ECOLOGY AND EVOLUTION OF LEPIDOPTERA-MICROBE SYMBIOSIS

by

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ABSTRACT

Microorganisms are now recognized to be integral components of the biology of many animal and plant taxa. These symbionts can play critical roles in host development, physiology, and in the mediation of ecological interactions among hosts. The study of insect-associated microbial symbionts is of particular interest, as insects comprise an enormous fraction of terrestrial biodiversity, provide important ecosystem services, and include destructive pests and disease vectors. However, symbiotic microbial functions are well-understood for only a limited set of insect taxa, and the broader ecological relevance of insect-microbe symbiosis is often unclear. Here, I describe four studies I undertook to investigate the structure and function of insect-associated microbiomes, with a particular emphasis on Lepidoptera (butterflies, moths, and their larvae). First, I conducted a comprehensive review of the literature pertaining to interactions between herbivorous insect gut microbes and the chemical compounds with which plants defend their tissues; these interactions may explain insect feeding patterns and diversification. Second, I found that antibiotic treatments, commonly administered to livestock, have unintended negative consequences: antibiotics retained in cattle dung affect the microbiome of dung beetles as well as modulate emissions of microbially produced greenhouse gases. Third, I investigated the composition and developmental dynamics of bacterial communities inhabiting a butterfly, finding that the butterfly microbiome undergoes a restructuring analogous to host metamorphosis. Fourth, I explored the gut microbiomes of a diversity of larval Lepidoptera, and found evidence for their generally transient nature. Collectively, these findings demonstrate that microbiomes can be highly diverse in form and function both within species and across major clades of hosts. They may also inform management practices, such as antibiotic use or pest control strategies.

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CHAPTER I

INTRODUCTION AND OVERVIEW

Introduction

The ecology and evolution of many animal lineages is now recognized to be influenced by the microbiome, the community of microbes symbiotically inhabiting the body of an animal or other "macrobial" host (McFall-Ngai et al. 2013). The pervasive effects of microbial symbionts on animal development, physiology, immunity, and ecological interactions have been most thoroughly explored in certain groups such as aphids and other true bugs, social corbiculate bees, termites, ruminant herbivores, and, especially, humans. Likely driven to a large degree by both the Human Microbiome Project (Turnbaugh et al. 2007) and the expanding availability of tools such as next-generation sequencing, scientific research on the microbiome has risen exponentially in the last 20 years (measured by citations in NCBI PubMed). There is now a widely held belief that all animals, not just the aforementioned model systems, comprise "holobionts" that are dependent upon a resident microbiome for many aspects of their biology (e.g., Zilber-Rosenberg and Rosenberg 2008; Gilbert et al. 2012; Douglas 2015). However, the published literature on which this generalization is founded represents a nonrandom, and possibly nonrepresentative, sample of animal diversity.

My thesis has mainly focused on the microbiomes of Lepidoptera (butterflies, moths, and their caterpillar larvae), a hyperdiverse (est. ~500,000 species), ecologically and economically important group of herbivores and pollinators (Scoble 1992; Kristensen et al. 2007). Thus, the study of Lepidoptera-associated microbiomes both adds to our understanding of the biology of this important group of insects, and tests the idea that all animals depend on microbial symbionts. Most Lepidoptera are herbivorous as larvae and, in many groups, as adults (on floral resources); as there are many prominent examples of microbially dependent insect herbivores (Hansen and Moran 2014), it could be

expected that lepidopteran herbivores would follow suit. However, at least in the larval stage, Lepidoptera present a number of distinctive digestive and nutritional traits (Dow 1986; Martin et al. 1991) which have been proposed to lessen their requirements for microbial symbionts (Appel 1994).

That Lepidoptera are often infected by pathogenic microorganisms has been known for at least 150 years (Steinhaus 1956). Following commonly used definitions (Douglas 1994; Lederberg and McCray 2001), pathogens can be considered as symbionts comprising part of the Lepidoptera microbiome. However, despite a large number of studies aiming to address this question (Paniagua Voirol et al. 2018), it has been unclear whether beneficial symbionts are also present—in other words, whether Lepidoptera depend on microbes for normal function. The vast majority of research on Lepidoptera-associated microbiomes has been conducted on larvae, often of pest species maintained as laboratory colonies. Furthermore, tests of microbial function have typically relied on *in vitro* assays or metagenomic sequence-based inference (e.g., Pinto-Tomás et al. 2007; Indiragandhi et al. 2008; Belda et al. 2011; Xia et al. 2017). These approaches have severe limitations for understanding how Lepidoptera interact with microbes *in vivo* and under field conditions. For example, a bacterium isolated from a caterpillar gut may be able to degrade pectin on agar plates, but it may only perform this function while infecting an intact leaf or while decomposing leaf litter. Also, domesticated colonies of caterpillars maintained in the laboratory can exhibit reduced immune function (Diamond and Kingsolver 2011), which may allow normally transient microbes to proliferate.

Therefore, my dissertation research has investigated the structure and function of Lepidoptera-associated microbiomes across life stages, *in vivo*, and in wild populations spanning a wide range of taxa and habitats. This research has shown first, how metamorphosis can enable the same insect to interact with microbes in very different ways across its development, and provided foundational information on the diversity, density, ecological role, and distribution of microbes associated with adult butterflies. Second, it has provided evidence that caterpillar herbivory does not

depend on microbial symbionts, a finding which raises new questions about the prevalence, drivers, and macroevolutionary consequences of asymbiotic lifestyles. Finally, I have also investigated the broader ecological consequences of antibiotic use, a common practice in livestock agriculture. This research has demonstrated that manipulating the microbiomes of cattle has downstream consequences for dung beetles, important members of agroecosystems, and for emissions of greenhouse gases.

Thesis Overview

Chapter II: *Gut microbial roles in the chemical ecology of insect-plant interactions*. Herbivorous insects represent an enormous fraction of macrobial biodiversity and have major impacts on terrestrial food webs. Some of these insect lineages have long been known to rely on microbial symbionts to digest plant material or supply nutrients missing from their diet. In this chapter, I call attention to an additional, relatively neglected microbial service—the detoxification of harmful chemical compounds plants use to defend their tissues. Understanding whether and how microbes underlie the ability of insects to circumvent plant chemical defenses may help explain insect diversification, and has implications for pest control. While my subsequent work shows that caterpillars do not require microbes to feed on toxic plants, a growing number of studies suggest that the insect-microbe-plant-chemical interactions I discuss here are prevalent in other herbivores and certain pollinators.

Chapter III: *Ecological consequences of antibiotic use in livestock agriculture*. Antibiotics are heavily used for disease treatment and growth promotion in agricultural systems around the world. While the evolution of antibiotic resistance is a well-studied and critical concern, manipulating microbes in agricultural food webs may have additional negative effects. In this project, I collaborated with researchers in Finland to test whether oxytetracycline treatment of cattle may affect downstream consumers and gas emissions of microbial origin. We found that antibiotics restructured dung beetle

microbiomes and increased methane fluxes from cattle dung, thus demonstrating the potential for antibiotic use to exhibit unintended ecological consequences.

Chapter IV: *Microbial dynamics over the life cycle of the butterfly* Heliconius erato. Butterflies and moths (Lepidoptera) are key herbivores and pollinators, and are of both scientific and popular interest for their remarkable ability to restructure their morphology, feeding ecology, and behavior through metamorphosis. However, the microbial communities of pupal and adult Lepidoptera had not been studied using modern molecular methods, and thus microbial dynamics across the life cycle had not been addressed. In a member of the genus *Heliconius* (Nymphalidae), which has served as a model for the study of mimicry evolution and adaptive radiation, I found that developmental changes in the composition of the whole-body microbiome parallel changes in the structure of the butterfly itself: across metamorphosis, the microbiome simplifies in terms of diversity, and reorganizes in terms of composition. I also found that compositional variation in *H. erato* caterpillar microbiomes was correlated with that of their food plants, suggesting that microbes in the caterpillar gut are derived from leaves. Lastly, *H. erato* feces were microbially similar to whole, homogenized caterpillars, indicating both that caterpillars lack abundant symbionts outside their gut, and that feces can be used as a proxy for the gut microbiome.

Chapter V: *Observational and experimental evidence for a lack of resident gut microbes in leaf-feeding caterpillars*. Despite the ecological and agricultural importance of caterpillar herbivory, whether caterpillars rely on gut microbial symbionts for feeding and development has long been an unresolved question. Similarity between caterpillar gut microbial composition and leaf microbial composition had been reported by myself (in *H. erato*) and by others, but this pattern could arise from distinct mechanisms with very different implications for microbial roles in caterpilar herbivory. The most common interpretation is that caterpillars acquire resident gut microbes from their diet; these symbionts then assist the host with digestion, detoxification, or nutrient supplementation of leaf

material. Alternatively, leaf microbes could be inactive or dead as they pass transiently through the caterpillar gut. By characterizing the composition and absolute abundance of gut microbiomes across a wide diversity of wild caterpillars, I found that resident gut microbes are largely absent. An experimental test on *Manduca sexta* (Sphingidae) showed that, for this species, feeding and development are independent of potential gut microbial activity. Caterpillars thus demonstrate how herbivory may evolve without the aid of microbial symbionts, and raise new questions about the prevalence and macroevolutionary consequences of asymbiotic lifestyles among animals.

CHAPTER II

GUT MICROBES MAY FACILITATE INSECT HERBIVORY OF CHEMICALLY DEFENDED PLANTS

(Hammer, T.J & Bowers, M.D. (2015). Gut microbes may facilitate insect herbivory of chemically defended plants. *Oecologia*)

Abstract

The majority of insect species consume plants, many of which produce chemical toxins that defend their tissues from attack. How then are herbivorous insects able to develop on a potentially poisonous diet? While numerous studies have focused on the biochemical counter-adaptations to plant toxins rooted in the insect genome, a separate body of research has recently emphasized the role of microbial symbionts, particularly those inhabiting the gut, in plant–insect interactions. Here we outline the "gut microbial facilitation hypothesis," which proposes that variation among herbivores in their ability to consume chemically defended plants can be due, in part, to variation in their associated microbial communities. More specifically, different microbes may be differentially able to detoxify compounds toxic to the insect, or be differentially resistant to the potential antimicrobial effects of some compounds. Studies directly addressing this hypothesis are relatively few, but microbe–plant allelochemical interactions have been frequently documented from non-insect systems—such as soil and the human gut—and thus illustrate their potential importance for insect herbivory. We discuss the implications of this hypothesis for insect diversication and coevolution with plants; for example, evolutionary transitions to host plant groups with novel allelochemicals could be initiated by heritable changes to the insect microbiome. Furthermore, the ecological implications extend beyond the plant and insect herbivore to higher trophic levels. Although the hidden nature of microbes and plant allelo-

chemicals make their interactions difficult to detect, recent molecular and experimental techniques should enable research on this neglected, but likely important, aspect of insect-plant biology.

Introduction

The interactions between plants and insects make up a large fraction of the interaction biodiversity on our planet, and exert a key control on the function of terrestrial ecosystems (Strong et al. 1984; Didham et al. 1996; Price 2002; Thompson 2002). Decades of research have shown that these interactions are mediated by plant toxins, secondary metabolites common to all plants that deter or poison some herbivores while serving as attractants or feeding stimulants for others (e.g., Rosenthal and Berenbaum 1992; Schoonhoven et al. 2005; Iason et al. 2012). These allelochemicals are important in explaining insect diet breadth, preference and performance, as well as in understanding the evolution of contemporary patterns of insect-plant associations. However, insects and plants coexist with other organisms that may influence how these chemically based interactions function and evolve (Zhu et al. 2014). One such group is the microorganisms that inhabit the insect gut and are thus exposed and respond to plant allelochemicals ingested by the insect.

The herbivorous insect microbiome—including bacteria, fungi, and protozoa—is increasingly recognized as a major force in shaping the ecological and evolutionary interactions between insects and plants (Feldhaar 2011; Biere and Bennett 2013; Douglas 2013). To date, the overwhelming majority of research has focused on two main roles for microbial symbionts in insect herbivory: breaking down plant structural compounds such as lignin or cellulose, and supplementing amino acids or vitamins missing from a plant-based diet (Douglas 2009). However, plant material is not only difficult to digest and nutritionally imbalanced, but also contains allelochemicals that a successful herbivore and its microbiome must be able to tolerate or detoxify; thus, microbes may have a third key role in insect herbivory by providing counter-defenses to plant toxins (Berenbaum 1988; Dowd 1991).

As there is relatively little direct support for such a role (but see Richerson 1992; Mason et al. 2014), some have argued that the scarcity of published evidence points to a lack of importance in nature (Douglas 2013; Hansen and Moran 2014). We argue that, on the contrary, interactions between plant chemical toxins and the insect gut microbiome are likely to be common and have far-reaching implications for the ecology and evolution of herbivorous insects. Here, we expand upon and update the hypothesis that the gut microbiome can facilitate insect herbivory by detoxifying, or being resistant to, plant allelochemicals (Jones 1984; Berenbaum 1988). We also suggest that, by enabling the insect host to overcome novel plant chemical defenses, heritable changes to an insect lineage's microbiome could alter its host plant range and underlie patterns of herbivorous insect diversification commonly attributed to changes in the insect's genome. Drawing on examples from pharmacology, soil microbiology, and the plant- and vertebrate-microbiome literature, we highlight the potential for gut microbes to mediate insect detoxification, and for plant allelochemicals to act as antibiotics targeting the insect gut microbiome. We propose that these interactions may have tritrophic implications as well, including mediating sequestration of plant toxins by herbivores and the evolution of predator detoxification systems. We also outline some methods that can be used to document associations between insects, microbes, and plant allelochemicals and detail their underlying mechanisms.

Main text

Who interacts with plant allelochemicals?

All plants contain some type of secondary metabolites that provide, among other functions, defense against herbivory (Wink 2003; Mithöfer and Boland 2012). The classic view of how insects feed on plants and cope with these compounds treats the herbivore as a single entity. It has generally been assumed that selection imposed by plant chemical defenses favors insect genomic adaptations that enable the herbivore to circumvent those defenses, and that genomic differences between insect

herbivore species underlie their remarkable variation in host plant use. Indeed, the insect genomic underpinnings of many chemically based insect-plant interactions have been well-established (e.g., Després et al. 2007). However, insects are not composed solely of insect cells and genomes, but also are intimately associated with dense and diverse populations of microbes that can contribute to their host's phenotype. In many herbivorous insects, the gut is colonized by microbial communities that co-occur and interact with both insect cells and ingested plant material, and could thus be crucial in shaping the ability of insects to feed on a chemically defended plant. If so, microbial variation could be responsible for some of the variation among insect lineages in their responses to plant allelochemicals. It is now well-recognized that insect symbionts, when secreted into plant tissue, modulate phytohormone signaling and defense regulation (Spiteller et al. 2000; Chung et al. 2013; Casteel and Hansen 2014). Here, however, we focus solely on microbial responses to allelochemicals and their consequences for herbivory.

In order to fully understand the chemically based interactions between herbivorous insects and plants it is crucial to address the contributions of the insect gut microbiome (Letourneau 1988). Cases in the literature where an insect genomic mechanism for coping with plant allelochemicals has been tested but not found are prime candidates for microbial investigation. Additionally, counter-defenses are rarely completely effective even for chemically specialized insects with genetically encoded detoxification or tolerance mechanisms, and may be enhanced by contributions from gut microbes. For example, tobacco hornworms (*Manduca sexta*) and the larvae of monarch (*Danaus plexippus*) and buckeye (*Junonia coenia*) butterflies suffer reduced growth at high dietary concentrations of the particular allelochemicals they are adapted to tolerate (Parr and Thurston 1972; Agrawal et al. 2012; Richards et al. 2012). Furthermore, the microbiome of chemical specialists might still need its own counter-defenses against ingested toxins to maintain digestive or nutritional services for the host.

Among the high diversity of herbivorous insects and symbionts found in nature, those most exposed to plant allelochemicals may be the most likely to engage in these kinds of interactions. Regarding symbionts, those associated with the gut are expected to be more important in detoxification than those inhabiting non-gut tissues or living within insect cells, which are relatively buffered from ingested plant toxins (Hansen and Moran 2014). However, their within-gut localization, both along (e.g., foregut versus hindgut) and across (e.g., outside versus inside the peritrophic membrane) the intestinal tract, will also affect exposure levels. For insects, those that consume whole plant tissue are generally more exposed to allelochemicals than piercing-sucking insects such as hemipterans (Mullin 1986). Chewing insect herbivores, including lepidopteran and sawfly larvae, beetles, and orthopterans, might be particularly reliant on microbes for detoxification, and their symbionts should have a higher need for toxin resistance. These groups not only comprise a large fraction of insect diversity, but are also very well-represented in the chemical ecology literature. Below- as well as above-ground chewing herbivores should be considered, given the frequent occurrence of secondary compounds in root tissue (Flores et al. 1999).

Interactions inside and outside the herbivore gut

Some older studies found evidence for insect gut microbial detoxification or inhibition by plant allelochemicals (Jones et al. 1981; Richerson 1992), while others did not (Andrews and Spence 1980; Howard and Bush 1989). However, these studies predate the modern era of environmental DNA sequencing and relied on culturing methods. As most microbes in nature are not readily cultured in the laboratory (Pace 1997), it is unknown whether these studies were based on organisms that are truly abundant and active inside the host insect or a small, possibly unrepresentative, easily culturable subset. More recently, several culture-independent studies using molecular methods have investigated these interactions, although the evidence is often circumstantial. For example, the stinkbug, *Megacopta*

punctatissima, harbors gut bacteria with genes for the decarboxylation of oxalate, a common plant secondary metabolite (Nikoh et al. 2011). Bacteria associated with mountain pine beetles have genomes enriched in terpene detoxification genes (Adams et al. 2013), and can metabolize terpenes *in vitro* (Boone et al. 2013). In the midguts of gypsy moth larvae, dietary phenolic glycoside levels were positively associated with the relative abundance of a plant-derived *Acinetobacter* species that could metabolize them (Mason et al. 2014). There are surely many other examples waiting to be discovered among the hundreds of thousands of herbivorous insect species on earth, all of which feed on chemically protected plants, and all of which harbor microbial symbionts.

In contrast to the relatively few concrete examples of insect gut microbe-allelochemical interactions, there are numerous examples of microbes interacting with plant toxins outside the herbivore gut. Bacteria and fungi associated with living leaves are often exposed to allelochemicals present in the tissues they infect. Foliar microbes, both mutualistic and pathogenic to plants, are known to have mechanisms for degrading or tolerating plant secondary compounds, many of which are active against microbes (Swain 1977; Bennett and Wallsgrove 1994). Decomposer microorganisms must also contend with allelochemicals retained in and leached from leaf litter, and tannins and phenolics have been shown to affect litter and soil microbes and, consequently, nutrient cycling processes (e.g., Fierer et al. 2001; Chomel et al. 2014). Analogously, plant toxicity against herbivore gut microbes could affect their ability to break down plant polysaccharides and synthesize nutrients, with consequences for host nutrition. Furthermore, many soil bacteria have been isolated that can degrade plant toxins such as nicotine (Wada and Yamasaki 1954), cyanide (Hardy and Knight 1967), and flavonoids (Wang et al. 2013). Such detoxification processes are likely to be occurring in the insect gut as well.

In pharmacology, inter-individual variation in the gut microbiome is recognized as an important source of variation in the activity or toxicity of drugs (Nicholson et al. 2005; Haiser and Turnbaugh 2012). For example, gut microbes can ferment or degrade pharmaceutical compounds, rendering them inactive

(Akao et al. 1994; Jung et al. 2012; Haiser et al. 2013), or produce structurally similar molecules that compete for binding sites on host enzymes (Clayton et al. 2009). What is not often acknowledged is that many drugs are derived from, or structurally similar to, plant secondary metabolites (Cragg and Newman 2013); thus, the gut microbiome-drug interactions observed in human and other mammals may be paralleled in herbivorous insects.

Research on vertebrate herbivores has illustrated how the gut microbiome can be involved in herbivory of chemically defended plants. For example, rumen microorganisms have been implicated in the detoxification of a wide range of plant allelochemicals, including cyanogenic glycosides, cardenolides, flavonoids, nonprotein amino acids, and alkaloids (Freeland and Janzen 1974; Jones and Megarrity 1986; Smith 1992). On the other hand, plant toxins can themselves directly suppress the growth of rumen bacterial populations (Anderson 1993; Wallace 2004; Krause et al. 2004), and thus might select for toxin resistance in the rumen. Foregut bacteria in herbivorous *Neotoma* woodrats can degrade oxalate (Miller et al. 2013), and appear to be under selection by the secondary chemical composition of the woodrats' diets (Kohl and Dearing 2012). Bacteria have been isolated from the guts of pikas and root voles that can degrade tannins *in vitro* (Dai et al. 2014). Among vertebrates, these phenomena are not limited to mammals; for example, herbivorous hoatzin birds harbor microbes in their crop that can detoxify plant saponins (Garcia-Amado et al. 2007).

These examples provide further evidence that similar microbe-plant allelochemical interactions could occur inside the gut of herbivorous insects, where the outcomes would have important consequences for insect herbivory. Furthermore, some of these studies may provide more than proof of principle: microbes with characterized relationships with plant toxins, particularly those associated with leaves or soil, may in fact reside in an insect gut for part of their life cycle. For example, soil bacteria directly colonize the gut of the bean bug, *Riptortus pedestris*, and endow their hosts with resistance to a xenobiotic, the insecticide fenitrothion (Kikuchi et al. 2012). More generally, microbes—which could be

adapted for dealing with plant secondary compounds—are known to disperse from soil to leaves (Herre et al. 2007; Bizzarri and Bishop 2008; Monnerat et al. 2009), from leaves to herbivores (Lilley and Hails 1997; Degnan et al. 2011; Mason and Raffa 2014), and between different herbivore individuals or even species (Fig. 2.1; Caspi-Fluger et al. 2011; Le Clec'h et al. 2013; Fürst et al. 2014).

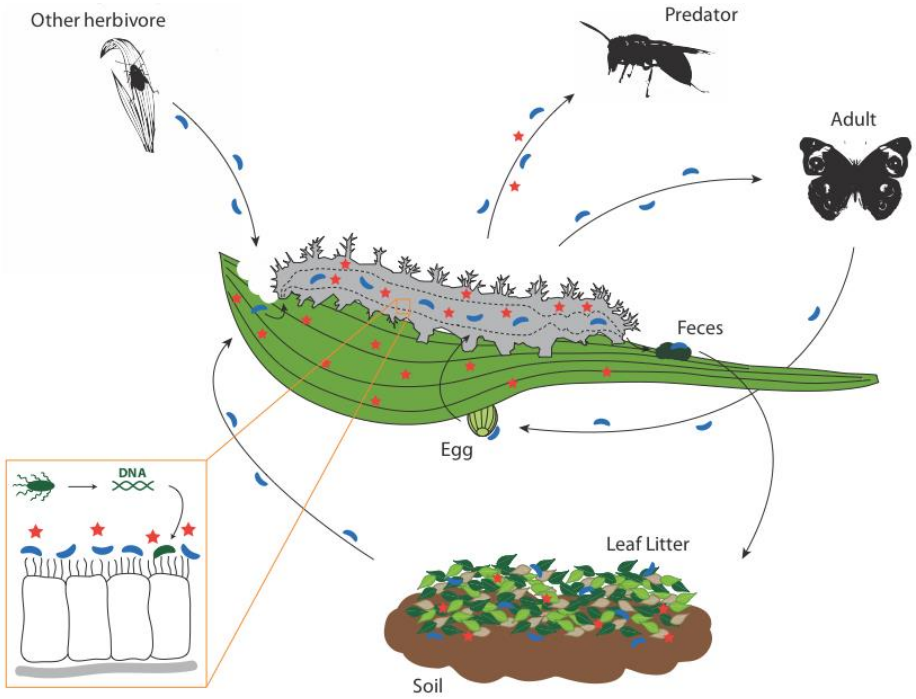

Figure 2.1. Potential microbial dispersal routes between different locations, herbivore life stages, herbivore species, and trophic levels. Here, red stars represent the presence or movement of iridoid glycosides, and the macroorganismal interactions are drawn from the literature. Traits relevant to allelochemical detoxification or resistance may be transferred along these conduits. In the buckeye caterpillar-*Plantago* system shown, plant allelochemicals are sequestered by the herbivore and lost in the adults, but in other herbivores they may be retained in subsequent life stages or not sequestered at all. Inset: horizontal gene transfer from an ingested microbe into a native member of the insect gut community.

Why microbes?

Microbes have a number of unique traits that could allow them to cope with or detoxify plant allelochemicals in situations where the host insect itself cannot. First, microbes have an exceptionally high diversity of pathways for acquiring energy and catabolizing chemical substrates, which could influence how they interact with plant toxins. For example, cyanogenic glycosides release cyanide upon

being metabolized, which binds to and inhibits cytochrome oxidase of the respiratory chain, thus shutting off the ability of aerobic respirers (such as animals) to derive energy from oxidative phosphorylation (Solomonson 1981). However, many bacteria and archaea can make a living without aerobic respiration—e.g., through fermentation—and might therefore be resistant to cyanogenic glycosides' toxic effects. Microbes are renowned for their biodegradative potential, and are frequently used in industrial or environmental applications to transform xenobiotics into less toxic or more useful end products (Knackmuss 1996; Díaz 2004; Zhang and Bennett 2005). Some enzymes unique to microorganisms are also known to modify plant allelochemicals; for example, bacterial nitrogenases can convert cyanide (as well as dinitrogen gas) to ammonia (Li et al. 1982), and only microbes are capable of fully mineralizing aromatic compounds (which include phenolics and some alkaloids, cyanogenic glycosides, and glucosinolates) (Boll et al. 2013). Many bacteria and fungi can metabolize plant secondary compounds, and in some cases use them as their sole source of carbon and energy (Laskin and Lechevalier 1984).

Second, many microbes have relatively short generation times, which can facilitate rapid responses to plant allelochemicals through changes in microbial community structure and adaptation within populations (Letourneau 1988). A number of cases exist where animals unexposed to a particular plant secondary compound perform poorly on diets containing that compound, whereas experienced animals are able to perform well, due in part to diet-induced changes in the microbiome (Smith 1992; Weimer 1998; Kohl et al. 2014). In one example, naïve goats were poisoned by a metabolic derivative of the alkaloid mimosine, but gained resistance when cross-infected with rumen contents from adapted goats (Jones and Megarrity 1986), which contain a bacterial species capable of degrading the compound *in vitro* (Allison et al. 1992). Dietary changes can alter gut microbial community structure within a matter of days (David et al. 2013), and ingested plant toxins may favor the growth of microbes able to tolerate or degrade them. In addition to the ability to rapidly turn over in response to environmental stimuli,

host-associated microbial populations can acquire novel traits within the host's lifespan, including antibiotic resistance (Smith et al. 2006; Morelli et al. 2010; Young et al. 2012; Price et al. 2013). Thus, allelochemical resistance or detoxification capabilities in the gut microbiome could emerge quickly in response to selection by the host insect's diet.

Third, in comparison to multicellular eukaryotes such as insects, microbes are especially effective at horizontal gene transfer, with consequences for their response to chemical compounds in the environment. Perhaps the most famous example is the rapid spread of antibiotic resistance between distantly related taxa or between bacteria from different environments (Normark and Normark 2002; Lester et al. 2006). Horizontal gene transfer is widespread among host-associated and free-living microbes (Smillie et al. 2011), and microbes in and outside of an insect gut could be exchanging genes encoding resistance to the antibiotic effects of plant toxins. Environmental microbes ingested with plant material can, through horizontal gene transfer, endow the resident microbiome with enzymes useful for processing it. For example, genes coding for enzymes degrading sulfated polysaccharides from seaweed appear to have jumped from seaweed-colonizing marine bacteria to the gut microbiome of Japanese individuals (Hehemann et al. 2010; Hehemann et al. 2012). The same process could occur with plant allelochemicals: normally leaf-dwelling microbes that only transiently pass through an herbivore's gut may donate genes to co-occurring resident microbes, conferring detoxification potential on the microbiome and its host (Fig. 2.1). More broadly, the dispersal routes taken by microbes into and out of the herbivorous insect gut (Fig. 2.1) could serve as conduits for the transfer of traits relevant to interactions with plant allelochemicals.

Microbial roles in insect-plant chemical interactions

Phenotypic diversity among animals can be driven by microbiome diversity (McFall-Ngai et al. 2013). Microbes could serve as one source of the extensive phenotypic variation among herbivorous

insects in the types and numbers of plants on which they feed – both of which are known to be closely linked to plant secondary chemistry (e.g., Fraenkel 1959; Schultz 1988). Specifically, insect individuals or lineages may be more or less able to feed on a particular plant because their microbiomes are more or less adept at resisting or detoxifying that plant's secondary chemical compounds. We discuss how two microbially mediated processes can facilitate insect herbivory on chemically defended plants (Fig. 2.2).

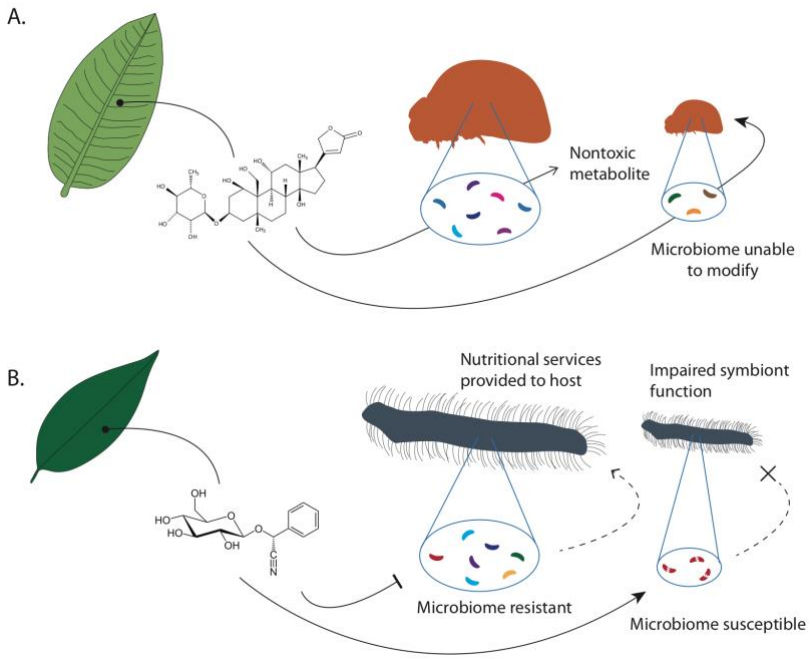

Figure 2.2. Interactions between plants and insects mediated by allelochemicals and gut microbes. In this example, variation in herbivore performance, indicated by differences in insect body sizes, is caused by variation in gut microbial community structure and not the insect genome. A) Facilitation by microbiome breakdown of an insect toxin into a harmless metabolite. Here, the original compound does not necessarily have any negative effect on the microbes. Depicted is the allelochemical ouabain, present in the leaves of common milkweed (*Asclepias syriaca*), consumed by larvae of the swamp milkweed beetle (*Labidoderma clivicollis*). B) Facilitation by microbiome resistance to an antibiotic toxin, leading to higher nutritional services provided to the host insect. Here, the compound does not necessarily have any negative effect on the herbivore itself. This example is illustrated with the cyanogenic glycoside prunasin, present in black cherry leaves (*Prunus serotina*) consumed by the eastern tent caterpillar (*Malacosoma americanum*).

First, variation in gut microbial resistance to allelochemicals can generate variation in the ability of herbivores to feed on toxic plants. The microbiome has been referred to as a virtual organ system integral to host physiology (O'Hara and Shanahan 2006); plant secondary compounds may have evolved

to target the microbiome in much the same way as certain compounds disrupt the nervous or endocrine systems. For example, an herbivore that relies on gut microbes for vitamin synthesis or the conversion of hemicellulose to short-chain fatty acids would suffer if the plant it consumes contains antimicrobial compounds. Over 50 years ago, Fraenkel (1959, p. 1470) speculated that "the vast array of pathogenic and commensal microorganisms, from bacteria to fungi, which inhabit plants, are affected…by secondary plant substances," and the antimicrobial effects of plant allelochemicals are now well-documented (Cowan 1999; Wallace 2004). The extension of these effects to insect-inhabiting microbes was made by culture-based studies suggesting allelochemical inhibition of gut flora in boll weevils (Hedin and Lindig 1978) and silkworm larvae (Jones et al. 1981), with concomitant suppression of host growth in the latter case. More recently, pine monoterpene composition has been shown to affect fungal symbionts isolated from bark beetles (Davis and Hofstetter 2012). These studies support the idea that, much as antibiotics select for antibiotic-resistant pathogens, the secondary chemistry of an insect herbivore's diet could select for allelochemical-resistant gut microbes, with beneficial impacts on the insect's nutrition.

Second, gut microbial interactions may reduce plant allelochemical toxicity to the host, and thus improve the host's ability to consume plant material (Dowd 1991). As discussed above, many microbes isolated from animal and plant hosts, or other environmental sources, are able to enzymatically transform plant allelochemicals into nontoxic metabolites (Fig. 2.2). For example, using culturing methods, Richerson (1992) isolated bacteria from soil, locoweed plant material, and insect herbivore guts that could break down locoweed alkaloids *in vitro*; when insects were reared aseptically, they were unable to complete development on alkaloid-containing diets. However, microbial detoxification is not limited to simple degradation of the compounds in question. One other potential mechanism is the microbial production of compounds that compete with plant toxins for the binding site of toxin-activating enzymes (e.g., myrosinases and beta-glycosidases); human gut bacteria have been shown to

repress acetaminophen activity in an analogous fashion (Clayton et al. 2009). Alternatively, microbes could eliminate toxic byproducts resulting from host allellochemical metabolism. For example, one pathway known to be involved in animal detoxification of cyanogenic glycosides generates sulfide ions; a means by which herbivorous insects deal with these byproducts has not been found (Brattsten 1992), but bacterial sulfide oxidation is common among some marine invertebrates (Grieshaber and Völkel 1998) and might also operate within the insect gut. Another possibility is that microbes could release molecules that complex with plant allelochemicals, rendering them less toxic. For example, caterpillar digestive juices are rich in free glycine, which binds to and inactivates ingested polyphenolics (Konno et al. 1997); gut microbes are a possible source of this extra glycine, as insect symbionts commonly provision their hosts with amino acids – a function usually interpreted in a nutritional context (Douglas 2009). However, in this case, the main function of microbial amino acid biosynthesis would be in detoxification, rather than nutrition *per se*. Additionally, gut microbes can take these detoxification services one step further, by turning allelochemicals—many of which contain valuable nitrogen—into nutrients for their host (and themselves).

Chemical and microbial cascades up the food chain

Interactions between herbivorous insects and other trophic levels—e.g., predators and parasitoids—can also be mediated by plant allelochemicals (Price et al. 1980; Barbosa and Saunders 1985; Ode 2006), and could be modified by gut microbiota. For example, successful consumers of herbivorous insects that have sequestered plant toxins must themselves be able to contend with those toxins. If insect prey contain microbes that confer detoxification, then their enemies could acquire the same ability in ecological or evolutionary time through consumption of the insects and acquisition of their microbes (Fig 2.1). These gut microbial dynamics are a neglected but potentially important aspect of predator physiology, which has been suggested to play a role in the evolution of prey toxicity (e.g.,

sequestration) (Speed and Ruxton 2014). Another means by which gut microbes could affect herbivory on chemically defended plants is through involvement or interference with the sequestration process itself, which some specialist insects depend upon for defense against enemies. Microbes could participate in the metabolic conversion of ingested allelochemicals to less-toxic forms, or to compounds able to cross the gut wall for storage in the body cavity. On the other hand, microbes could prove costly to these insects if gut detoxification reduces allelochemical supply, rendering them vulnerable to attack.

Implications for the diversification of herbivorous insects

The majority of multicellular organisms on earth are insects, of which most are herbivorous (Grimaldi and Engel 2005), and a large body of work has implicated coevolutionary interactions with plants as a primary driver of this diversity (e.g., Ehrlich and Raven 1964; Berenbaum 1983; Mitter et al. 1988; Farrell 1998; Winkler et al. 2009). In turn, the chemical nature of these interactions—the evolutionary interplay between plant allelochemical defenses and insect counterdefenses—appears to have had a major role in promoting adaptive diversification (Ehrlich and Raven 1964; Berenbaum 1983; Becerra 1997; Wheat et al. 2007). According to a key component of the escape and radiate model of coevolution, insects radiate onto an array of plants with novel defenses after evolving the ability to circumvent those defenses (Ehrlich and Raven 1964; Thompson 1999). Among plant defenses, secondary chemical compounds appear to be particularly important in driving the repeated bursts of insect and plant diversification that this model predicts (Cornell and Hawkins 2003; Futuyma and Agrawal 2009). For example, diversification rates increase after the shifts of butterflies in the Pierinae onto the glucosinolate-containing Brassicaceae, and of ithomiine butterflies from the Apocynaceae onto the chemically distinct Solanaceae (Fordyce 2010).

Janson et al. (2008) argued that symbionts may be involved in insect diversification by enabling their hosts to overcome barriers to establishing populations on novel host plants, an event that could

lead to ecological speciation. If host plants can be regarded as evolutionary islands (Janzen 1968), then microbes could be thought of as the rafts by which herbivorous insects reach new shores. Following our previous arguments, gut microbes could facilitate the formation of insect host races on a novel plant—potentially resulting in speciation (Drès and Mallet 2002)—by mediating the evolution of resistance to, or detoxification of, that plant's secondary chemical compounds. However, there are few empirical tests for a microbial breaching of the chemical barriers considered particularly important to insect-plant coevolution.

There are at least three processes by which heritable microbiome change could occur. First, a vertically inherited gut microbial population could adapt to the chemical compounds in a host's novel diet, benefiting its host's (and its own) fitness by detoxifying those compounds and/or becoming resistant to their antimicrobial effects. Adaptive genetic variants could arise from mutation, or from horizontal gene transfer from foreign microbes (see above). Second, an insect could acquire a microbe that then becomes vertically inherited. Likely candidates would be preadapted for dealing with a novel plant's allelochemicals by having inhabited, for example, a different herbivore consuming that plant, or the plant's own tissues (Fig. 2.1). These two processes do not require any change to the insect's nuclear or mitochondrial genomes, but do require that the microbial genomes are vertically transmitted. Long-term vertical transmission of insect gut microbiota can occur by multiple routes (Engel and Moran 2013), for example, by nestmate contact in social bees (Koch and Schmid-Hempel 2011), or symbiont capsules in plataspid stinkbugs (Hosokawa et al. 2006). A third process could operate when offspring acquire gut microbes from the environment rather than inheriting them from their parents (common among many insects, (Engel and Moran 2013)). Here, adaptation to plant allelochemicals takes the form of insect genomic changes that affect which microbes are sourced from the environment. A number of examples exist for vertebrate genes that control microbial colonization through their effects on metabolism, host behavior, or immunological tolerance in the gut (Spor et al. 2011). Parallel cases may exist for insect

herbivores, where the resistance or detoxification ability of the resulting microbial communities is under natural selection.

Gut microbial symbiosis may have played a role in herbivorous insect diversification at multiple scales. At broader scales, microbes may enable the origin of major clades with similar, chemically novel diets (e.g., terpenoid-feeding *Blepharida* beetles (Becerra 1997)). This phenomenon is well-documented for symbionts that help their hosts overcome broad nutritional or digestive obstacles (Hansen and Moran 2014); for example, the common ancestor of the Auchenorrhyncha appears to have acquired an amino-acid supplementing symbiont that enabled the origin of this sap-feeding group (Moran et al. 2005; Wu et al. 2006). At finer scales, gut microbiota could be involved in the subsequent diversification of those clades. Although insect speciation often occurs via transitions to new plant species that are closely related to their former host plants (Janz and Nylin 1998; Winkler and Mitter 2008), allelochemicals can vary significantly even between congeneric plants, and so insect host shifts and host plant specificity are tightly linked to plant secondary chemical profiles (Fraenkel 1959; Feeny 1992). By mediating host shifts onto plant species with novel, specific plant allelochemicals, gut microbes may be important to insect species-level diversification.

Testing the gut microbial facilitation hypothesis

Despite many lines of evidence that suggest gut microbiota may mediate insect-plant allelochemical interactions, strong empirical support is still relatively scant. Here we detail a number of approaches for testing the hypothesis outlined above.

Broad-scale observational studies are useful for generating hypotheses regarding microbe-plant allelochemical relationships. These studies might include microbial community barcoding surveys (e.g., of the 16S ribosomal RNA gene) of the guts of an array of insect species feeding on host plants with well-characterized secondary chemistry, to find microbes that have consistent associations with particular

compounds. Metagenomic sequencing may be a necessary complement to barcoding, given that ecologically relevant genomic variation is often lumped together within single gut microbial species (Engel et al. 2012), including strain-level differences in genes relevant to allelochemical metabolism (Haiser et al. 2013). Gut or fecal samples should be collected in the field, or at least from insects reared from wild stock, to avoid laboratory effects on insect microbiota (e.g., Chandler et al. 2011; Hammer et al. 2014). Combined genome sequencing of herbivores and their gut microbiota may suggest cases where genes known to participate in detoxification are lacking from the former, while present in the latter. Insect genomic studies have the additional benefit of detecting horizontal gene transfer events from past symbionts into the host genome; for example, Wybouw et al. (2014) found a bacterial cyanide-detoxification gene in the genome of a two-spotted spider mite adapted for cyanogenic plants.

Experiments will be necessary for unequivocally demonstrating microbial roles in detoxification or the antibiotic effects of allelochemicals on gut flora. One starting point is to manipulate the chemical composition of an insect's diet and assay its gut microbial response at the taxonomic or genetic level. This could take the form of artificial diets supplemented with relevant concentrations of plant toxins; however, as these diets are nutritionally and microbiologically distinct from natural host plants (and often contain antibacterial or antifungal compounds), a more rigorous approach is to use plants genetically engineered to lack toxins (e.g., Steppuhn et al. 2004). Cultures will inevitably prove useful for testing the sensitivity of microbes to certain compounds, or for assaying their ability to metabolize them, but culture-independent data—such as DNA sequencing or fluorescence microscopy—are needed to verify that those cultures are indeed stably associated with the host insect. Furthermore, as responses to, and effects on, plant allelochemicals may involve coordination among multiple community members, assays involving single isolates may miss important interactions (Mason et al. 2014).

Antibiotics are sometimes used to test for microbial symbiont function, but these may have direct negative effects on the host immune system that could confound effects due to altered

microbiota (Willing et al. 2011). An alternative route, recently used in the woodrat-creosote system (Kohl et al. 2014), is to transplant gut communities (via feces) from allelochemical-experienced donors into allelochemical-naïve recipients, in order to test whether performance in the latter group is improved on a toxin-containing diet. We suggest that this approach be expanded to include herbivores of different species, or even orders, that consume plants with different types of allelochemicals. For example, fecal transplants from a terpenoid-feeding beetle into a terpenoid-intolerant caterpillar, in conjunction with diet treatments of varying terpenoid concentrations, could determine the degree to which terpenoid-specific feeding abilities are conferred by the microbiota.

Conclusions

Plants present herbivorous insects with the formidable challenge of dealing with toxic secondary metabolites. Here, we have argued that the ever-present community of gut microorganisms may help insects meet this challenge. Furthermore, in light of the often critical function of symbionts in other aspects of insect herbivory (e.g., nutrient supplementation or cell wall digestion), plant allelochemicals may have evolved antibiotic functions to disrupt this community. These phenomena might even extend beyond plants and insects to higher trophic levels; for example, unlike the insect genes long assumed to be the main players in detoxification, microbes could be easily passed up the food chain to help carnivores avoid poisoning by sequestered allelochemicals. Finally, plant toxins are also central to insect-plant coevolution by acting as barriers to insect host shifts and subsequent diversification; by helping insects circumvent these barriers, gut microbial symbiosis could have had a hand in generating the extraordinary diversity of herbivorous insects on earth today.

Interactions between insect gut microbiota and plant toxins operate on a normally invisible level, hidden within plant and herbivore tissues, and are thus not easily observed. Given this difficulty, it may not be surprising that studies treating these interactions are relatively few, but modern techniques

in microbial ecology have made experimental investigation much more tractable. We call for further research on this underappreciated component of insect-plant (and enemy) interactions, which we have argued may be a ubiquitous and important aspect of insect ecology and evolution.

CHAPTER III

TREATING CATTLE WITH ANTIBIOTICS AFFECTS GREENHOUSE GAS EMISSIONS, AND MICROBIOTA IN DUNG AND DUNG BEETLES

(Hammer, T.J., Fierer, N., Hardwick, B., Simojoki, A., Slade, E., Taponen, J., Viljanen, H., & Roslin, T. (2016). Treating cattle with antibiotics affects greenhouse gas emissions, and microbiota in dung and dung beetles. *Proc. Roy. Soc. B*)

Abstract

Antibiotics are routinely used to improve livestock health and growth. However, this practice may have unintended environmental impacts mediated by interactions among the wide range of micro- and macroorganisms found in agroecosystems. For example, antibiotics may alter microbial emissions of greenhouse gases by affecting livestock gut microbiota. Furthermore, antibiotics may affect the microbiota of nontarget animals that rely on dung, such as dung beetles, and the ecosystem services they provide. To examine these interactions, we treated cattle with a commonly used broad-spectrum antibiotic and assessed downstream effects on microbiota in dung and dung beetles, greenhouse gas fluxes from dung, and beetle size, survival and reproduction. We found that antibiotic treatment restructured microbiota in dung beetles, which harbored a microbial community distinct from those in the dung they were consuming. The antibiotic effect on beetle microbiota was not associated with smaller size or lower numbers. Unexpectedly, antibiotic treatment raised methane fluxes from dung, possibly by altering the interactions between methanogenic archaea and bacteria in rumen and dung environments. Our findings that antibiotics restructure dung beetle microbiota and modify greenhouse gas emissions from dung indicate that antibiotic treatment may have unintended, cascading ecological effects that extend beyond the target animal.

Introduction

Antibiotics are extensively used in agriculture to promote growth and to treat or prevent livestock disease (Mellon et al. 2001; McEwen and Fedorka-Cray 2002; Sarmah et al. 2006; Krishnasamy et al. 2015; Van Boeckel et al. 2015), yet they may have major consequences for human and environmental health (Gustafson and Bowen 1997; Kumar et al. 2005; Van Boeckel et al. 2015). For example, the use of antibiotics in agriculture can favor the evolution of antibiotic resistance among pathogens and the spread of antibiotic resistance genes to surrounding environments (e.g., (Schwarz et al. 2001; Teuber 2001; Aarestrup 2005)). In addition, this practice can have other ecosystem-level ramifications which are likely important, but less appreciated. In particular, antibiotic treatment could affect two distinct, but potentially interacting ecological processes: the removal and recycling of livestock dung by decomposer organisms, and the release of greenhouse gases from dung.

Livestock dung provides a source of nutrients, organic matter, and microbes to pasture ecosystems (Eghball et al. 2002; Aarons et al. 2009; Yoshitake et al. 2014). Dung can also act as a source of pathogens (Mawdsley et al. 1995) and emit significant quantities of greenhouse gases, including methane (Jarvis et al. 1995; Saggar et al. 2004). However, these effects can be modified by the diverse communities that inhabit, consume, and/or interact with dung, of which dung beetles have been particularly well-studied (Yokoyama et al. 1991; Penttilä et al. 2013; Nichols and Gomez 2014; Slade et al. 2015; Manning et al. 2016). In general, dung beetles play a critical role in carbon and nitrogen cycling and the maintenance of soil fertility in both natural and agricultural pasture ecosystems (Bang et al. 2005; Nichols et al. 2008). Veterinary pharmaceuticals can harm beetles and other downstream consumers of livestock dung, which may depress dung decomposition and reduce the diversity of dung-based communities. But while the effects of antiparasiticides have been relatively well studied in this context (e.g., (Wall and Strong 1987; Madsen et al. 1990; Floate et al. 2005; Pérez-Cogollo et al. 2015)), the effects of antibiotics on dung beetles remain poorly known (Schmitt and Römbke 2008).

In contrast to antiparasiticides, which may directly act on the physiology of dung consumers (e.g., (Verdú et al. 2015)), broad-spectrum antimicrobial compounds could have far-reaching, microbially mediated ecological effects. For example, as microbial symbionts ("microbiota") are often critical to insect health and reproduction (Engel and Moran 2013; Douglas 2015), antibiotics retained in dung could affect beetle performance by altering beetle microbiota. Furthermore, antibiotic-induced restructuring of livestock gut microbiota could change the nutritional, chemical, and microbiological properties of dung, the diet of dung beetles.

Antibiotics could also directly or indirectly modulate greenhouse gas emissions from livestock dung. Antibiotics have been shown to alter the structure and activity of mammalian gut and fecal microbiota (Looft and Allen 2012; Looft et al. 2012; Maurice et al. 2013). In livestock such as cattle, certain members of the gut microbiota are responsible for producing key greenhouse gases such as methane, nitrous oxide, and carbon dioxide (Moss et al. 2000; Kebreab et al. 2006; Martin et al. 2010). Despite interest in reducing emissions of these microbially mediated greenhouse gases, and the frequent use of antimicrobial compounds in agriculture, the relationship between the two remains unclear. Furthermore, the effects of antibiotics on greenhouse gas fluxes and on dung beetles could be linked. For example, dung beetles can reduce methane production from dung to an extent that could have impacts in some agricultural systems (Slade et al. 2016). As methanogenesis is thought to be inhibited by oxygen entering the dung pat through beetle tunnels (Penttilä et al. 2013), it could be sensitive to the size and number of dung beetles. In turn, if beetle performance and fitness are influenced by antibiotic-sensitive microbiota, the effect of dung beetles on greenhouse gas emissions could depend upon the antibiotic treatment.

We hypothesized that antibiotics administered to cattle alter dung beetle microbiota and, as a consequence, depress beetle fitness. In addition, we hypothesized that antibiotics modulate gas emissions from dung and the extent to which beetles influence emissions. To experimentally test these

hypotheses, we treated cattle with and without a broad-spectrum antibiotic and assayed microbial communities in cow dung and in field-collected dung beetles (*Aphodius fossor* L.). We also tested the effect of antibiotics on beetle size and numbers, and on fluxes of multiple greenhouse gases (carbon dioxide, nitrous oxide, and methane) from dung. Our findings demonstrate that the common practice of administering antibiotics to livestock can have important, unintended impacts on dung biota and the biogeochemical processes they mediate in agroecosystems.

Methods

Experimental setup

Ten cows were randomly assigned to two treatments: five were given a standard three-day course of tetracycline and five were left as controls. None of the cows had undergone antibiotic treatment within the previous six months, except for one cow in the antibiotic group, which had received a course of penicillin six weeks prior to the experiment (for the full history of each cow, see Appendix A3 Methods). From each cow, we collected fresh dung on a single day, beginning one hour after the last administration of antibiotics. That afternoon, we separated the dung from each cow into six 1 L pats and placed each pat in a mescosm (an open-bottom, mesh-covered plastic bucket) in the field. We also included four control mesocosms without dung to measure background fluxes of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) from the soil. More detail on experimental design is given in Appendix A3 Methods.

To examine the effects of antibiotics on dung beetle microbiota, on the performance of beetles, and on beetle-mediated effects on gas fluxes from cow pats, we focused on the dung beetle *Aphodius fossor* (L.). *A. fossor* is a regionally widespread and locally common species (Roslin 2001), and its ecology and interactions with dung have been extensively studied (e.g., (Vessby 2001; Penttilä et al. 2013; Slade et al. 2015)). The beetles were collected in the field in early June from different localities and stored at 4°C until they were added to dung pats.

Dung beetles were added to four of the six dung pats produced by each cow (randomly chosen). Gas measurements and dung samples for microbial analysis were taken from two intact pats with beetles and two without beetles. The two additional pats with beetles were used for more invasive sampling of beetles for microbial characterization and to measure beetle reproduction and development. Based on beetle densities recorded in the field and on a previous study (Vessby 2001), we added 12 beetles to each pat, maintaining a sex ratio of 1:1.

Gas flux measurements

Gas fluxes of CO_2, N_2O and CH_4 were measured from dung mesocosms in the field at five time points over the course of the experiment (more information given in the Appendix A3 Methods). Net gas fluxes emitted from each dung pat were calculated as in Penttilä et al. 2013 (Penttilä et al. 2013).

Dung and beetle sampling

All dung and beetle samples were preserved for subsequent characterization of microbiota using 95% ethanol, which is an effective storage medium for microbial community analysis (Hammer et al. 2015). On days 2, 11, and 23 of the experiment, dung samples of approx. 1 ml were taken and stored in ethanol (see Appendix A3 Methods).

To test whether antibiotic treatment affected beetle microbiota, two parent beetles from each pat were preserved in ethanol on day 7 of the experiment. To measure antibiotic effects on beetle size, reproduction and survival, we sampled pats on day 43 (for half-grown larvae) and days 71 and 73 (for the next generation of adult beetles). Larvae recovered were weighed while fresh and the width of their head capsule was measured. Total offspring counts by the end of the experiment were used as an integrated measure of both adult reproduction and offspring survival.

Molecular protocol and sequence data processing

To characterize the overall beetle-associated microbial community, whole adults were homogenized and DNA was extracted from ~100 mg of homogenate with the MoBio PowerSoil kit, following similar studies (Hammer et al. 2014, 2015). Approx. 100 mg subsamples of dung were used for DNA extraction with the same kit. Using barcoded primers, we PCR-amplified the ~300 bp V4 region of the bacterial and archaeal 16S rRNA gene, with the amplicons sequenced on an Illumina MiSeq platform as previously described (Ramirez et al. 2014; Hammer et al. 2015). Detail regarding sequence data processing and taxonomic identification is given in the Appendix A3 Methods.

Statistical analysis

All statistical analyses were performed in R v. 3.2.2 (R Core Team 2016) and plots were constructed with the ggplot2 package (Wickham 2009). The vegan package (Oksanen et al. 2013) was used to analyze microbial community data. Following previous work (e.g., (Ramirez et al. 2014; Hammer et al. 2014, 2015)) we used the Bray-Curtis dissimilarity metric to quantify variation in overall microbial community structure among samples. Non-metric multidimensional scaling plots were constructed to visualize Bray-Curtis dissimilarity in two dimensions. To avoid pseudoreplication of beetle data, dissimilarities (multivariate analyses) or performance variables (univariate analyses) were averaged between the beetles in each mesocosm; thus, multilevel statistical tests were conducted on the effects of antibiotic treatment and cow individual.

The effects of antibiotic treatment and cow individual on beetle and dung microbiota were tested using nested permutational multivariate ANOVA in the BiodiversityR package (Kindt and Coe 1995), with cow nested within treatment (999 permutations). Unless noted otherwise, below we report results from dung sampled on day 2 of the experiment, which we expected to most closely represent microbiota in the cow gut, but analyses of dung sampled on days 11 and 23 are shown in the Fig. A3.1.

With each of the gases measured, we used ANOVA to assess effects of antibiotics and beetle presence on cumulative gas fluxes over the course of the experiment, as well as the interaction of the two factors. We analysed the proportion of methanogens in dung microbiota (all time points) with the same procedure, but with prior log transformation to meet the assumption of normality.

To assess antibiotic effects on the performance of larval offspring, we chose to focus on weight, which was highly correlated with head size (Spearman's rank correlation, rho = 0.68, $p = 0.005$). Weight can be considered as a proxy for performance as it is correlated with adult fecundity in both *Aphodius* (Hirschberger 1999) and other insects (e.g., (Honek 1993; Tammaru et al. 1996)). A nested ANOVA (cow individual within antibiotic treatment) was used to test for effects on larval weight; normality of model residuals was confirmed visually. The effect of antibiotic treatment on total counts of beetle offspring recovered from each dung pat was analyzed by fitting a generalized linear mixed model with cow individual as a random effect, Poisson-distributed errors and a log link function.

Results and Discussion

Microbial community structure in beetles

Dung beetles are extraordinarily diverse (Hanski and Cambefort 1991) and both scientifically (Simmons and Ridsdill-Smith 2011) and ecologically important (Nichols et al. 2008), yet only one previous study has used DNA sequence-based methods to characterize their microbiota (*Onthophagus taurus*, (Estes et al. 2013)). Despite feeding on a microbe-rich substrate, we found that *A. fossor* adults transform dung microbiota during digestion or host symbionts not present in their diet. Overall, beetles and dung had compositionally distinct microbiota ($F_{1,51} = 17.32$, $p = 0.001$; Fig. 3.1c and Fig. 3.2), regardless of the dung sampling time point (Figs. A3.1a,b), and beetles contained lower microbial diversity than their diet ($F_{1,51} = 45.12$, $p < 0.0001$; Fig. A3.2). While the dung communities were dominated by Bacteroidia, Clostridia, and Spirochaetes, the dung beetle microbiota were dominated by

Gammaproteobacteria and Bacilli. Less than 25% of the operational taxonomic units (OTUs) that were relatively abundant in beetle communities (overall proportion ≥1%) were also abundant in dung, implying that the dominant beetle microbes are either vertically transmitted or rare in dung. Indeed, the genus *Acinetobacter* was highly abundant in *A. fossor* (Fig. 3.2) and also found in *O. taurus* fed sterilized dung (Estes et al. 2013), suggesting that *Acinetobacter* could be vertically transmitted symbionts common among dung beetles.

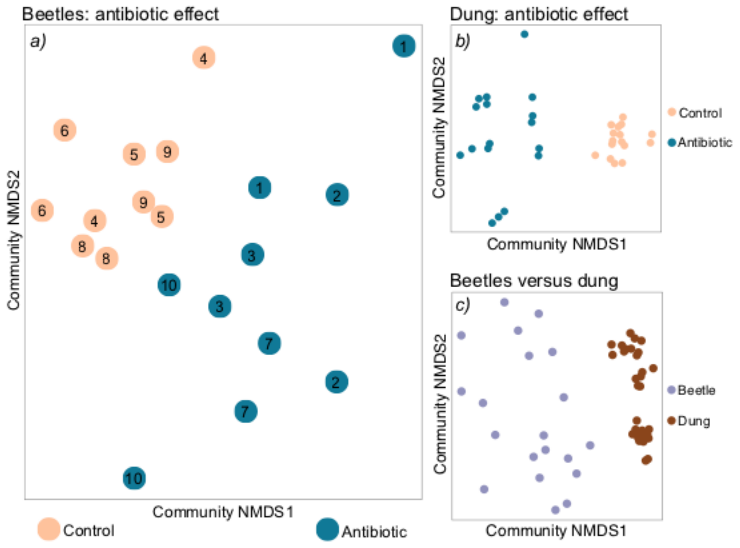

Figure 3.1. Microbial communities diverge upon antibiotic treatment and cluster according to sample habitat (beetle versus dung). Non-metric multidimensional scaling ordinations of microbiota in: a) parental dung beetles, where cow individual is indicated by numbers and treatment by color, b) dung samples only, and c) beetles versus dung. The ordinations visually represent Bray-Curtis dissimilarities among samples in two dimensions.

Effects of antibiotics on dung and dung beetle microbiota

As expected from previous work (Grønvold et al. 2011; Looft and Allen 2012; Looft et al. 2012; Maurice et al. 2013), we found a clear effect of antibiotic treatment on dung microbiota ($F_{1,23}$ = 4.84, p = 0.01; Fig. 3.1b, Fig. A3.2), and this effect persisted even 23 days after defecation and sample collection (Figs. A3.1c,d). As in other animal taxa that maintain individual-specific microbiota (e.g., (Costello et al. 2009; Grønvold et al. 2011)), we also found that dung microbial communities clustered by cow individual

($F_{8,23}$ = 4.87, p = 0.001; Fig. 3.2). Antibiotic treatment administered to cattle also affected dung beetle microbiota ($F_{1,10}$ = 2.39, p = 0.007; Fig. 3.1a, Fig. A3.2), and mirroring the patterns found for dung, the microbial communities in beetles also clustered by cow individual ($F_{8,10}$ = 1.38, p = 0.001; Fig. 3.1a).

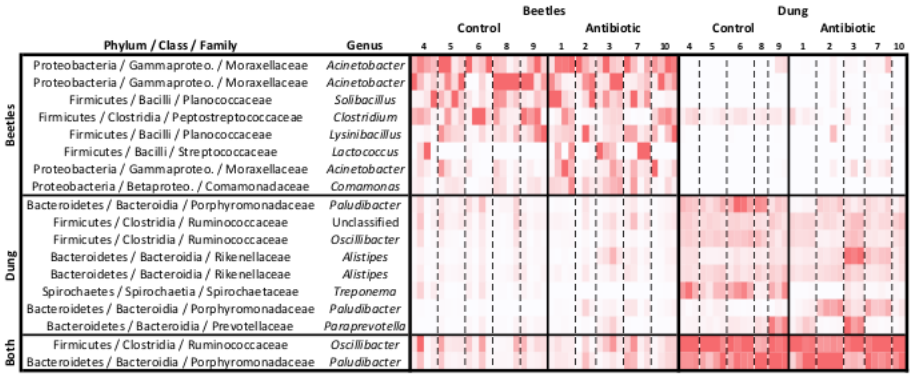

Figure 3.2. Variability in dominant microbial operational taxonomic units (OTUs) between individuals, sample habitats, and treatments. The heatmap shows OTU relative abundance in each sample (columns) where red indicates a high proportion; the range of values is scaled separately for each sample. Numbers above columns denote cow individuals. For clarity, only the top 10 OTUs for each sample type are shown (two OTUs were in the top 10 of both beetles and dung). All OTUs shown are bacteria, as archaea were less abundant overall.

Antibiotics could alter beetle microbiota by multiple mechanisms. For example, antibiotic-induced changes in dung microbiota (Fig. 3.1b) could change the nutritional quality of the beetles' diet, or provide an altered inoculum to the beetle community. Alternatively, antibiotic residues present in dung and consumed by beetles may act on beetle gut microbiota. Concern has been raised over the persistence of antiparasiticides in the environment, where—as eukaryote-specific pharmaceuticals—they are directly toxic to animals (Schmitt and Römbke 2008; Puniamoorthy et al. 2014; Verdú et al. 2015). Our data suggest that even antibacterials such as tetracycline may have similar far-reaching effects, by altering the microbiota of nontarget animals.

Beetle size and numbers

Despite clear antibiotic effects on beetle microbiota (Fig. 3.1a), and the often critical role of microbiota in insect biology (Engel and Moran 2013; Douglas 2015), antibiotics did not influence dung beetle size, reproduction or survival. Larval weight was unaffected by antibiotic treatment ($F_{1,13}$ = 0.018, p = 0.90), and the total number of beetle offspring recovered from dung pats by the end of the experiment was similar between treatments (Poisson GLMM, z = -0.35, p = 0.73). An average of 4.2 ± 1.5 versus 3.0 ± 0.71 offspring were collected from dung pats of control and antibiotic treatments, respectively (mean ± SEM, n = 10 per treatment). As not all members of the beetle microbiota responded to antibiotics (Fig. 3.2), one possible explanation is that the antibiotic effect on overall microbiota was driven by commensal and not mutualistic species. For example, *Acinetobacter* abundance was not sensitive to antibiotic treatment ($F_{1,10}$ = 0.35, p = 0.57, Fig. 3.2), and tetracycline resistance has been documented among *Acinetobacter* in other environments (Henwood et al. 2002; Coyne et al. 2011). Alternatively, microbial symbionts may simply not be important to the nutrition or development of dung beetles (Byrne et al. 2013). However, given the wide variety of processes that can be influenced by microbiota (e.g., (Brownlie and Johnson 2009; Feldhaar 2011; Lizé et al. 2013)), the antibiotic-induced microbial restructuring we observed could affect unmeasured aspects of dung beetle biology.

Greenhouse gas emissions from dung

The presence of beetles decreased methane fluxes from dung ($F_{1,36}$ = 7.49, p < 0.01; Fig. 3.3a), an effect reported previously and likely due to oxygenation of the pat caused by beetle tunnels (Penttilä et al. 2013; Slade et al. 2016). In contrast, antibiotic treatment consistently increased methane emissions ($F_{1,36}$ = 22.21, p < 0.0001; Fig. 3.3a), which is likely related to changes in dung microbiota (Fig. 3.1b). Contrary to our expectations, the two effects were unrelated (interaction $F_{1,36}$ = 0.004, p = 0.95),

suggesting that beetle tunnelling activity is not affected by antibiotic modification of dung (and its impact on the beetle microbiome).

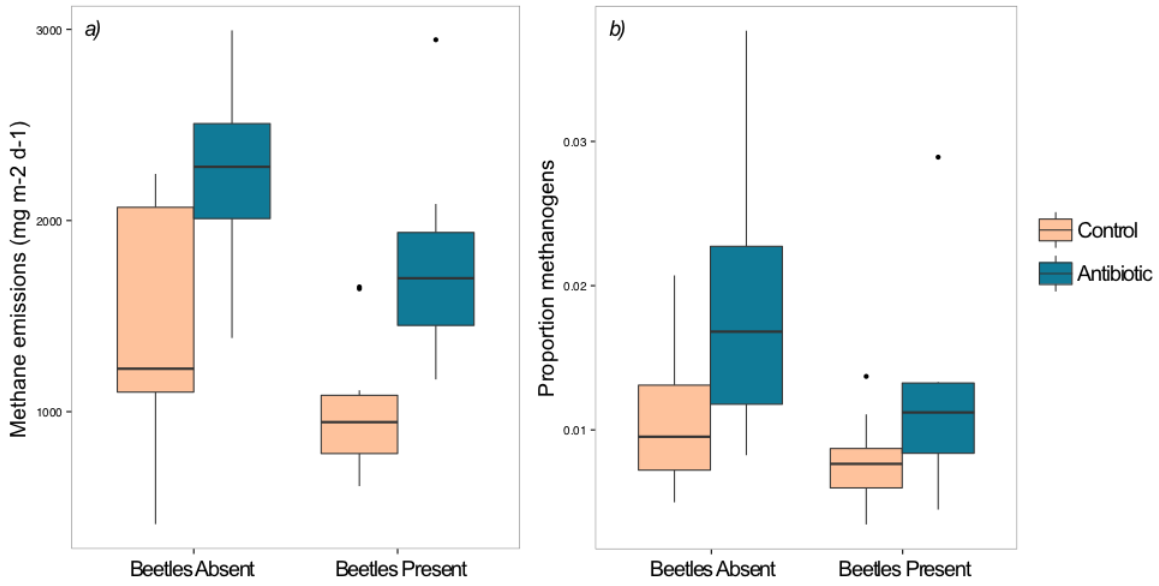

Figure 3.3. Dung methanogenesis is raised by antibiotic treatment and lowered by the presence of dung beetles, a pattern matched by methanogen abundance in dung microbiota. a) Boxplot showing the factors affecting total methane emissions quantified from dung pats. b) Boxplot showing the proportion of methanogenic archaea in DNA sequence libraries from the same dung samples. Bold horizontal lines indicate medians, box limits show first and third quartiles, whiskers extend to the most extreme values within 1.5 * the inter-quartile range, and dots show outlying data points.

Carbon dioxide emissions were similar between treatments ($F_{1,36}$ = 0.001, p = 0.98), and there was no effect of beetle presence ($F_{1,36}$ = 0.02, p = 0.88) nor an interaction between the two factors ($F_{1,36}$ = 0.22, p = 0.64), suggesting that antibiotics do not affect overall microbial decomposition rates. This result indicates that the antibiotic effect on methane production is not simply due to an increase in overall microbial activity. In contrast, fluxes of nitrous oxide, another potent greenhouse gas, were influenced by beetles in an antibiotic-dependent manner (Fig. A3.3). Specifically, the presence of beetles raised nitrous oxide emissions ($F_{1,36}$ = 17.31, p < 0.001), in agreement with a previous report (Penttilä et al. 2013) (but see (Slade et al. 2016)). In support of our original hypothesis, antibiotic treatment appeared to weaken this beetle-mediated increase (interaction $F_{1,36}$ = 5.85, p = 0.02). There was no main

effect of antibiotics alone ($F_{1,36}$ = 1.99, p = 0.17), despite clear antibiotic-induced changes to dung microbiota (Fig. 3.1b). It remains to be determined whether antibiotics modulate the effect of beetle presence on nitrous oxide specifically through their impact on beetle microbiota (Fig. 3.1a).

Implications and potential mechanism of methane effect

The large (1.8-fold) increase in methane emissions from the dung of cattle treated with antibiotics (Fig. 3.3a) has not been documented, despite the considerable literature on methane production from agricultural systems (e.g., (Johnson and Johnson 1995; Moss et al. 2000; Kebreab et al. 2006; Martin et al. 2010; Shibata and Terada 2010)) and the long-standing and increasing administration of antibiotics to livestock. Previous studies have found either a short-lived decrease (Johnson and Johnson 1995), or no effect following antibiotic treatment (Hashimoto et al. 1981; Shibata and Terada 2010); to our knowledge, this is the first report of antibiotics increasing methane emissions. While dung emissions of methane are typically lower than those released from belching (Tubiello et al. 2013), they still comprise a substantial proportion of total agricultural methanogenesis in pasture systems (Slade et al. 2016). Moreover, as the effects of antibiotics apparently derive from microbial interactions within the gut (explained below), they likely extend to gas emissions from enteric fermentation as well. Hence, we suggest that future research should be focused on antibiotic effects on methane emissions from belching.

The pattern of methane emissions (Fig. 3.3a) was qualitatively similar to that of the relative abundance of methanogens in the same dung pats (Fig. 3.3b). As with gas fluxes, antibiotics raised the proportional abundance of methanogens ($F_{1,35}$ = 8.72, p = 0.006), and there was no interaction of antibiotics with beetle presence ($F_{1,35}$ = 0.26, p = 0.62). We suggest that these patterns may be explained by competitive dynamics among gut microbiota. In the gut of ruminants and other mammals, methanogenic archaea may compete with bacteria for hydrogen, which is often scarce (Strocchi et al.

1994; Morvan et al. 1996). Tetracycline and some other broad-spectrum antibiotics are generally less effective against archaea, including methanogens isolated from mammalian digestive tracts (Dridi et al. 2011; Khelaifia and Drancourt 2012). Therefore, we propose that by specifically suppressing bacteria in the gut and subsequently in dung, antibiotic treatment enables methanogens to outcompete bacteria for hydrogen, increasing their concomitant methane output.

Conclusions

The routine practice of administering antibiotics to livestock can have unexpected consequences for dung biota and greenhouse gas emissions from agriculture. First, antibiotics altered the composition of microbial communities associated with dung beetles, an ecologically important group of insects in many environments. This finding highlights a unique feature of antibacterial pharmaceuticals: even if not directly toxic to nontarget animals, they may have a range of unanticipated effects by altering the microbiota of both livestock and wildlife. Second, we provide the first demonstration that antibiotics can increase dung emissions of methane, a potent greenhouse gas. Our findings call for analyses at larger scales [e.g., (Slade et al. 2016)] that take other factors into account, such as the relative importance of dung versus belching in gas emissions, and the global extent and purpose of antibiotic use in livestock production. Improved monitoring and estimates of agricultural antibiotic use will be necessary to identify whether antibiotics may impact the overall contribution of livestock production to global warming. Finally, further research into this effect will require unravelling the ecological interactions between microbes in the gut of livestock, and their susceptibilities to antibiotic disturbance.

CHAPTER IV

METAMORPHOSIS OF A BUTTERFLY-ASSOCIATED BACTERIAL COMMUNITY

(Hammer, T.J., McMillan, W.O., & Fierer, N. (2014). Metamorphosis of a butterfly-associated bacterial community. *PLoS One*)

Abstract

Butterflies are charismatic insects that have long been a focus of biological research. They are also habitats for microorganisms, yet these microbial symbionts are little-studied, despite their likely importance to butterfly ecology and evolution. In particular, the diversity and composition of the microbial communities inhabiting adult butterflies remain uncharacterized, and it is unknown how the larval (caterpillar) and adult microbiota compare. To address these knowledge gaps, we used Illumina sequencing of 16S rRNA genes from internal bacterial communities associated with multiple life stages of the neotropical butterfly *Heliconius erato*. We found that the leaf-chewing larvae and nectar- and pollen-feeding adults of *H. erato* contain markedly distinct bacterial communities, a pattern presumably rooted in their distinct diets. Larvae and adult butterflies host relatively small and similar numbers of bacterial phylotypes, but few are common to both stages. The larval microbiota clearly simplifies and reorganizes during metamorphosis; thus, structural changes in a butterfly's bacterial community parallel those in its own morphology. We furthermore identify specific bacterial taxa that may mediate larval and adult feeding biology in *Heliconius* and other butterflies. Although male and female *Heliconius* adults differ in reproductive physiology and degree of pollen feeding, bacterial communities associated with *H. erato* are not sexually dimorphic. Lastly, we show that captive and wild individuals host different microbiota, a finding that may have important implications for the relevance of experimental studies using captive butterflies.

Introduction

Butterflies are important herbivores and pollinators and are used as model systems in a variety of ecological and evolutionary fields (Boggs et al. 2003). Like all animals, butterflies also host internal communities of microorganisms, yet their associations with these symbionts remain poorly understood. This knowledge gap persists despite a large and rapidly growing body of work on other insect groups demonstrating that microbes can have important effects on host nutrition, digestion, detoxification, and defense from predators, parasites, and pathogens (Douglas 2009; Brownlie and Johnson 2009; Feldhaar 2011; Engel and Moran 2013). Studies of butterfly-associated microorganisms therefore have the potential to advance our understanding of the biology of butterflies and their ecological and evolutionary interactions with plants and natural enemies.

Unfortunately, even basic information on butterfly microbial symbionts is lacking, making it difficult to identify the potential impacts that these microbes may have on butterfly ecology and evolution. While various bacteria have been isolated from the adult butterfly intestinal tract (Steinhaus 1941; Kim et al. 2012), and the presence of *Wolbachia* and *Spiroplasma* has been reported in the adults of some species (Tagami and Miura 2004; Narita et al. 2006; Russell et al. 2012), there are no community-level descriptions of the dominant microbial taxa present. Kingsley (Kingsley 1972) cultured multiple bacterial populations from the gut of newly emerged adult monarch butterflies, but such cultivation-based surveys are well known to misrepresent the community structure *in situ* (Pace 1997). To our knowledge, there have been no previous culture-independent studies of microbial communities associated with adult butterflies.

Additionally, while the larval gut microbiota of a handful of butterfly species have been described (Broderick et al. 2009; Robinson et al. 2010), it is not known how microbial communities associated with larvae compare with those in the adult stage, nor how they may change during metamorphosis. In fact, this question has not been addressed in any lepidopteran since the advent of

molecular tools for characterizing microbial diversity. Kingsley's survey of monarch gut bacteria (Kingsley 1972) included multiple developmental stages, but owing to a dependence on culturing and physiology-based taxonomic assignments, it is uncertain whether those findings are generalizable. We do know from work on other holometabolous insect groups that larvae may have few or no microbial symbionts (Lauzon et al. 2009; Martinson et al. 2012), different microbiota (Vasanthakumar et al. 2008; Wong et al. 2011; Brucker and Bordenstein 2012), or similar microbiota as adults (Arias-Cordero et al. 2012; Colman et al. 2012). We expected that butterfly larvae and adults would host distinct bacterial communities owing to the radical switch in diet from the larval to the adult stage of butterflies, as well as the changes in internal morphology and physicochemical conditions that accompany metamorphosis. Diet is a major factor structuring microbiota across animal taxa [22,23], and diet shifts may also underlie patterns of microbial variation across developmental stages of a single host. For example, nutritional or chemical differences between the diets of larvae and adults may differentially select for microbial taxa best able to grow at each stage. Conversely, those particular microbial taxa may aid the host in utilizing life-stage-specific resources by providing functions related to digestion, detoxification, and/or nutrient supplementation.

Perhaps the most striking contrast in feeding biology between butterfly larvae and adults is in the neotropical genus *Heliconius*. *Heliconius* larvae consume leaves and stems of cyanogenic glycoside-rich passion-flower vines (Engler-Chaouat and Gilbert 2007), while adults visit flowers to feed on pollen as well as nectar. Among butterflies, pollen feeding is an evolutionary innovation unique to *Heliconius*, and has led to major changes in reproductive biology and life history traits (Gilbert 1972). We therefore focused on *Heliconius* to test for a possible differentiation in microbial community structure between the larval and adult stages. Additionally, *Heliconius* butterflies represent an ideal model system for microbial symbiosis research as they are collectable in the wild and experimentally tractable, and as a wide array of relevant ecological, evolutionary, and genomic information is available [27,28]. In contrast,

almost nothing is known about their microbiota, besides the sporadic presence of *Wolbachia* (Werren et al. 1995; Muñoz et al. 2011). Given their distinctive larval and adult diets, *Heliconius* butterflies also provide an opportunity to test whether associations with microbial symbionts have been important in the evolution of host traits related to herbivory and pollen feeding.

In addition to investigating how *Heliconius*-associated microbial communities change across different life stages, we also wanted to determine how wild and captive *Heliconius* butterflies may differ with respect to their microbiota. Many experimental studies of *Heliconius* (and other butterflies) have used lab- or insectary-reared subjects. Evidence from moth larvae (Xiang et al. 2006; Belda et al. 2011) and other insects (Lehman et al. 2009; Chandler et al. 2011) suggests that symbiont community structure can change when hosts are brought from the wild into captivity, an effect possibly mediated by artificial diets or selection history. Testing whether captive and wild butterflies are different in terms of their microbiota is important not only for future microbial investigations, but also for other types of studies on captive butterflies where the phenomena under question may be influenced by microbial symbionts (including, but not limited to, host plant use and defense against parasites or parasitoids).

We used a high-throughput DNA sequencing-based approach to characterize internal bacterial communities associated with the butterfly *Heliconius erato*, thus providing a foundation for future studies of microbial symbiosis in *Heliconius* and other butterflies. To test the hypothesis that the microbiota varies across the butterfly life cycle, we compared bacterial community structure in replicate larvae, pupae, newly emerged adults, and mature adults of *H. erato*. We also assessed variation in bacterial community diversity and composition between wild adults sampled from the field, wild adults maintained in an insectary, and the reared adult offspring of the latter to determine whether captive butterflies harbor bacterial communities representative of their wild counterparts.

Materials and Methods

Insect collection and rearing

In April and May 2012, adult *Heliconius erato* butterflies were collected from a wild population as they visited flowers in Parque Nacional Soberanía, Panama (9°7'20"N, 79°42'54"W), for which permission was provided by the Panamanian Environmental Authority (ANAM) under permit #SE/A-92-11. Voucher specimens have been deposited at the Fairchild Invertebrate Museum of the University of Panama. Thirteen individuals (nine males and four females) were stored at −20°C directly after field collection. All samples described below were preserved in the same manner.

We relocated nine additional wild-caught females to a nearby insectary, where they were housed under semi-natural conditions in separate mesh cages. They were supplied with flowers frequently visited by wild *H. erato* in this area (*Psychotria elata*, *Lantana camara*), and with an autoclaved sucrose and honeybee pollen solution. Potted *Passiflora biflora*, the main host plant of the specialist *H. erato* (Smiley 1978), were placed in the cages to elicit oviposition. Eggs were removed and placed individually in plastic cups. The parental females were sampled after a sufficient number of eggs were obtained, corresponding to appx. 2-4 weeks in captivity. Given that females of *H. erato* only very rarely mate more than once in the wild (Walters et al. 2012), it is likely that the individuals in each brood are full siblings.

We reared larvae on plant material collected from potted *P. biflora* grown in an open-air greenhouse near the forest. One larva per brood was sampled two days into the fifth stadium, while it was actively feeding, as was the frass it had produced that day. Pupae were sampled midway through the pupal stage. Newly emerged adults were sampled immediately after they had excreted meconium. The rest of the adults were kept under identical conditions as described above for wild-caught parental females. One male and one female per brood were sampled four days after eclosion, by which point both sexes of this species have reached sexual maturity.

Sample processing

We used whole, surface-sterilized insects to describe the dominant bacterial taxa associated with the internal portion of the body. Insects were rinsed in sterile molecular-grade water (Sigma-Aldrich), soaked in 70% ethanol for 30 s followed by 10% bleach for 30 s, and rinsed again in sterile water. For adults, wings were clipped where they met the thorax prior to sterilizing the body. After surface sterilization the samples were ground under liquid N_2 with single-use, sterile mortar and pestles (Fisher Scientific). Frass samples were not surface sterilized.

DNA sequencing and data processing

Bacterial communities were characterized using barcoded Illumina sequencing of 16S rRNA genes. Total DNA was extracted from homogenized material using the MoBio PowerSoil kit as described previously (Fierer et al. 2008). We used the primer pair 515F/806R to amplify the V4 region of the 16S rRNA gene, and PCR conditions followed those described previously (Caporaso et al. 2012). Amplicons were sequenced on the Illumina MiSeq platform, resulting in an average of 1779 150-bp reads per sample after filtering with default parameters for sequence length and minimum quality score in QIIME v. 1.6.0 (Caporaso et al. 2010). Sequences were clustered into operational taxonomic units (hereafter, "phylotypes") at the 97% similarity level by reference-based picking with the QIIME implementation of UCLUST (Edgar 2010) against the October 2012 release of the Greengenes database (McDonald et al. 2012) with remaining sequences clustered *de novo*. The Ribosomal Database Project (RDP) classifier (Wang et al. 2007) set at a minimum confidence level of 0.5 was used to assign taxonomy to the phylotypes. The centroid (seed sequence) used by UCLUST was chosen as the representative sequence for each phylotype. With representative sequences from the 10 most abundant phylotypes across all *H. erato* samples, we used SeqMatch to find the best high-quality matches ≥ 1200 bp in the curated RDP 16S database (Cole et al. 2009).

Because this primer set can amplify non-bacterial rRNA gene sequences, phylotypes identified by the RDP classifier as chloroplast or mitochondrial 16S rRNA (which represented 24% of the sequences on average) were removed prior to downstream analyses. In order to standardize sequencing effort, all samples were rarefied by randomly selecting 500 sequences per sample. As the samples from which we obtained fewer than 500 bacterial sequences were excluded from further analysis, there are fewer replicates for pupae than were initially collected. This sequencing depth has been shown to be sufficient for detecting biological patterns in insect-associated bacterial communities (Jones et al. 2013) and other community types (Kuczynski et al. 2010). Amplicon sequences and associated metadata from this study are publicly available in the EMBL-EBI database (http://www.ebi.ac.uk/) under accession number ERP003400.

Statistical analyses

We used nonparametric Kruskal-Wallis tests in R v. 3.0.0 (R Core Team 2016) to determine whether there were significant differences in community richness or the relative abundances of individual bacterial taxa (families or phylotypes) with a Bonferroni correction applied to account for multiple comparisons. The family-level tests were conducted only on dominant families, defined as those contributing at least a median 2% of the sequences within any of the factor levels. To compare community composition between sample types, we used vegan (Oksanen et al. 2013) to compute a Bray-Curtis dissimilarity matrix after Hellinger transformation of the phylotype count data. Subsequent multivariate analyses were conducted in PRIMER (Clarke and Gorley 2006). Variation among samples in their bacterial taxonomic composition was visualized using constrained principal coordinates analyses (Anderson and Willis 2003). We used Mantel tests to determine whether patterns of compositional dissimilarities among larvae were correlated with dissimilarities among their frass. Permutational multivariate ANOVA tests (Anderson 2001) were used to assess differences in bacterial community

composition associated with several sample categories, with tests of life stage or frass versus larvae run using sample type as a fixed effect. Variation in the dissimilarity matrix linked to the level of relatedness among captive adults was tested using family as a random effect. Lastly, for all adult butterflies, a two-factor design was used to test the effects of captivity/rearing status and sex (both fixed).

Results

Bacterial community dynamics across the life cycle

Bacterial phylotype richness varied among life stages (Fig. 4.1a, $P < 0.01$). Median richness was similar between larvae and mature adults with 39 and 43 phylotypes per individual, respectively. In contrast, pupae and newly emerged adults were associated with roughly half as many phylotypes (median 17 and 22 phylotypes, respectively). Nearly identical patterns were observed when diversity was measured using the Shannon index, which takes relative abundances into account (Fig. A4.1, $P < 0.01$). A comparison restricted to only the numerically dominant phylotypes—those contributing at least 5 sequences per sample (1%)—produced a similar pattern: median richness of dominant phylotypes was 12 in both larvae and mature adults, and 4 and 5.5 in pupae and newly emerged adults, respectively.

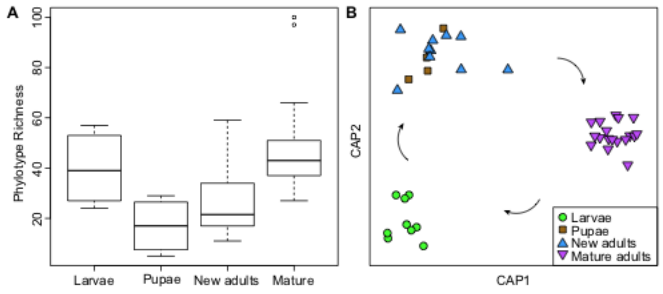

Figure 4.1. a) Boxplot of community phylotype richness. b) Constrained principal coordinates analysis showing variation in community composition over the life cycle. CAP1 and CAP2 are the canonical axes in principal coordinate space that best discriminate among life stages. Arrows indicate significant pairwise differences in composition.

Bacterial community composition also varied across life stages (Fig. 4.1b, $P = 0.001$). In agreement with the pattern shown in the constrained ordination (Fig. 4.1b), all pairwise comparisons were significant at $P < 0.05$ except that between pupae and newly emerged adults. On average, only 13% of the phylotypes present in either the larva or mature adults of each replicate brood were present in both stages.

Communities from frass samples and the individual larvae that produced them were not significantly different in composition (Fig. A4.2, $P = 0.16$). Additionally, variation in community composition among larvae was reflected in their frass (Fig. A4.2, $P < 0.05$, Mantel rho = 0.47).

The four life stages of *H. erato* analyzed here were dominated by six bacterial families: the Acetobacteraceae (Alphaproteobacteria), Moraxellaceae and Enterobacteriaceae (Gammaproteobacteria), Enterococcaceae and Streptococcaceae (Firmicutes), and an unclassified family in the Bacteroidetes phylum (Fig. A4.3). Although family-level bacterial community composition varied substantially between individuals of the same life stage in some cases, all of these families excluding the Enterococcaceae and Enterobacteriaceae shifted significantly in relative abundance across the life cycle (Fig. A4.3, Bonferroni-corrected $P < 0.05$).

The 10 most abundant phylotypes present across all *H. erato* samples are listed in Table 4.1. The split between larval and adult communities appears to be driven by the higher relative abundance of *Acinetobacter* in the larvae and of *Asaia*, *Lactococcus*, and an unclassified Bacteroidetes phylotype in the mature adults. Most of these phylotypes matched at 98-100% identity to named isolates in the RDP database. Two phylotypes had highest similarity to sequences obtained from uncultured bacteria in ground beetle and honeybee digestive tracts.

Taxonomic classification	% Total	Larvae (9)	Pupae (4)	New adults (10)	Mature adults (18)	Parental adults (9)	Wild adults (13)	Best-scoring RDP match	% ID
Firmicutes (Enterococcaceae)	16.04	16.96	67.70	31.40	9.17	3.78	5.72	*Enterococcus*	100
γ-Proteobacteria‡ (Enterobacteriaceae)	15.17	18.07	2.45	37.92	19.96	2.22	1.92	*Enterobacter*	100
γ-Proteobacteria (Enterobacteriaceae)	6.86	9.18	0.10	1.00	7.34	8.51	10.02	*Enterobacter*	100
Bacteroidetes** (Unclassified)	5.36	0.02	0.00	1.12	10.70	8.78	4.22	Unclassified (honeybee gut)	94.7
γ-Proteobacteria† (Orbaceae)	4.83	0.02	0.00	1.68	2.07	4.04	16.46	*Orbus*	100
Firmicutes** (Streptococcaceae)	4.50	0.69	0.00	3.56	7.93	5.73	3.62	*Lactococcus*	100
α-Proteobacteria‡ (Acetobacteraceae)	4.45	0.07	0.05	0.58	0.64	17.02	8.38	*Commensalibacter*	98
γ-Proteobacteria** (Moraxellaceae)	3.91	22.33	8.15	0.94	0.06	0.27	0.02	*Acinetobacter*	100
α-Proteobacteria* (Acetobacteraceae)	2.98	0.00	0.00	0.90	5.66	3.16	3.72	*Asaia*	100
γ-Proteobacteria (Pseudomonadaceae)	2.41	0.00	0.00	0.04	0.00	2.49	9.92	Unclassified (ground beetle gut)	99.3

Table 4.1. The 10 most abundant bacterial phylotypes (by percent of total sequences) across all *H. erato* samples, with phylum (or class for Proteobacteria) and family-level classification. The number of specimens sequenced per sample type is indicated in parentheses. Mean percent relative abundances are shown for each life stage and adult group. The genus-level taxonomic identification of the best match for each phylotype, using the RDP SeqMatch tool, is shown along with its percent sequence identity. If the best match is unclassified, the habitat from which it was sequenced is given. Asterisks indicate significantly different relative abundances across life stages (Bonferroni-corrected $P <$ *0.01, **0.001). The † symbols indicate significantly different relative abundances across adult groups (Bonferroni-corrected $P <$ †0.01, ‡0.001).

Factors structuring adult-associated microbiota

Bacterial phylotype richness did not differ between wild, captive wild-caught (parental), and reared mature adult butterflies ($P = 0.24$), although each group hosted bacterial communities distinct in composition (Fig. 4.2, $P = 0.001$; all pairwise comparisons significant at $P < 0.05$). Despite compositional differences, all adults clustered together to the exclusion of reared larvae (Fig. A4.4).

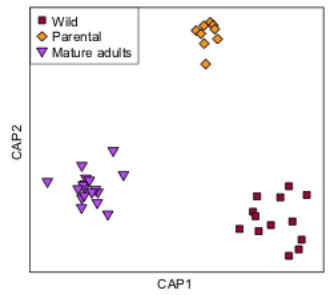

Figure 4.2. Constrained principal coordinates analysis showing differences in community composition between adults sampled directly from the wild, wild-caught females kept in an insectary ("Parental"), and their reared adult offspring ("Mature adults"). CAP1 and CAP2 are the canonical axes in principal coordinate space that best discriminate among adult groups.

Four of the six dominant adult-associated bacterial families differed in relative abundance between the three groups we analyzed (Fig. A4.5, Bonferroni-corrected $P < 0.05$). Specifically, an increase in Streptococcaceae and reduction in an unclassified Gammaproteobacterial family were associated with captivity, whereas an increase in Enterobacteriaceae and reduction in Acetobacteraceae were associated with rearing. Among all adult butterflies, sex did not have an effect on community composition ($P = 0.80$), and there was no interaction between sex and captivity/rearing status ($P = 0.33$). Among the butterfly individuals with known relatedness (i.e., captive females and their mature adult offspring), variation between families was not greater than variation within families ($P = 0.78$).

Discussion

Overall structure of the Heliconius erato microbiota

Heliconius erato larvae and adult butterflies host relatively simple bacterial communities, in agreement with previous reports of low diversity in other lepidopterans (Broderick et al. 2004; Zaspel and Hoy 2008; Robinson et al. 2010) and other insect orders (Colman et al. 2012; Jones et al. 2013) relative to vertebrate-associated and free-living microbiota. The uneven structure of these communities is illustrated by the observation that the 10 most abundant phylotypes contributed more than 65% of the sequences from all *H. erato* samples. The majority of these dominant phylotypes were highly similar to sequences from genera known to colonize the gut of lepidopterans and other insects. The phylotype with the highest abundance across all *H. erato* samples matched most closely to isolates in the genus *Enterococcus*. Enterococci are commonly present in the intestinal tract of lepidopteran larvae (Broderick et al. 2004, 2009; Brinkmann et al. 2008) and other insects (Martin and Mundt 1972), but are also found free-living in a variety of environmental habitats (Fisher and Phillips 2009). Evidence from other lepidopterans that enterococci in the larval gut can persist through metamorphosis (Bucher 1963) is supported by our finding that *Enterococcus* is prevalent in all stages of *H. erato*.

A phylotype matching with 100% sequence identity to an *Orbus* clone in the Orbaceae was also abundant. Although the natural history of this family is not well known, one member has been isolated from a butterfly gut (Kim et al. 2012), and two others are associated with the gut of honeybees (Engel et al. 2013; Kwong and Moran 2013). Another phylotype classified as *Acinetobacter* was variably present across life stages, but at highest relative abundance in the larvae. *Acinetobacter* sequences have been reported from the larval midgut of a number of insect species including cabbage white butterflies (Robinson et al. 2010) and saturniid moths (Pinto-Tomás et al. 2011), although their possible role in host herbivory is not well understood.

Phylotypes belonging to the bacterial family Acetobacteraceae were overrepresented in mature adults relative to earlier stages (Fig. A4.3). Bacteria in this family are commonly associated with the

intestinal tract of insects with sugar-rich diets, such as adult mosquitoes, bees, fruit flies, and sugarcane mealybugs (Crotti et al. 2010). We discovered two dominant Acetobacteraceae phylotypes in *H. erato*, one of which matches to *Asaia sp.*, which in other insects can form biofilms on the midgut epithelium and colonize egg surfaces and reproductive structures (Crotti et al. 2009). As members of the *Drosophila* gut flora, acetic acid bacteria have been shown to prevent colonization by pathogens (Ryu et al. 2008), affect development and insulin signaling (Shin et al. 2011), and influence dietary carbohydrate utilization (Ridley et al. 2012). Such bacteria are likely to be broadly associated with nectar- and fruit-feeding adult butterflies, in which they may have similar functions, and their role in the biology of *Heliconius* clearly warrants further investigation.

Another dominant phylotype in the adult stage, a member of the Bacteroidetes phylum, appears to be only distantly related to taxa reported from insects or other habitats. Interestingly, its closest match was to a clone from honeybee intestines (Babendreier et al. 2007). We do not know if this phylotype is uniquely associated with *Heliconius*, but given that a similar bacterium has been found in honeybees, which also feed on pollen, it is possible that this taxon is involved in *Heliconius* pollen feeding. For example, certain honeybee gut bacteria can produce enzymes that degrade pectin, a major structural component of pollen walls (Engel et al. 2012). In *Heliconius*, which digest pollen grains attached to the proboscis using exuded saliva (Eberhard et al. 2009), symbionts with similar functions could reside in the salivary gland.

Because we sampled the entire internal portion of the insect, the exact location of these taxa within the host is unknown. Bacteria could reside in other structures besides the gut, such as reproductive organs and the salivary gland. However, the observation that frass samples were not different in composition from the whole larvae that produced them indicates that, for the larval stage at least, we have primarily sequenced gut bacteria. Likewise, previous studies have found that

communities from whole homogenized insects can closely resemble those sampled from the gut alone (Sabree et al. 2012; Sudakaran et al. 2012).

Effects of captivity and rearing on adult butterfly microbiota

Studies of microbial symbionts in Lepidoptera and other insects commonly use hosts reared in the laboratory where they are often maintained for multiple generations on artificial diets. We found that *H. erato* butterflies sampled directly from the wild were different in bacterial community composition from individuals from the same population housed in an insectary for 2-4 weeks. Although the reasons for this microbial community shift remain unknown, altered adult diet—specifically, access to artificial sucrose/pollen solution, and the absence of certain flowers normally visited by *H. erato* in the wild—could underlie this difference, as could altered exposure to microbial inocula from their environment.

Reared four-day-old adult offspring were also different in composition from their wild-caught mothers, despite being maintained under identical conditions in the insectary. Although the average age of the wild-caught group is unknown, a difference in adult age could be partly responsible for these differences. As the wild-caught mothers spent all of the larval stage and some period of the adult stage in the wild prior to capture, there could be additional effects of diet and exposure to microbial inocula in both stages.

Generally, these results support previous findings of captive-wild differences in insect-associated microbial communities (Xiang et al. 2006; Lehman et al. 2009; Belda et al. 2011; Chandler et al. 2011) and they suggest that caution should be taken when inferring evolutionary history or ecological function from microbiota associated with captive insects without an explicit comparison to wild populations. Altered bacterial community composition in captive individuals may also affect host nutrition, detoxification, and defense from natural enemies, as these traits can be mediated by

microbial symbionts. The use of captive experimental subjects may consequently render studies of these phenomena less relevant to natural conditions. Although not tested here, these changes in the microbiota could partly account for the observations that reared *Heliconius* butterflies exhibit lower success in courtship and pollen collection compared with wild-caught individuals (Brown 1981).

Community dynamics across metamorphosis

Bacterial diversity dropped by approximately 50% from the larval to the pupal stage, remained low in the newly emerged adults, and redoubled in the mature adults after feeding. Likewise, bacterial communities changed in composition from the larval to the pupal stage, remained similar in the newly emerged adults, and changed again in the mature adults. Thus, butterfly-associated bacterial communities appear to both simplify and reorganize over metamorphosis, a pattern that can be explained by multiple possible mechanisms. The reduction in richness during metamorphosis could be due to larval voiding of the gut prior to pupation (Nijhout and Williams 1974) and/or secretion of antibacterial proteins into the pupal gut lumen (Russell and Dunn 1996), both of which could selectively eliminate or reduce the abundance of gut-associated bacteria. Degeneration of the larval gut and its contents, in tandem with the development of a morphologically distinct adult gut (Judy and Gilbert 1969; Hakim et al. 2010; Lowe et al. 2013) and new structures such as the adult salivary gland and reproductive organs, could also facilitate the successional patterns observed here. After adult emergence, feeding by the host might stimulate the growth of bacteria persisting through the pupal stage, or add new taxa sourced from the diet, restoring community richness—though not composition—to pre-metamorphosis levels.

Differences in diet presumably drive the remarkable difference in bacterial community composition between *H. erato* larvae and adults (Figs. 4.1b and A4.4). Diet could directly impact life-stage-specific microbiota as an inoculum, as a resource supporting the differential growth of resident

bacteria, and as a source of chemical compounds with selective antimicrobial activity. Diet may also directly affect the environmental conditions within the host—for example, by inducing gut pH changes (Schultz and Lechowicz 1986; Appel and Maines 1995). Additionally, diet could indirectly impact the microbiota through the morphological and biochemical adaptations hosts have evolved to utilize different resources in different life stages (here, foliage versus nectar and pollen).

Impact of holometaboly on insect microbiota

The spectacular success of the Holometabola, of which the Lepidoptera are one of the most diverse groups (Kristensen 1999), has been attributed to the differentiation in form and function between larvae and adults (Hennig 1981). This divergence enables specialization on different diets in the larval and adult stages and reduces competition between immature and mature conspecifics for resources (Whiting 2003; Grimaldi and Engel 2005). We propose that the evolutionary innovation of holometaboly also created distinct niches for colonization by distinct microbial symbionts. Over the holometabolous host life cycle, variation in diet and internal physicochemical conditions could support communities functionally specialized for a particular life stage. It remains to be determined whether holometabolous species—especially those whose adults feed, and on diets distinct from the larvae—are thus associated with more diverse microbial symbiont communities than other insects.

Conclusions

We have identified a relatively simple bacterial community associated with *H. erato* that differs in composition between larvae and adults. This difference in taxonomic membership may reflect divergent functional roles in life-stage-specific resource use. These results will be valuable in designing genomic studies and experimental manipulations to test how *Heliconius*-associated bacteria may be involved in their host's distinctive feeding biology. Additionally, the overall compositional similarity

between frass and whole larvae, as well as the finding that community differences among larvae are maintained in their frass, indicate that frass could be used in the future as a way to sample the larval gut microbiota nondestructively. As with temporal surveys of the human gut (Costello et al. 2009), this would allow an analysis of gut communities from the same individual over larval development and into the adult stage.

Furthermore, we found that both captivity and rearing are associated with a compositional change in the microbiota from wild *H. erato* individuals of the same population. This change could be partly responsible for observed differences in performance between wild-caught and captive butterflies, and has implications not only for future studies of butterfly symbionts, but also for other kinds of studies on captive butterflies where microbial differences may influence experimental results.

We have demonstrated that the internal bacterial community of *H. erato* simplifies and reorganizes across host development. Presumably, different life stages represent habitats that selectively favor the growth of certain bacterial taxa. This ability of the microbiota to undergo a structural “metamorphosis,” in tandem with its host, might entail an overall greater diversity in microbial community form and function within a given holometabolous species relative to other insect groups.

CHAPTER V

CATERPILLARS LACK A RESIDENT GUT MICROBIOME

Hammer, T.J., Janzen, D.H., Hallwachs, W., Jaffe, S.P., & Fierer, N. (2017). Caterpillars lack a resident gut microbiome. *Proc. Natl. Acad. Sci. U.S.A.*)

Abstract

Many animals are inhabited by microbial symbionts that influence their hosts' development, physiology, ecological interactions, and evolutionary diversification. However, firm evidence for the existence and functional importance of resident microbiomes in larval Lepidoptera (caterpillars) is lacking, despite the fact that these insects are enormously diverse, major agricultural pests, and dominant herbivores in many ecosystems. Using 16S rRNA gene sequencing and quantitative PCR, we characterized the gut microbiomes of wild leaf-feeding caterpillars in the United States and Costa Rica, representing 124 species from 15 families. Compared with other insects and vertebrates assayed using the same methods, the microbes we detected in caterpillar guts were unusually low-density and variable among individuals. Furthermore, the abundance and composition of leaf-associated microbes were reflected in the feces of caterpillars consuming the same plants. Thus, microbes ingested with food are present (though possibly dead or dormant) in the caterpillar gut, but host-specific, resident symbionts are largely absent. To test whether transient microbes might still contribute to feeding and development, we conducted an experiment on field-collected caterpillars of the model species *Manduca sexta*. Antibiotic suppression of gut bacterial activity did not significantly affect caterpillar weight gain, development, or survival. The high pH, simple gut structure, and fast transit times that typify caterpillar digestive physiology may prevent microbial colonization. Moreover, host-encoded digestive and detoxification mechanisms likely render microbes unnecessary for caterpillar herbivory. Caterpillars

illustrate the potential ecological and evolutionary benefits of independence from symbionts, a lifestyle which may be widespread among animals.

Introduction

Many animals are colonized by microbial symbionts that have beneficial and fundamentally important impacts on host biology. Microbes can regulate animal development, immunity and metabolism, mediate ecological interactions, and facilitate the evolutionary origin and diversification of animal clades (Moran 2002; Janson et al. 2008; Frago et al. 2012; Sommer and Bäckhed 2013; McFall-Ngai et al. 2013; Douglas 2014; Sudakaran et al. 2017). These integral host-microbe relationships have led to a conceptualization of animals as "holobionts" (Zilber-Rosenberg and Rosenberg 2008; Gilbert et al. 2012; Bordenstein and Theis 2015), superorganism-like entities composed of the host plus its microbiome—defined here as the entire assemblage of commensal, pathogenic, and mutualistic microorganisms (Lederberg and McCray 2001). Furthermore, the recent proliferation of microbiome surveys supports a widely held assumption that microbial symbioses are universal across animals (Russell et al. 2014; Vavre and Kremer 2014).

The Lepidoptera (butterflies, moths, and their caterpillar larvae), despite being key components of most terrestrial foodwebs and extraordinarily diverse (Scoble 1992), are one group in which the role of microbes remains ambiguous. Here we focus on caterpillars, which are the main—and in some Lepidoptera, the exclusive—feeding stage, and which have long been intensively studied in many fields (Stamp and Casey 1993). The vast majority of caterpillars are herbivores, and some herbivores rely on microbes to supplement nutrients, neutralize toxins, or digest plant cell walls (Douglas 2009; Hammer and Bowers 2015). However, considering caterpillars' simple gut morphology and rapid digestive throughput, it has been speculated that microbes cannot persist in the caterpillar gut and do not contribute to digestion (Bernays and Janzen 1988; Appel 1994). Indeed, microscopy-based studies

report no, or minimal, microbial growth in caterpillar guts (Kukal and Dawson 1989; Shannon et al. 2001; Vilanova et al. 2016).

DNA- and culture-based investigations of caterpillar gut microbiomes have produced mixed findings, with conflicting implications for microbial involvement in caterpillar biology. Some studies report a highly abundant and consistent bacterial community (Kingsley 1972; Broderick et al. 2004; Anand et al. 2010), characteristics that may indicate a functional association with the host. Others report high intraspecific variability in composition, and similarity between diet- and gut-associated microbes (Priya et al. 2012; Mason and Raffa 2014; Staudacher et al. 2016; Whitaker et al. 2016). Inconsistencies could arise from methodological factors such as contamination of low-biomass samples (Salter et al. 2014), starvation prior to sampling, sequencing of extracellular DNA, and the use of laboratory-raised insects or artificial diets (Lighthart 1988; Hammer et al. 2014; Staudacher et al. 2016). As an additional complication, many microbiome surveys do not distinguish between dead or dormant passengers ("transients" (Berg 1996)) and persistent, living populations ("residents" (Berg 1996) or "symbionts" *sensu* (Douglas 1994)). Further, microbes in the latter category may be parasitic or pathogenic, as well as beneficial. While microbes were known to cause disease in caterpillars as early as Louis Pasteur's experiments on silkworms (Steinhaus 1956), their potential importance as mutualists remains unclear.

Do caterpillars depend on gut microbes for feeding and development? To answer this question, we first characterized gut microbial abundance and composition across a taxonomically and geographically broad array of wild caterpillars (Fig. A5.1). Our analyses are focused on the digestive tract, the most likely habitat for microbial colonization, as abundant microbes have not been observed elsewhere in the caterpillar body (Bucher 1967; Hammer et al. 2014). We applied the same methods to 24 additional insect, bird, and mammal species that we expected to have functional microbiomes, to assess the reliability of our protocol and to contextualize our findings. We then conducted a field-based

experiment testing whether gut bacteria impact larval growth and survival of the model species *Manduca sexta* (Sphingidae). Our findings question the generality of animal-microbe symbioses, and may inform a multitude of research programs based on caterpillar herbivory in both natural and managed ecosystems (e.g., (Ehrlich and Raven 1964; Pearse and Altermatt 2013; I-M-Arnold et al. 2016)).

Results

Using quantitative PCR and sequencing of the 16S rRNA gene, we found that wild caterpillars representing a broad diversity of Lepidoptera had gut bacterial densities multiple orders of magnitude lower than the microbiomes of other insects and vertebrate feces measured using identical methods (one-way ANOVA, $F_{1,145} = 228.2$, $p < 0.0001$, Fig. 5.1a). Some animals host symbiotic fungi (Gibson and Hunter 2010), but fungal biomass was also lower in caterpillar guts relative to other insects and vertebrates (median 6.1×10^2 vs. 9.5×10^4 rRNA gene copies per gram, respectively; one-way ANOVA, $F_{1,145} = 36.03$, $p < 0.0001$). While mitochondrial rRNA genes from fungi and other eukaryotes (as well as chloroplasts) are detectable using primers designed for bacteria and archaea (Rastogi et al. 2010), more targeted characterization of fungi in caterpillar guts is warranted. As another indicator of low microbial biomass, in most caterpillars over 80% of fecal 16S rRNA gene sequences were from plant chloroplasts or mitochondria, versus ~0.1% for other herbivores or omnivores with plant-rich diets (Wilcoxon rank-sum test, $p < 0.0001$, Fig. 5.1b). In a subset of caterpillars from which we sampled whole, homogenized midgut and hindgut tissue, plant DNA represented an even higher proportion of sequences in guts than in feces (Fig. A5.2a). This pattern is more likely a function of plant DNA degradation during intestinal transit than of bacterial proliferation, as bacterial density remained similar or decreased slightly from midgut to feces, depending on the caterpillar species (Fig. A5.2b).

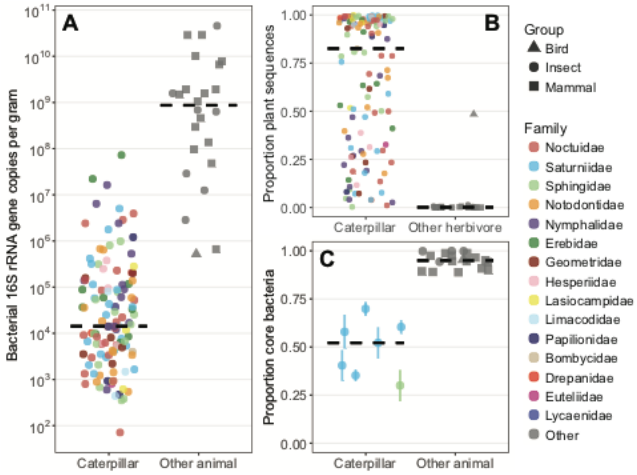

Figure 5.1. Comparisons of bacterial density, relative abundance of plant DNA, and intraspecific variability between caterpillars and other animals expected to host functional microbiomes. Medians are indicated by black dashed lines, and points are horizontally jittered. One caterpillar species yielding <100 total sequences was excluded. For species with multiple replicates, the median is plotted. a) The density of bacterial 16S rRNA gene copies in caterpillar feces versus fecal (vertebrates) or whole-body homogenate (other insect) samples of other animals (N=121 caterpillar species, 24 other species). Two caterpillar species with lower amplification than DNA extraction blanks are not shown. b) The proportion of sequence libraries assigned to plant chloroplast or mitochondrial rRNA (N=123 caterpillars, 21 other herbivores). c) The proportion of bacterial sequences belonging to core phylotypes, defined for each species as those present in the majority of conspecific individuals analyzed. Included are species with at least three replicates with >100 bacterial sequences each (N=7 caterpillars, 19 other animals). For species with more than three, points show the median core size across all combinations of three individuals, and error bars show the interquartile range.

Animals with functionally important, resident microbiomes tend to host a high abundance of microbial taxa shared among conspecific individuals (e.g., (Moran et al. 2012; Sudakaran et al. 2012; Falony et al. 2016)). Indeed, within species of the other insects and vertebrates analyzed here, microbiomes were largely made up of a common set of bacterial phylotypes (Fig. 5.1c). For example, >99% of sequences in any one honeybee belonged to phylotypes found in the majority of honeybees included in the analysis. In contrast, even when consuming the same species of food plant under similar conditions, caterpillars had a much lower proportion of their gut bacterial assemblage belonging to core phylotypes (one-way ANOVA, $F_{1,24} = 165.3$, $p < 0.0001$, Fig. 5.1c). In *Schausiella santarosensis*, which among the seven caterpillar species (mostly Saturniidae) examined had the highest median core size of ~70%, four of its six core phylotypes were *Methylobacterium*, a typical inhabitant of leaf surfaces

(Vorholt 2012). This observation hints that many of the core taxa found in caterpillar guts may be transient, food-derived microbes.

Caterpillar gut microbiomes are dominated by leaf-associated bacteria, further suggesting that resident, host-specific symbionts are sparse or absent. The bacterial phylotypes present in the feces of at least half of the sampled individuals are *Staphylococcus*, *Escherichia, Methylobacterium, Klebsiella*, *Enterococcus*, and *Sphingomonas* (Table A5.1). Of these, all but *Staphylococcus*—a potential caterpillar pathogen (Haloi et al. 2016) or a transient from human skin (Grice et al. 2009)—are also among the ten most common phylotypes found in paired leaf samples. Across caterpillars, a median 89.6% (interquartile range: 80.2-99.0%) of fecal bacterial sequences belonged to leaf-associated phylotypes. However, bacterial assemblages were not identical between leaves and caterpillar feces (PERMANOVA, pseudo-$F_{1,196}$ = 12.54, R^2 = 0.06, p = 0.001). Besides the potential growth of parasites and/or mutualists in the gut, this difference could arise from digestion filtering out subsets of the leaf microbiome.

Low precision may partly explain the extensive variation in caterpillar gut bacterial loads (Fig. 5.1a), as these estimates are a product of bacterial sequence composition and total 16S rRNA gene counts (Appendix A5 Methods), both of which contain measurement error. However, transient inputs of leaf microbes also generate variation among caterpillar species and individuals. Leaf bacterial densities differed greatly within (tomato) and between (milkweed, eggplant, tomato) plant species, and these differences were reflected in the feces of monarch (*Danaus plexippus*) and *M. sexta* caterpillars feeding on them (linear regression, R^2 = 0.24, p = 0.031; Fig. 5.2a). Furthermore, bacterial densities dropped by a median of 214-fold from leaves to feces (Fig. 5.2a), suggesting that any potential bacterial growth within the gut is relatively minor. The extent of this reduction varied widely (from 5 to 8385-fold, Fig. 5.2a), possibly because of inter-individual or interspecific differences in physiological traits that eliminate leaf microbes, such as gut pH. Variation in bacterial taxonomic composition among leaves and caterpillar feces was also correlated (Mantel test, r = 0.28, p = 0.001; Fig. 5.2b). In other words, caterpillars

consuming leaves with more distinct bacterial assemblages have more distinct bacterial assemblages in their feces, as would be expected if gut microbes are diet-derived and only transiently present. Moreover, this process can explain a relationship between host relatedness and microbiome structure, a pattern sometimes termed "phylosymbiosis" (Brooks et al. 2016). Specifically, although confamilial caterpillars in Costa Rica had marginally more similar gut bacterial assemblages than did caterpillars in different families (PERMANOVA, pseudo-$F_{6,43}$ = 1.47, p = 0.053), they had also been feeding on plants with especially similar leaf microbiomes (PERMANOVA, pseudo-$F_{6,42}$ = 1.73, p = 0.005).

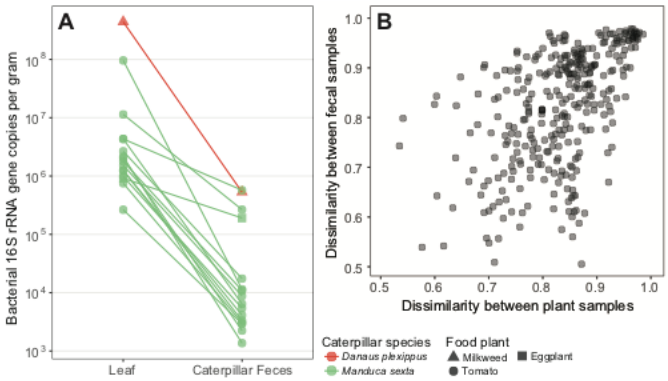

Figure 5.2. The abundance and composition of caterpillar fecal bacteria as compared with paired diet (leaf) samples. a) The density of bacterial 16S rRNA gene copies in ground leaves versus feces, for 16 caterpillars collected in Colorado. Parallel lines indicate an association between plant and fecal bacterial abundances across pairs. b) The correlation between beta diversity (Bray-Curtis dissimilarity) across caterpillar fecal samples collected in Costa Rica, and paired leaf surface samples (N=24 caterpillar species, 19 plant species; 26 individuals each). Here only samples with >2,000 sequences are shown, to facilitate visualization.

Supporting our claim that caterpillars lack resident gut microbiomes, we show experimentally that the growth and survival of field-collected *Manduca sexta* caterpillars are not dependent on gut bacterial activity. As measured by qPCR, wild *M. sexta* contain ~61,000-fold lower bacterial loads than expected from allometric scaling relationships based on animals with resident microbiomes ((Kieft and Simmons 2015) and Fig. A5.3). Feeding *M. sexta* antibiotics reduced this already low number of gut bacteria by 14- to 365-fold (range of medians across dosages), as measured using culture-dependent methods (linear regression, R^2 = 0.13, p = 0.003; Fig. A5.4a). Bacterial colony counts were correlated

with the number of 16S rRNA gene copies (Pearson correlation, $r = 0.38$, $p = 0.003$; Fig. A5.4b). Suppression of viable bacteria had no effect on pupal weight (linear regression, antibiotics: $p = 0.45$; sex: $p = 0.014$; interaction: $p = 0.70$; Fig. 5.3), which is predictive of fecundity in insects (Honek 1993), nor on development time (linear regression, antibiotics: $p = 0.19$; sex: $p = 0.023$; interaction: $p = 0.63$; Fig. A5.5a). Likewise, antibiotic treatment did not affect survival from larval hatching to adult emergence (logistic regression, 95% CI of odds ratio = 0.76-9.39, $p = 0.19$; Fig. A5.5b), nor generally impact total feces production, a metric integrating leaf consumption and assimilation efficiency (linear regression, antibiotics: $p = 0.07$; sex: $p = 0.002$; interaction: $p = 0.048$). As expected with *M. sexta* (Stillwell and Davidowitz 2010) we found clear sexual size dimorphism, suggesting our experimental design had sufficient power to detect biologically meaningful differences. Given that antibiotics reduced fecal bacteria to a variable extent within and among treatments (Fig. A5.4a), we repeated the aforementioned analyses using gut bacterial abundance as the predictor variable. In all cases there was no significant relationship with host performance ($p > 0.1$), further indicating that reducing or eliminating gut bacteria from caterpillars does not reduce *M. sexta* fitness.

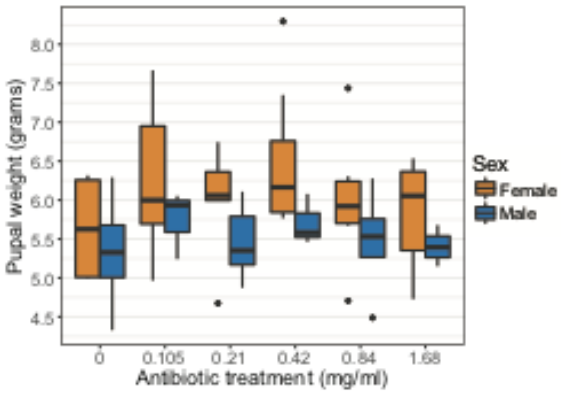

Figure 5.3. The abundance and composition of caterpillar fecal bacteria as compared with paired diet (leaf) samples. a) The density of bacterial 16S rRNA gene copies in ground leaves versus feces, for 16 caterpillars collected in Colorado. Parallel lines indicate an association between plant and fecal bacterial abundances across pairs. b) The correlation between beta diversity (Bray-Curtis dissimilarity) across caterpillar fecal samples collected in Costa Rica, and paired leaf surface samples (N=24 caterpillar species, 19 plant species; 26 individuals each). Here only samples with >2,000 sequences are shown, to facilitate visualization.

Discussion

Consistent with previous microscopy-based (Kukal and Dawson 1989; Shannon et al. 2001; Vilanova et al. 2016) and molecular studies (Priya et al. 2012; Mason and Raffa 2014; Staudacher et al. 2016; Whitaker et al. 2016), we found that microbial symbionts are generally absent or present only in low numbers in caterpillar guts. As expected for herbivores consuming microbe-rich leaf tissue, diet-derived microbes are transiently present in caterpillar guts, wherein they may be dead or inactive. That the microbial biomass in caterpillar guts is far lower than in the guts or whole bodies of many other animals (Fig. 5.1a), and also lower than in leaves (Fig. 5.2a), suggests a lack of persistent microbial growth. Moreover, any potential microbial metabolism might be too limited to substantially affect digestive processes, as illustrated by our observation that *Manduca sexta* caterpillars contain microbial loads orders of magnitude lower than comparably sized animals with resident microbiomes (Fig. A5.3). Caterpillar gut microbiomes also exhibit high inter- and intraspecific variability in both abundance and composition (Fig. 5.1, Fig. 5.2). Lacking resident populations, they may be easily influenced by the idiosyncrasies of which microbes are present on a given leaf and in what abundance, and which can survive transit through the digestive tract. Ingested microbes which die within the host could still be beneficial as food or by stimulating the immune system, but are not themselves symbionts (following the original definition of symbiosis as the “living together of different species” (referenced in (Douglas 1994)).

In tandem with the transient nature of gut microbiomes across caterpillar species, the experiment on *M. sexta* suggests that microbes are unlikely to have cryptic, but essential, functions in caterpillar guts. Antibiotic suppression of viable gut bacteria had no apparent negative consequences for *M. sexta*, contrasting sharply with the many examples of major reductions in host growth or survival upon removal of beneficial symbionts (e.g., (Salem et al. 2012; Ceja-Navarro et al. 2015; Raymann et al. 2017)). If anything, caterpillars treated with antibiotics showed slight (but not statistically significant)

increases in performance (Fig. 5.3, Fig. A5.5b). Antibiotics increase weight gain of laboratory-bred caterpillars (Murthy and Sreenivasaya 1953; van der Hoeven et al. 2008; Visôtto et al. 2009), and commercially made caterpillar diets often contain antibiotics. This effect might reflect microbial parasitism occurring in even apparently healthy caterpillars, or costly immune responses to the presence of pathogens (Rolff and Siva-Jothy 2003). Aside from known leaf specialists, many of the most frequently detected bacterial genera in this study (Table A5.1) have been reported to cause disease in caterpillars (Bucher 1967; Bulla et al. 1975; Peleg et al. 2009; Haloi et al. 2016). Additionally, even normally transient gut microbes can negatively affect caterpillars under certain circumstances, such as after ingestion of insecticidal toxins (Broderick et al. 2009), and thus may be important to understanding caterpillar herbivory and to pest management.

The lack of a resident gut microbiome in caterpillars may directly result from a digestive physiology that is unfavorable to microbial growth (Appel 1994). The midgut, the largest section of the digestive tract wherein caterpillars digest leaf material and absorb the resulting nutrients (Dow 1986), is a particularly hostile environment for microbes (Broderick et al. 2004). It is highly alkaline, with pH values often >10 (Johnson and Felton 1996) and as high as 12 (Dow 1984), and contains host-encoded antimicrobial peptides (Jiang et al. 2010). Additional attributes of the caterpillar gut that may hinder microbial colonization include a simple tube-like morphology without obvious microbe-housing structures (Appel 1994), a continually replaced lining (the peritrophic matrix) covering the midgut epithelium (Hegedus et al. 2009), and short retention times (food transit takes ~2 hours in *M. sexta* (Brinkmann and Tebbe 2007)). Although some insects harbor symbionts in specialized organs (Buchner 1965), to our knowledge, similar structures have not been reported in caterpillars. Buchner's foundational survey of animal endosymbiosis describes Lepidoptera only as "a group in which no symbiont bearers have been discovered" ((Buchner 1965), p. 817). Moreover, previous studies did not find microbes that were abundant outside of the gut (Bucher 1967; Hammer et al. 2014), though in

infected populations the reproductive parasite *Wolbachia* may inhabit other larval tissues (Narita et al. 2007).

Without the aid of microbial symbionts, how are caterpillars able to overcome the dietary challenges posed by herbivory? Caterpillars use a combination of mechanical disruption, endogenously produced digestive enzymes, and high pH to extract easily solubilized nutrients, primarily from the contents of plant cells (Santos et al. 1983; Barbehenn 1992; Appel 1994). Although this method of processing leaves is relatively inefficient, essential nutrients are not totally absent, so that caterpillars can compensate by simply eating more (Dow 1986; Appel 1994). Some insects likely require microbes for detoxification (Hammer and Bowers 2015), but many caterpillars possess host-encoded mechanisms for degrading or resisting plant allelochemicals (Després et al. 2007). However, there may be a vestigial role for microbes in these processes, as genomes of many Lepidoptera contain microbial genes encoding enzymes with related functions (Sun et al. 2013; Wybouw et al. 2014). These gene acquisitions may have enabled a symbiont-free feeding strategy.

The caterpillars surveyed here are likely to be representative of most externally leaf-feeding Lepidoptera, as we included a range of diet breadths, from monophagous to highly generalist, and many of the most diverse families (Fig. A5.1). However, a lack of resident gut microbiome in the caterpillar may not apply to the adult butterfly or moth. For example, adult honeybees have abundant gut microbes, while the larvae do not (Martinson et al. 2012). As compared with caterpillars, adult butterflies host distinct bacterial communities (Hammer et al. 2014) and high gut microbial loads (Ravenscraft et al. 2017). On the other hand, unlike honeybees or butterflies, many Lepidoptera do not feed as adults, and in these groups microbes may be altogether irrelevant to digestion or nutrition. However, we cannot exclude the possibility that microbial symbionts influence host fitness by their potential activities in eggs or pupae.

The extraordinary diversity and abundance of Lepidoptera (Scoble 1992) indicates that a symbiont-independent feeding strategy can be highly successful. Perhaps such success reflects a release from constraints imposed on other animals that do host and depend on symbionts. There are costs to engaging in mutualisms (e.g., (Frederickson et al. 2012; Nougué et al. 2015; Simonsen et al. 2017)), and in a gut microbiome context one cost includes nutrient competition between host and microbes (Gaskins et al. 2002). A high availability of food allows caterpillars to "skim the cream" (Dow 1986), assimilating simple nutrients that might otherwise be used by gut microbes and excreting recalcitrant material. In other words, "Why not do the digestion yourself rather than pay someone else to do it?" ((Janzen 1985), p. 53). Another cost is the risk of gut microbes becoming pathogenic (Broderick et al. 2009; Young et al. 2017) or of foodborne pathogens exploiting a gut environment that is hospitable to mutualists. The extreme conditions in the caterpillar midgut may lower these risks by limiting the growth of both pathogens and potential mutualists.

Dependence on microbes with different physiological tolerances than the host constrains overall niche breadth (Moran 2002; Nougué et al. 2015). As compared with groups lacking functional microbiomes, animals whose biology is heavily influenced by microbial mutualists may be less able to switch to new food plants or new habitats over evolutionary time. Indeed, it has been argued that while microbial symbioses can provide novel ecological functions, they may also increase the extinction risk of host lineages (Moran 2002; Bennett and Moran 2015). As Lepidoptera represent one of the most species-rich animal radiations (Wiens et al. 2015), a conspicuous question is whether independence from microbes may, in some cases, facilitate host diversification.

Caterpillars do not appear to be unique in lacking a resident microbiome that is important for feeding and development. Microbiomes of walking sticks (Shelomi et al. 2013), sawfly larvae (Whittome et al. 2007; Lucarotti et al. 2011), a saprophagous fly (Šustr et al. 2014), a parasitic horsehair worm (Hudson and Floate 2009), a leaf beetle (Taylor 1985; Hammer et al. 2015), and certain ants (Sanders et

al. 2017) display features similar to those we observed in caterpillars. Our data suggest that some vertebrates also have minimal gut microbiomes, and these species may feed relatively autonomously. Feces of the herbivorous goose *Branta bernicla* had low bacterial loads and a high proportion of plant DNA, and the insectivorous bat *Myotis lucifugus* had similarly low fecal bacterial loads (Fig. 5.1a, Fig. 5.1b). These species exhibit caterpillar-like physiological traits such as a short gut and rapid digestive transit (Buchler 1975; Buchsbaum et al. 1986). Additional examples in the literature might be obscured by contaminants masquerading as mutualists (Lauder et al. 2016), a frequent absence of quantitative information (Sanders et al. 2017) and experimental validation of microbial function *in vivo*, and publication bias against "negative results."

While recent literature has documented extraordinary variation in the types of services provided by microbial symbionts, less explored is variation in the degree to which animals require any such services. Animals likely exist on a spectrum from tightly integrated host-microbe holobionts to simply animals, *sensu stricto*, in which a microbial presence is only relictual (i.e. mitochondria and horizontally transferred genes). Documenting the existence of microbially independent animals, as well as their ecological, physiological and phylogenetic contexts, is a first step toward understanding the causes and consequences of evolutionary transitions along this continuum.

Materials and Methods

Sampling and Sequencing. Caterpillar fecal samples (N=185) were obtained from actively feeding, field-collected individuals in AZ, CO, MA, and NH, USA, and Área de Conservación Guanacaste, Costa Rica. To sample plant microbiomes, we collected leaves from the same branch used to feed caterpillars prior to fecal or gut sampling. All species were identified by morphology. Non-caterpillar animals were included if microbiome samples were available in our laboratory or were readily collectable during caterpillar sampling. We extracted DNA, PCR-amplified the 16S rRNA V4 gene region and sequenced amplicons on

an Illumina MiSeq in the same manner as previous insect microbiome studies (Hammer et al. 2014, 2015). These DNA extracts and primers were also used for quantitative PCR, which provides microbial biomass estimates concordant with those from microscopy (Sanders et al. 2017) and culturing (A5.4b). We did not find evidence that low amplification of caterpillar fecal bacteria is due to primer bias, PCR inhibitors, or storage methods (Appendix A5 Methods).

Antibiotic Experiment. We collected *Manduca sexta* eggs from *Datura wrightii* plants near Portal, AZ, USA. 72 newly hatched larvae were randomly and evenly divided among six treatments varying from 0-1.68 mg total antibiotics per ml distilled water, and reared in separate unused plastic bags on *D. wrightii* foliage. Water with or without antibiotics was sprayed onto leaves, which were then briefly dried prior to feeding. The compounds used here (rifampicin, tetracycline, streptomycin, in a 1:2:4 ratio) have been shown to suppress bacterial symbionts in other insect herbivores (Wilkinson 1998; Ceja-Navarro et al. 2015). More detail is provided in Appendix A5 Methods.

Data Analysis. Statistical analyses were conducted in R (R Core Team 2016). Differences in microbial loads, core sizes, and *M. sexta* performance were tested using linear models. *M. sexta* survival was analyzed using logistic regression. We used a Mantel test to estimate the rank correlation between leaf and fecal microbiome dissimilarities. A Wilcoxon rank-sum test was used for proportions of plant DNA. Differences in community composition were analyzed using PERMANOVA. DNA sequences, metadata, and R code available at https://doi.org/10.6084/m9.figshare.4955648.

CHAPTER VI

CONCLUSIONS

My dissertation research has provided a new perspective on interactions between microbial communities and Lepidoptera, one of the most diverse and ecologically important insect orders. First, my finding that caterpillars do not rely on microbes for feeding and development sheds light on the biology of Lepidoptera, and also opens new research directions into the causes and consequences of variation in microbial dependence. Future work combining microbiome data with host phylogenetic, ecological, physiological, and life history information across a diverse suite of animals could help reconstruct the timing and potential causes of asymbiosis. Second, my research on the butterfly *Heliconius erato* suggests that the way in which Lepidoptera interact with microbes may be dramatically different in the adult stage. This decoupling of larval and adult microbiomes is likely enabled by metamorphosis, prompting the question of whether holometabolous insects exhibit distinct forms of microbial symbioses as compared with other animals. Ongoing and future research based on my work with *H. erato* will address the structure of adult gut microbiomes across multiple genera of passion-vine butterflies (Heliconiini), and their potential functional roles in butterfly nutrition and pollination. This highly diverse butterfly clade may prove to be a valuable model system for the study of gut microbiome evolution and plant-microbe-insect interactions. Finally, the discovery that antibiotics can have unintended, far-reaching ecological effects in the environment has implications for both basic science and management. This study has also provided a glimpse into the many ways in which we are altering the microbes with which we are surrounded, with often surprising—and negative—consequences. As environmental issues continue to mount, microbiome research will become an even more important tool for ecosystem management and conservation.

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APPENDIX

CHAPTER III APPENDIX

Appendix A3 Methods

Experimental setup

On June 7th 2014, ten cows were randomly assigned to two treatments: five were given a standard three-day course of tetracycline (Engemycin LA vet 100 mg/ml, manufacturer Intervet International B.V., dosage 10 mg/kg by intravenous injection administered once daily, "AB+") and five were left as controls ("AB-"). In the control group, only one cow had received antibiotic therapy in its lifetime, whereas the others had never been treated with antibiotics. Eleven months prior to the experiment, this cow had received "dry cow therapy" (DCT), a mixture of penethamate hydroiodide, benethamine penicillin, and framycetin administered locally as a single, low-level dose to the udder, with low absorption. In the antibiotic group, three of the cows had previously been treated with antibiotics: one with a course of penicillin six months prior to the experiment, the second with penicillin six weeks prior and DCT 11 months prior, and the third with two courses of penicillin and two DCT three years prior. A permit for medicating healthy cows was obtained from the Animal Experiment Board in Finland (ELLA) in accordance with the Finnish Act on Animal Experimentation (6/2/2006). The ten cows ranged in weight from 580 to 810 kg and were housed in a shed at the Viikki Experimental Farm at the University of Helsinki and grazed outdoors daily.

From each cow, we collected 7-10 kg of dung rectally into a clean 20 L tub. To minimize variation, we collected all dung within a single day (June 9, 2014), between 10 AM and 2 PM, beginning one hour after the last administration of antibiotics. That afternoon, we started the experiment by first homogenizing and separating the dung from each cow into six 1 L pats using a scoop replaced between cows. Each pat was placed in a mesocosm under semi-natural field conditions. These mesocosms were constructed from open-bottom plastic buckets (58 cm diameter, height 32cm) that had been dug into the soil in May 2014. To keep the grass at a length typical of pastures, it was cut inside and outside the

mesocosms. We also included four control mesocosms without dung, which were used to measure background fluxes of carbon dioxide (CO2), methane (CH4), and nitrous oxide (N2O) from the soil.

To examine the effects of antibiotics on dung beetle microbiota, on the performance of beetles, and on beetle-mediated effects on gas fluxes from cow pats, we focused on the dung beetle *Aphodius fossor* (L.). *A. fossor* is a regionally widespread and locally common species (Roslin 2001), and its ecology and interactions with dung have been extensively studied (e.g., Vessby 2001, Penttilä et al. 2013, Slade et al. 2015). The beetles were collected in the field in early June and stored in moist paper at 4°C until they were added to dung pats. Beetles from different localities were randomized among mesocosms.

Dung beetles were added to four of the six dung pats produced by each cow (randomly chosen). Gas measurements and dung samples for microbial analysis were taken from two intact pats with beetles and two without beetles. The two additional pats with beetles were used for more invasive sampling of beetles for microbial characterization and to measure beetle reproduction and development. Based on beetle densities recorded in the field and on a previous study (Vessby 2001), we added 12 beetles to each pat, maintaining a sex ratio of 1:1. Each mesocosm was covered by a mesh lid for the full course of the experiment to prevent beetle escape and corvid predation.

Gas flux measurements

Gas fluxes of CO2 were measured in the field with an EGM-4 Environmental Gas Monitor for CO2, and a SRC-1 soil respiration chamber (PP Systems, Amesbury, USA). For each sampling event, the gas flux chamber was pushed 5 cm into the ground around the pat. The sampling time was 80 seconds and the measuring interval 4.8 seconds. Temperature inside the chamber was recorded during the sampling of all gases, for later scaling of gas fluxes to temperatures.

For N2O and CH4, we took four sequential samples 5, 10, 20 and 30 minutes after pushing the chamber into the ground and sealing it. Samples were taken with syringes into 3-ml Exetainer® vials with

double-wadded septa (Labco Ltd., Lampeter, UK). Gas flux measurements of N2O and CH4 were taken on days 1, 5, 10, 24 and 43 of the experiment (experimental setup being conducted on day 0). CO2 measurements were taken on the same days apart from the first, when we had to measure CO2 on day 2 instead due to rain. The composition of gas samples was quantified within five days of collection with a gas chromatograph (Agilent 7890B Series Custom GC GHG-Analyser, Agilent Technologies, Santa Clara, USA). Net gas fluxes over the course of the experiment were calculated as in Penttilä et al. 2013.

Dung and beetle sampling

All dung and beetle samples were preserved for subsequent characterization of microbiota using 95% ethanol, which is an effective storage medium for microbial community analysis (Hammer et al. 2015). On days 2, 11, and 23 of the experiment, samples of approx. 1 ml were taken from the underside of pats (to avoid breaking the hard crust surface) and stored in ethanol. On day 2 we collected one sample per mesocosm, and on the other days four samples were taken and later pooled before DNA extraction.

To test whether antibiotic treatment affected beetle microbiota, two parent beetles from each of the two beetle-sampling pats per cow were collected and preserved in ethanol on day 7 of the experiment. To measure antibiotic effects on beetle size, reproduction and survival, we sampled pats on day 43 (for half-grown larvae) and days 71 and 73 (for the next generation of adult beetles). Larvae recovered were weighed while fresh and the width of their head capsule was measured. Total offspring counts by the end of the experiment were used as an integrated measure of both adult reproduction and offspring survival.

Molecular protocol and sequence data processing

To characterize the overall microbial community associated with dung beetles, whole adult beetles were thoroughly homogenized with autoclaved, stainless steel beads in a shaker (SPEX Geno/Grinder), and DNA was extracted from ~100 mg of homogenate with the MoBio PowerSoil kit, following similar studies (Hammer et al. 2014, 2015). Dung samples were drained of ethanol before subsampling ~100 mg for DNA extraction with the same kit. Using barcoded primers, we PCR-amplified the ~300 bp V4 region of the bacterial and archaeal 16S rRNA gene, with the amplicons sequenced on an Illumina MiSeq platform as previously described (Ramirez et al. 2014, Hammer et al. 2015).

Paired-end sequence reads were merged, quality-filtered and clustered into operational taxonomic units ("OTUs") using the UPARSE pipeline (Edgar 2013; see Ramirez et al. 2014 for more detail). Taxonomic affiliation was assigned using the RDP classifier (Wang et al. 2007) and the August 2013 GreenGenes database (McDonald et al. 2012). OTUs that were relatively abundant in DNA extraction blanks and PCR no-template controls were removed, as were OTUs identified as mitochondria or chloroplasts. For the most abundant OTUs in beetles and dung (Fig. 3.2), we used the representative sequence of each OTU and RDP SeqMatch (Cole et al. 2009) to refine genus-level identities. Prior to all statistical analyses, we rarefied (randomly subsampled) each sample to an even sequencing depth of 5000 reads/sample. Quality filtering and rarefaction resulted in dropping one beetle sample and 16 dung samples from subsequent analyses, leaving 39 beetles and 103 total dung samples.

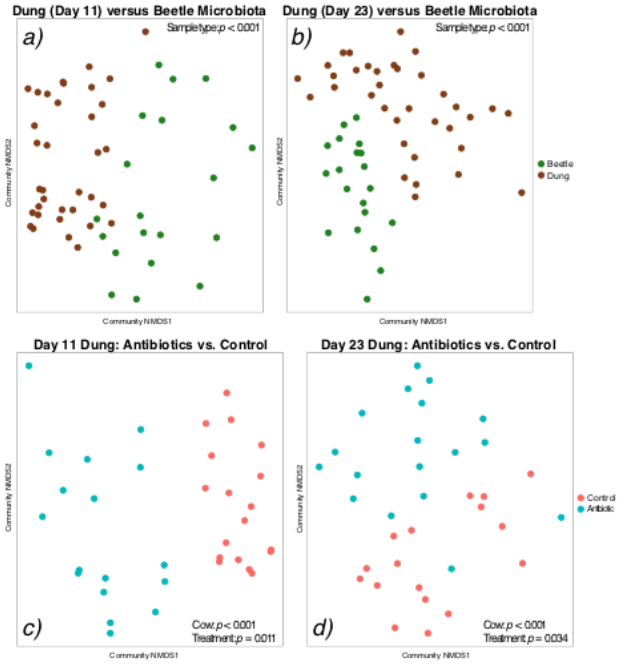

Figure A3.1. Regardless of the sampling point (number of days into the experiment), dung microbiota are distinct from dung beetle microbiota, and remain affected by antibiotic treatment and cow individual. Shown are non-metric multidimensional scaling ordinations of microbial communities in dung versus beetles (both treatments combined; *a* and *b*), and in dung from control cows versus antibiotic-treated cows (*c* and *d*). The ordinations visually represent Bray- Curtis dissimilarities among samples in two dimensions. In the corners of each plot, we show P values associated with each tested predictor variable from permutational multivariate ANOVAs.

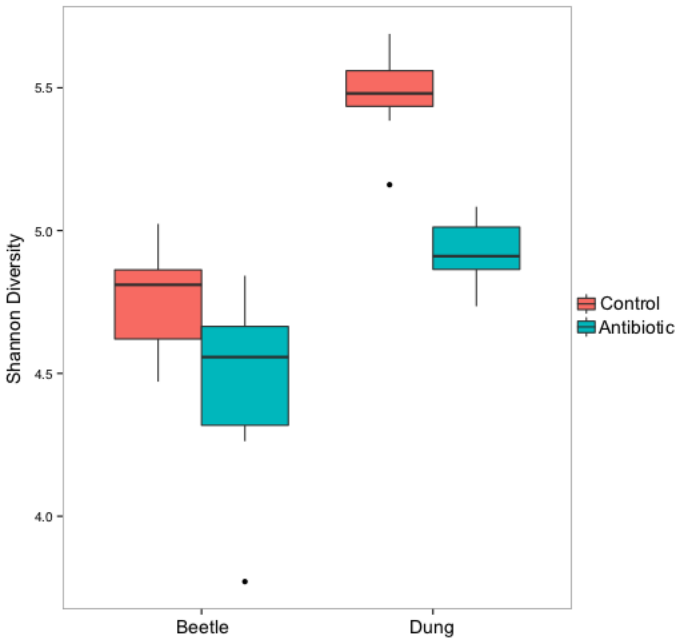

Figure A3.2. Microbial community diversity (Shannon index) is lower in beetles than in dung, and is also lowered by antibiotic treatment. Shown are boxplots with bold horizontal lines indicating the median, box limits showing first and third quartiles, whiskers extending to the most extreme values within 1.5 * the inter-quartile range, and dots showing outlying data points.

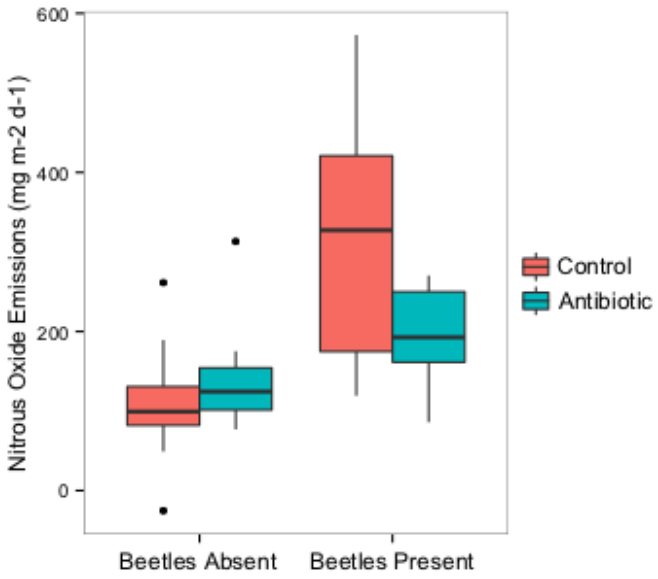

Figure A3.3. Total dung pat emissions of nitrous oxide are generally raised by the presence of dung beetles, but to an extent that is mediated by antibiotic treatment. Antibiotics limit the degree to which beetles increase nitrous oxide fluxes. Shown are boxplots with bold horizontal lines indicating the median, box limits showing first and third quartiles, whiskers extending to the most extreme values within 1.5 * the inter-quartile range, and dots showing outlying data points.

CHAPTER IV APPENDIX

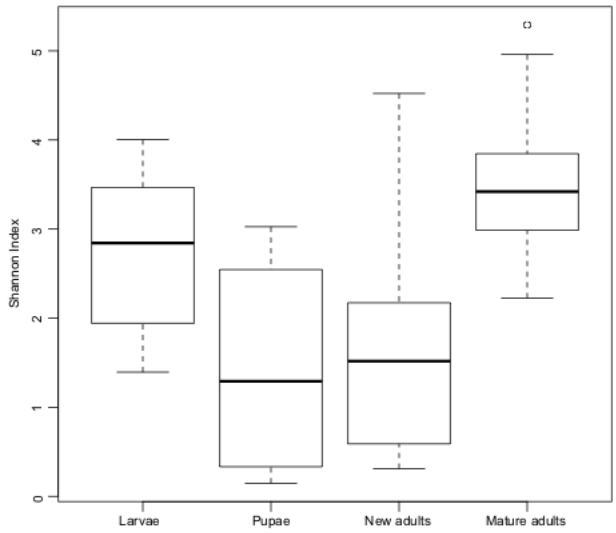

Figure A4.1. Boxplot of Shannon Diversity Index values from *H. erato* larvae, pupae, newly emerged adults, and mature adults, standardized at 500 sequences per sample.

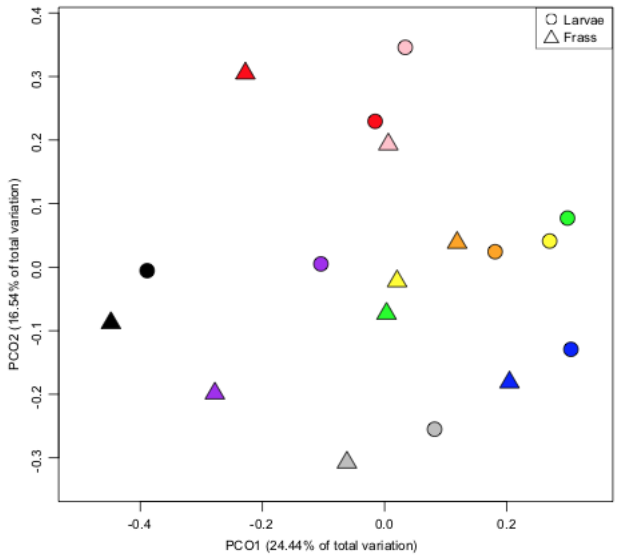

Figure A4.2. Principal coordinates analysis of bacterial communities in whole larvae and their frass, colored by individual, showing clustering by individual rather than sample type.

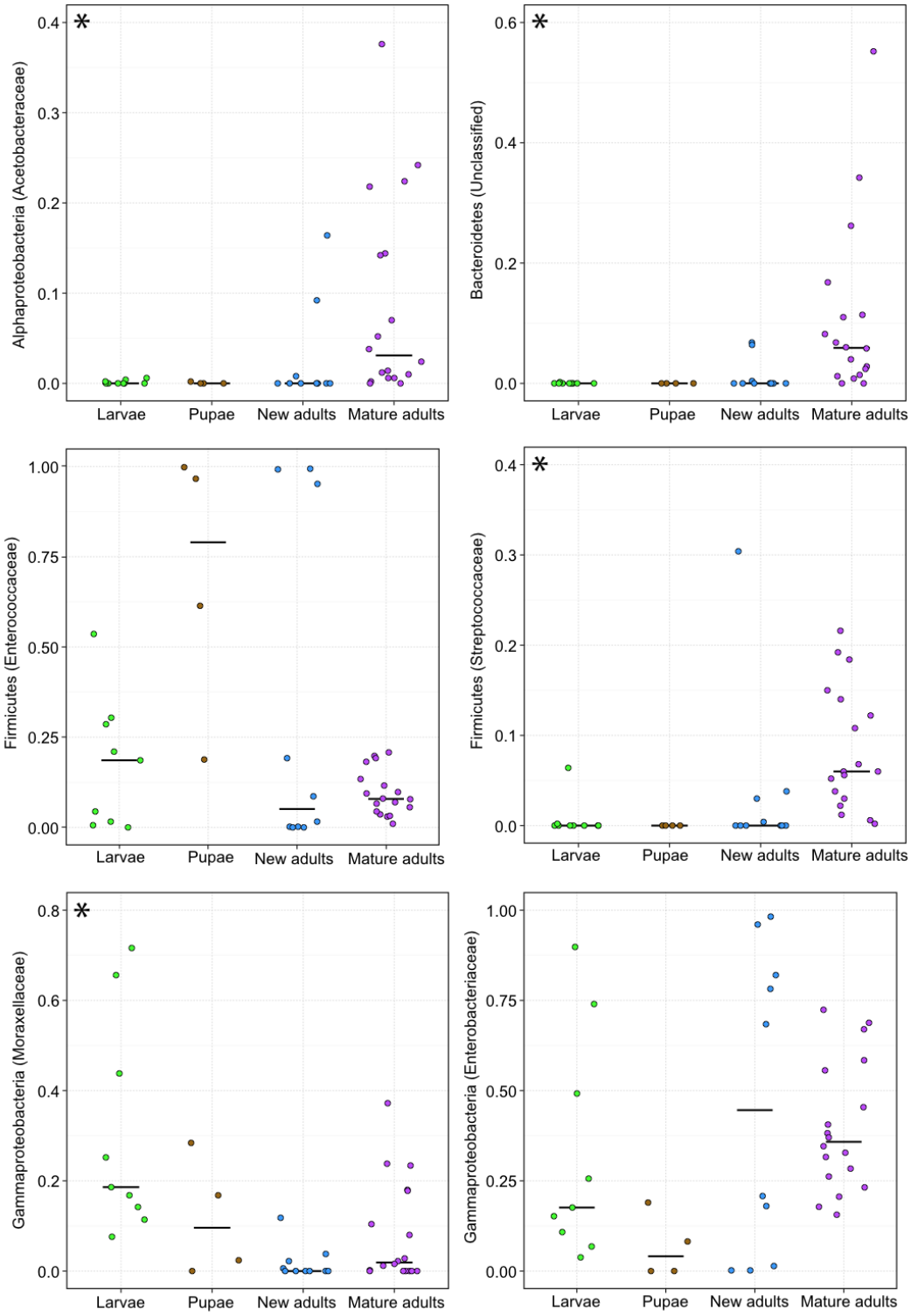

Figure A4.3. Relative abundances of the six dominant bacterial families among *H. erato* life stages, defined as those with a median abundance over 2% within any life stage. Points represent individual samples and are laterally jittered to display within-stage variability more clearly. Bars show median relative abundances. Asterisks indicate bacterial families whose relative abundances differed significantly across life stages.

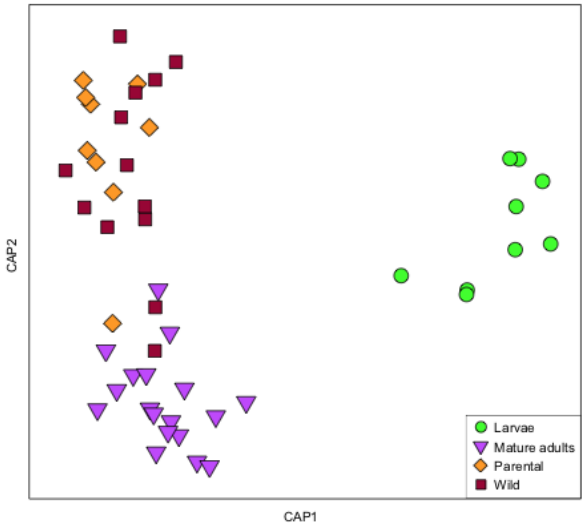

Figure A4.4. Constrained principal coordinates analysis of bacterial community composition in *H. erato* larvae and all adult groups. CAP1 and CAP2 are the axes in principal coordinate space that best discriminate among sample types.

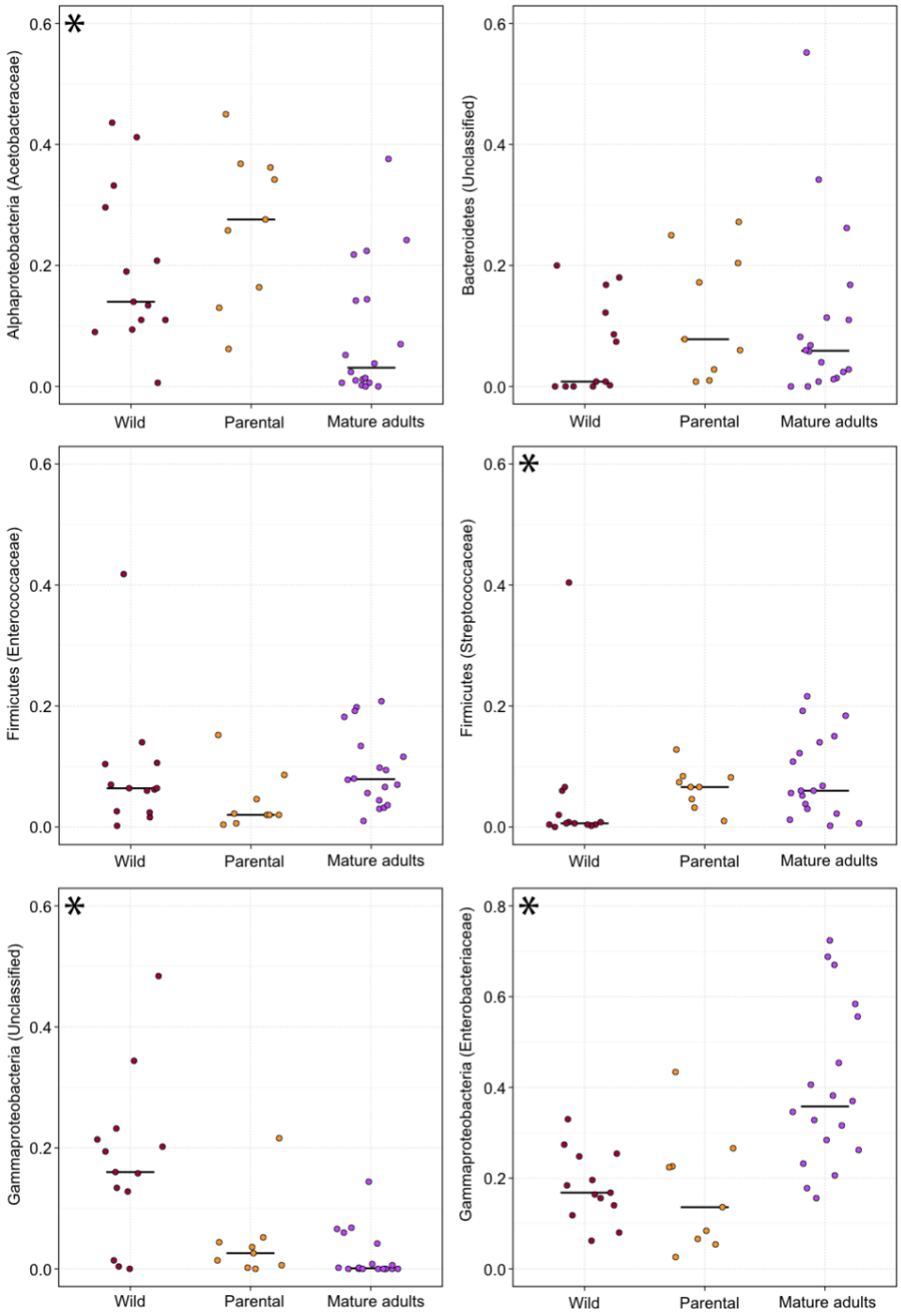

Figure A4.5. Relative abundances of the six dominant bacterial families among *H. erato* adult groups, defined as those with a median abundance over 2% within any group. Points represent individual samples and are laterally jittered to display within-group variability more clearly. Bars show median relative abundances. Asterisks indicate bacterial families whose relative abundances differed significantly across groups.

CHAPTER V APPENDIX

Appendix A5 Methods

Sampling

Fecal samples were obtained from wild populations of caterpillars in four regions: Área de Conservación Guanacaste (Costa Rica), New Hampshire and Massachusetts (USA), Boulder County, Colorado (USA), and Portal, Arizona (USA). Caterpillars were collected in ACG under permit #ACG-PI-027-2015 and in Arizona under a Scientific Use Permit from the United States Forest Service. For more details about the ACG landscape and collection, rearing, and identification protocols, see (Janzen et al. 2009; Janzen and Hallwachs 2011; Janzen and Hallwachs 2016). Most species were collected as caterpillars, but some ACG specimens were reared from eggs either found on foliage or laid by females caught at light traps (see file "Additional_ACG_SampleData" at https://doi.org/10.6084/m9.figshare.4955648). For some caterpillars we had information on whether they died of parasitoids or disease after sampling, and these samples were discarded in order to focus on apparently healthy individuals. Most caterpillars were sampled in the final or penultimate instar.

All samples were preserved within 30 minutes of defecation, as preliminary evidence suggested rapid (by 6-12 hours) bacterial and fungal growth in excreted fecal pellets, which would render old feces unsuitable as a proxy for gut microbial communities. In five caterpillar species, we did not find evidence for abundant bacterial populations in the midgut (including both ecto- and endoperitrophic spaces) or hindgut that were not captured in feces (Fig. A5.2a), supporting a previous finding that caterpillar feces approximates the whole-body microbial community (Hammer et al. 2014). Further supporting the use of fresh feces to sample microbes in the caterpillar gut, we found that the inter-individual variation in sequence composition (including nonbacterial DNA) was reflected in fecal samples (Mantel tests: midgut $r = 0.33$, $p = 0.001$; hindgut $r = 0.39$, $p = 0.001$).

We preserved gut and fecal samples using either dry storage at -20°C or 95% ethanol (see file "SampleData" at https://doi.org/10.6084/m9.figshare.4955648); both methods are suitable for storing insect microbiome samples and do not substantially alter community composition (Hammer et al. 2015). Approximately 50 mg (fresh weight) of sample was used for DNA extraction. Prior to DNA extraction, ethanol-preserved samples were dried in a vacuum centrifuge; since this also evaporated water, their fresh weight equivalent was estimated using percent water content calculated from *M. sexta* guts or feces. To test whether microbial biomass estimates may have been biased by ethanol storage, we compared PCR amplification for paired ethanol-stored and frozen fecal pellets from eight *M. sexta* individuals. From a collection of pellets defecated by each individual during a 1-2 hour window, separate pellets were randomly chosen for each storage type (note that pre-storage inter- pellet microbial variation is possible even under these relatively controlled conditions). As assessed by a linear mixed-effects model treating individual as a random effect, there was no significant influence of storage method on 16S rRNA gene copy number ($\chi 2(1) = 1.09$, $p = 0.30$).

For caterpillars in Costa Rica and Colorado, we also sampled microbes from leaves of the same branch as that fed to the caterpillar prior to feces collection. With this strategy we aimed to maximize microbial similarity between the leaves that were sampled and those consumed by the caterpillar, although leaf microbiomes can also vary substantially within a branch (Espinosa-Garcia and Langenheim 1990). These leaves appeared clean and had not, to our knowledge, come into contact with any caterpillars prior to sampling. Leaves from Colorado plants were frozen dry at -20°C and ground under liquid N2 with a mortar and pestle prior to DNA extraction (thus including endophytes as well as surface-associated microbes). Leaves from Costa Rican plants were stored in 95% ethanol, and surface-associated microbes were concentrated in a vacuum centrifuge and resuspended in molecular grade water prior to DNA extraction. As this sampling method was not quantitative, we did not perform qPCR on plant samples from Costa Rica and used them only for analyses of microbial composition.

Non-lepidopteran animals were sampled using the same procedures outlined above, with five species preserved in ethanol, two in 15% glycerol at -80°C, and 17 preserved dry at -20°C or -80°C (see file "Other_animal_Metadata" at https://doi.org/10.6084/m9.figshare.4955648). With the exception of two dung beetles feeding on herbivore dung, and the insectivorous bat *M. lucifugus*, these species are either predominantly or exclusively herbivorous, although the type of plant matter consumed (sap, leaves, seeds, fruit, pollen, etc.) varies. We extracted DNA from feces for vertebrates and from subsamples of homogenized whole bodies for insects (as some insects house the majority of symbionts in organs outside the gut). By including all tissue from these insects, we may have underestimated bacterial densities in the particular organs where microbes are housed (Fig. 5.1a).

DNA extraction, PCR and sequencing

Following previous studies of insect microbiomes (Hammer et al. 2014; Hammer et al. 2015; Hammer et al. 2016), we used the MoBio Powersoil kit to extract DNA (100 µl eluate) from measured amounts of sample material. We then PCR-amplified a portion of the 16S rRNA gene with barcoded 515f/806r primers (Caporaso et al. 2012). PCR products were cleaned and normalized (up to 25 ng DNA/sample) using the SequalPrep Normalization kit (Thermo Fisher Scientific), and then sequenced on an Illumina MiSeq. Paired-end sequences of 16S rRNA amplicons were merged, quality-filtered, and clustered into operational taxonomic units ("phylotypes") at the 97% sequence similarity level using UPARSE (Edgar 2013), and classified using the RDP classifier and Greengenes (Wang et al. 2007; McDonald et al. 2012) as previously described (Ramirez et al. 2014). The representative sequences of phylotypes unclassified at this stage, and mitochondrial rRNA phylotypes (which could be from plant, insect, fungal or other mitochondria) were aligned to the NCBI nonredundant nucleotide database (nt) using BLAST for taxonomic identification.

As bacterial DNA is ubiquitous in laboratory reagents used for DNA extraction and PCR, and especially problematic with low- biomass samples (such as caterpillar feces) (Salter et al. 2014), we removed contaminants from our samples using information from the 22 DNA extraction blanks and PCR no-template controls that yielded >100 bacterial sequences. Importantly, phylotypes detected in these blanks are not exclusively composed of reagent contaminants, because they receive some input from sample DNA during laboratory processing (Lazarevic et al. 2016). As high-biomass samples are both least likely to experience reagent contamination (Salter et al. 2014), and themselves most likely to be the source of "real" sample phylotypes identified in blanks, they can be used to distinguish between laboratory contaminants and true sample sequences (Lazarevic et al. 2016). We classified contaminants as phylotypes present at ≥1% abundance in one or more blank samples, excepting phylotypes present at ≥1% abundance in one or more of the best-amplifying samples (the top third in 16S rRNA gene copy number as measured by qPCR). These 25 phylotypes were removed from the dataset prior to analyses of bacterial abundance and composition (they are retained only in Fig. A5.2a). This approach does not include other types of contaminants introduced prior to DNA extraction, such as those from human skin. Finally, we note that the high relatedness between microbes commonly present in laboratory reagents (listed in Salter et al. 2014) and those present in soil, water and leaves—all possible genuine microbial inputs to the caterpillar gut—precludes a taxonomy-based approach to removing contaminants.

Sequence Data Analysis

All analyses were conducted in R version 3.3.2 (R Core Team 2016) and are available in the file "Hammer2017_Rcode_June2017.R" at https://doi.org/10.6084/m9.figshare.4955648. Analyses involving bacterial composition were limited to samples with at least 100 bacterial sequences. To calculate phylotype-level overlap between fecal and plant samples, "phylotypes detected on leaves" are defined as those present at any abundance in any plant sample in our dataset. New England and Arizona fecal

samples which lack paired plant samples were excluded from this comparison. In measuring core microbiome size in caterpillars and other animals, we excluded species with fewer than three replicate individuals. Further, to be conservative, only caterpillars sampled from the same location, and feeding on the same species of plant were compared. As the number of replicates could affect this metric, and varied among species, we iterated these analyses over multiple combinations of only three replicates per species.

Quantitative PCR

We measured 16S rRNA gene copy number using quantitative PCR with the same primers and DNA extracts as above. Reaction conditions and other details are specified in (Carini et al. 2016). Each sample was run in triplicate (except 11 non-caterpillar species for which limited DNA was available, which were run singly) and the mean of these technical replicates was used for subsequent analyses. Standard curves were calculated using purified genomic DNA from *E. coli* DH10B, which has seven 16S rRNA operons per genome (Durfee et al. 2008). The median copy number of 31 qPCR'd DNA extraction blanks was subtracted from sample copy numbers. Resulting counts of total 16S rRNA genes in samples were then multiplied by the proportion of bacterial sequences identified from the same DNA extract—excluding contaminants and DNA from plants and other eukaryotes—resulting in estimates of bacterial 16S copy numbers.

It is unlikely that the low amplification we found in caterpillar samples results from primer bias against abundant bacterial taxa. First, these primers successfully amplified bacteria in non-lepidopteran animals, even when in some cases (such as aphids, Clark et al. 1999), the dominant symbiont has been strictly vertically transmitted between hosts for tens of millions of years. Even in this case, divergence from free-living relatives has not been so great that its 16S rRNA gene is un-amplifiable using 515f/806r primers. Second, the caterpillar gut-associated microbial taxa we found are similar to those reported as

being relatively (i.e., in terms of the proportion of sequence libraries) abundant in metagenomic surveys (Belda et al. 2011; Xia et al. 2017) and amplicon-based studies using different 16S rRNA-targeting primer pairs (e.g., (Broderick et al. 2004; Brinkmann et al. 2008; Pinto-Tomás et al. 2011; Mason and Raffa 2014; Staudacher et al. 2016)).

To estimate the relationship between body size and whole-animal microbial loads (Fig. A5.3), we combined published data from (Kieft and Simmons 2015) with body mass data we calculated directly or derived from other studies (see file "Body_mass_data" at https://doi.org/10.6084/m9.figshare.4955648). To restrict the allometric scaling relationship for noncaterpillar animals to those species likely to harbor resident microbiomes, we removed species that had bacterial densities < 1/100th of the group median. These species were the goose *Branta bernicla*, the bat *Myotis lucifugus*, and the dung beetle *Geotrupes stercorosus*. The body size of two *M. sexta* individuals from Arizona was not recorded and so we substituted the median from other *M. sexta*. Furthermore, as we only had direct gut mass measurements for *M. sexta* (30-40% of body mass), for species sampled using feces (including *M. sexta*) we calculated total microbial loads by multiplying 16S rRNA gene density in feces by body mass. This procedure is likely to have slightly overestimated the microbial load for these species. Despite the numerous methodological uncertainties, microbial counts from (Kieft and Simmons 2015) and our qPCR-based data, and their allometric scaling relationship with body size (excepting *M. sexta*) were remarkably similar (compare solid and dashed line in Fig. A5.3).

PCR inhibition assays

To examine whether low 16S rRNA gene copy number estimates in caterpillar samples are an artifact of caterpillar-specific PCR inhibitors, we used two distinct approaches. First, we tested whether diluting extracted DNA improves PCR amplification by minimizing inhibitor effects (Nechvatal et al. 2008). However, 1:10 and 1:100 dilutions of fecal DNA from eight *M. sexta* individuals did not have this

effect (Fig. A5.6a). Second, we individually added the twelve lowest-amplifying caterpillar fecal samples—which might be especially likely to contain PCR inhibitors—to qPCR reactions with targeted primers and a template highly unlikely to be present in caterpillar feces (rDNA ITS region of *Batrachochytrium dendrobatidis* strain JEL270, a chytrid fungus pathogenic to amphibians). As compared to replicate reactions with pure molecular-grade water, adding caterpillar fecal DNA reduced amplification of *B. dendrobatidis* rDNA by 7.4% (Fig. A5.6b). This inhibition effect, which is also present in feces of humans (Nechvatal et al. 2008) and likely many other species, is miniscule relative to the difference in bacterial loads between caterpillars and non-lepidopterans spanning multiple orders of magnitude (Fig. 5.1a). Therefore, the relatively low PCR amplification of 16S rRNA genes from caterpillar feces is most likely due to low microbial biomass rather than high PCR-inhibitory substances.

Additional information on the antibiotic experiment

M. sexta larval feces production was measured by collecting, drying (50°C for 24 hours), and weighing all fecal pellets in the final instar. Pupae were weighed six days after pupation and monitored daily for adult eclosion. We collected a fresh fecal pellet from each caterpillar midway through the final instar, from which one subsample was cultured on LB media, and another used for qPCR and sequencing using the aforementioned protocol. To culture bacteria, we plated a dilution series (in sterilized phosphate-buffered saline) of weighed (10-20 mg) subsamples of feces, incubated in aerobic conditions at 37°C. After 24 hours, visible colonies were counted and then, if present, collected *en masse* from the agar surface for sequencing using a sterile swab. This plate-scrape method produces a list of the most abundant bacterial phylotypes potentially culturable using our approach. It should be noted that the presence of fecal bacteria in culture demonstrates that these taxa were viable, but not necessarily growing or metabolically active, while in the caterpillar gut.

Comparison of biomass estimates and evidence of extracellular DNA

Among *M. sexta* fecal samples collected during the antibiotic experiment, we found that qPCR-estimated bacterial abundances were correlated with the number of cultured bacterial colonies (see Results; Fig. A5.4b). Eleven individuals' fecal pellets did not produce any bacterial colonies whatsoever, but did contain measurable levels of DNA (Fig. A5.4b), and excluding these "zero-colony" samples yielded a stronger association between bacterial colony counts and 16S rRNA gene copy number (Pearson correlation, $r = 0.51$, $p = 0.0002$). This result could stem from the presence of bacteria that cannot grow aerobically or on LB. Alternatively, it may be due to PCR amplification of extracellular DNA or DNA from dead or otherwise nonviable cells (Carini et al. 2016). To evaluate these possibilities, we compared the phylotypes (identified by 16S rRNA gene sequencing) in zero-colony fecal samples to those from other samples that did yield colonies, in which bacterial biomass was swabbed directly from the agar surface and sequenced. Most of the 16S rRNA gene sequences in the zero-colony fecal samples (median 84%, interquartile range: 74-95%) belong to phylotypes cultured from other samples, suggesting that qPCR may have overestimated viable bacterial loads by amplifying DNA from lysed or nonviable cells. If the fraction of the gut microbiome originating from dead or nonviable cells is disproportionately high in caterpillars in general (e.g., due to their digestive physiology – see Discussion), then the difference in living, active microbial biomass between caterpillars and other animals (Fig. 5.1a) may have been underestimated.

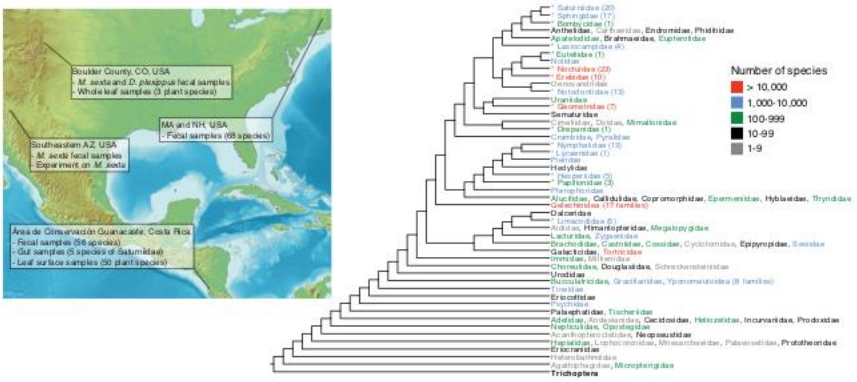

Figure A5.1. The caterpillar species included in this study and their phylogenetic placement (cladogram adapted from the "nt123_degen1" tree of Regier et al. 2013). Asterisks show sampled families of Lepidoptera, with the number of sampled species given in parentheses. To indicate the distribution of diversity (and taxonomic effort) across the tree, family names are colored by the number of described species given in (van Nieukerken et al. 2011). Inset: caterpillar sampling or experiment localities. Map from Wikimedia Commons.

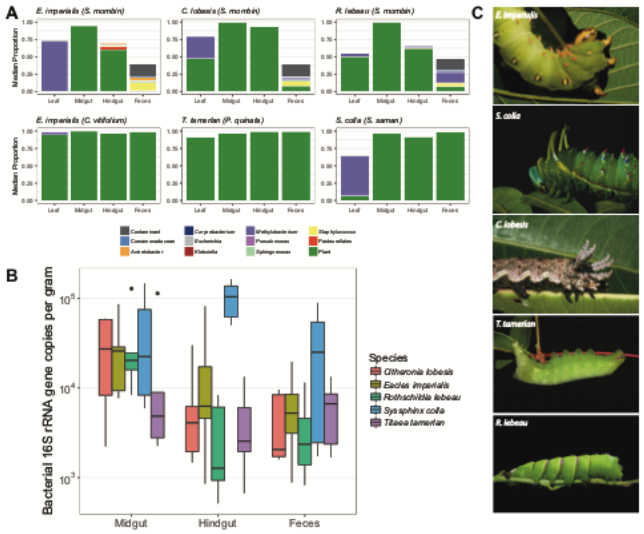

Figure A5.2. Bacterial community composition and density in five caterpillar species (Saturniidae). a) The composition of sequence libraries from the leaf surface, midgut, hindgut, and feces. The median across five replicate individuals is displayed. The food plant species is indicated in parentheses. Note that one species, *Eacles imperialis*, was reared separately on two plant species. Only plant chloroplast or mitochondrial sequences, reagent contaminants, and the top 10 bacterial genera (among the dissected individuals only) are shown; the remainder of the community (summing to 1) represents sequences from a variety of low-abundance taxa. b) The number of bacterial 16S rRNA gene copies per gram (fresh weight) in homogenized midgut or hindgut tissue and feces (N=5 individuals per species, except *E. imperialis* with 10 individuals (5 each on two plant species)). C) Photographs of each species taken in ACG, Costa Rica.

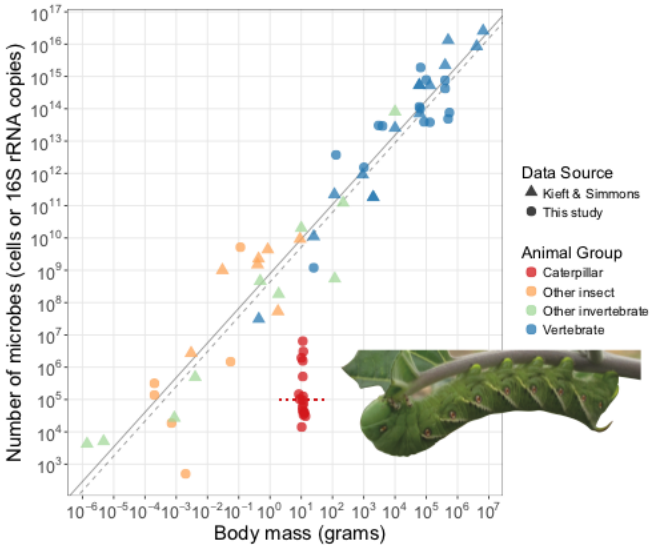

Figure A5.3. Allometric scaling of whole-individual microbial loads with body size. Triangles and the solid line show data replotted from (Kieft and Simmons 2015), which were originally measured using microscopy or culturing. Circles show data generated in this study, using quantitative PCR. The dashed regression line is calculated from a model only including non-caterpillar species analyzed in this study, limited to those species with bacterial densities not less than 1/100 of the group median. The red horizontal dotted line indicates the median per-caterpillar bacterial load for *Manduca sexta* individuals collected in Colorado (N=15) or Arizona (N=2). The photograph is *M. sexta* feeding on *D. wrightii*.

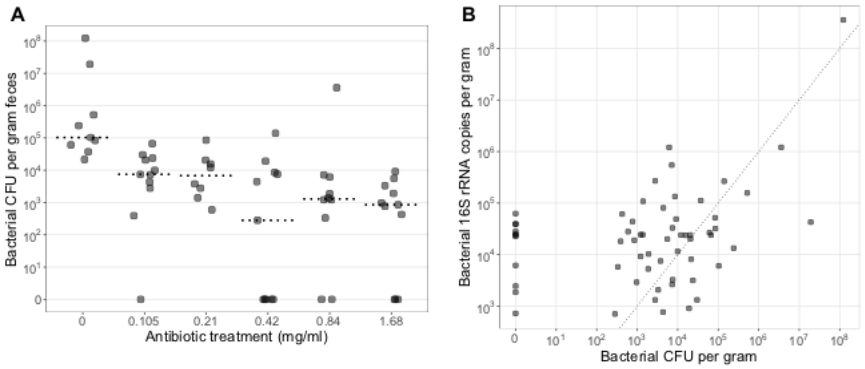

Figure A5.4. The relationships between antibiotic dose, the number of bacterial colony-forming units cultured on LB media, and the number of bacterial 16S rRNA gene copies measured by qPCR. Fecal samples that yielded no cultured colonies are plotted at 100 on log10 axes; for these, nonzero estimates of 16S rRNA gene copies are likely due, in large part, to amplification of DNA from dead or nonviable cells (see Appendix A5 Methods). a) Effect of antibiotic treatment on the number of culturable bacteria in caterpillar feces. Points are individual caterpillars (N=60) and are horizontally jittered for clarity. Dashed lines are medians for each treatment. b) Correlation of bacterial density as measured by culturing versus by DNA quantification. The 1:1 line between the two variables is shown.

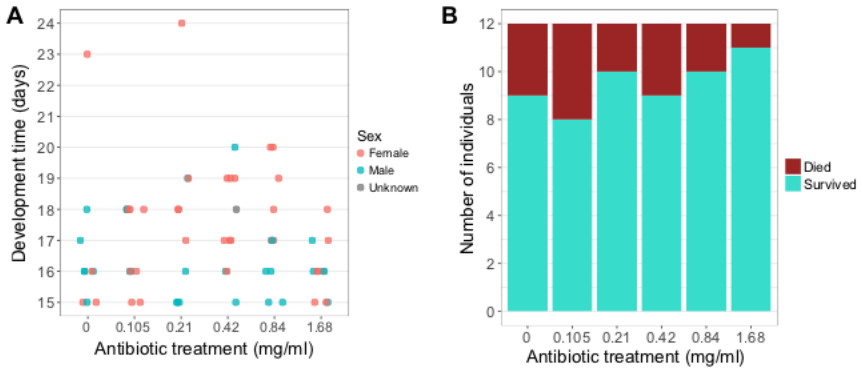

Figure A5.5. The relationship between antibiotic treatment and other components of *M. sexta* fitness. A) Number of days from larval hatching from eggs to the cessation of feeding, which marks the beginning of the prepupal stage. Shown are the 64 individuals that survived to this point. We were unable to identify the sex of two individuals that died as a prepupa or pupa. B) The proportion of individuals surviving from larval hatch to adult eclosion, for the control group and each antibiotic treatment.

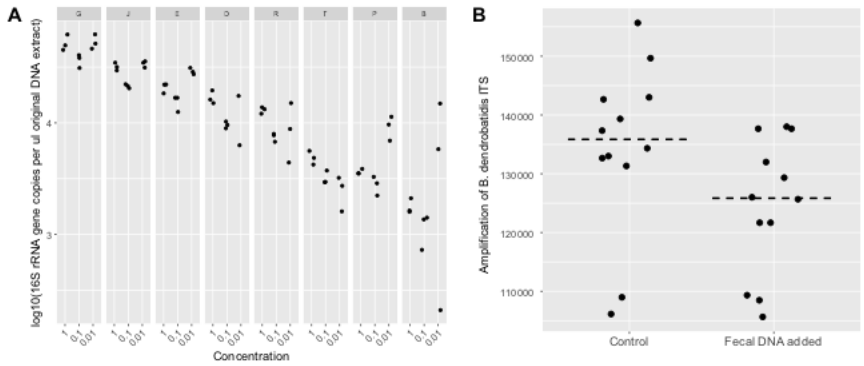

Figure A5.6. Two tests for PCR inhibitory substances in caterpillar feces. a) Fecal DNA from eight *M. sexta* individuals, arranged left-right by decreasing 16S rRNA gene copy number in original extracts. For each individual, log10(16S rRNA gene copies) is shown for the original sample, and for and extracts diluted 1:10 and 1:100 in pure water. Copy number estimates are standardized per ul of original DNA extract. Note that variability between technical replicates increases with low concentrations of template DNA. One sample, D-0.01, had less amplification than negative controls and is not shown. b) Amplification (arbitrary units) of rDNA ITS of *B. dendrobatidis*, a chytrid fungus of amphibians, showing 12 replicate controls (PCR-grade water only) versus 12 reactions to which 5 µl of caterpillar fecal DNA was substituted for water. Means of triplicate reactions are shown. The twelve caterpillar species with the lowest total 16S rRNA gene copy number were used for this test. Dashed lines show medians for each group.

Phylotype	Phylum	Family	Genus	Prop. samples detected in	Median rRNA copies per g when present	Among top 10 plant phylo-types?	Top BLAST hits to named isolates	Highest seq. identity
OTU_19	Firmicutes	Staphylococcaceae	*Staphylococcus*	0.79	280	No	*Staphylococcus*	100%
OTU_23	Proteobacteria	Enterobacteriaceae	*Escherichia*	0.73	132	Yes	*Escherichia coli*	100%
OTU_11	Proteobacteria	Methylobacteriaceae	*Methylobacterium*	0.72	692	Yes	*Methylobacterium*	100%
OTU_2	Proteobacteria	Enterobacteriaceae	*Klebsiella*	0.65	202	Yes	*Klebsiella, Enterobacter, others*	100%
OTU_5	Firmicutes	Enterococcaceae	*Enterococcus*	0.65	109	Yes	*Enterococcus*	100%
OTU_79	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.54	155	Yes	*Sphingomonas*	100%
OTU_8	Firmicutes	Lachnospiraceae	*Clostridium*	0.49	40	Yes	*Clostridium*	93%
OTU_16	Proteobacteria	Moraxellaceae	*Acinetobacter*	0.44	86	No	*Acinetobacter*	100%
OTU_160	Actinobacteria	Corynebacteriaceae	*Corynebacterium*	0.42	108	No	*Corynebacterium*	100%
OTU_36	Actinobacteria	Microbacteriaceae	unclassified	0.42	141	Yes	*Curtobacterium, Flavobacterium*	100%

Table A5.1. The taxonomic classification from Greengenes is given in columns 2-4. For each phylotype, the median absolute abundance was calculated only including samples in which it was present. We also indicate whether each phylotype was one of the 10 most common among all leaf samples. The representative sequence was used in a BLAST search against the NCBI database, restricted to named bacterial isolates, and the sequence identity and genus-level classification of the top hit(s) are shown.