefaction depth of 2000, the highest rarefaction depth of 21000, a step size of 1000, and a replicate number of ten, which normalizes the data at each depth. Alpha diversity was calculated using the alpha_diversity.py script with the metrics observed species (species richness) and Shannon Indices (evenness) [114,116,117]. Alpha diversity data was not averaged between replicate mosquitoes as they have been averaged by resampling-replicates and the complications and validity of this is still being considered[118,119]. Metrics were summarized using the collate_alpha.py script.

Statistical analyses

Statistical analyses were performed using R (the R Foundation for Statistical Computing, version 3.1.1). Following processing through the QIIME pipeline, "Permutational MANOVA" (PERMANOVA) in the Vegan package [120] was used to compare the OTU data. Independent variables were instar (n=3), PPCP treatment (n=4) and the interaction of the two, with three replicates (n=3) of each instar in each PPCP treatment and control (n= 36). PERMANOVA is analogous to MANOVA but is suited to address the non-normality that is commonly associated with count data in ecological community and genetic data [72,73]. Microbial community data were further examined via principal component analysis (PCA) performed in the FactoMineR package [74]. PCA and PERMANOVA were conducted on each instar in the individual PPCP and control treatment groups. Following PCA, variables were examined for their contributions and correlation to each of the first two dimensions. Those variables (OTUs) that were ≥ 85% correlated were included in subsequent pairwise comparisons by instar in their respective treatment. Generalized linear hypotheses was used to perform pairwise comparisons in the multcomp package[121]. P values were adjusted using the p.adjust command. Alpha diversity data was analysed using a negative binomial generalized linear models at a sequence depth of 3000 sequences/sample to normalize data to the highest number where all sample mosquitoes were present. The alpha level for all tests was 0.05.

Results

The mosquitoes' microbiomes were significantly affected by both PPCPs (PERMANOVA: F= 3.78; df= 3, 32; p< 0.001) and instar (PERMANOVA: F= 8.64; df= 2, 33; p< 0.001). Additionally, there was a significant interaction of PPCP treatment and instars (PERMANOVA: F= 7.63; df= 6, 29; p< 0.001). The microbiome of the control mosquitoes changed significantly between instars (PERMANOVA: F= 10.39; df= 2, 8; p< 0.01). In pairwise comparisons of only larvae from the control treatments, second instar larvae were significantly different from third instar (p<0.01) but were not different than fourth instars (p= 0.1) while, third and fourth instars were not significantly different (p= 0.1014) from each other. Families with at least 1% proportionality of the microbiome in at least one instar were also examined via pairwise comparisons. The results are included in table 3.2.

When PPCP treatments were examined for differences among instars, second (PERMANOVA: F= 4.05; df= 3, 8; p< 0.001), third (PERMANOVA: F= 10.25; df= 3, 8; p<0.001), and fourth (PERMANOVA: F= 9.63; df= 3, 8; p< 0.001), were significantly affected by PPCP treatments. When subjected to pairwise comparisons, antibiotic and hormone treated mosquitoes' microbiomes were significantly different between second and fourth instar only (p< 0.01), and there was no significant differences in any instars in the mixture treated mosquitoes (p= 0.3736).

Table 3.2: Mean percentage of bacterial families by instar with at least 1% proportionality in the relative control mosquito instar. [a] denotes significant difference between second and third instar; [b] denotes significant difference between third and fourth instar; and [c] denotes significant difference between second and fourth instar.

Bacterial Phylum	Bacterial Family	Percentage Second Instar	Percentage Third Instar	Percentage Fourth Instar
Bacteroidetes	*Cytophagaceae* [a, b, c]	56.25	1.19	3.33
Actinobacteria	*Microbacteriaceae* [a, b, c]	0.00	15.84	34.89
Proteobacteria	*Rickettsiaceae*	36.72	37.49	28.15
	Enterobacteriaceae [a, b, c]	1.84	41.81	20.10
	Sinobacteraceae [a, b, c]	0.04	0.53	3.95
	Acetobacteraceae [b]	0.00	0.11	1.05
	Comamonadaceae [a, b]	0.86	0.08	1.02
Various	Unknown Family [a, b, c]	0.0598	1.300	6.336
	Sum Percentages	95.77	98.35	98.83
	Mean Total OTU	13821.00	15773.00	12741.67

PCA (Fig. 3.1) demonstrates that various instars separate from each other in the four treatment groups with third and fourth instar loading similarly on the first dimension and all three instars loading distinctly in the second dimension. Bacterial families *Oxalobacteraceae* and *Aeromonadaceae* closely follow the separation pattern in the first dimension and *Cryomorphaceae* follows in the second (Table 3.3). In the antibiotic treatments second instar loaded separately from third and fourth on the second dimension (Fig. 3.1). The bacterial family *Propionibacteriaceae* also follows this trend and was the only family with at least 85 % correlation in either of the first two dimensions (Table 3.3). For hormone treated mosquitoes, second instars loaded separately from fourth

instars on the first dimension (Fig. 3.1). The bacterial families, which follow this trend, are *Enterobacteriaceae* and *Pseudomonadaceae*. As shown in Figure 3.2 and Table 3.2, control treatments' bacterial families changed with instar; starting with *Cytophagaceae* (56.25%) in second instars, changing to *Enterobacteriaceae* (41.81%) in third instar and finally the control fourth instars' most predominant family was *Microbacteriaceae* (34.89%). There were a total of eight bacterial families with proportionalities greater then 1% in at least one instar of the control treatment. In fourth instars there is a resurgence of bacterial families from second instars, which were overshadowed by the third instar bacterial families (Table 3.2). Interestingly, the family *Rickettsiaceae* was the second most predominant family in all control instars. Operational taxonomic units (OTUs) assigned to the family *Rickettsiaceae* were found in most treatments and instars, although they were reduced in many hormone treated samples relative to the controls and increased in the antibiotic treatments. Notably, antibiotic and mixture treatment groups' most predominant family was *Rickettsiaceae* over all instars. The second most predominant family in antibiotic and mixture treatment groups was *Sphingobacteriaceae* in all instars. Hormone treatments changed bacterial communities between instars but not as drastically as the control group. Second instars exposed to hormones predominately contained *Oxalobacteraceae*, which changed to *Microbacteriaceae* and *Rickettsiaceae* in the third instar. The predominant family of fourth instar hormones was *Microbacteriaceae*, although some proportion of *Rickettsiaceae* was still present.

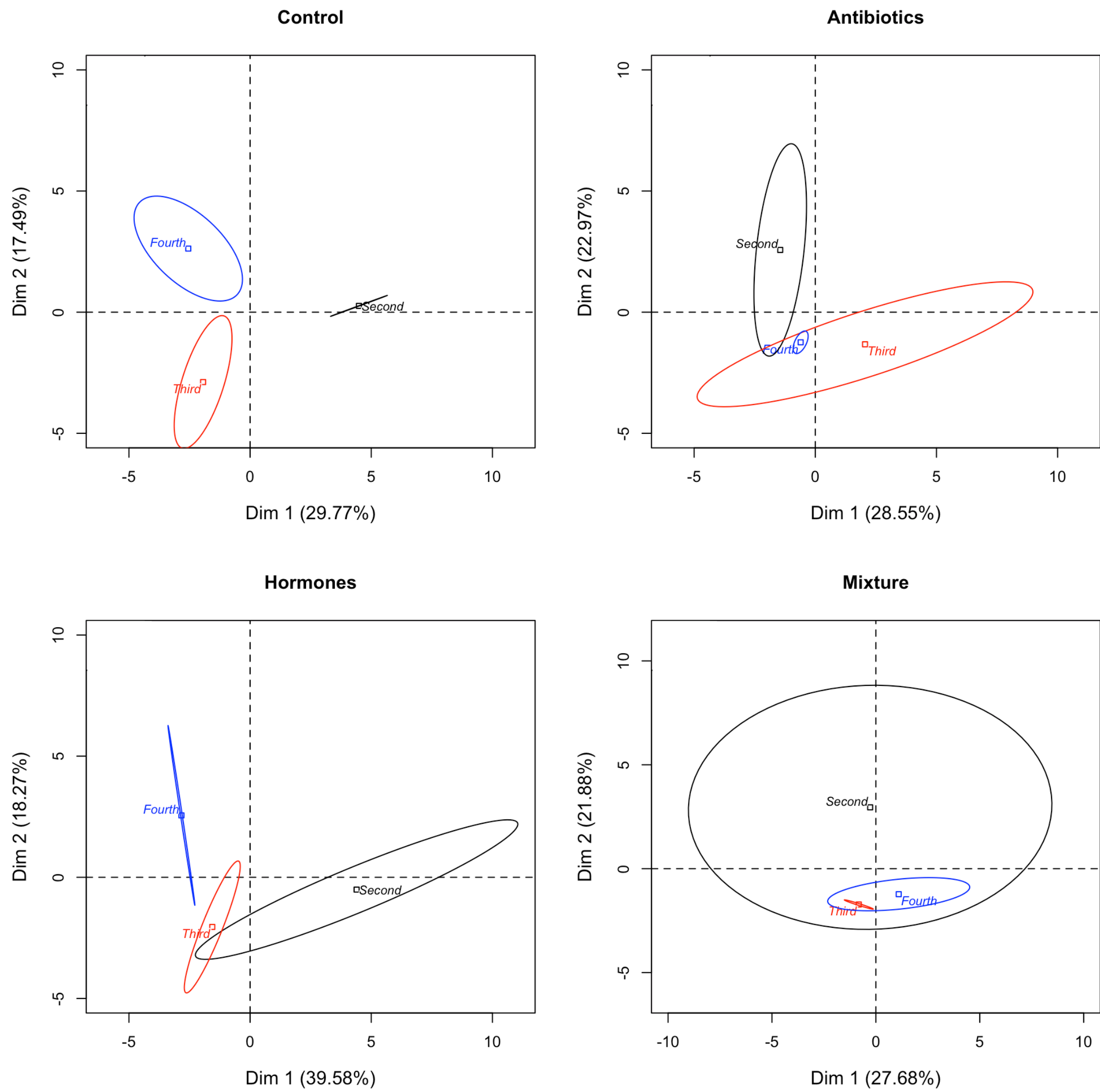


Figure 3.1: Principle component analysis of control and PPCP treatments by instar. Ellipses incorporate all instar replicates (n=3) with the centre of the ellipses displaying the mean analysis.

Table 3.3: All bacterial families with at least 85% familial correlation to one of the first two PCA dimensions. * denotes a significant difference between the instars.

Treatment	Phylum	Family	Dim	Second-Third	Second-Fourth	Third-Fourth
Control	*Proteobacteria*	Oxalobacteriaceae	1	*	*	
		Aeromonadaceae	1	*	*	
	Bacteroidetes	Cryomorphaceae	2	*		
Antibiotics	*Proteobacteria*	Propionibacteriaceae	2	*	*	
Hormones	*Bacteroidetes*	Weeksellaceae	1	*	*	
		Flavobacteriaceae	1	*	*	*
	Firmicutes	Bacillaceae	1		*	
		Paenibacillaceae	1	*	*	
		Leuconostocaceae	1		*	
	Proteobacteria	Sphingomonadaceae	1	*	*	*
		Oxalobacteraceae	1		*	
		Enterobacteriaceae	1	*		
		Pseudomonadaceae	1	*		
		Xanthomonadaceae	1	*	*	
Mix	*Proteobacteria*	Enterobacteriaceae	1	*	*	*
	Bacteroidetes	Cytophagaceae	1			

In the alpha diversity analysis, richness (number of different species in one location) was examined as mean observed species (Fig. 3.3) and evenness (distribution of species' proportionalities in one location) was measured by mean Shannon's index (Fig. 3.4). For mean observed species at a sequencing depth of 3000 sequences/sample there was a significant difference between treatments (X^2: 870.40; df: 3; p< 0.001), instars (X^2: 80.02; df: 2; p< 0.001) and a significant interaction of treatment and instar (X^2: 136.76; df: 6; p< 0.001). Mosquitoes treated with antibiotics had lower richness and fewer total sequences per sample than all other treatments with the richness decreasing as larvae age. This is evident in Fig. 3.2, as there are proportionally fewer bacterial families outside of

Rickettsiaceae and *Sphingobacteriaceae* than in other treatments. In contrast, mosquitoes reared in the mixture of hormones, antibiotics and the common contaminants, acetaminophen and caffeine; demonstrate a relatively constant richness over time (Fig. 3.3).

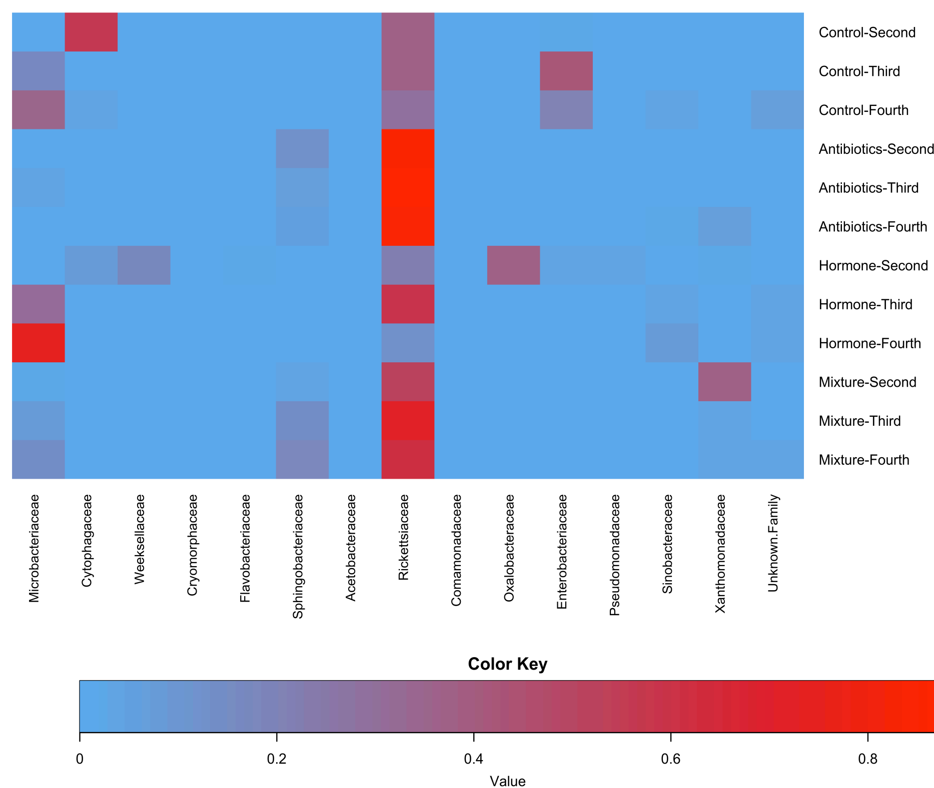


Figure 3.2: The top 15 families with relative proportions ≥1% by family and samples by PPCP treatment- instar on the X and Y-axes respectively. More predominant families appear darker on the map

The control groups and hormone treatments fluctuate more than the mixture and antibiotic treatment groups but demonstrate consistently higher richness (Fig. 3.4). The mean Shannon's diversity index suggests that antibiotics alone substantially reduced

diversity. The mixture treatments also display reduced diversity, however, they are more diverse than their antibiotic treatment counterparts.

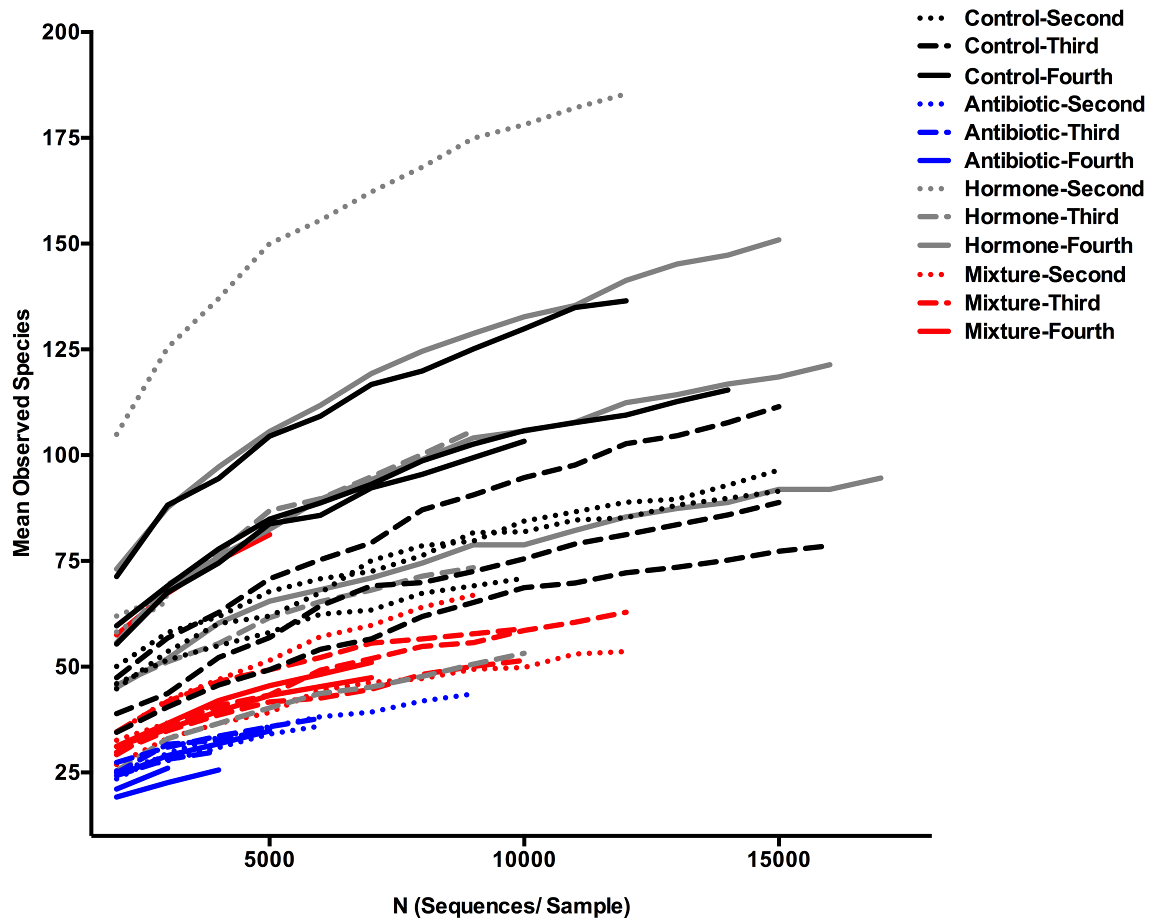


Figure 3.3: Mean observed species (n=10 resampling events at a given subsampling interval) by the number of sequences per sample. Antibiotic, control, hormone and mixture treatments are shown by blue, black, grey, and red colours respectively. Dotted, dashed-dotted, and dashed line types represent second, third, and fourth instars respectively.

The control groups display a greater diversity than both the antibiotic alone and mixture treatments when compared by increasing instar, where as the hormone treatment group, had no discernable pattern. The mixture also displays no discernable pattern

compared by instar, which is likely due to the effects of the hormones added to antibiotics. Finally, it is notable that in some treatments, the mean species number (Figure 3.3) failed to reach an asymptote.

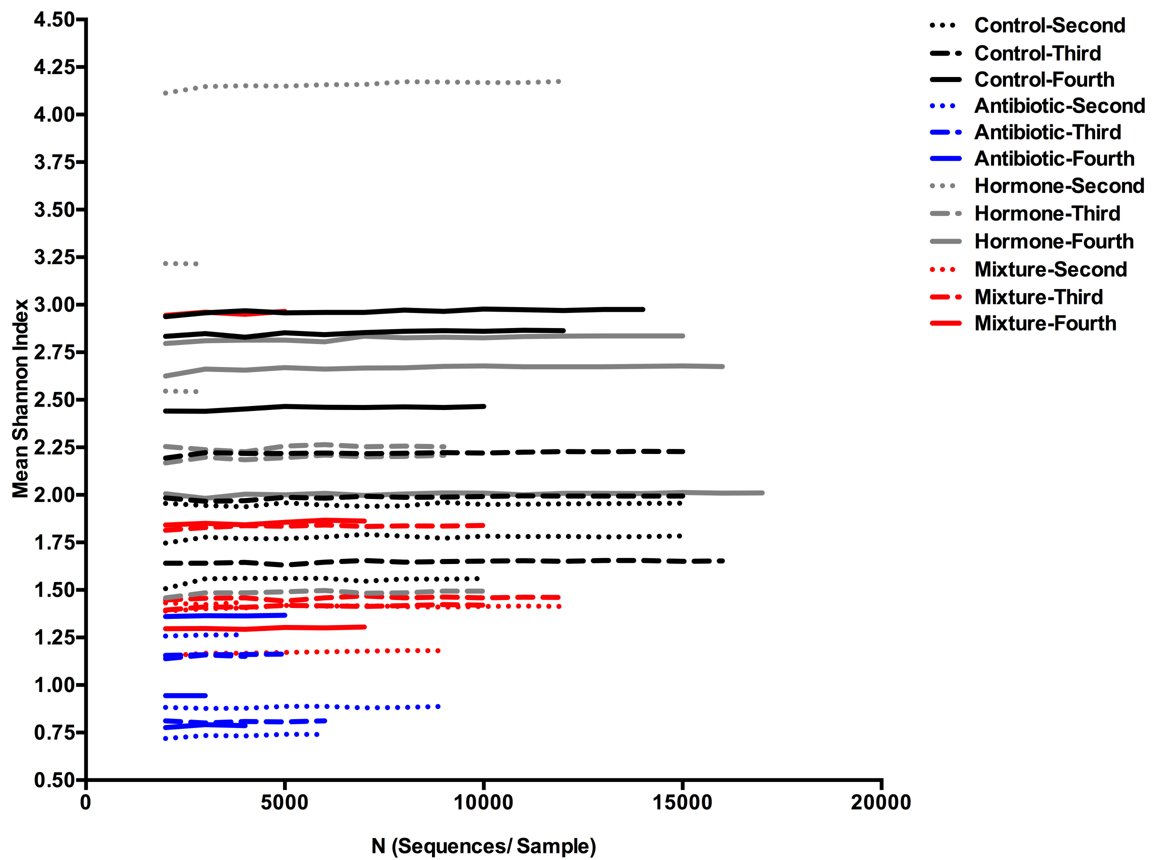


Figure 3.4: Mean Shannon's index (n=10 resampling events at a given subsampling interval) by the number of species sequences per sample. Antibiotic, control, hormone and mixture treatments are shown by blue, black, grey, and red colours respectively. Dotted, dashed-dotted, and dashed line types represent second, third, and fourth instars respectively.

This may indicate that an inadequate sample size for detecting extremely rare species, or that the reduced diversity of some samples allowed for the entire community to be described.

Discussion

Here we have demonstrated that the microbiome of larval *Culex* mosquitoes changes throughout development, and variation between instars is affected when exposed to various PPCPs. It has previously been demonstrated that mosquitoes rely on their microbiomes to aid in development and that removing certain symbionts can significantly slow larval development [63,82,122]. Pennington et al. [107] demonstrated that PPCPs at environmentally relevant concentrations, which are significantly lower than those used in most laboratory studies, can alter the microbiome of mosquitoes and slow their development.

In the field, Duguma et al. [111] showed pooled *Culex* species' microbiomes will change from early (first and second) to late (third and fourth) instars. Coon et al. [82] and Wang et al. [122] showed that the microbiome of mosquitoes will change as the insects advance from fourth instar larvae, to pupae, to the adult stage, and after adults fed on a blood meal. We have shown that the microbiome of early (second and third) instars' will change from one instar to the next even without exposure to PPCPs (control group). In the second, third, and fourth instars, predominant families change from *Cytophagaceae* to *Enterobacteriaceae* and finally to *Microbacteriaceae*. However similar to Pennington et. al [107], third and fourth instar were not significantly different and our findings also correlate to what Duguma et al. [111] found in their laboratory reared *Culex tarsalis* late instars. However, as their third and fourth instars were pooled, only the *Enterobacteriaceae* family predominates. These families were all removed in the antibiotic and mixture treatments. Fourth instar larvae in the control group match what

was described in *Aedes aegypti* by Coon et al. [82]. Coon et al. [82] also described the microbiome of two other mosquito species (*Anopheles gambiae* and *Georgecraigius atropalpus*) during the fourth instar. Their microbiomes had different proportions of familial microbiota between each other and both were different from the findings in our *C. quinquefasciatus* fourth instars. To our knowledge ours is the first study to look at the microbiome changes of individual early instars in mosquitoes. This suggests the possibility of a new strategy for mosquito control targeting the critical microorganisms essential for development at specific stages. Specifically, additional research targeting key symbionts found in earlier instars would determine if the younger larvae can be controlled more effectively, as has been seen with pesticides such as Bti [123].

A number of mosquitoes are common carriers of the bacterial genus *Wolbachia,* which usually acts as a reproductive parasite in the ovaries of the females, and is suspected to be in at least 20% of all insect species [124]. As in Pennington et al. [107], *Rickettsiaceae*, the family containing *Wolbachia pipientis,* continuously holds the majority count of the antibiotic and mixture treatments' microbiome. When the OTUs mapped to the family *Rickettsiaceae* was examined at the level of genus the predominant and sole genus detected was *Wolbachia*. *Rickettsiaceae* is vertically transmitted from mother to offspring [125]; however, for many of the other bacterial families present, it is difficult to discern the source or how they are incorporated into the insects' microbiome. This is made further complicated since these traits may vary by species or genus and mapping OTUs to finer taxonomic levels was generally not possible. Similarly, it may be possible to determine some origins via comparison with the water in rearing pans over

time, although there was no DNA found in water at the start of the experiments. However, our focus was not on the origin of bacterial species in these mosquitoes, and we do not have these data. Analyses or the microbial community in such pans would be an interesting follow-up study. Interestingly, *Enterobacteriaceae*, which includes the genus *Buchnera* and other common endosymbionts, is the predominant family of the third instars in the control treatment. For example, the gut symbiont of the plataspid stinkbug (*Megacopta punctatiss*) is phylogenetically similar to *Buchnera* species [126]. In potato psyllids (*Bactericera cockerelli*) various genera of the family *Enterobacteriaceae* have been reported in the life stages and faeces accounting for at least 21% of the microbiome [127,128]. *Enterobacteriaceae* is one of, if not the most important family of endosymbionts in the pea aphid (*Acyrthosiphon pisum*) [64], and is commonly used in research regarding the effects of antibiotics on insect-symbiont interactions [97]. Similarly, Chouaia et. al (2012) [63] described a slowing of larval development in *Anopheles* mosquitoes when they removed *Asaia* bacteria from the family *Acetobacteraceae*. However this family has one of the lowest proportionalities in all of the mosquitoes including control treated. This suggests it is not an endosymbiont of this *Culex* mosquito specie. However, reports of the effects of other PPCPs on insect-symbiont interactions are rare.

It is interesting to note that the hormones found in wastewater from treatment plants (and in the hormone treatment groups) are all mammalian female sex hormones and would not be expected to affect bacteria. We would not expect an effect of these hormones on bacteria, as there is no endocrine system; nonetheless, substantial changes

in the microbiome occurred in response to exposure to these hormones (Fig. 3.1). Similarly, caffeine and an antihistamine, would not be expected to effect biofilms, but were shown to repress respiration in stream biofilms [51]. We think there may be some influence the hormones have on bacterial gene expression however that is not in the scope of this paper and thus, specific effects for each PPCP or combination of contaminants will need to be determined from more experimental data.

The increased bacterial diversity during mosquito ontogeny could result from either bacterial replication during development or by acquisition through ingestion. In the hormone and control treatment groups, we are unsure if bacteria are lost during development, or if the change in bacterial diversity was caused by differential growth among taxa. Interestingly, in pairwise comparisons of the hormone treated mosquitoes (Table 3.3), the majority of the significant differences were between second and fourth instar larvae. The hormone treated mosquitoes also had the most families that were correlated to the first principal component at a minimum of 85%. Combined this suggests that the mosquitoes exposed to hormones had the most diverse microbial communities and that this diversity increases over time.

Mammalian hormones change the microbiome of *C. quinquefasciatus* mosquitoes and it is possible they are responsible for the increased richness and diversity seen in the mixture treatments compared to antibiotics alone; however more studies will need to be conducted to confirm this conclusion. Regardless, our results indicate that reclaimed wastewater has the potential to impact mosquito ecology. Considerably more research will be required to discern how mixtures of PPCPs could affect bacterial microbiomes for

important medical pests. If similar results are found for agriculturally important insects (including either pests and/or beneficial insects) exposed to these emerging contaminants, additional research documenting the effects of increasing use of reclaimed water and associated changes to the insect microbiome will become even more important. Similarly, because insects are a critical food source for higher trophic level organisms in terrestrial surface waters, releases of PPCPs in aquatic environments have the potential to modify the ecology of these ecosystems.

Chapter 4

Effects of contaminants of emerging concern on *Megaselia scalaris* (Lowe, Diptera: Phoridae) and its microbial community

Abstract

Drought, rising temperatures and expanding human populations are increasing water demands. Many countries are extending potable water supplies by irrigating crops with wastewater. Unfortunately, wastewater contains biologically active, long-lived pharmaceuticals, even after treatment. Run-off from farms and wastewater treatment plant overflows contribute high concentrations of pharmaceuticals to the environment. This study assessed the effects of common pharmaceuticals on a cosmopolitan saprophagous insect, *Megaselia scalaris* (Diptera: Phoridae). Larvae were reared on artificial diets spiked with contaminants of emerging concern (CECs) at environmentally relevant concentrations. Female flies showed no oviposition preference for treated or untreated diets. Larvae exposed to caffeine in diets showed increased mortality, and larvae fed antibiotics and hormones showed signs of slowed development, especially in females. The normal sex ratio observed in *M. scalaris* from control diets was affected by exposure to caffeine and pharmaceutical mixture treatments. There was an overall effect of treatment on the flies' microbial communities; notably, caffeine fed insects displayed higher microbial variability. Eight bacterial families accounted for approximately 95% of the total microbes in diet and insects. Our results suggest that CECs at environmentally relevant concentrations can affect the biology and microbial communities of an insect of ecological and medical importance.

Introduction

Pharmaceuticals have been increasingly prescribed for the past 30 years, and prescription rates have almost tripled in the past 14 years [40,41]. In food-producing animals alone, there were 9.1 million kg of medically important antibiotics (antibiotics used in both humans and animals) used in 2013. Of those 9.1 million kg used, 73.6% was used for the purpose of increasing production of the animals, and this use continues to increase [129]. Many antibiotics and other common Contaminants of Emerging Concern (CECs) (acetaminophen, mental stimulants, heartburn medications, allergy, and bacterial infection treatments), are excreted by both humans and animals with little change in their chemical structure [42]. It is no surprise pharmaceuticals have been appearing in wastewater, and in some cases tap water, over the past few years [130,131].

Standard wastewater treatment facilities are ill equipped to remove pharmaceuticals [132,133]. Many pharmaceuticals are released during heavy storms in the untreated wastewater, due to overflow, which then flows directly to the environment [46]. These pharmaceuticals are now found at biologically active concentrations in surface waters around the world [4–7,134]. In addition to runoff, there is an increasing effort to use reclaimed wastewater in drought affected areas, such as Southern California [24,135]. In agriculture/livestock operations, pharmaceuticals are also found in manure that is then used as fertilizer, effectively compounding the pharmaceutical concentrations [7,136,137]. Current research shows these chemicals tend to be both long lived in soil and detrimental to soil microbes [4,48,49,138,139].

Recent studies on the effects of pharmaceuticals on aquatic insects show that at environmentally relevant concentrations they can alter development of the mosquito *Culex quinquefaciatus*, its susceptibility to a common larvicide, and its larval microbial communities [107,140]. Watts et al. [11] showed alterations and deformities in the midge *Chironomus riparius* after treatment with a common birth control agent, 17α-ethinylestradiol, and a common plasticizer, Bisphenol-A. Interestingly, many chemicals used by humans, which are not intended for use on microbial communities, have been shown to affect microbes. For example, caffeine, a common mental stimulant, alters biofilm respiration, and an antihistamine, diphenhydramine, has been demonstrated to modify the microbial community and respiration of lake biofilms [51]. Because of unexpected pharmaceutical effects, it is relatively difficult to predict what will occur in model organisms. This problem is exacerbated by a lack of information regarding pharmaceuticals' effects on terrestrial insects: no available publications report the effects on any terrestrial insects' microbial community.

Arthropods, such as insects and crustaceans, rely on hormones to grow, develop, mate and even produce pigmentation [53–55]. However, many pharmaceuticals, especially hormones, resemble chemicals that these organisms rely on for growth and development. These pharmaceuticals then bind to receptors and either over-express or suppress their counterparts' natural function. This has been reported in birds, reptiles, and arthropods where endocrine disruption occurs, primary and secondary sexual characteristics are modified, and courtship behaviors change [9,10,18,52,53,141]. While most arthropod hormones do not closely match those of mammals, their molting hormone (ecdysone), is very

similar to 17β-estradiol (the mammalian female sex hormone). In crustaceans, mammalian hormones have been known to cause both increased molting events and inhibition of chitobiase, the enzyme responsible for digestion of the cuticle during insect molting [56,57]. In insects, 17α-ethinylestradiol, a common synthetic birth control hormone, has been shown to alter molting and lead to deformities of *C. riparius*. Also, Bisphenol-A, a common plasticizer, can bind and activate estrogen receptors in humans, and the ecdysone-binding protein in insects [11,33]. In addition to these effects, pharmaceuticals have been shown to cause effects to insects over multiple generations [32].

Megaselia scalaris (Lowe, Diptera: Phoridae) is a common saprophagous pest. They are known to infect living humans (myiasis), provide important ecological roles as detritivores, and because they often feed on human corpses are commonly used in forensic entomology to determine time of death [142,143]. This species will generally feed on a variety of decomposing plant and animal tissues, and acts as a vector of pathogens [142,144]. These insects are both fecund and hardy because females can lay over 650 eggs in 16 days and are tolerant of heavy metals [145,146]. The white, roughly football-shaped eggs, hatch after approximately 24 hours into white translucent larvae. When they have matured to third instar (life-stage) they pupariate [142,143]. Their detritivorous larval life history exposes them to a wide diversity of microorganisms that may act as pathogens, commensals, and symbionts. There is currently no record of how *M. scalaris* acquires their microbiota or if any symbionts are required. However, it stands to reason that they, like so many other insects, would rely on microbial symbionts [63,147]. There are many

ways insects acquire symbionts: from their diet, the environment, their social network, or vertical transmittance (maternally inherited) [81,98,102,148].

Currently there is little to no information regarding pharmaceutical effects at the concentrations found in reclaimed water on the growth or microbial community composition of any terrestrial detritivore. These detritvores become exposed to contaminants after the CECs enter surface waters, soil, and plants from overflow and wastewater reuse. There are studies involving antibiotics at high doses to determine necessity of microbiota in several insects, but these have not tested relevant concentrations found in reclaimed water or joint effects of other pharmaceuticals, which often coexist with antibiotics [28,63]. To assess potential effects of common pharmaceuticals, we used a series of bioassays to determine the possibility of individual and joint contamination on development, mortality and population sex ratios of *M. scalaris*. Any effects would have potentially important implications from medical, ecological, and forensic perspectives. Also, as there is currently no information on *M. scalaris*' microbial community, information generated from this study could serve as novel information into the role possible symbionts play in *M. scalaris* development.

Methods and Materials

Chemicals

Test compounds included: acetaminophen, caffeine, three antibiotics, and four estrogenic steroidal hormones. Six treatments were examined: acetaminophen, caffeine, an antibiotic mixture (lincomycin, oxytetracycline, and ciprofloxacin), a hormone

mixture (estrone, 19-norethindrone, 17β- estradiol, and 17α- ethynylestradiol), a mixture of all chemicals (as would be found in overflow or wastewater effluent), and a control, consisting of only distilled water. Distilled water was tested for CECs and found to not contain any. Treatment groups were chosen as representative compounds for pain relievers, mental stimulants, antibiotics commonly used on humans and livestock, hormones normally either produced or prescribed to humans, and as a mixture that would be simple, yet representative of wastewater effluent or reclaimed wastewater. Artificial diets were prepared at room temperature to negate any decomposition of the CECs. Acetaminophen (10 μg/L), caffeine (6 μg/L), estrone (0.112 μg/L), 19-norethindrone (0.872 μg/L), 17β- estradiol (0.2 μg/L), 17α- ethynylestradiol (0.831 μg/L), lincomycin (0.73 μg/L), and oxytetracycline (72.9 μg/L) concentrations were chosen based on the maximum concentrations measured by Kolpin et al. [5]. Ciprofloxacin (6,500 μg/L) concentration was chosen from the maximum lake water concentration reported by Mutiyar and Mittal[6].

The chemicals used were purchased as follows: acetaminophen with a purity of ≥ 90%; (MP Biomedicals, LLC, Santa Ana, CA); caffeine at laboratory grade purity (Fisher Scientific, Hanover Park, IL); lincomycin, oxytetracycline, and ciprofloxacin with purities of ≥98% (Alfa Aesar, Ward Hill, MA); estrone, 19-norethindrone, 17β- estradiol, and 17α- ethynylestradiol at ≥98% purity (Sigma-Aldrich, St. Louis, MO). Blue formula 4-24® instant *Drosophila* medium, hereafter known as 'blue diet', was purchased from Carolina Biological Supply Company (Burlington, NC). Hydrochloric acid (12.1 M) was obtained from Fisher Scientific. Sodium hydroxide was acquired from Sigma-Aldrich (St.

Louis, MO) as anhydrous pellets. Stock solutions were prepared by adding powdered chemicals to deionized water. Approximately 5 mL 80% ethanol was added to 250 mL steroidal hormone solutions to facilitate dissolution. Hydrochloric acid (1 M) was added to antibiotic chemical solutions to facilitate dissolution and pH was adjusted using NaOH (1 M) to pH 4.00. Compounds were added to distilled water to the desired concentrations for each treatment and then an equal amount of blue diet flakes was added as described by the manufacturer. In all experiments, preparations and concentrations of treatment groups were prepared as stated previously.

Insects

A *M. scalaris* colony was established in 2015, and the species verified at the Los Angeles Natural History Museum by Ms. Emily Hartop. The colony was maintained on an alfalfa diet as in Mandeville et al. [149] . For all experiments, eggs were collected from adults and were stored in an incubator (model 818: Precision Scientific Inc., Buffalo, NY) at 26 °C, approximately 70% RH, and a light: dark cycle of 16:8. In order to standardize the age of larvae in all of the following experiments, eggs were collected by placing 9 cm Petri dishes, containing blue diet, inside the colony for ~12 hr. Petri dishes were wrapped in aluminum foil in a funnel shape to exclude colony larvae. Eggs were then transferred, by microspatula, to bifurcated 9 cm Petri dishes or individual 50 mL centrifuge tubes each containing 25 mL or 2 mL, respectively, of treated or control blue diet. Nine centimeter Petri dishes contained blue diet only on one side of the bifurcation to allow larvae to migrate to the empty side for pupariation. Centrifuge tubes allowed for

monitoring of individual larvae, while the Petri dishes were used to rear multiple insects for microbial community analyses.

Oviposition choice assay

Following blue diet preparation, size 12 cork-borer plugs were taken from each Petri dish. Ten individual 9 cm Petri dish arenas were prepared by placing one plug of each treatment and the control in a circle (6 plugs per dish), with plugs placed equidistantly. Ten male/female pairings were added to each arena and allowed to choose and oviposit for 24 h. Eggs on each plug in each replicate were then counted and recorded.

Mortality, days to pupariation, and sex differences

Individual eggs were transferred to 50 mL centrifuge tubes by microspatula. There were 10 centrifuge tubes per replicate, and 5 replicates for each treatment (n=50/ n= 300 across all treatments). Inside each centrifuge tube, a strip of filter paper was placed inside to reduce excess moisture and provide a pupariation surface. Individuals were monitored daily for pupariation and adult emergence until all individuals had emerged or died. Adults were then sacrificed at -60 °C, and their sexes were determined based on the structure of genitalia.

Insect rearing for bacterial analysis

Eggs were transferred, by microspatula, to the blue diet of each 9 cm Petri dish. There were 3 replicates per life stage for each of the 6 treatments (n= 54). Lids contained a size 7 cork-borer hole affixed with 2 layers of fine organza mesh to allow for moisture and gas exchange. Following egg placement, Petri dish lids and bottoms were aligned and secured with parafilm. Petri dishes were monitored daily for development. Six individuals were collected at third instar, pupa, and adult life-stages, triple washed with 200 proof ethanol, and stored in clean 200 proof ethanol at -60 °C until DNA extraction. During the collection of each treatment group and life-stage, blanks in triplicate of DDI H_2O were used to monitor contamination. Before extraction, triplicate blanks were pooled.

DNA extractions and Illumina sequencing of whole body Megaselia scalaris *bacteria*

All DNA extractions and Illumina preparations were performed as in McFrederick and Rehan [148]. Briefly, DNA extractions were performed using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). An individual from each life-stage (n= 3), each treatment group (n= 6), and replicate group (n= 3), along with triplicates of the pooled blank (DDI H_2O), for each treatment group (n= 18), were acquired (n= 72) and placed in individual wells of a 96-well plate provided in the kit. Into each well, we added 180 µL of buffer ATL, a sterile 3.2 mm chrome-steel bead and 100 µL of 0.1 mm glass (Biospec, Bartlesville, OK). A Qiagen tissuelyzer was then used to bead-beat each sample for 6 min

at 30 Hz. We then added 20 μL of Proteinase K to each sample and incubated at 57 °C overnight. The standard DNeasy extraction protocol was then followed.

Following extraction, dual-index inline barcoding was used to prepare libraries for sequencing on the Illumina MiSeq. We used primers that included either the forward or reverse Illumina sequencing primer, a unique eight-nucleotide long barcode, and the forward or reverse genomic oligonucleotide as in Kembel et al. [150]. For the bacterial 16S rDNA sequences we used the primers 799F-mod3 CMGGATTAGATACCCKGG [151] and 1115R AGGGTTGCGCTCGTTG [150], which have been shown to minimize contamination from plastids.

We used these primers to generate 16S rRNA gene amplicons for Illumina sequencing using PCR. PCRs were performed using 10 μL ultrapure water, 10 μL of 2x Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 0.5 μL of each 10 μM primer stock, and 4 μL of DNA. We used a 52 °C annealing temperature, 35 cycles, and negative controls for each reaction. To remove unincorporated primers and dNTPs, we used the Ultraclean PCR clean up kit (MoBio, Carlsbad, CA). We used 1 μL of the clean PCR product as a template for another PCR, using HPLC purified primers to complete the Illumina sequencing construct as in Kembel *et al.* [150]: CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC, and AATGATACG GCGACCACCGAGATCTACACTCTTTCCCTACACGACG. For the reactions, we used a 58 °C annealing temperature, 35 cycles and negative controls. Once the PCR cycles were finished, we used 18 μL of the PCR product and SequalPrep Normilization plates (ThermoFisher Scientific, Waltham, MA) to normalize the amount

of DNA in each sample. We pooled 5 μL of each normalized sample, performed another cleanup, and then used a 2100 Bioanalyzer (Agilent, Santa Clara, CA) to assess our library quality. After quality control, we sequenced the libraries using the MiSeq Reagent kit v3 with 2 X 300 cycles. Raw data are available on the NCBI Sequence Read Archive (SRA) accession number SRP099221.

Bioinformatics

All genomic information was processed using macQIIME ver. 1.9.1-20150604 [152]. We used USEARCH v6.1 [115] to identify and remove chimeric sequences, and SUMACLUST [153] to cluster OTUs and remove any with less than two reads per sample. We used 97% sequence identity to bin OTUs and choose representative OTUs. We then performed standard alpha and beta diversity analyses in QIIME. To assign taxonomy to OTUs, Greengenes taxonomy [154] and the RDP Naïve Bayesian Classifier [155] were utilized, and we also performed BLASTN searches against NCBI's online Nucleotide Collection (nr/nt) and 16S ribosomal RNA sequences (Bacteria and Archea) databases (accessed January 17, 2017). Taxonomy was then used to identify any mitochondria or chloroplast OTUs, which were removed from the dataset as in McFrederick & Rehan [148]. We aligned the quality-filtered dataset using the pynast aligner [156] and the Greengenes database [154]. We then reconstructed the phylogeny of the bacterial OTUs using FASTTREE version 2.1.3 [157]. Next we performed weighted and unweighted UniFrac analyses [158] using the generated phylogeny and OTU tables. Using the generated distance matrices, we performed Adonis [120] and created PCA [74] graphs in R version 3.3.1 [159]. For

alpha diversity, we plotted rarefaction curves in GraphPad Prism version 6.00 software (La Jolla, California), and used gplots [160] to create a heatmap of the most abundant bacterial families; a 0.025 proportional abundance in at least one sample was used as the cutoff.

Statistics

All statistical analyses were performed using R (version 3.3.1). Normality was determined using Shapiro-Wilk normality tests. Mortality was determined using a generalized linear model with a binomial family. Differences in days to pupariation were determined using the 'survival' and the 'OIsurv' packages [161,162]. In all cases, when data were not considered normal, either a Poisson distribution or a negative binomial generalized linear model was used and the best fitting model was determined from Akaike's 'An Information Criterion'. Adonis within the R package "vegan" [120] was used for all PERMANOVA analyses. As there is no post-hoc [163] test for Adonis, we used adjusted p values obtained from metagenomeHIT_zig in R through QIIME [152,164] to determine differentially abundant OTUs in treatments between life stages.

Results

Oviposition choice, mortality, days to pupariation, and sex ratio differences

These insects did not respond to these contaminants because pharmaceuticals in reclaimed water did not affect oviposition preferences (χ^2= 3.66, df= 5, p=0.60). Mortality was increased (χ^2= 23.21, df= 5, p< 0.001) when *M. scalaris* were fed diets

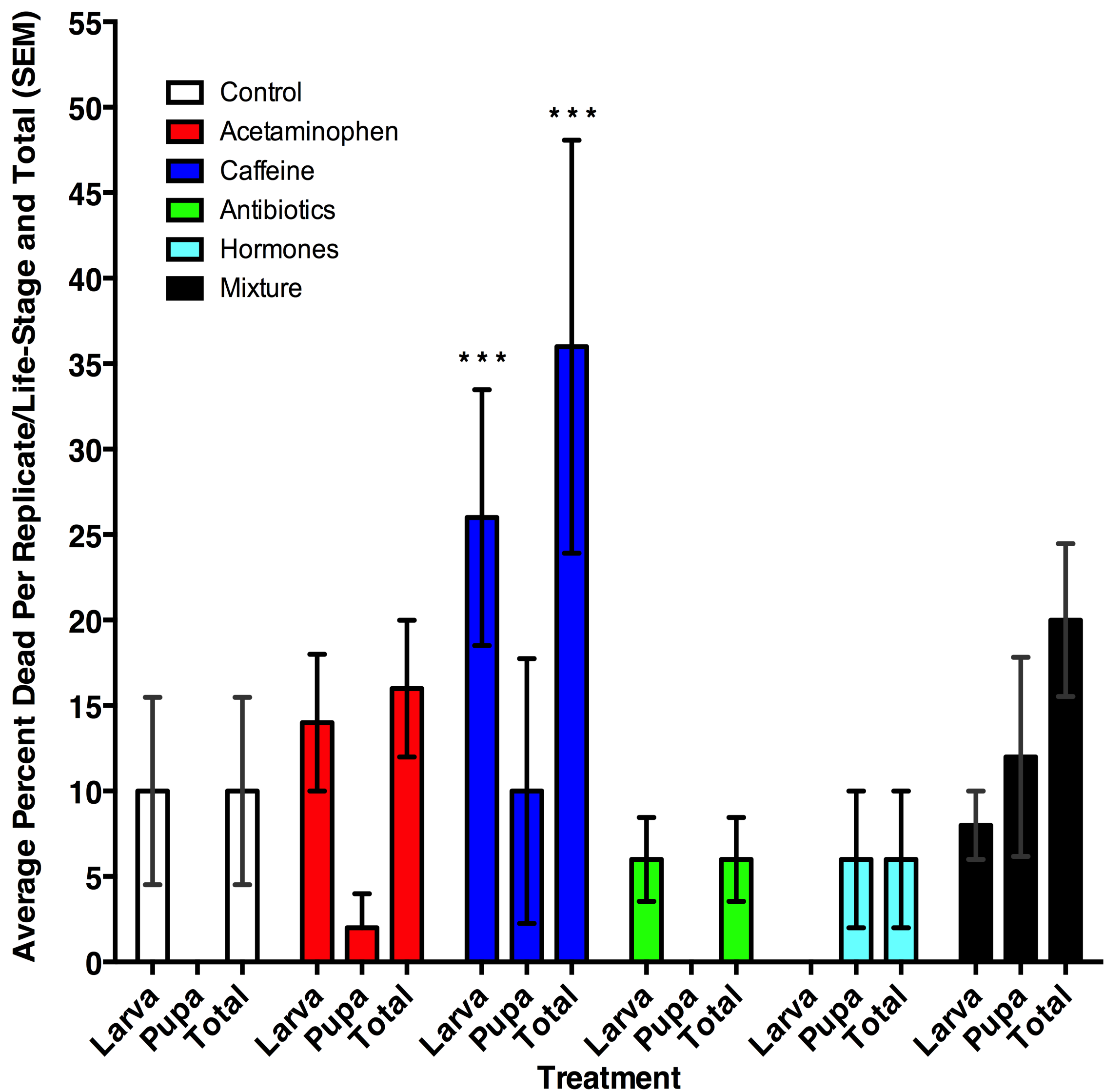


Figure 4.1: Average (SEM) mortality of larvae, pupae, and total insects for each treatment group. *** denotes significant difference (α= 0.05) relative to the control

containing caffeine ($p < 0.01$) (Fig. 4.1). Increased mortality was evident both in the larval life-stage ($\chi^2 = 22.81$, df= 5, $p < 0.001$) and the pupal stage ($\chi^2 = 17.41$, df= 5, $p < 0.01$). The largest increase in mortality was in larval-stage caffeine treatments ($p < 0.05$).

There was an increased time to pupariation (χ^2= 24.71, df= 5, p< 0.001) for *M. scalaris* fed antibiotic (p< 0.01) or hormone (p< 0.001) containing diets (Fig. 4.2).

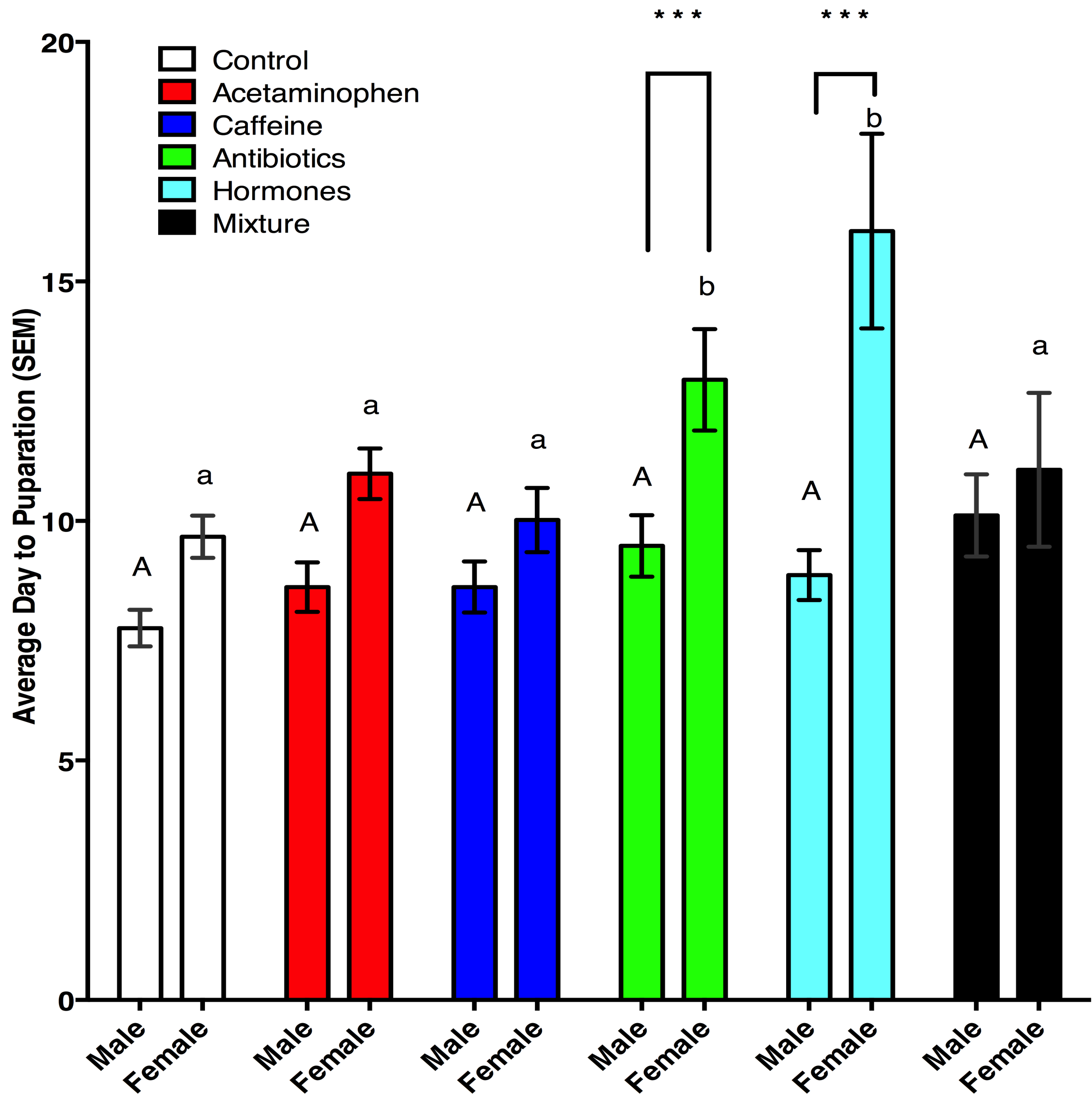


Figure 4.2: Average day to pupariation of male and female *Megaselia scalaris* by treatment. Upper case letters denote significant differences in days to pupariation (DTP) from male control. Lower case letters denote significant differences in DTP from female control. * * * denotes an overall day to pupariation difference from controls

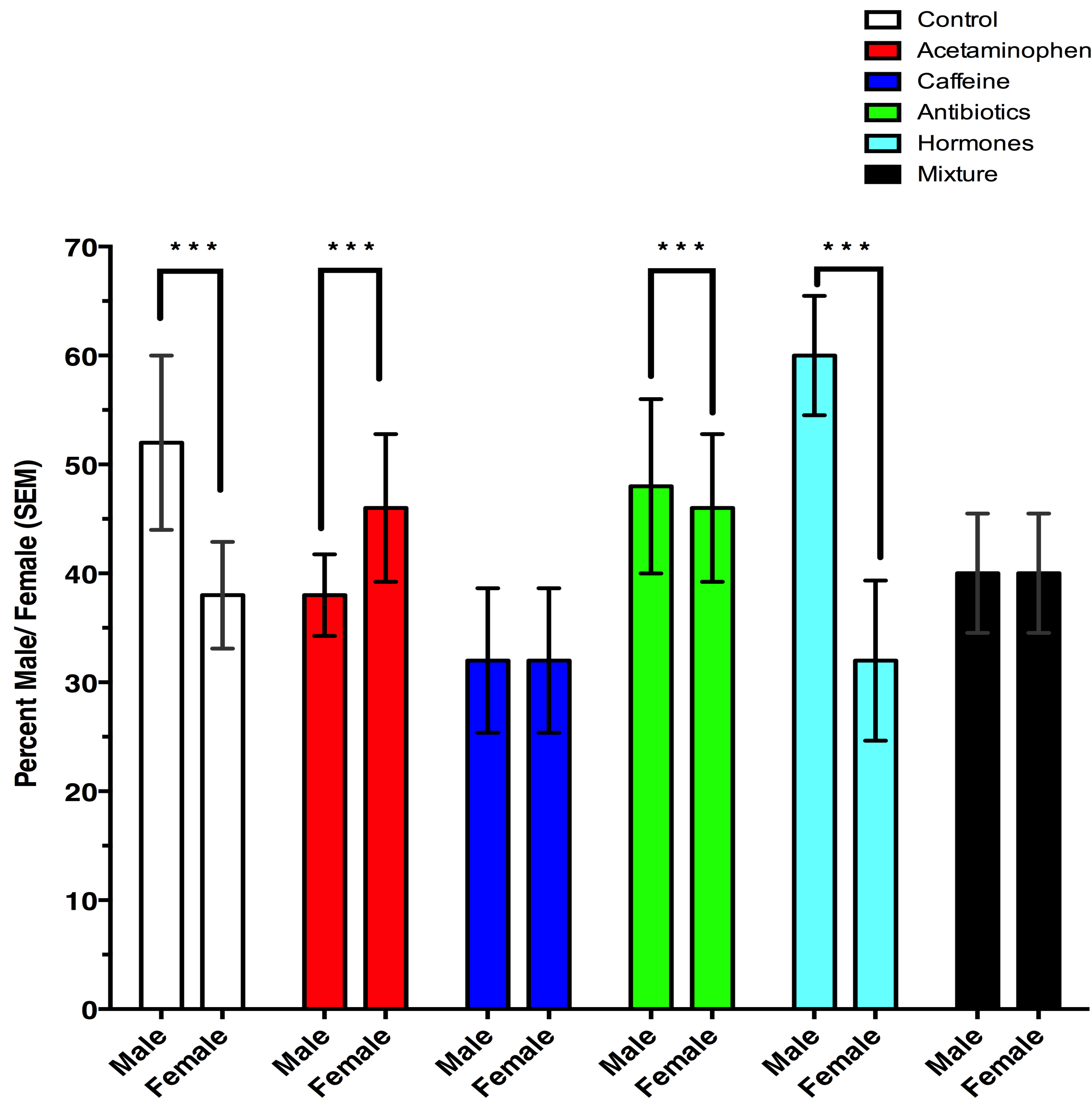


Figure 4.3: Male: female ratios of *Megaselia scalaris* fed diets contaminated with common pharmaceuticals found in reclaimed water. * * * denotes a significant difference in sex ratio with respect to treatment

While there were no overall differences of sex ratio (χ^2= 4.54, df= 5, p= 0.48), sex did have an effect on pupariation time (χ^2= 52.59, df= 1, p< 0.001), and there was an

interaction of treatment and sex (χ^2= 26.88, df= 5, $p< 0.001$), which was most evident in the mixture treatment ($p< 0.01$).

Within individual treatments, however, there were sex ratio differences (Fig. 4.3) for *M. scalaris* exposed to diets in the control ($p< 0.05$), acetaminophen ($p< 0.05$), antibiotics ($p< 0.001$), and hormone ($p< 0.001$) treatments. Interestingly, there were no sex ratio differences in the caffeine ($p= 0.15$) and mixture ($p=0.88$) treatments. Comparing the time to pupariation of opposite sexes (χ^2= 44.25, df= 5, $p<0.001$), male times to pupariation in treatments were not different (χ^2= 7.34, df= 5, $p=0.20$) than the controls. However, female (χ^2= 44.25, df= 5, $p< 0.001$) development in the antibiotic ($p< 0.01$) and hormone ($p< 0.001$) treated diets took significantly longer to pupariate than the control females (Fig. 4.2).

Bacterial community analysis

There were 752,855 total raw reads, with an average of 10,456 reads per sample, and a total of 772 distinct operational taxonomic units (OTUs), DNA sequences which are at least 97% identical, after removing OTUs identified as mitochondria, chloroplast, and obvious contaminant DNA through BLAST analysis. Overall, there was an effect of treatment (Adonis PERMANOVA: $F = 1.92$; $df = 5, 44$; $p < 0.05$) on the bacterial community of *M. scalaris*. Based on adjusted p-values (BH False Discovery Rate), a majority of differences in the treatments occurred between third instars and adults (Table 4.1). There were 30 different OTUs responsible for these differences and of those 28 were different in the third instar/adult stage comparisons.

Table 4.1: Bacterial families and genera in each treatment that are significantly different in at least one life-stage pairing. *denotes adjusted p value of <0.05 in those genera for each life-stage pairing in a treatment.

Treatment	Phylum	Family	Genus	Species	Fourth Instar-Pupa	Fourth Instar-Adult	Pupa-Adult
Control	Actinobacteria	Corynebacteriaceae	*Corynebacterium*	sp.	*		
	Bacteroidetes	Chitinophagaceae	*Sediminibacterium*	sp.	*	*	
	Proteobacteria	Alcaligenaceae	*Achromobacter*	sp.	*	*	
		Caulobacteraceae	*Caulobacter*	sp.	*	*	
		Enterobacteriaceae	*Klebsiella*	sp.	*	*	
			Enterobacter	sp.	*		
		Erwiniaceae	*Erwinia*	sp.	*	*	
		Hyphomicrobiaceae	*Pedomicrobium*	sp.		*	
		Methylobacteriaceae	*Methylobacterium*	sp.	*	*	
		Pseudomonadaceae	*Pseudomonas*	*veronii*	*	*	
				sp.	*	*	
				sp.	*	*	
		Sinobacteraceae	*Steroidobacter*	sp.		*	
Acetaminophen	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	sp.		*	
Caffeine	Actinobacteria	Cornebacteriacea	*Corynebacterium*	sp.			*
	Proteobacteria	Bradyrhizobiaceae	*Afipia*	sp.		*	*
		Burkholderiaceae	*Burkholderia*	sp.		*	*
		Enterobacteriaceae	*Enterobacter*	sp.	*	*	
		Erythrobacteraceae	*Altererythrobacter*	sp.		*	*

		Methylobacteriaceae	*Methylobacterium*	sp.		*	*
				sp.		*	*
		Pseudomonadaceae	*Pseudomonas*	sp.	*	*	
				sp.		*	*
Antibiotics	Actinobacteria	Microbacteriaceae	*Microbacterium*	sp.		*	
		Micrococcaceae	*Micrococcus*	sp.		*	
	Firmicutes	Lactobacillaceae	*Lactobacillus*	*brevis*	*	*	
	Proteobacteria	Burkholderiaceae	*Burkholderia*	sp.		*	*
Hormones	Actinobacteria	Mycobacteriaceae Nocardioidaceae	*Mycobacterium* *Nocardioides*	sp. sp.		* *	*
	Bacteroidetes	Sphingobacteriaceae	*Sphingobacterium*	*multivorum*	*	*	
	Proteobacteria	Alcaligenaceae	*Achromobacter*	sp.	*	*	
		Enterobacteriaceae	*Klebsiella*	sp.		*	
			Enterobacter	sp.	*	*	
		Erwiniaceae	*Erwinia*	sp.	*	*	
		Methylobacteriaceae	*Methylobacterium*	sp.	*	*	
		Moraxellaceae	*Acinetobacter*	*lwoffii*	*	*	
		Pseudomonadaceae	*Pseudomonas*	*veronii*	*	*	
				sp.	*	*	
				sp.	*	*	
		Rickettsiaceae	*Rickettsia*	sp.		*	*
Mixture	Proteobacteria	Burkholderiaceae	*Burkholderia*	sp.		*	*
		Erwiniaceae	*Erwinia*	sp.	*	*	

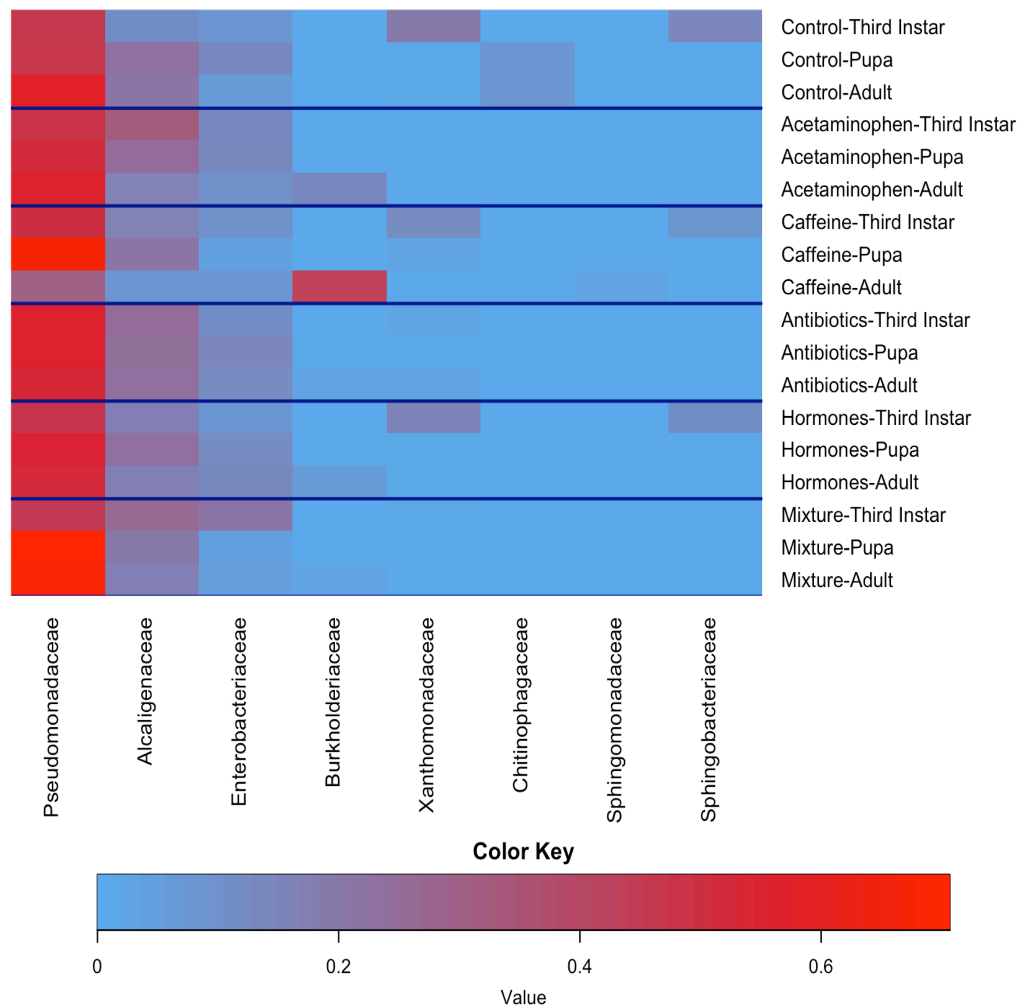


Figure 4.4: Heatmap of the most abundant bacterial families (each accounting for at least 2.5% of the total OTUs) by average OTUs of treatment life-stage pairing. Increased red coloration is an increase in indicative of increased proportional abundance.

There were eight bacterial families that accounted for at least 2.5% (by proportional abundance of OTUs) of the total bacterial families found in at least one sample (Fig. 4.4 and Table 4.2) and, collectively, they account for at least 94% of the total microbial community found in all life-stages. Six of the eight families in Figure 4 showed differences between third instars and adults (Table 4.1). Only *Burkholderiaceae* did not

show a difference between third instar and pupal stages. *Xanthomonadaceae* and *Sphingomonadaceae* were the only families on Figure 4.4 not also in Table 4.1. According to a non-metric multidimensional scaling (NMDS) plot (Supp. Fig. 4.2), the least dissimilarities were observed among 1) pupae and diets from hormone treatments, 2) the adults, pupae, and diets, from mixture treatments and 3) the individuals exposed to antibiotics.

Table 4.2: Average percentage of bacterial families by insect life-stage.

Bacterial Phylum	Bacterial Family	Avg. Percentage Fourth Instar	Avg. Percentage Pupa	Avg. Percentage Adult
Proteobacteria	Alcaligenaceae	26.41	22.90	17.23
	Enterobacteriaceae	13.31	10.64	9.42
	Pseudomonadaceae	50.61	58.02	52.26
	Burkholderiaceae	0.00	0.02	12.25
	Xanthomonadaceae	3.57	1.63	1.21
	Sphingomonadaceae	0.16	0.19	0.67
Bacteroidetes	Chitinophagaceae	> 0.01	1.40	1.49
	Sphingobacteriaceae	1.42	0.00	0.02
	Sum Percentages	95.48%	94.80%	94.55%
	Average OTU Count	4746.50	6579.78	8662.17

Discussion

Megaselia scalaris, a common detritivore, has been known to develop on substances as diverse as human wounds and corpses [165,166], modeling clay, and emulsion paint [167,168]. Their ability to grow and mature on these diets, with minimal effect on their survival, and their tolerance to heavy metals [145] makes any effect of pharmaceuticals at very low doses found in reclaimed water even more surprising. In our study, the females had no preference for untreated diets versus any treated diets. This poses a problem for the insect population, as there was higher larval mortality when developing on a caffeine-contaminated food source. Because females require an additional 24 hours [142] after

emergence in order to be receptive to males, populations exposed to hormones or antibiotics would be adversely affected. If females require an extra six days to emerge and become receptive, there is a reasonable possibility the males would leave the area or perish before mating. In addition, the suitability of decaying food sources tends to be temporary [169]. Collectively, these factors could likely negatively influence population growth. Also, these changes in population growth rate could hinder forensic scientists from determining an accurate time of death if there were long lasting or even moderate concentrations of these pharmaceuticals in the body at death.

Sex ratios of emergent adults were also affected in the caffeine and mixture treatments. The sex ratios found in control treatments in our study are similar to those reported in Benner & Ostermeyer [170] of a male: female sex ratio at 25° C of 1.18:1. However, sex ratios from the acetaminophen, caffeine, and mixture treatments differed significantly from the controls. A major difference in sex ratio would change the reproductive capacity of a population. It is unclear why acetaminophen and caffeine would alter sex ratios, however acetaminophen as been recorded to hinder the production of arachadonic acid in mosquitoes, another Dipteran, and it could be playing a similar role here [171]. Ibuprofen, another analgesic and antipyretic has been shown to alter the sex ratio in another mosquito[172].

Many insects rely on their microbial communities and endosymbionts to grow and develop [173]. However, Adonis, the statistical method used to analyze these data, does not have a post hoc test available that would allow direct pairwise comparisons between treatments. Nonetheless, there are changes in the bacterial community (Fig. 4.4 and Table

4.1) based on adjusted p-values evaluating differential abundance. We found significant shifts in the microbial community in the various life stages examined within the control treatments. A similar result has been reported for mosquitoes [140] and other insects [127,174]. Not surprisingly, insects that undergo complete metamorphosis and also rely on a different food source as adults would require a different bacterial community; however there is one family, *Pseudomonadaceae*, which appears in all treatments and life-stages. Species in this family are gram-negative Proteobacteria that cannot survive in acidic environments [96]. They are fairly common in insects [175], and can be responsible for 90+% of the bacterial community [176]. They are resistant to antibiotics [96], which potentially explains why they are so prevalent in many of our treatments. *Pseudomonadaceae* is responsible for ~ 50% of the bacteria in all life-stages, followed by *Alcaligenaceae*, *Enterobacteriaceae*, and *Xanthomonadaceae*. *Pseudomonadaceae* and *Enterobacteriaceae* families contain known symbionts in insects [81,177–179] and could be filling the same role in *M. scalaris*.

When *Pseudomonadaceae* is removed from the heatmap (Supp. Fig 4.1), it becomes clear how the next three highly proportional families change with life-stage. *Alcaligenaceae* tends to become more proportionally abundant in pupae and adults than in larvae. Species in the family *Alcaligenaceae* are oxidase- and catalase-positive and utilize a variety of organic and amino acids as carbon sources [95]. *Enterobacteriaceae* has higher proportions in larvae than in adults. Species of *Enterobacteriaceae* are likely to be either symbionts or a pathogen to their hosts [96]. *Enterobacteriaceae* includes *Buchnera*, an important endosymbiont of aphids [64], and other species that inhabit various insects to

provide facultative benefits [81,180]. *Xanthomonadaceae*, like *Enterobacteriaceae*, is more prominent in larvae than in other life-stages except in acetaminophen, antibiotic, and mixture containing diets, where *Enterobacteriaceae* dominate. Most species in *Xanthomonadaceae* are plant pathogens [96], and have been known to make use of chitin as a carbon source and utilize insects as vectors [181,182]. It is possible that some of the bacteria in this family may act as symbionts with insects as they have been found in a variety of insect orders [75,76,183].

In the NMDS plot (Supp. Fig. 4.2) there is distinct clustering in the microbiota by treatment. In individuals exposed to antibiotics in their diets, there is a lack of dissimilarity among their microbial communities. Insects reared on diets containing hormones had less microbial diversity in pupae. Unfortunately, we do not know if this is due to similarity in the microbial communities of third instar individuals as the statistical process of rarefication removed that particular life-stage in larvae exposed to hormones, controls, and caffeine treatments. However, the insects feeding on the mixture treatments show a distinct clustering of microbial groups in the pupal and adult stages, whereas the larval stage contains individuals with more variable microbiota. This is likely due to the early instars being exposed directly to the microbe-laden diet, while the later life-stages are only exposed to a subset of bacteria left after the gut contents were expelled at time of pupariation. The greatest dissimilarity was found in the caffeine-treated adults (Fig. 4.5, Supp. Fig. 4.2). The adult stage, regardless of treatment, also seems to be where the majority of variation in microbiota occurs (Fig. 4.5).

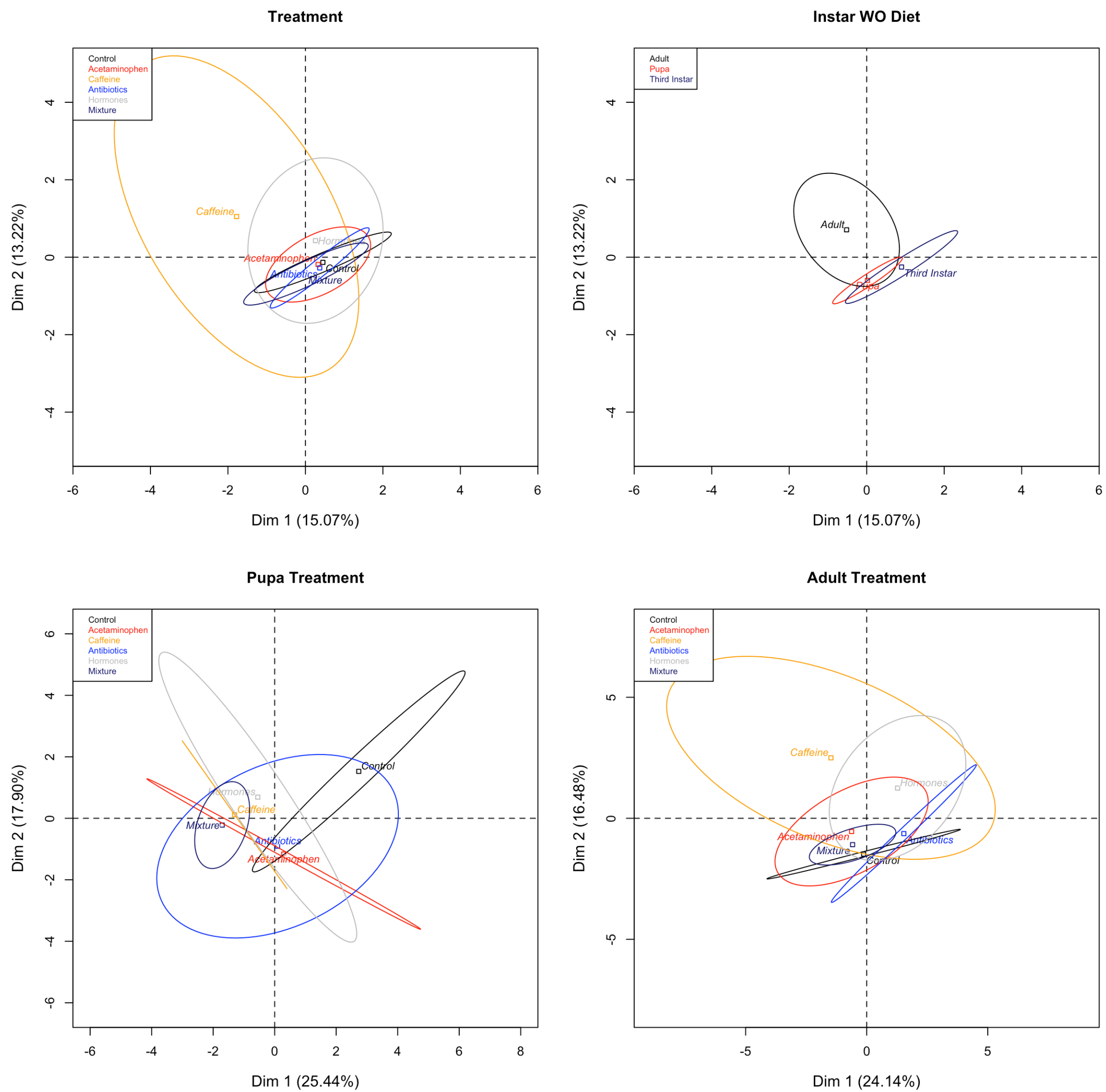


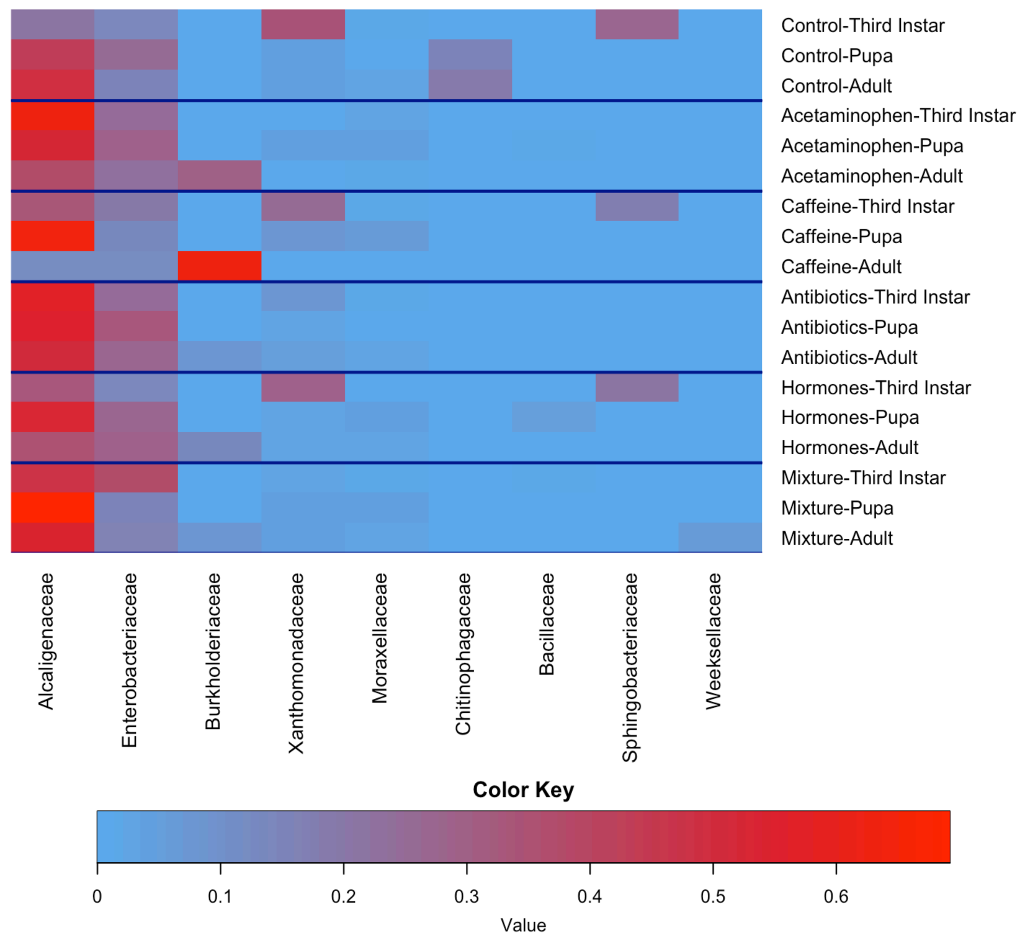
Figure 4.5: Principal Component Analysis of treatments, life-stage, pupa by treatment, and adult by treatment. Ellipses denote range of individuals around a centroid barycentre

Megaselia scalaris has been suggested as a model organism for bioassays for drugs and pollutants [142], and our findings support this claim. However, our results also suggest that the presence of even very low concentrations of some pharmaceuticals could affect the forensic estimation of time of death based on emergence patterns of adult *M.*

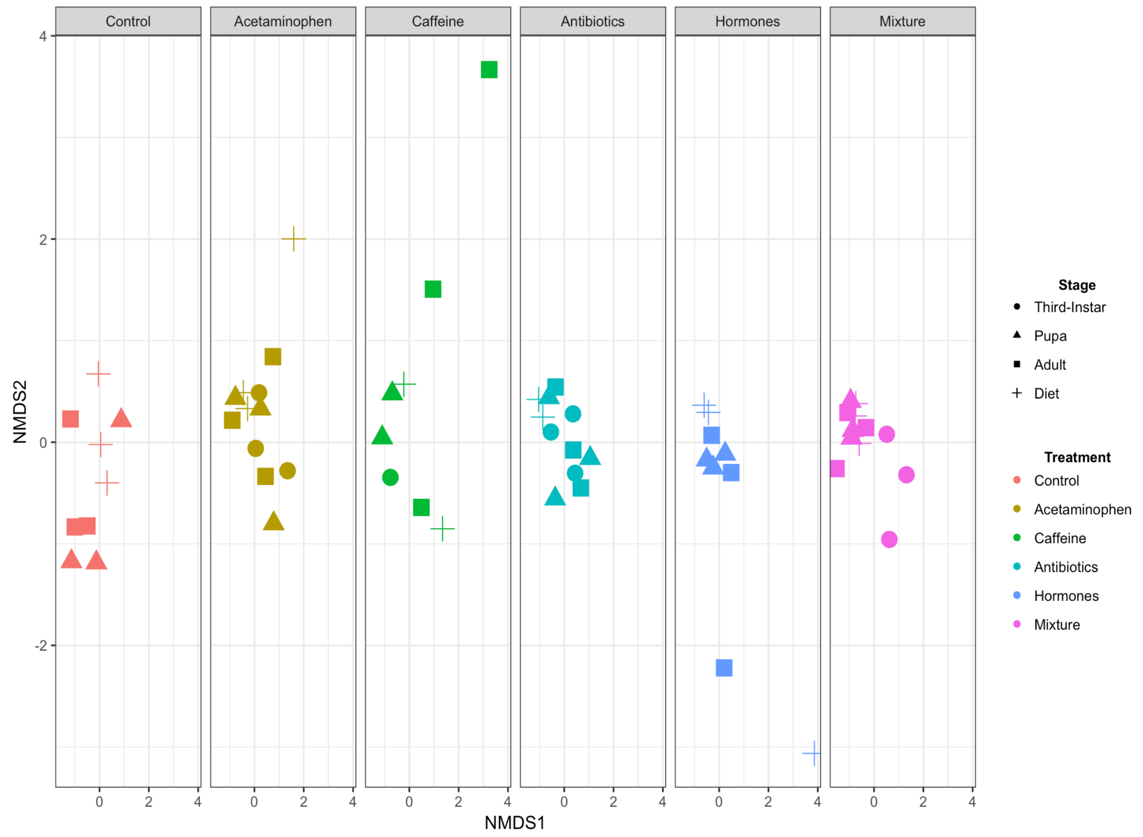
scalaris. We also caution that the pharmaceuticals used in this trial were at low concentrations found in wastewater and could be much higher in cadavers, as pharmaceuticals in humans tend to be higher than what is found in the environment [184–187]. Also, due to increases in concentrations caused by water loss (on a weight/weight basis), pharmaceuticals could have higher toxicity in decaying matter. Perhaps most importantly, pharmaceuticals in reclaimed water are having unintended effects on the microbial community of these flies, which could lead to decreased viability of these ecologically useful detritivores.

Supplemental Information

Supp. Figure 4.1: Heatmap, after removing the family *Psedomonadaceae*, of the top 5% proportionately most abundant families by average OTUs of treat life-stage pairing. Increased red coloration is an increase in proportional abundance.



Supp. Figure 4.2: Non-metric multidimensional scaling plots of dissimilarities in treatments by life-stage.



Chapter 5

Contaminants of emerging concern affect *Trichoplusia ni* (Hübner, Lepidoptera: Noctuidae) biology on artificial diets and a key host plant

Abstract

Many countries are utilizing reclaimed wastewater for agriculture because drought, rising temperatures, and expanding human populations are increasing water demands. Unfortunately wastewater often contains biologically-active, pseudopersistant pharmaceuticals, even after treatment. Runoff from farms and output from wastewater treatment plants also contributes high concentrations of pharmaceuticals to the environment. This study assessed the effects of common pharmaceuticals on an agricultural pest, *Trichoplusia ni* (Lepidoptera: Noctuidae). Larvae were reared on artificial diets spiked with contaminants of emerging concern (CECs) at environmentally relevant concentrations. *Trichoplusia ni* showed increased developmental time and mortality when reared on artificial diets containing antibiotics, hormones, or a mixture of contaminants. Mortality was also increased when *T. ni* were reared on tomatoes grown hydroponically with the same concentrations of antibiotics. The antibiotic-treated plants translocated ciprofloxacin through their tissues to roots, shoots, and leaves. Microbial communities of *T. ni* changed substantially between developmental stages and when exposed to CECs in their diets. Our results suggest that use of reclaimed wastewater for irrigation of crops can affect the developmental biology and microbial communities of an insect of agricultural importance.

Introduction

Many antibiotics and other common Contaminants of Emerging Concern (CECs) (pharmaceuticals, mental stimulants, surfactants, etc.), can be excreted by both humans and animals with little change in their chemical structure [42]. Standard wastewater treatment facilities are not equipped to completely remove pharmaceuticals [132,133] resulting in these compounds being found in effluent. In addition, even higher concentrations of many pharmaceuticals are released during heavy storms in the untreated wastewater overflow, which then directly contaminate the environment [4–7,46].

Recent studies of the effects of pharmaceuticals on aquatic insects show that at concentrations found in reclaimed water these CECs can alter development of the mosquito *Culex quinquefasciatus*, its susceptibility to a common larvicide, and its larval microbial communities [107,140]. However, because larval forms of aquatic insects develop directly in the contaminated water, their constant exposure is likely greater than most terrestrial insects. Interestingly, many CECs, which were not designed specifically to impact microbes, have been shown to affect microbial communities. For example, caffeine, a common mental stimulant, can alter biofilm respiration, and diphenhydramine, an antihistamine, have been shown to modify the microbial community of lake biofilms [51]. Due to such unexpected effects, accurately predicting the consequences of specific CECs, even in model insects, is not yet possible. This problem is exacerbated by a lack of information regarding effects of pharmaceuticals and other CECs on the microbial communities of any terrestrial insects.

Arthropods rely on hormones to grow, develop, mate, and produce pigmentation [53,55]. However, many pharmaceuticals, especially mammalian sex hormones, are structurally similar to chemicals that these organisms rely on for growth and development. These pharmaceuticals then bind to receptors and either over-express or suppress their counterparts' natural function. This has been seen in birds, reptiles, and arthropods where endocrine disruption occurs, primary and secondary sexual characteristics are modified, and courtship behaviors are changed [9,10,18,52,53,141]. While most arthropod hormones do not closely match those of mammals, their molting hormone (ecdysone), is very similar to the mammalian female sex hormone 17β-estradiol. In insects, 17α-ethinylestradiol, a common synthetic birth control hormone, has been shown to alter molting and lead to deformities of *Chironomus riparius* [11,32]. In addition to these effects, pharmaceuticals have been shown to have delayed cross-generational effects [32].

The cabbage looper (*Trichoplusia ni*) is a well-studied polyphagous insect native to North America, and found throughout much of the world [188,189]. This species is a pest on many agricultural crops including crucifers and a variety of other vegetables in both field and greenhouse settings [190]. Potential agricultural losses are exacerbated by a history of pesticide resistance development [191–193].

Currently there is little to no information regarding pharmaceutical effects at the concentrations found in reclaimed water on the growth or microbial community composition of any terrestrial herbivore. To investigate the function of the gut microbes in insects, several studies have used antibiotics applied at high doses [28,63]. To test the hypothesis that common pharmaceuticals affect mortality, development, and microbial

communities of *Trichoplusia ni*, we conducted a series of bioassays in artificial diet and on a key host plant.

Methods and Materials

Insect rearing

Insects were acquired from Benzon Research (Carlisle, PA). Eggs clusters were cut on wax-paper sheets, and taped to the lids of 237 mL Styrofoam cups containing artificial diet (Southland Products, Inc.; Lake Village, AR) to emerge. For experiments that required larvae to be moved, loopers were allowed to develop to second instars before initiating trials in order to minimize handling damage.

Artificial diet

Dry *T. ni* artificial diet mix and raw linseed oil was purchased from Southland Products, Inc. (Lake Village, AR) and mixed following the manufacturer's directions. For artificial diet treatments, 100 mL of DI water was treated with one of five CECs treatments or an untreated control. Environmentally relevant CEC treatments were used as in Pennington et al. [140], and based on concentrations described by Koplin et al. [5], and Mutiyar and Mittal [6] (Table 1). Briefly, treatment groups consisted of a control with only artificial diet for *T. ni*; an acetaminophen treatment (MP Biomedicals, LLC, Santa Ana, CA; purity ≥ 90%); a caffeine treatment (Fisher Scientific, Hanover Park, IL; laboratory grade purity); an antibiotic treatment of lincomycin, oxytetracycline, and ciprofloxacin (Alfa Aesar, Ward Hill, MA; purity ≥98%); a hormone treatment of estrone, 19-

norethindrone, 17β- estradiol, and 17α- ethynylestradiol (Sigma-Aldrich, St. Louis, MO; purity ≥98%); and a mixture of all pharmaceuticals.

Table 5.1: Contaminants of Emerging Concern (CEC) treatment group components and concentration.

Contaminant	**Concentration (μg/L)**	**Reference**
Antibiotics		
Oxytetracycline	72.90	[5]
Lincomycin	0.730	[5]
Ciprofloxacin	6,500	[6]
Hormones		
17α-Ethynylestradiol	0.831	[5]
17β- Estradiol	0.200	[5]
19- Norethindrone	0.872	[5]
Estrone	0.112	[5]
Mixture		
Acetaminophen	10.00	[5]
Caffeine	6.000	[5]
Antibiotics	Concentration as above	
Hormones	Concentration as above	

Hydrochloric acid and NaOH (prepared to a 1 M stock solution) (Fisher Scientific; 12.1 M, anhydrous pellets respectively) were used to adjust the final pH of all treatments and experimental solutions to 7± 0.5. Approximately twenty *T. ni* eggs were taped on the lids of the artificial diet cups. After emergence, larvae were removed until only ten were left. Data including day to adult and mortality were taken daily until all larvae reached the adult stage or died. Adult mass was recorded and adults were then frozen at -62 ± 2°C.

Three separate replicates (n=20 per replicate, total *T. ni* =360) of artificial diet for each of the six treatment groups were used for microbial DNA extractions. At least three individuals from three extra diets cups treated similarly were removed at third instar, sixth instar, the pupal stage, and the adult life-stages. Following removal of pupae from

the artificial diet, diet samples were stored at -62 ± 2°C for microbial DNA extractions. Each individual was washed three times in 200 proof EtOH and stored in clean 200 proof EtOH at -62 ± 2°C until DNA extraction.

Host plant

Tomatoes (*Solanum lycopersicum* L., variety 'Yellow Pear') were grown from seeds in 10.16 cm pots in UC soil mix No. 3 [194] and fertilized with Miracle Gro nutrient solution (Scotts Company, Marysville, OH) at labeled rate. At approximately 10 cm, tomato plants' roots were washed with water and transplanted to sand culture as in Hladun et al. [195]. Transplants were treated with CECs in hydroponic growth media (Oasis Hydroponic Fertilizer 16-4-17; Oasis Grower Solutions; Kent, OH) with concentrations described in Table 1. Plants were watered every 2 hr from 6 am- 6 pm and every 4 hr thereafter. Hydroponic solutions were kept in 120 L containers. Each container included one of five CEC treatments or an untreated control hydroponic solution. Plants grew 4-6 wk before cabbage loopers were bagged onto whole leaves with white mesh organza bags (20.32 cm x 30.48 cm). If *T. ni* devoured the entire leaf they were immediately moved to a leaf of similar location on the plant. Data regarding growth and development and mortality were collected daily. Growth index data were calculated as in Zhang et al. [196]. Data were then analyzed in R. Plants were separated into parts (roots, stems, old leaves, etc.) and frozen at -62 ± 2°C until analyzed.

DNA extractions and Illumina sequencing of whole body Trichoplusia ni *bacteria*

All DNA extractions and Illumina preparations were performed as in McFrederick and Rehan [148]. Briefly, DNA extractions were performed using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). An individual from each life-stage (n= 4), each treatment group (n= 6), and replicate group (n= 3), along with triplicates of a pooled blank for each treatment group (n= 24; total n=96), were placed in individual wells of a 96- well plate provided in the kit. We then added 180 µL of the supplied buffer ATL, a sterile 3.2 mm chrome-steel bead and 100 µL of 0.1 mm glass beads (Biospec, Bartlesville, OK) to each well. A Qiagen TissueLyzer was then used to bead-beat each sample for 6 min at 30 Hz. After addition of 20 µL of Proteinase K to each sample, samples were incubated samples at 57°C overnight. The standard DNeasy extraction protocol was then followed.

Dual-index inline barcoding was used to prepare libraries for sequencing on the MiSeq sequencer (Illumina Inc.; San Diego, CA). We used primers that included either the forward or reverse Illumina sequencing primer, a unique eight-nucleotide long barcode, and the forward or reverse genomic oligonucleotide as in Kembel *et al.* [150]. The bacterial 16S rDNA sequence primers used were 799F-mod3 CMGGATTAGATACCCKGG [151] and 1115R AGGGTTGCGCTCGTTG [150], which have been shown to minimize contamination from plastids.

Following the generation of 16S rDNA amplicons by these primers, PCR was performed to generate Illumina amplicons. PCRs were performed using 10 µL ultrapure water, 10 µL 2x Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich,

MA), 0.5 µL of each 10 µM primer stock, and 4 µL of DNA. We used a 52 °C annealing temperature, 35 cycles, and negative controls for each reaction. We used the Ultraclean PCR clean up kit (MoBio, Carlsbad, CA) to remove unincorporated primers and dNTPs. Then, 1 µL of the clean PCR product was used as a template for another PCR, using HPLC purified primers to complete the Illumina sequencing construct as in Kembel *et al*. [150]: CAAGCAGAAGACGGCATAC GAGATCGGTCTCGGCATTCCTGC and AATGATACG GCGACCACCGAGATCTACACTCTTTCCCTACACGACG. For these reactions, a 58 °C annealing temperature for 15 cycles and negative controls were used. Once the PCR cycles were finished, 18 µL of the PCR product and SequalPrep Normilization plates (ThermoFisher Scientific, Waltham, MA) were used to normalize the amount of DNA in each sample. Five microliters of each normalized sample were pooled together and used to performed another cleanup. Next, a 2100 Bioanalyzer (Agilent, Santa Clara, CA) was used to assess library quality. After quality control, the libraries were sequenced using a MiSeq sequencer (Illumina) and MiSeq Reagent kit v3 (Illumina) with 2 X 300 cycles. Raw data are available on the NCBI Sequence Read Archive (SRA) accession number SRP099237.

Bioinformatics

All genomic data were processed in macQIIME ver. 1.9.1-20150604 [114,152]. USEARCH v6.1 [115] was used to identify and remove chimeric sequences, and SUMACLUST [153] was used to cluster Operational Taxonomic Units (OTUs) with at least 97% sequence identity, and remove any with less than two reads per sample [197]. We then

used macQIIME to perform standard alpha and beta diversity analyses. To assign taxonomy to OTUs, Greengenes taxonomy [154] and the RDP Naïve Bayesian Classifier [155] were utilized. Because training set can influence these taxonomic assignments [198], BLASTN searches against NCBI's online Nucleotide Collection (nr/nt) and 16S ribosomal RNA sequences (Bacteria and Archea) databases (accessed 03/02/2017) were performed. Any mitochondria or chloroplast OTUs and other obvious contaminants (as determined by the blank controls) were removed from the dataset [148]. We aligned the quality-filtered dataset using the pynast aligner [156] and the Greengenes database [154]. The phylogeny of the bacterial OTUs was reconstructed using FASTTREE version 2.1.3 [157], and generated weighted and unweighted UniFrac distance matrices [158] using the phylogeny and OTU tables. Adonis analyses [120] and PCA [74] graphs were performed or created in R, version 3.3.1, [159] utilizing the UniFrac matrices. For alpha diversity, rarefaction curves were plotted in GraphPad Prism version 6.00 software (La Jolla, California), and the R package 'gplots' [160] to create a heatmap of the most abundant bacterial families; a 2.5% abundance in at least one sample was used as the cutoff.

Solid-Phase Extraction (SPE)

Dried plant material (0.25 ± 0.05 g) was ground by mortar and pestle with liquid nitrogen to a fine powder and extracted by EPA guidelines 1694 [199]. Breifly, powdered plant material was spiked with deuterated standards to determine recovery of chemicals and extracted with two rounds of sonication (30 min.) 20 mL of HPLC grade acetonitrile (Fisher Scientific, Hanover Park, IL) then centrifuged in a Beckman model J2-21M

Induction Drive Centrifuge (Indianapolis, IN) with a JA-17 tube holder for 15 min. at 10,000 RPM. Supernatant was decanted into a glass 40 mL vial and dried under N_2 gas in a hot water bath at 32.5 ± 2.5°C. In between both rounds of organic solvent extraction plant material was sonicated for 30 min. with 20 mL of phosphate buffer at pH 2.0± 0.2, centrifuged as before, and the supernatant decanted into a separate 40 mL glass vial. Extracts from dried acetonitrile were re-suspended in 1 mL of HPLC grade methanol (Fisher Scientific, Hanover Park, IL) and added to the phosphate buffer extracts. Phosphate buffer was then loaded a Waters™ SPE column (Milford, MA), pre-conditioned with 6 mL HPLC methanol then 12 mL DI water, and eluted under gravity. After all of the phosphate buffer had passed, the SPE column was dried under vacuum for 45 min. Once dried, 20 mL of HPLC methanol was passed through the SPE column by gravity into a glass 20 mL vial. The methanol was then dried under N_2 gas in a hot water bath (32.5 ± 2.5°C). Dried extracts were re-suspended in 1.5 mL of Ultima grade H_2O (18 Ω):MetOH (95:5 by v/v) and transferred to a 2 mL centrifuge tube. Extracts were centrifuged at 150,000 RPM for 15 min. in a tabletop SciLogex d2012 (Rocky Kill, CT). Supernatants were passed through a 13 mm polytetrafluoroethylene syringe filter with a 0.22 μm pore size (Restek; Bellefonte, PA) and stored at -4.0 ± 2.0°C until UPLC LC MS/MS analysis.

LC MS/MS analysis

Instrument analysis was preformed on a Waters ACQUITY ultra- performance liquid chromatography (UPLC) combined with a Waters Micromass Triple Quadrupole

mass spectrometer (qQq) equipped with an electrospray ionization (ESI) interface (Waters, Milford, MA). Separation was achieved using an ACQUITY UPLC C18 column (2.1 mm × 100 mm, 1.7 µm, Waters.) at 40°C and a binary gradient system of mobile phase A , DI water (18 Ω) acidified using 0.2% formic acid (FA), and mobile phase B composed of MeOH: acetonitrile (50:50 v/v) and 0.2% FA was used to separate analytes. The solvent gradient program, in terms of Mobile phase A, was as follows: initial condition began with 80% until 1 min when it was decreased linearly to 60% for 0.5 min, it was further decreased to 15% where it was held for 3 min then decreased linearly to 10% for 1 min, then increased linear to 90% for 1.5 min and held for 1 min after which time it equilibrated for 1 min for a total run time of 7:00 min. The flow rate was 0.3 mL min^{-1} and the injection volume was set to 5 µL. Mass data were acquired using Intellistart® (Waters) in the multiple reactions monitoring (MRM) mode and product ion scan in the positive ESI mode. The specific instrument settings were as follows: capillary source voltage 1.34 kV, dwell time 0.008 s, source temperature 150°C, desolvation temperature 500°C, desolvation gas 900 L h^{-1} and cone gas 50 L h,$^{-1}$ the collision gas was Argon 99.9% pure. Cone voltage and collision voltage for each compound are described in sup. table 5.1.

Statistics

All statistical analyses were performed using R (version 3.3.1). Normality was determined using Shapiro-Wilk normality tests, Quantile-quantile Plots, and histograms. Mortality was determined using a generalized linear model with a binomial family.

Analysis of variance (ANOVA) was used to determine differences of growth index. Days to adulthood were determined using the 'survival' and 'OIsurv' packages [161,162]. In all cases, when data were not considered normal, either a Poisson distribution or a negative binomial generalized linear model was used and best fit was determined from Akaike's 'An Information Criterion'. Adonis within the R package 'vegan' [120] was used for all PERMANOVA analyses. As there is no post-hoc [163] test for Adonis, adjusted p-values were obtained from metagenomeHIT_zig in R through macQIIME [152,164] to determine differentially abundant OTUs in treatments between life-stages. All Adonis analyses were conducted on weighted and unweighted UniFrac distance matrices. Weighted UniFrac places more emphasis on more abundant microbiota, whereas unweighted UniFrac is based solely on presence or absence; therefore, rare microbiota have similar weight to common microbiota. The Adonis groups that were significantly different were broken into their component differences, which were compared by adjusted p-values using p.adjust function and Benjamini-Hochberg adjustment in R. For UPLC work, individual compound peaks were detected and integrated using TargetLynx XS software (Waters).

Results

When *T. ni* were reared on artificial diet mortality was increased (χ^2= 44.99; df= 5; $p < 0.001$) in the antibiotic ($p < 0.05$), hormone ($p < 0.05$) and mixture ($p < 0.05$) treatments as compared to the controls (Figure 5.1A). The time to adulthood (χ^2= 63.43; df= 5; $p < 0.05$) was increased in the mixture treatments ($p < 0.05$) (Figure 5.1B). When reared on plants there was no significant difference for growth index ($p > 0.99$)

between treatment groups, however there was a significant effect on mortality (χ^2: 11.69; df=5; p< 0.05), predominately when reared on plants treated with antibiotics (p< 0.05) (Figure 5.1C). Chemical extraction data show increased concentrations of ciprofloxacin in leaves of the tomato plants (Table 5.2). There was no significant effect of treatment on time to adult for *T. ni* when reared on plants (χ^2= 7.76; df= 5; p= 0.17) (Figure 5.1D). This was likely due to dilution effects in the tomatoes and because those larvae that survive to pupation generally reach the adult stage.

Treatments (PERMANOVA: F = 7.17; df = 5, 79; p < 0.001) and life-stages (PERMANOVA: F = 25.26; df = 3, 81; p < 0.001) had significant effects on the *T. ni* microbial communities. However, there was no overall difference between microbial community types (insect or diet) (PERMANOVA: F = 0.42; df = 1, 83; p = 0.67). There were also significant interactions of treatment by life-stage (PERMANOVA: F = 2.84; df = 15, 69; p < 0.001) and by type of community (PERMANOVA: F = 5.35; df = 5, 79; p < 0.001).

Table 5.2: Concentration of antibiotics in treated plants through SPE and measured by UPLC. ND denotes non-detectable and NQ denotes detectable but non-quantifiable.

Chemical Extraction Data of Tomatoes var. 'Yellow Pear' (ng/Kg (**SEM**))										
		Plants without *T. ni*						**Plants with *T. ni***		
Treatment	**Chemical**	**Roots**	**Stems**	**Old Leaves**	**New Leaves**	**Flowers**	**Fruit**	**Leaves**	**Unrip e Fruit**	**Ripe Fruit**
Control	Lincomycin	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Ciprofloxacin	NQ	NQ	NQ	NQ	NQ	NQ	50951.66 **18938.88**	NQ	NQ
	Oxytetracycline	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Antibiotics	Lincomycin	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Ciprofloxacin	23524577.05 **4131781.70**	NQ	23781.97 **3212.40**	161700.38 **59534.52**	NQ	NQ	66295.59 **8237.10**	ND	ND
	Oxytetracycline	274724.56 **64315.22**	NQ	NQ	NQ	NQ	NQ	ND	NQ	NQ
Mixture	Lincomycin	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Ciprofloxacin	13589399.25 **5654303.13**	NQ	124356.25 **26436.56**	16050.92 **15567.80**	17314.47 **10664.73**	NQ	42321.95 **8110.49**	NQ	NQ
	Oxytetracycline	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ

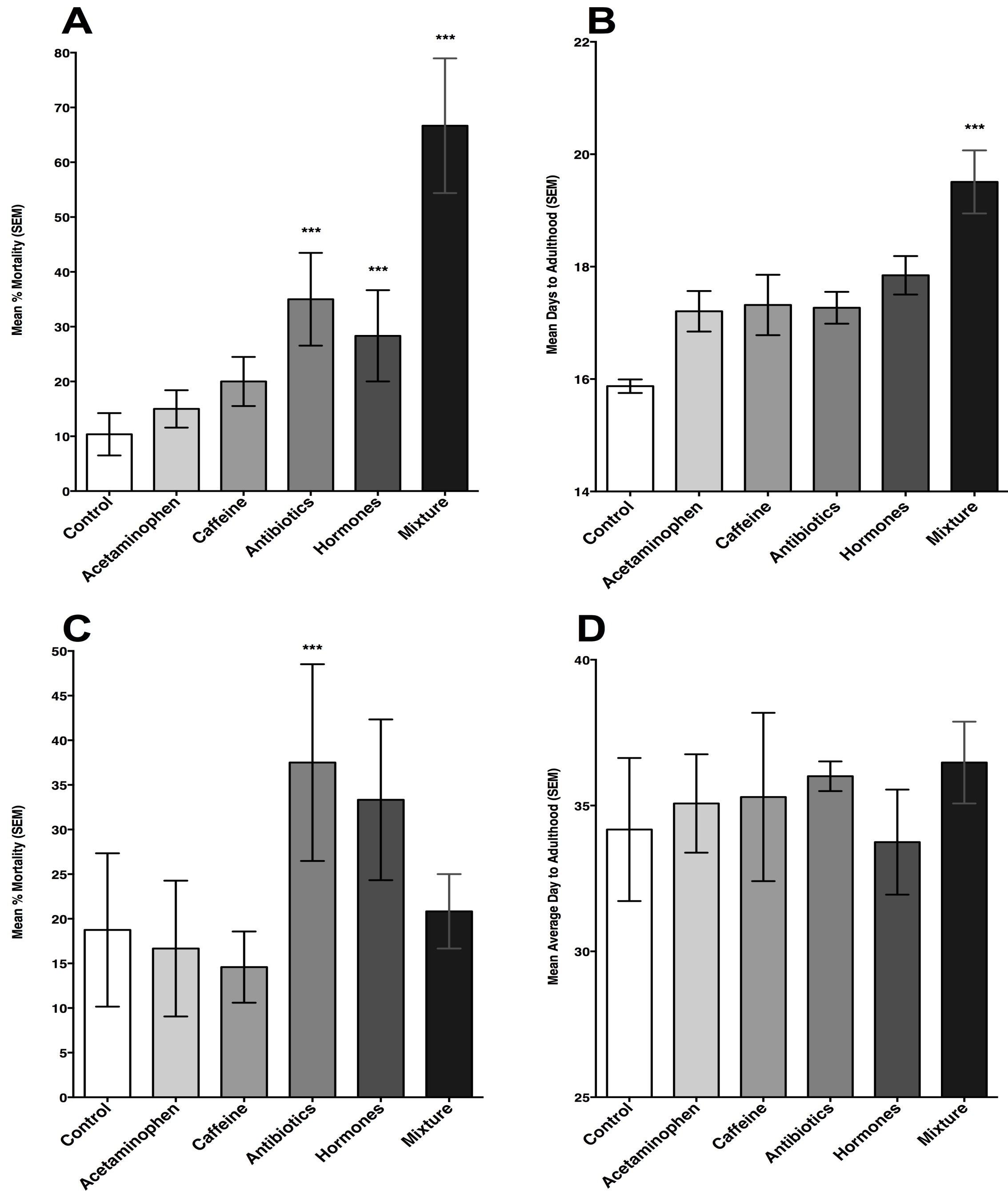


Figure 5.1: Effects of treatment on A) mortality of *Trichoplusia ni* reared on artificial diets; B) days to adulthood of *T. ni* on artificial diets; C) mortality of *T. ni* reared tomato plants treated with contaminated hydroponic growth solution; and D) days to adulthood of *T. ni* reared on tomato plants grown in contaminated hydroponic solution. *** denotes a significant difference relative to the control.

When more rare microbes were taken into account, there was a significant effect of treatment (PERMANOVA: $F = 1.62$; $df = 5, 79$; $p < 0.01$) and stage (PERMANOVA: $F = 3.26$; $df = 3, 81$; $p < 0.001$), and an interaction of treatment and type of microbial community (PERMANOVA: $F = 1.44$; $df = 5, 79$; $p < 0.01$). There was no significant effect of type (PERMANOVA: $F = 0.54$; $df = 1, 83$; $p = 0.96$), and there was no significant interaction of treatment and stage (PERMANOVA: $F = 5.35$; $df = 15, 69$; $p = 0.14$). As treatment was not significantly different in either weighted or unweighted UniFrac analyses, it was removed from further analyses.

When only third instar was considered, all treatments had significantly ($P_{adj} < 0.01$) different microbial communities. However, sixth instars, pupae, and adult life-stages ($P_{adj} \geq 0.84$, 0.56, 0.14 respectively) were not significantly different between treatments for both weighted and unweighted UniFrac. In all of the life-stages the treatments acetaminophen, caffeine, antibiotics, and hormones were all significantly different ($p < 0.05$) for weighted UniFrac. For unweighted UniFrac matrices, control and mixture treatment fed insects were significantly different ($P_{adj} < 0.01$).

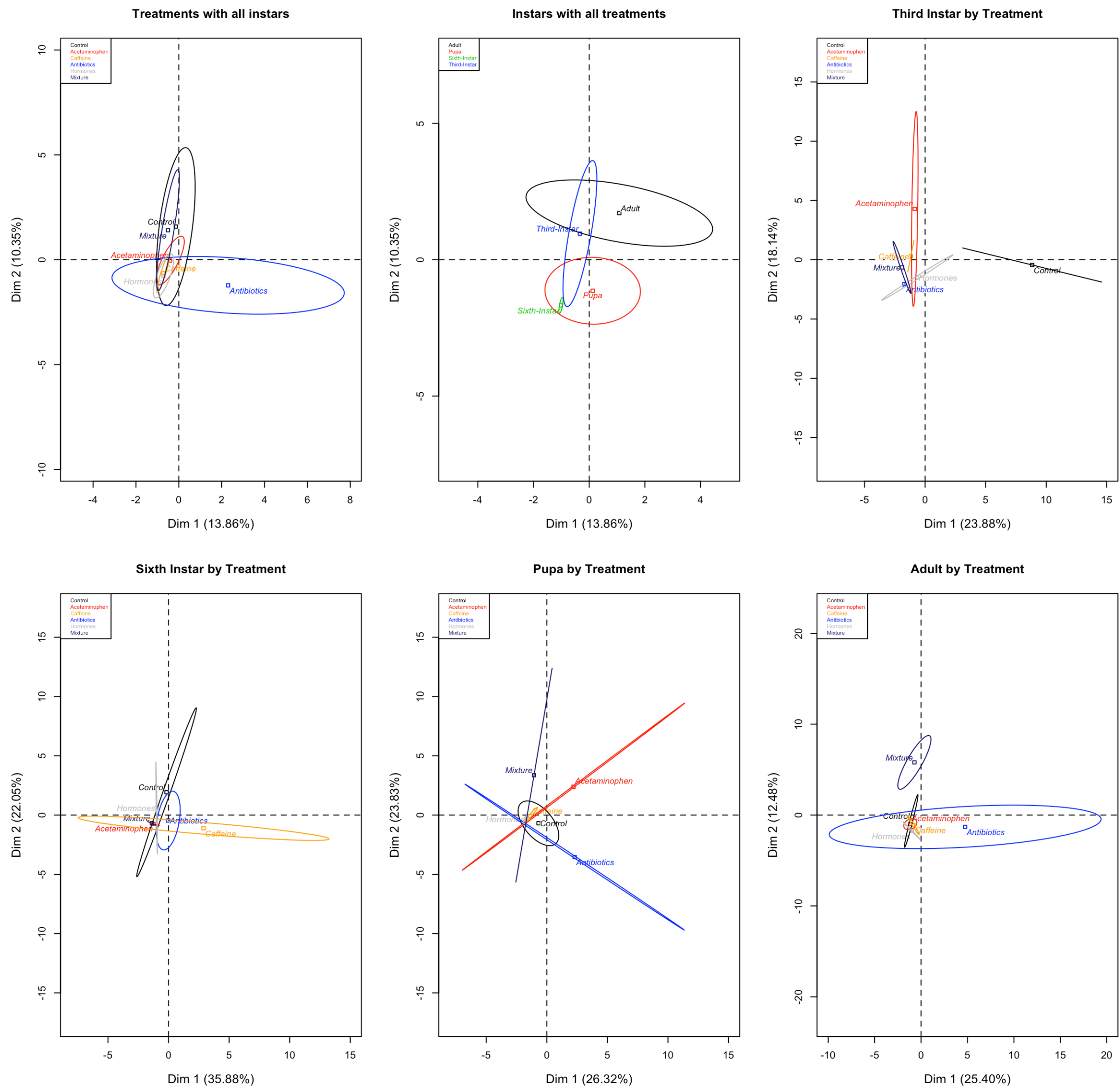


Figure 5.2: Principle Component Analyses of treatments with all instars, instars with all treatments, and by individual instars with all treatments. Ellipses denote the range of individuals around a centroid barycentre.

Figure 5.2 and Tables 5.3 and 5.4, describe the overall communities of *T. ni* reared on treated artificial diets. The top ten families, by average proportional abundance (Figure 5.3), account for over 86% of the entire microbial community of *T. ni* (Tables: 5.3 and 5.4).

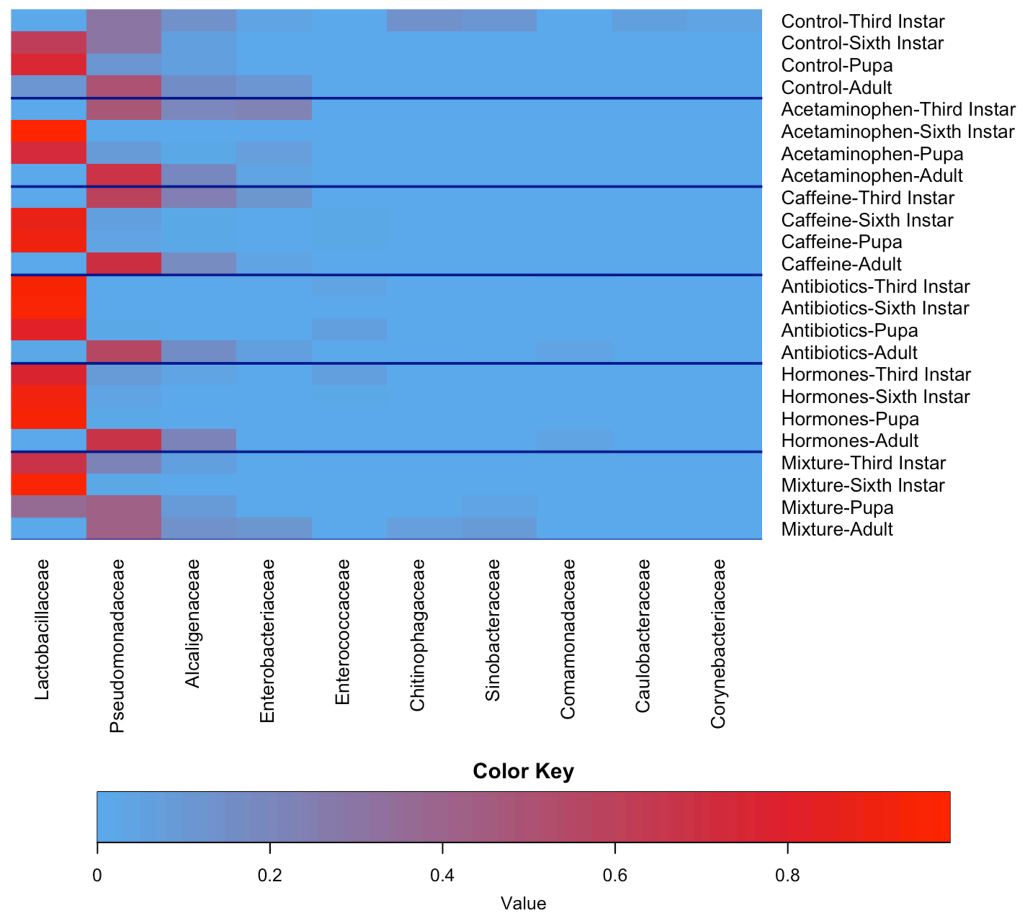


Figure 5.3: Heatmap of the top 10 most proportionally abundant bacterial families by average OTUs of treatment life-stage pairing. Increased red coloration is indicative of increased proportional abundance.

For the first three life-stages (third instars, sixth instars, and pupae) in all treatments, the majority of microbes belong to the family *Lactobacillaceae* (Table 5.3). *Lactobacillaceae*'s proportional abundance increases in third and sixth instar; remain high, but decrease in the pupae; and then decrease further in adult insects where the majority of microbes were *Pseudomonadaceae*. *Alcaligenaceae*, *Pseudomonadaceae*, and *Enterobacteriaceae,* the next three families with the highest average percentages of rarefied OTU counts, have similar patterns of high percentages in third instars followed by a decline in sixth instars and pupae, and then a spike in the adult life-stage. This same pattern was seen in both *Chitinophagaceae* and *Sinobacteraceae*, as well. For the control, acetaminophen, caffeine, and mixture treatment groups containing all life-stages (Table 5.4), the trends of average percentages follow the same patterns. The families with highest average percentage of rarefied OTU counts were *Lactobacillaceae*, *Pseudomonadaceae*, *Alcaligenaceae*, and *Enterobacteriaceae*, respectively. Interestingly, this pattern changes for antibiotic, hormone, and mixture treatment groups. For insects fed diets containing antibiotics and hormones, the average most proportionate families were *Lactobacillaceae*, *Pseudomonadaceae*, *Alcaligenaceae*, and *Enterococcaceae*.

Table 5.3: Average proportional abundance of bacterial families by life-stage.

Phylum	**Family**	**Third Instar**	**Sixth Instar**	**Pupa**	**Adult**
Acidobacteria	*Corynebacteriaceae*	0.56	0.05	0.06	0.39
Bacteriodetes	*Chitinophagaceae*	1.75	0.00	0.1	1.40
Firmicutes	*Enterococcaceae*	2.11	1.50	1.86	0.28
	Lactobacillaceae	43.07	88.70	75.45	2.56
Proteobacteria	*Alcaligenaceae*	10.44	1.89	3.52	15.6
	Caulobacteraceae	0.64	0.02	0.12	0.27
	Comamonadaceae	0.65	0.12	0.35	1.93
	Enterobacteriaceae	6.53	0.09	1.86	6.85
	Pseudomonadaceae	26.39	7.02	9.79	56.53
	Sinobacteraceae	1.36	0.00	0.39	1.61
	Percentage Total OTUs	93.5	99.39	93.5	87.42
	Average OUT Count	4075.25	4079.56	4062.23	4057.89

When examining the differential abundance of the individual OTUs by life-stage in each treatment (Table 5.5), an interesting pattern appears. There were significant differences ($P_{adj} < 0.05$) in controls between third instars and all other life-stages, between acetaminophen fed third instars and sixth instars, and between caffeine-fed third instars and sixth instars and pupae. However, in these three treatment groups, there were no significant differences ($P_{adj} > 0.05$) between sixth instar and pupae versus their adult life-stages. In the *T. ni* treated with antibiotics and the mixture treatment, microbes were significantly different ($P_{adj} < 0.05$) between any early life-stage and adults. For insects fed a hormone-contaminated diet, microbes were only different ($P_{adj} < 0.05$) for third instars when compared to adults.

Table 5.4: Average proportional abundance of bacterial families by treatment

Phylum	**Family**	**Control**	**Acetaminophen**	**Caffeine**	**Antibiotics**	**Hormones**	**Mixture**
Acidobacteria	*Corynebacteriaceae*	0.69	0.16	0.17	0.23	0.24	0.10
Bacteriodetes	*Chitinophagaceae*	2.37	0.16	0.00	0.27	0.01	2.36
Firmicutes	*Enterococcaceae*	0.87	0.61	1.41	2.58	2.39	0.32
	Lactobacillaceae	41.88	36.95	47.31	65.37	63.71	50.04
Proteobacteria	*Alcaligenaceae*	9.77	11.16	9.35	4.00	7.08	7.12
	Caulobacteraceae	0.90	0.04	0.02	0.01	0.00	0.66
	Comamonadaceae	0.61	0.96	0.51	0.88	0.89	0.85
	Enterobacteriaceae	4.42	9.66	3.79	1.26	0.39	4.41
	Pseudomonadaceae	28.82	34.09	34.18	11.87	21.48	24.72
	Sinobacteraceae	1.92	0.00	0.04	0.00	0.01	3.49
	Percentage Total OTUs	92.25	93.79	96.78	86.47	96.2	94.07
	Average OTU Count	3440.69	3448.69	3448.38	3464.85	3496.29	3394.25

There were similar trends when individual OTUs were examined for each life-stage comparing differences in treatments (Table 5.6). For third instar insects, controls had significant differences ($P_{adj} < 0.05$) in all other treatment groups; acetaminophen was significantly different ($P_{adj} < 0.05$) when compared to antibiotic, hormone, and mixture treatments, but not the caffeine treatment; caffeine-fed insect microbes followed a similar trend to acetaminophen. For third instar, there were no significant differences between antibiotic, hormone, and mixture treatments. Between all of the multiple treatment comparisons with OTUs, there were no significant differences in the sixth instar insects. Microbe composition in the pupal and adult life-stages followed similar trends. For pupae, acetaminophen and caffeine-fed insects' microbes were different compared to antibiotic treatments. The microbial communities in the antibiotic treatment were significantly different from hormone and mixture treatments. Adults had significant differences ($P_{adj} < 0.05$) in microbe composition in all treatments versus the mixture treatment. Principle Component Analyses (PCA) visualize these findings (Figure 5.2).

Table 5.5: Bacterial families and genera in each treatment that are significantly different in at least one life-stage pairing. *denotes adjusted p-value of <0.05 for that life-stage grouping in the genera treatment pairing.

Treatment	Phylum	Family	Genus	Species	3rd Instar-6th Instar	3rd Instar-Pupa	3rd Instar-Adult	6th Instar-Pupa	6th Instar-Adult	Pupa-Adult
Control	Acidobacteria	Bryobacteraceae	*Bryobacter*		*	*	*			
		Corynebacteriaceae	*Corynebacterium*	sp.1, 2	*	*	*			
	Bacteroidetes	Chitinophagaceae	*Sediminibacterium*	sp.1	*	*	*			
	Firmicutes	Peptoniphilaceae	*Finegoldia*		*	*	*			
	Proteobacteria	Bradyrhizobiaceae	*Bradyrhizobium*	sp.1, 2	*	*	*			
		Burkholderiaceae	*Burkholderia*		*	*				
		Caulobacteraceae	*Caulobacter*		*	*	*			
		Hyphomicrobiaceae	*Hyphomicrobium*		*	*	*			
		Methylobacteriaceae	*Methylobacterium*		*					
		Pseudomonadaceae	*Pseudomonas*	sp.1	*	*	*			
		Rhodospirillaceae	*Reyranella*		*	*	*			
		Sinobacteraceae	*Nevskia*		*	*	*			
			Steroidobacter	sp.1, 2	*	*	*			
Acetaminophen	Proteobacteria	Alcaligenaceae	*Achromobacter*		*					
		Enterobacteriaceae	*Erwinia*		*					
		Pseudomonadaceae	*Pseudomonas*	sp.2, 3	*					
		Xanthomonadaceae	*Stenotrophomonas*		*					
Caffeine	Firmicutes	Enterococcaceae	*Enterococcus*		*	*				
	Fusobacteria	Leptotrichiaceae	*Leptotrichia*		*	*				
	Proteobacteria	Enterobacteriaceae	*Erwinia*	sp.1, 2	*	*				
			Klebsiellia		*	*				
Antibiotics	Firmicutes	Enterococcaceae	*Enterococcus*				*			*
		Lactobacillaceae	*Lactobacillus*						*	*
	Proteobacteria	Acetobacteraceae	*Commensalibacter*	sp.1					*	
				sp.2					*	
		Alcaligenaceae	*Achromobacter*				*		*	*
		Comamonadaceae	*Pelomonas*				*		*	*
		Enterobacteriaceae	*Erwinia*	sp.1			*		*	*
				sp.2					*	
			Klebsiella						*	
			Salmonella						*	
		Methylobacteriaceae	*Methylobacterium*				*		*	*
		Pseudomonadaceae	*Pseudomonas*	sp.2, 4			*		*	*
		Xanthomonadaceae	*Stenotrophomonas*				*		*	
Hormones	Proteobacteria	Comamonadaceae	*Pelomonas*				*			
			Variovorax				*			
Mixture	Bacteroidetes	Chitinophagaceae	*Ferruginibacter*	sp.2			*		*	*
			Sediminibacterium	sp.1			*		*	*
	Proteobacteria	Bradyrhizobiaceae	*Bradyrhizobium*				*		*	*
		Caulobacteraceae	*Calobacter*				*			
		Ectothiorhodospiraceae	*Ectothiorhodospira*							*
		Geobacteraceae	*Geobacter*				*		*	*
		Methylobacteriaceae	*Methylobacterium*				*			
		Sinobacteraceae	*Steroidobacter*				*			

Table 5.6: Bacterial families and genera in each treatment that are significantly different in at least one treatment pairing. Lettering (AC, CA, AN, H, M) denotes adjusted p-value of <0.05 for that treatment group (Acetaminophen, Caffeine, Antibiotics, Hormones, and Mixture, respectively) in the genera life-stage pairing

Stage	Phylum	Family	Genus	Species	Control	Acetaminophen	Caffeine	Antibiotics	Hormones
3rd Instar	Acidobacteria	Bryobacteraceae	*Bryobacter*		AC, CA, AN, H, M				
		Corynebacteriaceae	*Corynebacterium*	sp.1	AC, CA, AN, H, M				
				sp.2	AN, M				
	Bacteroidetes	Chitinophagaceae	*Sediminibacterium*	sp.1	AC, CA, AN, H, M				
	Firmicutes	Enterococcaceae	*Enterococcus*		AN, H	AN, H, M	AN, H		
		Lactobacillaceae	*Lactobacillus*	sp.1	AN	AN, M	AN		
				sp.2	AN, H, M	AN, H, M	AN, H, M		
		Peptoniphilaceae	*Finegoldia*		AC, CA, AN, M				
	Fusobacteria	Leptotrichiaceae	*Leptotrichia*		CA		AN		
	Proteobacteria	Alcaligenaceae	*Achromobacter*		AN	AN	AN		
		Bradyrhizobiaceae	*Bradyrhizobium*	sp.1	AC, CA, AN, H, M				
				sp.2	AC, AN, H, M				
		Burkholderiaceae	*Burkholderia*		AC, CA, AN, H, M				
		Caulobacteraceae	*Caulobacter*		AC, CA, AN, H, M				
		Enterobacteriaceae	*Erwinia*	sp.1	AC, AN, H	AN, H, M	AN, H		
				sp.2		AN, H, M	AN		
			Klebsiellia		AN, H	AN, H	AN, H		
		Hyphomicrobiaceae	*Hyphomicrobium*		AC, CA, AN, H, M				
		Methylobacteriaceae	*Methylobacterium*		AN, M				
		Moraxellaceae	*Acinetobacter*		AC	H			
		Pseudomonadaceae	*Pseudomonas*	sp.1		AN	AN		
				sp.2		AN, H, M			
				sp.4	AC, AN, H, M				
		Rhodospirillaceae	*Reyranella*		AC, CA, AN, H, M				
		Sinobacteraceae	*Nevskia*		AC, CA, AN, H, M				
			Steroidobacter	sp.1, 2	AC, CA,				

					AN, H, M				
		Xanthomonadaceae	*Stenotrophomonas*			AN			
6th Instar	/	/	/	/	/	/	/	/	/
Pupa	Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	sp.1		AN	AN	H, M	
	Bacteroidetes	Chitinophagaceae	*Sediminibacterium*	sp.1				M	
	Firmicutes	Enterococcaceae	*Enterococcus*					H, M	
		Lactobacillaceae	*Lactobacillus*	sp.2		AN	AN	H, M	
	Proteobacteria	Acetobacteraceae	*Commensalibacter*	sp.1, 2, 3		AN	AN	H, M	
		Burkholderiaceae	*Burkholderia*					M	
		Caulobacteraceae	*Caulobacter*					M	
		Comamonadaceae	*Pelomonas*					M	
		Enterobacteriaceae	*Salmonella*			AN	AN	H, M	
		Methylobacteriaceae	*Methylobacterium*					M	
		Rhodospirillaceae	*Reyranella*					M	
		Sinobacteraceae	*Nevskia*		M	M	M	M	M
Adult	Bacteroidetes	Chitinophagaceae	*Flavihumibacter*		M	M	M	M	M
			Sediminibacterium	sp.1	M	M	M	M	M
	Proteobacteria	Bradyrhizobiaceae	*Bradyrhizobium*	sp.1	M	M	M	M	M
		Burkholderiaceae	*Burkholderia*			M	M	M	M
		Caulobacteraceae	*Caulobacter*		M	M	M	M	M
		Comamonadaceae	*Variovorax*				M		M
		Geobacteraceae	*Geoalkalibacter*		M	M	M	M	M
		Oxalobacteraceae	*Massilia*		AC, CA, AN, H, M				
		Rhodospirillaceae	*Reyranella*		M	M	M	M	M
		Sinobacteraceae	*Nevskia*		M	M	M	M	M
			Steroidobacter	sp.2	M	M	M		M

Discussion

In our study, CECs at concentrations found in reclaimed wastewater were shown to increase mortality of *T. ni*, especially on artificial diets contaminated with antibiotics, hormones, and a mixture of the chemicals. The mortality effect was also evident when *T. ni* were reared on plants grown in antibiotic-contaminated hydroponic growth media. Because plants grown in the hydroponic system contained quantifiable levels of ciprofloxacin in the leaf tissue, and the antibiotic treatments significantly changed the microbial community of the insect, we think this is possibly a cause of the mortality but we cannot exclude direct effects of the CECs on the insects or indirect effects through the plants. Interestingly we did not see the increased time to adulthood in *T. ni* reared on plants as compared with those reared on contaminated artificial diet. We postulate the discrepancy is possibly due to a number of factors such as dilution of CECs, as they were acquired from the water by the plants or there was biodegradation of the chemicals occurring in the plant [200] or by photodegradation. More studies would be needed to determine how CECs at concentrations found in reclaimed water for agriculture would interact with current integrated pest management (IPM) strategies (particularly pesticide application and use of beneficial insects), and how soil matrices would effect the chemical acquisition and translocation by plants.

Many insects rely on microbial communities and endosymbionts to grow and develop [173]. While Adonis does not have a post hoc test for direct pairwise comparisons, we can evaluate changes in the bacterial communities (Figure 5.2 and Table 5.3) based on adjusted p-values and PCA ellipses. We found significant shifts in the microbial

community in the various life-stages examined within the control treatments notably from third instar to subsequent life-stages. A similar result has been reported for mosquitoes [140] and other insects [127,174]. However, there were no significant differences in any of the later life-stages. This suggests that *T. ni* may require certain microbes to advance to later instar, but more information is needed to confirm this. Not surprisingly, insects that undergo complete metamorphosis and also rely on a different food source as adults would require a different bacterial community throughout the life stages; however there is one family, *Lactobacillaceae*, which appears in all treatments and life-stages in high proportions, except for adults. Species in this family are Gram positive Firmicutes that are known to produce lactate, formate, and succinate through fermentation [201]. They are fairly common in insects [79,103,147], and can be responsible for at least 70% of the bacterial community [148]. *Lactobacillaceae* is responsible for approximately 42% of the bacteria in all life-stages, followed by *Pseudomonadaceae*, *Alcaligenaceae*, and *Enterobacteriaceae*. *Lactobacillaceae* have been shown to act as beneficial bacteria in *Drosophila* and aid systemic growth when larvae are reared on nutrient-poor diet [202]. *Pseudomonadaceae* and *Enterobacteriaceae* families contain known symbionts in insects [81,177–179]. *Alcaligenaceae* has been shown to be present in other moths [79] but at a much lower proportionality than we found. These microbes could be commensal or mutualistic but more research would be needed to determine this.

There are clear patterns regarding the changes in microbial community proportionality according to the heatmap (Figure 5.3). In controls, third instar microbial communities are relatively evenly spaced by family. The microbial community becomes

predominately *Lactobacillaceae* for sixth instars and pupae. Once the insects reach the adult stage their most predominant family is *Pseudomonadaceae*. This pattern holds in the acetaminophen and caffeine treatment groups as well. Interestingly, the other treatment groups do not share this pattern. For antibiotic- and hormone-treated *T. ni*, *Lactobacillaceae* is the predominant microbial family in the immature stages, but at the adult stage microbial community reverts to predominantly *Pseudomonadaceae*. We suspect that this is because once the larvae undergo metamorphosis and shed their gut contents; they are no longer exposed to the pressures exerted by the CECs on the microbial community.

Figure 5.2 provides a visual indication of the changes in the bacterial communities over time. In larvae treated with antibiotics, the ellipses are relatively small in the third and sixth instars (indicative of low beta diversity), but become elongated (indicative of higher beta diversity) in pupae and adults. We suspect this is due to bacterial antibiotic resistance occurring in some of the insects' microbial communities but not the others. Interestingly, the hormone-treated *T. ni* follow a similar pattern to those exposed to antibiotics, but their ellipses are always much smaller, suggesting the entire insect population is showing a uniform response within their microbial communities. However, the mixture treated insects do not follow any of the patterns described above. Larvae exposed to mixtures of CECs display a greater diversity, on average, in their microbial community structure than either pupae or adults according to the heatmap (Figure 5.3). However, their ellipses are relatively small as adults (Figure 5.2), suggesting the microbial communities of the adult population are relatively similar

but still diversify after an extended period of time and through developmental stages. As this finding has not been shown in any treatment group containing only a single category of chemical (antibiotics, hormones, mental stimulants, analgesics), we suspect the microbes exposed to mixtures could be experiencing joint effects between chemical groups that could lead to possible synergism, additivity, potentiation, or antagonism. Such interactions should be the focus of future studies.

To the best of our knowledge, these results are the first to show that a terrestrial insect pest of commercial crops can be affected by CECs found in reclaimed wastewater for agricultural use. Our results suggest that CECs found in wastewater can impact *T. ni* growth and development, survivorship, and alter their microbial communities. Because *T. ni* is a common agricultural pest found around the world, feeds on a wide variety of plants, and has a history of resistance development, its ability to deal with toxins is likely higher than many other insect species. In addition, the responses we observed to CECs could have interesting implications for IPM practices such as lowering the amount of pesticides needed or increasing susceptibility to insect pathogens, as has been shown in mosquitoes [107]. We specifically want to note that ingestion of these compounds through uptake and translocation by a plant is not the only way *T. ni* or any other insect would be exposed to these compounds. Overhead sprinkler irrigation could cause contact absorption, and simply drinking water on leaves at contaminated sites could expose insects to higher concentrations than were found in plant tissues. Therefore, the effects reported here may be conservative, but additional studies with other insects, particularly

those with other feeding strategies, will be necessary before any patterns can be discerned.

Supplemental Information

Supp. Table 5.1: UPLC chemical analysis for each chemical and their deuterated standard.

Group	Compound	Formula	Tr [min]	Molecular ions [M + H]	Cone [V]	Fragment ions	CE [V]	ESI
Antibiotic	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	2.58 (±0.2)	332.0343	48	245.0868 231.0416	26 32	+
	Ciprofloxacin-D_8	$C_{17}D_8H_{10}FN_3O_3$	2.59 (±0.2)	336.1194	44	248.0708 235.0031	22 44	+
	Lincomycin	$C_{18}H_{34}N_2O_6S$	2.14 (±0.2)	407.1243	48	126.1087 41.8801	42 72	+
	Lincomycin-^{13}C-D_4	$C_{17}{}^{13}CH_{30}D_4N_2O_6S$	6.62 (±0.2)	408.2206	20	80.9941	16	+
	Oxytetracycline	$C_{22}H_{24}N_2O_9$	2.61 (±0.2)	461.0329	32	426.0248 201.0076	20 44	+
	Tetracycline-$^{13}C_2$- D_6	$C_{29}{}^{13}C_2H_{18}D_6N_2O_8$	2.49 (±0.2)	451.1181	32	416.1078 104.0699	18 44	+

Chapter 6

Effects of contaminants of emerging concern on

Myzus persicae (Sulzer, Hemiptera: Aphididae) biology

and on their host plant, *Capsicum annum*

Abstract

Many countries are utilizing reclaimed wastewater for agriculture as water demands due to drought, rising temperatures, and expanding human populations increase. Unfortunately, wastewater often contains biologically-active, pseudopersistant pharmaceuticals, even after treatment. Runoff from agriculture and effluent from wastewater treatment plants also contribute high concentrations of pharmaceuticals to the environment. This study assessed the effects of common pharmaceuticals on an agricultural pest, the aphid *Myzus persicae* (Sulzer, Hemiptera: Aphididae). Second instar nymphs were transferred to bell peppers (*Capsicum annum*) that were grown hydroponically. Treatment plants were spiked with contaminants of emerging concern (CECs) at environmentally-relevant concentrations found in reclaimed wastewater. *Myzus persicae* displayed no differences in population growth or microbial community differences. Plants however, displayed significant growth reduction in antibiotic and mixture treatments, specifically in wet root masses. Antibiotic treatment masses there were differences in antibiotic and mixture treatments. Antibiotic treatment masses were significantly reduced in the total and root wet masses. Mixture treatments displayed an overall reduction plant root wet mass. Our results suggest that the use of reclaimed wastewater for crop irrigation would not affect aphid populations, but could hinder or delay crop production.

Introduction

Pharmaceuticals have been increasingly prescribed for the past 30 years, and prescription rates have almost tripled in just the past 14 years [40,41]. In 2013 alone, over two-thirds of the antibiotics used (9.1 million kg) were employed for the purpose of increasing agricultural production [129]. Many antibiotics and other common 'Contaminants of Emerging Concern' (CECs, pharmaceuticals, mental stimulants, and surfactants), can be excreted by both humans and animals with little change in the CECs' chemical structure [203–205]. Not surprisingly, pharmaceuticals have been appearing in wastewater, and in some cases tap water, over the past few years [130,131].

Standard wastewater treatment facilities are not equipped to completely remove pharmaceuticals [132,133] resulting in these compounds being found in effluent. In addition, even higher concentrations of many pharmaceuticals are released during heavy storms in the untreated wastewater overflow, which then directly contaminate the environment [46]. These pharmaceuticals have been found at biologically active concentrations in surface waters around the world [4–7,134,136,206]. There is also an increasing effort to use reclaimed wastewater in drought affected areas, such as Southern California [24,135]. In agriculture/livestock operations, pharmaceuticals are found in manure that is used as fertilizer, effectively compounding the pharmaceutical concentrations [7,136,137]. Current research shows these chemicals tend to be both pseudopersistant in soil and detrimental to soil microbes [4,48,49,138,139].

Our recent studies of the effects of pharmaceuticals on aquatic insects show that at concentrations found in reclaimed water these CECs can alter development of the

mosquito *Culex quinquefasciatus*, its susceptibility to a common larvicide, and its larval microbial communities [107,140]. Female *Megaselia scalaris*, which are ecologically important detritivores, also displayed an increased developmental period, which could jeopardize the population's survival, when exposed to CECs [207]. Watts et al. [11] showed 17α-ethinylestradiol, a common birth control agent, and Bisphenol-A, a common plasticizer, can cause deformities in the midge *Chironomus riparius*. However, because larval forms of aquatic insects develop directly in the contaminated water, their constant exposure is likely greater than most terrestrial insects. Interestingly, many CECs that were not designed to specifically impact microbes have been shown to affect microbial communities. For example, the common mental stimulant caffeine can alter biofilm respiration, and diphenhydramine, an antihistamine, has been shown to modify the microbial community of lake biofilms [51]. Due to such unexpected effects, accurately predicting the consequences of specific CECs, even in model insects, is not yet possible. This problem is exacerbated by a general lack of information regarding effects of pharmaceuticals and other CECs on the microbial communities of terrestrial insects. Arthropods, such as insects and crustaceans, rely on hormones to grow, develop, mate, and produce pigmentation [53–55]. However, many pharmaceuticals, especially mammalian sex hormones, are structurally similar to chemicals that these organisms rely on for growth and development. These pharmaceuticals bind to receptors and either over-express or suppress their counterparts' natural function. Endocrine disruption has been noted in birds, reptiles, and arthropods where endocrine disruption occurs, primary and secondary sexual characteristics are modified, and courtship behaviors are changed

[9,10,18,52,53,141]. While most arthropod hormones do not closely match those of mammals, their molting hormone (ecdysone), is very similar in structure to the mammalian female sex hormone 17β-estradiol. In crustaceans, mammalian hormones have been known to cause both increased molting events and inhibition of chitobiase, the enzyme responsible for digestion of the cuticle during insect molting [56,57]. In insects, 17α-ethinylestradiol, a common synthetic birth control hormone, has been shown to alter molting and lead to deformities of *C. riparius* [11,32]. In addition to these effects, pharmaceuticals have been shown to have delayed cross-generational effects [32].

Aphids are phloem-limited hemimetabolous (immature insects resemble the adults and do not undergo complete metamorphosis) insects. *Myzus persicae* (Sulzer, Hemiptera: Aphididae) is polyphagous, highly cosmopolitan, and an efficient vector of viruses [208]. This insect overwinters in the egg stage on *Prunus* species and when their host plants are over-populated and/or stressed, they begin producing alates (winged-form) to disperse and colonize new plants [209–213]. The sexual forms are also alates and are formed in autumn temperatures wherever peaches or suitable host plants are available [208,213]. Economically, *M. persicae* is most damaging in the Spring, when the insects hatch and feed on new peach leaves, and serve as vectors of over 100 different plant viruses (both persistent and non-persistent) [208]. The aphid microbiome has been extensively studied and is well understood, making aphids excellent models for microbial community and biological research [210,212–214]. Previous research has determined antibiotics can reduce fecundity, reduce population growth, and increase mortality of aphids [64,215–217]. Previous

findings were usually due to the reduction of *Buchnera*, a key symbiont that provides required nutrients the aphids cannot make themselves or acquire from their diet [64].

Currently there is little information regarding pharmaceutical effects at the concentrations found in reclaimed water on the growth or microbial community composition of terrestrial herbivores. Many herbivores can be exposed to these contaminants after the CECs enter surface waters, soil, and plants from wastewater reuse and unintended discharge. To investigate the function of the gut microbes in insects, several studies have used antibiotics, but these chemicals were applied at much higher doses than found in reclaimed water [28,63]. There little to no information available regarding effects of CECs when translocated through plants to terrestrial insects. Depending on the acquisition and sequestration by their host-plant species, insects with limited feeding methods, such as aphids, could have either reduced or increased exposure to CECs. Because previous research demonstrated a substantial change in both the biology and microbial communities of other insects when treated with ecologically-relevant levels of CECs [107,140,207], and since aphid growth and development rely on symbionts, we hypothesized that aphids could be affected in similar ways. To test this hypothesis, we conducted bioassays of aphids reared on a key host plant, *Capsicum annum*, exposed to CECs at concentrations found in reclaimed water Any effects would have potentially important implications from agricultural perspectives. Also, as there is currently little information on effects of CECs on terrestrial insects acquired through a plant matrix, our findings would have possible interest for integrated pest management (IPM) research.

Methods and Materials

Insect rearing

Green peach aphids (*Myzus persicae*) were obtained from a colony maintained on bell peppers (*Capsicum annum*, variety 'Islander') in a UCR greenhouse. The insects were in colony for less than one year at the time of the experiments. Natural light was supplemented with artificial light to maintain a long day photoperiod (LD: 16-8). When transfer of insects was required, second instar aphids were moved to new host plants to eliminate mortality that occurred when first instar insects were handled.

Population growth

Bell peppers were grown from seeds in 10.16 cm pots in UC soil mix No. 3 [194] and fertilized with Miracle Gro nutrient solution (Scotts Company, Marysville, OH) at labeled rate and watered as needed in the UCR greenhouse. When plants were approximately 10 cm, their roots were washed with D.I. water and they were transplanted to a 475 mL Mason jars (Fischers, IN). Mason jars were coated with Folk Art Multi-Surface acrylic paint (Plaid Enterprises, INC.; Norcross, GA) on the outside to prevent root exposure to light. Jars were filled with hydroponic growth media (Oasis Hydroponic Fertilizer 16-4-17; Oasis Grower Solutions; Kent, OH) containing CEC concentrations described in Table 5.1 with average pH of 7.0 ± 0.5. Briefly, treatment groups consisted of a control with 500 µL of 5:45 (v:v) methanol (carrier): D.I. water; an acetaminophen

treatment (purity ≥ 90%; MP Biomedicals, LLC, Santa Ana, CA); a caffeine treatment (laboratory grade purity; Fisher Scientific, Hanover Park, IL); an antibiotic treatment of lincomycin, oxytetracycline, and ciprofloxacin (purity ≥98%; Alfa Aesar, Ward Hill, MA); a hormone treatment of estrone, 19-norethindrone, 17β- estradiol, and 17α-ethynylestradiol (purity ≥98%; Sigma-Aldrich, St. Louis, MO); and a mixture of all pharmaceuticals. Hydrochloric acid, NaOH, prepared to a 1 M stock solution, (12.1 M, anhydrous pellets respectively; Fisher Scientific) and a pH adjuster (JR Peters Laboratory, Allentown, PA) were used to adjust the final pH of all treatments and experimental solutions to 7± 0.5.

Table 6.1: Contaminants of Emerging Concern (CEC) treatment group components and concentration.

Contaminant	Concentration (µg/L)	Reference
Antibiotics		
Oxytetracycline	72.90	[5]
Lincomycin	0.730	[5]
Ciprofloxacin	6,500	[6]
Hormones		
17α-Ethynylestradiol	0.831	[5]
17β- Estradiol	0.200	[5]
19- Norethindrone	0.872	[5]
Estrone	0.112	[5]
Mixture		
Acetaminophen	10.00	[5]
Caffeine	6.000	[5]
Antibiotics	Concentration as above	
Hormones	Concentration as above	

Treatment media were prepared utilizing stock solutions of treatment compounds dissolved in 5:45 (v:v) methanol: D.I. water with aliquots of < 500 μL being dissolved in 18 L. Growth media was stored at room temperature in blackened 19 L tanks to protect the CECs from photodegradation and algal growth. Hydroponic growth media was drained, by Erlenmeyer filter flask and vacuum, and replaced every three days to hinder bacterial and fungal growth and maintain CEC concentrations. After filtering through a HEPA-CAP (Whatman, Inc.; Florham Park, NJ) air filter, house air was bubbled into jars through black irrigation tubing to aerate the hydroponic growth media. Each container included one of five CEC treatments or an untreated control hydroponic solution, and was used to water four plants. Plants grew three weeks before 10 *M. persicae* were placed evenly on two fully-expanded leaves per plant. There were four replicate hydroponic containers for each of the six treatments (n= 20 individuals per plant; n= 480 total *M. persicae*). Data regarding population growth were collected daily and the experiment was ended after two weeks. Three life-stage groupings (first and second, third and fourth, and adult life stages) were collected from each plant, with a minimum sample size of 20 individuals per life-stage (n= 20 individuals per plant; n= 480 total *M. persicae*), and stored in 200 proof ethanol at 62 ± 2°C until DNA extractions were performed. Plants were separated into parts (roots and leaves), weighed, and immediately frozen at -62 ± 2°C.

DNA Extractions and Illumina Sequencing of Whole Body Myzus persicae *Bacteria*

All DNA extractions and Illumina preparations were performed as in McFrederick and Rehan [148] within one month of -62 ± 2°C storage. Briefly, DNA extractions were performed using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Five pooled individuals from each life-stage (n= 3), each treatment group (n= 6), and replicate group (n= 4), along with triplicates of a pooled blank for each treatment group (n= 9) and three negative blanks (n=3; total n=84), were placed in individual wells of a 96- well plate provided in the kit. One-hundred eighty microliters of the supplied buffer ATL, a sterile 3.2 mm chrome-steel bead and 100 μL of 0.1 mm glass beads (Biospec, Bartlesville, OK) were added to each well. A Qiagen TissueLyzer was then used to bead-beat each sample for 6 min at 30 Hz. After addition of 20 μL of Proteinase K to each sample, they were incubated at 57°C overnight. The standard DNeasy extraction protocol was then followed.

Dual-index inline barcoding was used to prepare libraries for sequencing on the MiSeq sequencer (Illumina Inc.; San Diego, CA). We used primers that included either the forward or reverse Illumina sequencing primer, a unique eight-nucleotide long barcode, and the forward or reverse genomic oligonucleotide as in Kembel et al. [150]. The bacterial 16S rDNA sequence primers used were 799F-mod3 CMGGATTAGATACCCKGG [151] and 1115R AGGGTTGCGCTCGTTG [150], which have been shown to minimize contamination from plastids. PCRs were performed using 10 μL ultrapure water, 10 μL 2x Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 0.5 μL of each 10 μM primer stock, and 4 μL of DNA. We used a

52 °C annealing temperature, 30 cycles, and negative controls for each reaction. We used the Ultraclean PCR clean up kit (MoBio, Carlsbad, CA) to remove unincorporated primers and dNTPs. Then, 1 μL of the clean PCR product was used as a template for another PCR, using HPLC purified primers to complete the Illumina sequencing construct as in Kembel et al. [150]: CAAGCAGAAGACGGCATAC GAGATCGGTCTCGGCATTCCTGC and AATGATACG GCGACCACCGAGATCTACACTCTTTCCCTACACGACG. For these reactions, a 58 °C annealing temperature for 15 cycles and negative controls were used. Once the PCR cycles were finished, 18 μL of the PCR product and SequalPrep Normalization plates (ThermoFisher Scientific, Waltham, MA) were used to normalize the amount of DNA in each sample. Five microliters of each normalized sample were pooled together and used to performed another cleanup. Next, a 2100 Bioanalyzer (Agilent, Santa Clara, CA) was used to assess library quality. After quality control, the libraries were sequenced using a MiSeq sequencer (Illumina) and MiSeq Reagent kit v3 (Illumina) with 2 X 300 cycles. Raw data are available on the NCBI Sequence Read Archive (SRA) accession number SRR5929442.

Bioinformatics

All genomic data were processed in macQIIME ver. 1.9.1-20150604 [114,152]. USEARCH v6.1 [115] was used to identify and remove chimeric sequences, and SUMACLUST [153] was used to cluster Operational Taxonomic Units (OTUs) with at least

97% sequence identity, and remove any OTUs with less than two reads per sample [197]. We then used macQIIME to perform standard alpha and beta diversity analyses. To assign taxonomy to OTUs, Greengenes taxonomy [154] and the RDP Naïve Bayesian Classifier [155] were utilized. Because training set can influence these taxonomic assignments [198], BLASTN searches against NCBI's online Nucleotide Collection (nr/nt) and 16S ribosomal RNA sequences (Bacteria and Archea) databases (accessed 06/25/2017) were performed. Any mitochondria or chloroplast OTUs and other obvious contaminants (as determined by the blank controls) were removed from the dataset as in McFrederick & Rehan [148]. We aligned the quality-filtered dataset using the pynast aligner [156] and the Greengenes database [154]. The phylogeny of the bacterial OTUs was reconstructed using FASTTREE version 2.1.3 [157]. Weighted and unweighted UniFrac distance matrices were then generated [158] using the phylogeny and OTU tables. Adonis analyses [120] were performed in R, version 3.3.1, [159] utilizing the UniFrac matrices. The R package 'gplots' [160] was used to create a heatmaps of the most abundant bacterial families; a top ten abundance was used as the cutoff.

Statistics

All statistical analyses were performed using R. Normality was determined using Shapiro-Wilk normality tests, quantile-quantile Plots, and histograms. Effects of treatments on population growth were determined using a generalized linear model and post-hoc tests were performed using R's 'summary' function. In all cases, when data were not considered normal, either a Poisson distribution or a negative binomial

generalized linear model was used and best fit was determined from Akaike's 'An Information Criterion' and followed with R's 'summary' function for pair-wise comparisons of treatment. Adonis within the R package 'vegan' [120] was used for all PERMANOVA analyses. All Adonis analyses were conducted on weighted UniFrac distance matrices.

Results

Aphids reared on treated pepper plants showed no difference in population growth (χ^2: 4.68; df: 5; p= 0.46) or microbial communities (F: 1.88; df= 5,58; p= 0.068). There were significant differences of peppers' masses (wet weight) (χ^2: 12.94; df: 5; p= 0.024) specifically in the antibiotic (t value= -2.18; p= 0.043) treatments (Figure 6.1). When dissected into parts there were significant differences in leaf (χ^2: 12.90; df: 5; p= 0.024) and root mass (χ^2: 13.52; df: 5; p= 0.019; Figure 6.2). For root masses, differences were predominately in the antibiotic (t value= -2.81; p= 0.012) and mixture (t value= -2.32; p= 0.033) treatments (Figure 6.3). The most predominate family in the aphid microbial community was *Enterobacteriaceae* (genus *Buchnera*; Figure 6.4) across all treatments (accounting for at least 84%; Table 2) and all life stages (accounting for at least 82 %; Table 3). However, once that family is removed, there is no discernable pattern with treatment and microbial community (Supp. Fig. 6.1) and Adonis analysis verified a lack of significant difference (F: 1.88; df= 5,58; p= 0.068).

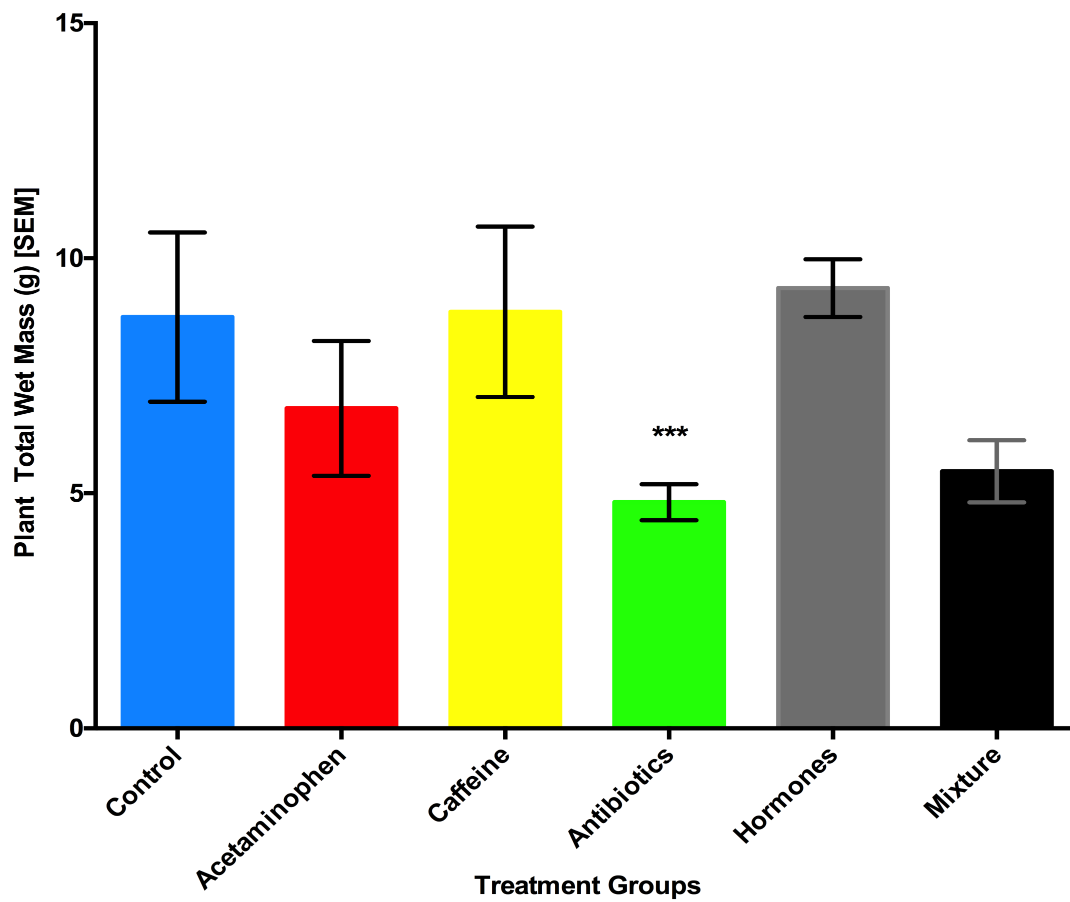


Figure 6.1: The average wet mass of the whole plants by treatment. *** denotes a significant ($\alpha < 0.05$) difference relative to the control

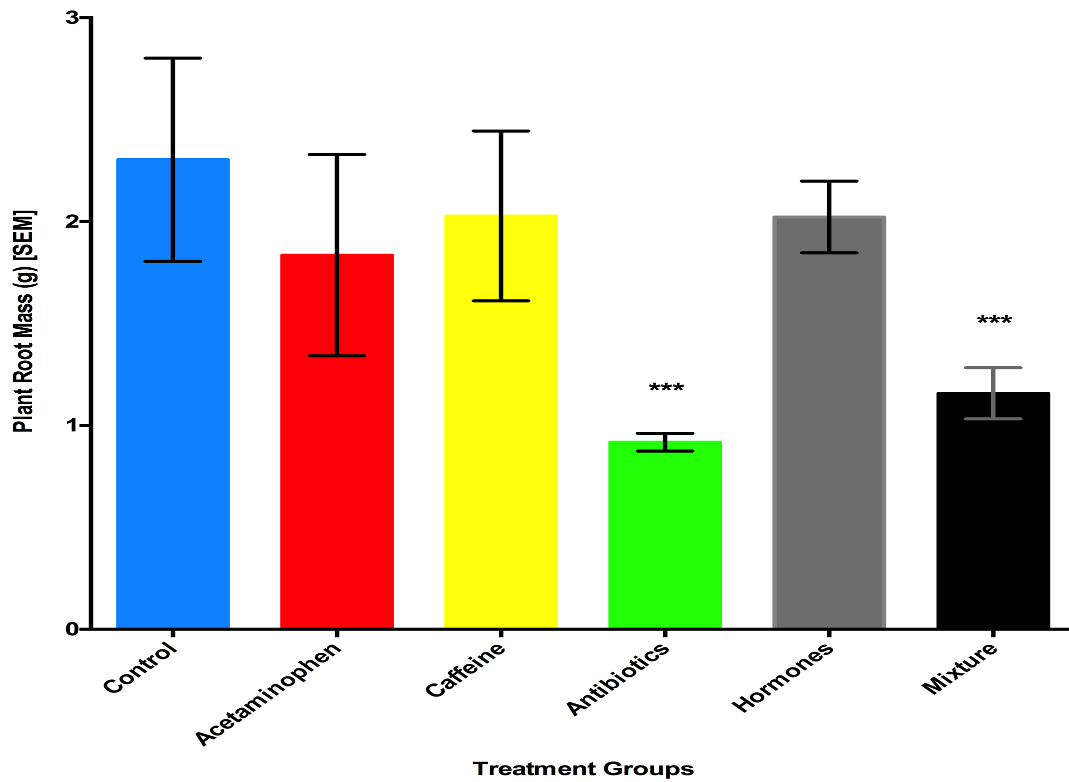


Figure 6.2: Average root wet mass. *** denotes a significant (α< 0.05) difference relative to the control.

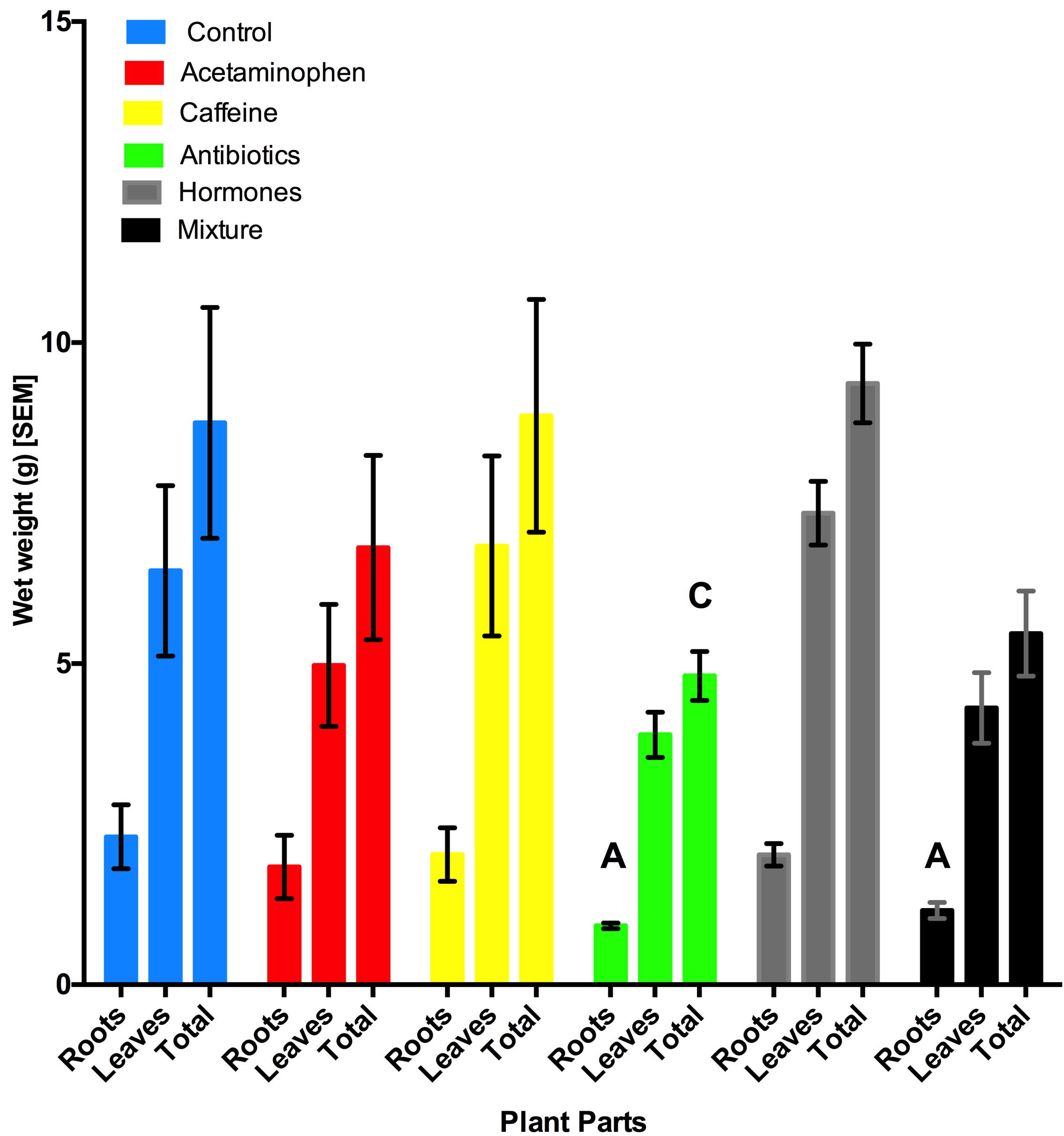


Figure 6.3: Average wet masses of plants as total and plant parts by treatment. Letters denote a significance ($\alpha < 0.05$) difference between the column and the relative control.

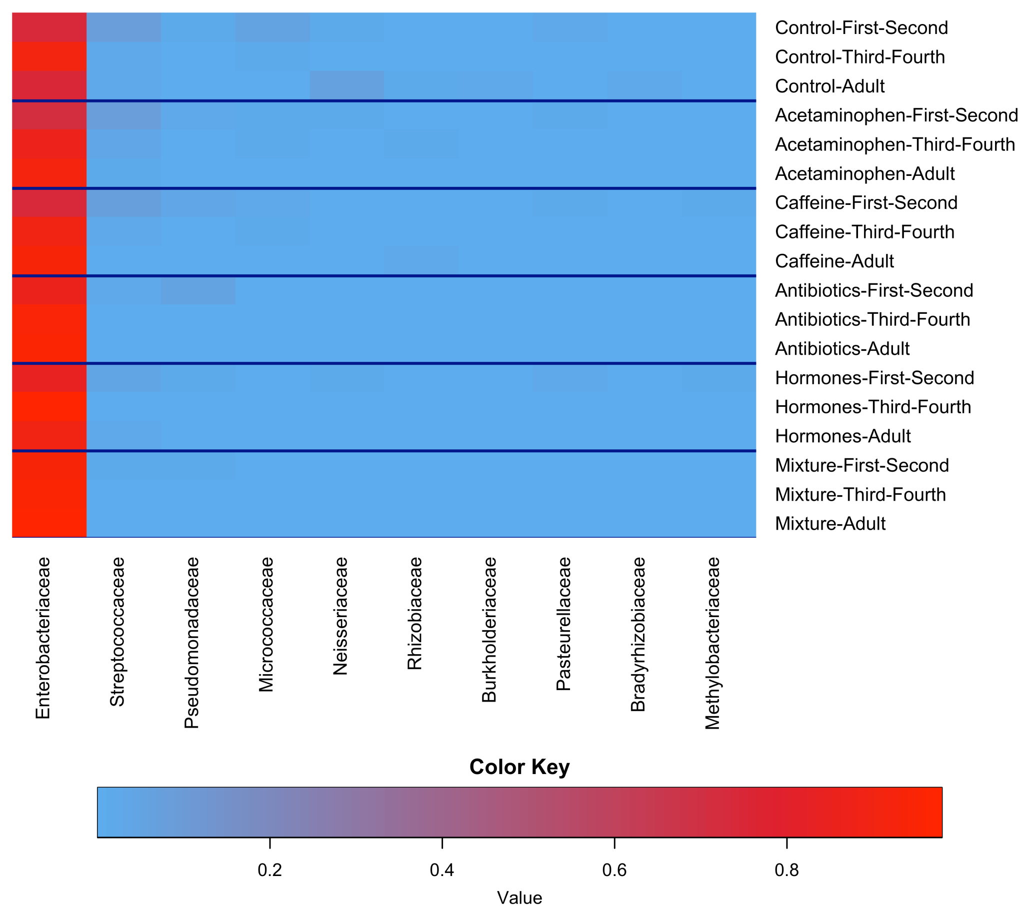


Figure 6.4: Heatmap of the top 10 most proportionally abundant bacterial families by average OTUs of treatment life-stage pairing. Increased red coloration is indicative of increased proportional abundance.

Table 6.2: Percentages of the top 10 bacterial families in each treatment (incorporating all life-stages).

Family	Control	Acetaminophen	Caffeine	Antibiotics	Hormones	Mixture
Enterobacteriaceae	80.52	84.69	86.48	91.83	91.08	95.31
Streptococcaceae	4.35	3.72	3.07	0.96	2.17	0.64
Pseudomonadaceae	1.01	1.10	1.36	2.03	0.58	0.91
Micrococcaceae	2.43	0.75	1.00	0.10	0.40	0.19
Neisseriaceae	2.70	0.76	0.29	0.07	0.59	0.05
Rhizobiaceae	0.38	0.78	1.38	0.27	0.20	0.08
Burkholderiaceae	1.12	0.63	0.54	0.21	0.37	0.20
Comamonadaceae	0.40	0.47	0.75	0.41	0.46	0.30
Pasteurellaceae	0.98	0.71	0.43	0.06	0.71	0.03
Bradyrhizobiaceae	0.79	0.65	0.45	0.42	0.26	0.24
Total Percentages	94.69	94.26	95.75	96.36	96.82	97.95
Total Reads	77031	95718	117524	104246	117125	93503

Table 6.3: Percentages of the top 10 bacterial families in each life-stage (incorporating all treatments).

Family	Adult	First-Second	Third-Fourth
Enterobacteriaceae	91.31	82.21	93.14
Streptococcaceae	1.09	4.62	1.34
Pseudomonadaceae	0.39	2.49	0.49
Micrococcaceae	0.19	1.53	0.44
Neisseriaceae	0.83	0.90	0.17
Rhizobiaceae	0.91	0.25	0.46
Burkholderiaceae	0.75	0.44	0.24
Comamonadaceae	0.23	0.79	0.39
Pasteurellaceae	0.12	1.02	0.22
Bradyrhizobiaceae	0.82	0.20	0.33
Total Percentages	96.63	94.45	97.22
Total Reads	206353	216040	182754

Discussion

Our work demonstrates that the selected CECs did not affect population dynamics or microbial communities of *Myzus persicae* reared on bell peppers. Many plants will translocate CECS [24,42,218,219]. However, some plants can metabolize and/or sequester

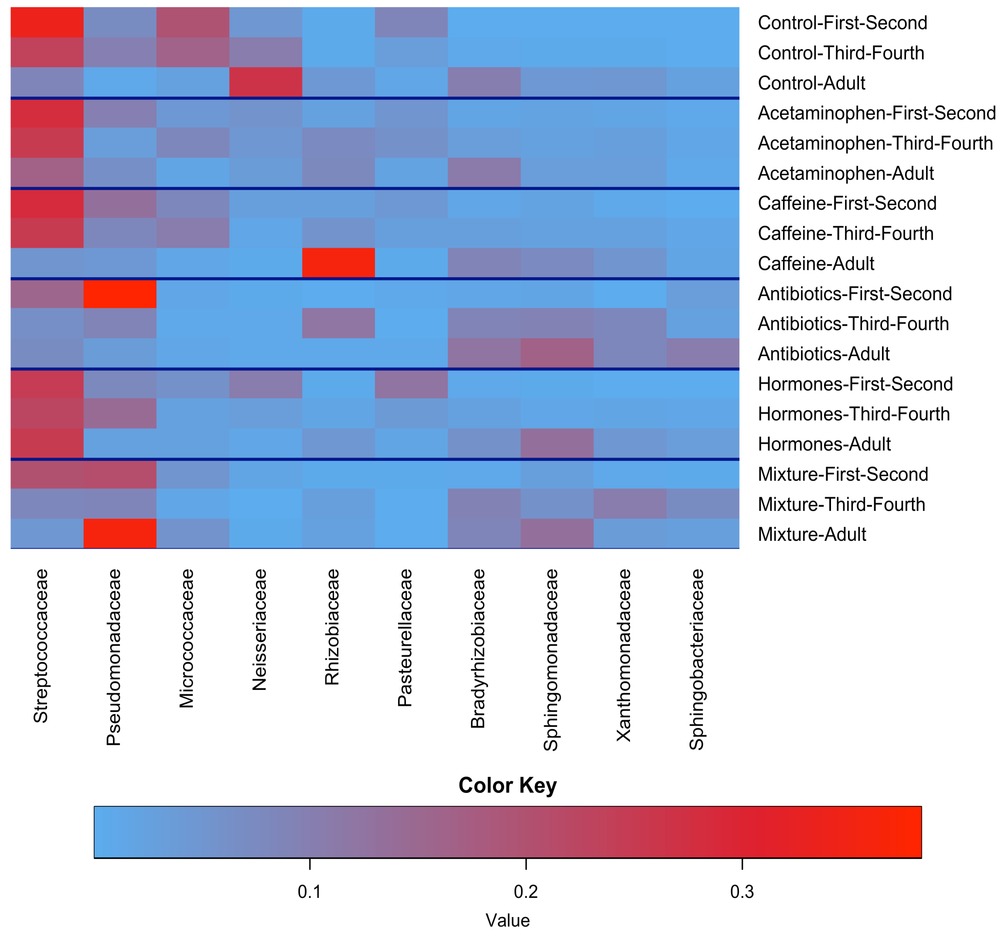
xenobiotics in tissues other than phloem, thereby removing the CECs from being accessed by the aphids [200,218]. As aphid species rely heavily on the endosymbiont *Buchnera* species to grow and develop, many aphid populations treated with antibiotics will not survive [215,216]. However, our treatments did not affect aphid microbial communities when treated with antibiotics and other CECs, which is possibly why there were no discernable effects on the aphid population as a whole. While treatments used in our study have previously been demonstrated to have negative effects for at least two other species of insects [107,140,207], this work suggests that aphids are either not exposed to CECs through their host plant, or their bacterial symbionts are not sensitive to, or depleted enough, to alter their basic biology.

Plants treated with antibiotics typically have lower levels of intracellular calcium due to chelation [22]. However, in our study we did not notice any obvious signs of calcium stress (discoloring or death of leaves), possibly due to the use of a hydroponic solution which contains more than enough metal ions to provide adequate nutrients to the plants, even with some chelating. We did notice an overall decrease in mass for plants treated with antibiotics likely due to a slowed growth rate from direct action of the antibiotics on plant growth [220].

Overall, there were no discernible effects of CECs on the aphids' populations and or microbes. However, there were reductions in plant growth when treated with antibiotics. This could pose a problem to growers, especially as antibiotics tend to be reapplied with each watering especially with manure from antibiotic-treated animals [218,221,222]. These antibiotics could also hinder the growth of the plant's rhizosphere which would add

another problem for growers who rotate crops to reintroduce nitrogen into the soil [223]. More studies will need to be performed to determine how CECs will affect root microbial communities in soil, the roots themselves in soil, and degradation of CECs in soil.

Supplemental Information



Supp. Figure 6.1: Heatmap of the top 10 most proportionally abundant bacterial families by average OTUs of treatment life-stage pairing after removing *Enterobacteriaceae*. Increased red coloration is indicative of increased proportional abundance.

Chapter 7

Conclusion

Drought, rising temperatures and expanding human populations are increasing water demands. Many countries are extending potable water supplies by irrigating crops with wastewater. Unfortunately, wastewater contains biologically active, long-lived pharmaceuticals, even after treatment. Run-off from farms and wastewater treatment plant overflows contribute high concentrations of pharmaceuticals to the environment. These pharmaceuticals then can be either directly ingested by insects or translocated through plants and then ingested by insects. I have determined the effects of CECs on four different insects, all with different feeding styles and exposure risk.

Culex quinquefasciatus larvae reared in water contaminated with environmentally relevant concentrations of common CECs were affected both directly and indirectly. Acetaminophen alone and a mixture of contaminants were found to increase developmental time of larvae. Susceptibility to *Bacillus thuringiensis* subsp. *israelensis* toxin increased in larvae exposed to antibiotics, acetaminophen, or a mixture of PPCPs. Overall there were significant differences in the microbial community of *C. quinquefasciatus* in treated water. Within control groups, the predominant families of bacterial symbionts change with each larval instar despite consistent diets and rearing conditions, an effect that has been described in older life stages but not in larvae. This trend was also seen in hormone treatments, but not in the antibiotic or the mixture treatments. Richness and evenness were reduced in both antibiotic and mixture treatments, suggesting that antibiotics remove certain bacteria or inhibit them from increasing to proportions seen in the control treatment. Interestingly, the mixture

treatments had greater richness and evenness compared to antibiotic alone treatments, possibly due to the other contaminants facilitating growth of different bacteria.

Megaselia scalaris larvae reared on artificial diets spiked with contaminants of emerging concern (CECs) at environmentally relevant concentrations displayed no oviposition preference for treated or untreated diets. Larvae exposed to caffeine in diets showed increased mortality, and larvae fed antibiotics and hormones showed signs of slowed development, especially in females. The normal sex ratio observed in *M. scalaris* from control diets was affected by exposure to caffeine and the pharmaceutical mixture treatments. There was an overall effect of treatment on the flies' microbial communities; notably, caffeine-fed insects displayed higher microbial variability.

Trichoplusia ni larvae showed increased developmental time and mortality when reared on artificial diets containing antibiotics, hormones, or a mixture of contaminants. Mortality was also increased when *T. ni* were reared on tomatoes grown hydroponically with the same concentrations of antibiotics. The antibiotic-treated plants translocated ciprofloxacin through their tissues to roots, shoots, and leaves. Microbial communities of *T. ni* changed substantially between developmental stages and when exposed to CECs in their diets.

Myzus persicae reared on bell peppers treated with CECs displayed no effects in population growth over nearly three generations and no effects on the aphids' microbial communities. The *M. persicae* retained their proportionately largest family *Enterobacteriaceae* across all life stages and across all treatment groups. Interestingly,

the greatest effect was noticed in the bell peppers themselves, which had decreased root and leaf growth in treatments containing antibiotics.

Overall, our findings indicate that at environmentally relevant concentrations, CECs in reclaimed water can have biologically important effects on important insects. They also, illuminate the complexity of the effects CECs can have on insects with different feeding methods. While the research reported in this dissertation suggests that CECs in reclaimed water could have positive effects on certain pests (mosquitoes and possibly caterpillars), they could have unintended negative effects on other insects and how humans interact with them. Effects described here have substantial potential consequences for forensics and IPM strategies. The results show increased developmental time and mortality in insects would alter biological time-clocks for forensic entomology and for dosage practices for IPM (possibly decreasing the number of pesticides needed). Determining whether or not these PPCPs could influence pathogen transmission in insect-plant interactions is also of concern. Because many of the insects studied here are lower trophic level organisms, the potential for biomagnification or unexpected chemical modification as CECs move through the food webs are serious concerns. There is also the possibility of pharmaceuticals affecting the rhizosphere of plants in soil and influencing plant health, but more research is still needed to determine precise effects.

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