Actinobacteria and the Vitamin Metabolism of Firebugs

- Characterizing a mutualism's specificity and functional importance -

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Das Promotionsgesuch wurde eingereicht und bewilligt am:

Gutachter:

1)
2)
3)

Das Promotionskolloquium wurde abgelegt am:

To Nagla and Samy, for ensuring that life's possibilities remain endless

*To Aly, for sharing everything**

And to Aileen, my beloved HERC2 *mutant*

* Everything except our first Gameboy (circa 1993). For all else, I am profoundly grateful.

CONTENTS

LIST OF PUBLICATIONS

The thesis is based on the following manuscripts:

Salem H, Florez L, Gerardo N, Kaltenpoth M (In review)
An out-of-body experience: The extracellular dimension for the transmission of mutualistic bacteria in insects
The American Naturalist (Chapter 2)

Sudakaran S, Salem H, Kost K, Kaltenpoth M (2012)
Geographic and ecological stability of the symbiotic mid-gut microbiota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae)
Molecular Ecology (Chapter 3)

Salem H, Kreutzer E, Sudakaran S, Kaltenpoth M (2013)
Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae)
Environmental Microbiology (Chapter 4)

Salem H, Bauer E, Strauss A, Vogel H, Marz M, Kaltenpoth M (In press)
Vitamin supplementation by gut symbionts ensures metabolic homeostasis in an insect host
Proceedings of the Royal Society B: Biological Sciences (Chapter 5)

Bauer E, Salem H, Marz M, Vogel H, Kaltenpoth M (In press)
Transcriptomic immune response of the firebug *Dysdercus fasciatus* to experimental elimination of vitamin-supplementing intestinal symbionts
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CHAPTER 1

SYMBIOSIS AND THE EVOLUTION OF BIOLOGICAL NOVELTY IN INSECTS

1.1 THE ORGANISM IN THE AGE OF THE HOLOBIONT: IT, ITSELF, THEY

Despite a seeming domination by plants and animals, the evolution of life on Earth has been driven by an ostensibly invisible, yet ecologically consequential force: microbes.

This notion, highlighting the impact of the microbial world on the biosphere's functionality (from molecular level to ecosystem structuring), is being rapidly and extensively integrated into the study of biology (McFall-Ngai *et al*., 2013). Following the technical and analytical breakthroughs of the last decades, our biological study of plants and animals is continuing to reflect an integrated model, one that takes into account the role of microbial partners in spurring various metabolic and defensive adaptations within multicellular organisms (Moran, 2007; Douglas, 2009; McFall-Ngai *et al*., 2013). It is a product of our expanding knowledge of the diversity, distribution and functional capacities of microorganisms, both in free-living and symbiotic contexts (Wu *et al*., 2009; Dillon and Dillon, 2004).

The establishment of a 'biological individual' has been integral towards our understanding of genetics, physiology, development, ecology and evolution. The 'individuality' criterion, however, has been tangled by decades-worth of research highlighting the role of heritable symbiotic microorganisms in their interactions with their eukaryotic hosts, thereby blurring previous lines characterizing what has been anatomically, physiologically and ecologically referred to as an individual (Gilbert *et al*., 2012).

Establishing that animals and plants are superorganisms comprised of eukaryotic cells and heritable bacterial symbionts was consequential both theoretically and empirically, mainly due to the perception that cooperation between unrelated individuals, and the distinct selective pressures exerted on the partners, violates the parsimonious and unilateral tenants of Darwinian evolution. As a result, the supplementing hologenome theory of evolution has been proposed by Rosenberg *et al*. (2007), stating that the object of natural selection is not the individual organism, but the organism together with its associated microbial community (the holobiont). The theory accounts for the heritable metabolic capacities encoded by the host's genome in addition to the genomes of its associated symbiotic microorganisms, thereby collectively reflecting in a hologenome that is more accurate representation of the symbionts' adaptive contributions towards the host's phenotype (Rosenberg *et al*., 2007).

Integrating this ecological paradigm for studying the host's interaction with its microbiome highlights the need to better understand the mechanisms and routes by which taxonomically and functionally diverse microbial communities colonize and, possibly, co-evolve with their eukaryotic hosts.

However, central challenges lie ahead of fully integrating the subdisciplines of zoology and botany to include a holistic view that accommodates microbiology. Most notably, as we consider classical examples of integrated partnerships between microbes and hosts, we must also evaluate which fractions of the host microbiome represents a random, transient condition, and which are maintained through the host's regulatory mechanisms and are truly intrinsic to (and relevant for) the host's

ecology and evolution, particularly when addressing questions relating to host-microbiome specificity. Considerable attention should be given to the processes that contribute towards the initiation and maintenance of a microbiome; including the elucidation of symbiont transmission routes, as well as the metabolic and behavioral adaptations that ensure its continuity.

1.2 ADAPTIVE SIGNIFICANCE OF SYMBIOSIS

Insights from comparative evolutionary genomics have revealed a widespread exchange of genetic material across taxonomically distinct lineages, thereby transcending traditional views on adaptation purely as a product of natural selection acting on existing genes within a species. This can be mediated by the acquisition of foreign genes that translate to beneficial traits for the receiving organism. For example, among closely related strains of *Escherichia coli*, up to 25% of a genome's composition can be unique to an individual strain, highlighting the prevalence of horizontal uptake of genes across interspecific lineages within the bacterial domain (Welch *et al*., 2002). While also prevalent in many eukaryotes (particularly unicellular protists), horizontal transfer of genes is rather limited in animals and plants, where gene duplication and divergence is by far the more common process instigating change within genomic profiles (Keeling and Palmer, 2008).

Alternatively, symbiotic interactions have also been viewed as an adaptive source and a mediating process of biological complexity (Moran, 2007). This view is founded on the observation that symbioses bind organisms from all of life's domains (Buchner, 1965; Douglas, 2009), thereby producing highly complementary partnerships that take advantage of the different genomic and metabolic capabilities of the contributing species. The notion that a single species can profit through mutualistic interactions therefore does not validate Darwin's (1859) concerns for how "Natural Selection cannot possibly produce any modification in a species exclusively for the good of another," since the complementary nature of many mutualisms ensures that the reward of providing a benefit usually outweighs the cost (Moran, 2007). However, the exchange of benefits within mutualisms can be molded by natural selection once the partnership is maintained across generations of host and symbiont, therefore highlighting the integral role of symbiont transmission as an important factor for the heritability and evolutionary stability of beneficial mutualisms (Sachs *et al*., 2004).

By integrating the diverse genetic repertoires of microbes, many animal lineages were able to expand their metabolic and physiological capacities, and, as a result, radiate and thrive in oligotrophic niches (Janson *et al*., 2008). Metazoans provide us with excellent models to study nutritional symbiosis due to the ancestral gene loss ubiquitously affecting the ability of animals to synthesize numerous metabolic compounds, including most vitamins as well as the 10 essential amino acids (Payne and Loomis, 2006), thereby predisposing them towards greater dependency on other organisms (Douglas, 2011). Alternatively, animals can also harness the ability of microorganisms to produce a broad range of bioactive compounds to offset attacks by natural enemies (Douglas, 2011). Similarly, symbionts may even extend defensive capabilities indirectly by immunologically priming the host, or by competitively excluding pathogenic microbes through occupying the same niche (e.g. the gut) and utilizing the same resources (e.g. ingested food) (Douglas, 2011).

Prior to the technological advancements that transformed molecular biology over the last decades, Paul Bucher conducted extensive microscopy-based surveys across numerous groups of invertebrates to elucidate the prevalence and role of symbiotic microorganisms towards provisioning essential nutrients to their hosts. Throughout his influential volume *Endosymbiosis of Animals with Plant Microorganisms* (1965), Buchner set out to specifically examine the shared evolutionary histories of

microbes and animal groups subsisting on nutritionally deficient diets. Among those, none later served as a better example for the metabolic interdependency of mutualisms than sap-feeding insects, most notably, aphids.

For the 4,400 known species of aphids, the vast majority engage in an intimate nutritional partnership with intracellular symbiotic bacteria of the genus *Buchnera* (Baumann, 2005). Within the body cavity of aphids is a bacteriome consisting of ~80 bacteriocytes, which are specialized cells capable of sustaining the obligate intracellular bacterium (Buchner, 1965; Baumann *et al*., 1995). Findings from bioassays as well as molecular and genomic studies revealed that *Buchnera* provides the aphid with the essential amino acids and vitamins that it cannot intrinsically produce (Gil et al., 2002; Douglas, 1998; Nakabachi and Ishikawa, 1997; Nakabachi *et al*., 2005). The adaptive significance of this mutualism is further highlighted by the feeding ecology of the insect host, which specializes on plant phloem. While rich in sugars, this nutritional resource is largely devoid of essential amino acids and many vitamins.

Despite possessing a drastically reduced genome (0.61-0.65Mb and ~560 protein coding genes) (Gil *et al*., 2002), *Buchnera*, nonetheless, retained the complete biosynthetic pathways required for the synthesis of all essential amino acids, supporting earlier physiological findings implicating the role of the bacterium in the amino acid metabolism of its host. Strikingly, the biosynthetic capacity for nonessential amino acids has been lost (Gil *et al*., 2002), in complement to the provisionment of these compounds by the host cells (Richards *et al*., 2010). The specific up-regulation of host genes involved in the synthesis of nonessential amino acids, and the import of essential amino acids highlights the molecular basis of nutritional interdependency between the aphid host and *Buchera* and is an exemplary testament to the metabolic alterations affecting partners in a strict nutritional association.

1.3 SYMBIONT-MEDIATED DIVERSIFICATION

Symbiotic interactions have often been credited as fundamental drivers of evolutionary change by conferring novel ecological traits, thereby increasing phenotypic diversity (Schluter, 2000). Symbioses, in particular, are thought to have mediated the ecological diversification of many organisms by assisting their radiation into novel ecological niches (Janson *et al*., 2008; Takiya *et al*., 2006). For example, the symbiotic acquisition of bacteria that subsequently gave rise to mitochondria permitted eukaryotes to spread from strictly anaerobic conditions and diversify into a myriad of unicellular protists and ultimately the three major multicellular kingdoms (animals, fungi and plants) (Baldauf, 2003). Similarly, the association of mycorrhizal fungi with more than 75% of plant taxa has often been considered as a prerequisite for the evolutionary transition of plants from aquatic to terrestrial habitats. This is supported by the vital mineral contributions of the fungi, the cosmopolitan distribution of the association (Newman and Reddell, 1987), as well as molecular and paleontological findings suggesting that the partnership evolved some 400 million years ago, around the time land plants first appear in the fossil record (Simon *et al*., 1993).

More recently, empirical tests explicitly linking symbioses to niche expansion and adaptive radiation have been performed (Janson *et al*., 2008). For gall midges of the Cecidomyiidae family (Diptera), Joy (2013) was able to demonstrate that lineages engaging in symbiotic partnerships with ambrosia fungi have undergone seven-fold expansion in the range of host-plant taxa that they can utilize relative to free-living lineages. Larval midges can utilize the fungal hyphae, which are internally creased throughout an array of plant structures, as food, as well as for defense against natural enemies (Rohfritsch, 2008). Remarkably, the diversification rate of symbiotic midges outpaced that of their

non-symbiotic counterparts by 17 times (Joy, 2013). Collectively, these findings (among others) support predictions implicating the role of symbiotic interactions in ecological niche expansion, and subsequently, species diversification.

Therefore, the incorporation of beneficial symbionts by animals may constitute a pivotal innovation, one that can permeate their ecological transition across a greater diversity of niches and habitats. By doing so, symbioses may allow organisms to colonize novel and seemingly uncompetitive resources en route towards expanding their ecological range and catalyzing their diversification rates (Losos, 2010).

1.4 REVISITING DARWIN'S MYSTERY OF MYSTERIES: THE ROLE OF SYMBIOSIS IN SPECIES FORMATION

Taken at face value, symbiosis and speciation appear as conflicting processes; the former describes the coming together of distinct lineages to form a greater, often singular entity, while the latter is the diversifying process by which a single species splits into two. However, a closer examination of the adaptive value conferred by many mutualisms highlights the potential of symbiosis in catalyzing the rate by which reproductive barriers between nascent species can originate (Brucker and Bordenstein, 2012a).

While nuclear genes have predominantly been considered the driving force behind speciation in eukaryotes (Coyne and Orr, 2004), recent studies have also implicated the host's microbiome as a critical contributor to this process (Miller *et al.*, 2010; Brucker and Bordenstein, 2013). The theoretical foundation for this assertion is based on four key observations: 1) microbial associations are ubiquitous among eukaryotes, 2) many of these partnerships have been demonstrated to exhibit high degrees of specificity, 3) bacterial symbionts have been previously described as instigators of reproductive barriers, and lastly, 4) the host's immune genes (known to be involved in hybrid incompatibility, and as a result, reproductive isolation) are coevolving with the host's microbial symbionts (Brucker and Bordenstein, 2012a; Miller *et al*., 2010).

Symbiont-mediated reproductive isolation has been demonstrated in *Drosophila melanogaster* where Sharon *et al* (2010) were able to attribute the differences observed in sexual attraction among genetically identical fruit flies to the variation in insects' microbiomes. Fly populations that were fed either molasses or starch were found to acquire distinct microbial communities, in addition to displaying significant mate discrimination (with attraction occurring within, but not across feeding groups). The described variation in mating preferences, however, was reversed when the flies were cleared of their microbiota through antibiotics. Strikingly, the condition could be selectively induced through reinoculation with bacteria originating from either fly population (molasses- or starch-fed). It was subsequently shown that many nuclear genes coding for sex pheromones in *D. melanogaster* are directly affected by bacterial-specific molecules (Ringo *et al*., 2011), thereby highlighting a mechanistic process by which the resident microbial community can affect the host's mating preferences, and ultimately, the degree of gene flow across populations in a prezygotic fashion.

In the neotropical *Drosophila paulistorum,* which is a super species consisting of numerous overlapping, yet reproductively isolated semipecies, females express strong premating seclusion against potential mates from other groups (Ehrman, 1968). Similar to the aforementioned examples, Miller and colleagues (2010) demonstrate that the observed reproductive isolation is in fact, at least partly, symbiotically-mediated. Here, the core endosymbiont *Wolbachia* was shown in hybrids to

transform into a pathogenic variety by causing embryonic inviability and male sterility. Experimental reduction of *Wolbachia* titers in hybrids reverses these effects and ultimately breaks down reproductive barriers between the semispecies of *D. paulistorum* (Miller *et al*., 2010).

The microbiota has also been demonstrated to affect postzygotic reproductive isolation across a number of taxa by contributing towards hybrid sterility or susceptibility. While hybridization in eukaryotes can promote the rates of generating genetic novelty by creating new arrangements of genes, it can also negatively affect fitness through sterility by contributing to the collapse of co-adapted gene complexes (Arnold and Hodges, 1995). Though the outcome of hybridization is complex, the influence of the microbiota can be examined in reference to the hybrid susceptibility hypothesis, which states that the effect of interspecific epistasis between immunity genes results in lower immunological resistance in hybrids compared to either parental species (Burke and Arnold, 2001; Brucker and Bordenstein, 2012a). In other words, if hybrids are more susceptible to pathogenic infection than non-hybrids, does the microbiota contribute to the induced vulnerability? For certain pteromalid wasps, the answer to that question is yes. A recent study from Brucker and Bordenstein (2013b) demonstrates that beneficial bacterial communities residing in the guts of *Nasonia* wasps (Hymenoptera: Pteromalidae) contribute towards the lethality observed among hybrids of this genus. *Nasonia* species harbor highly conserved gut microbiotas, to the extent that the interspecific microbial community relationships mirror the phylogeny of their hosts (Figure 1.1) (Brucker and Bordenstein, 2012b), coevolution of hosts and their associated microbial communities.

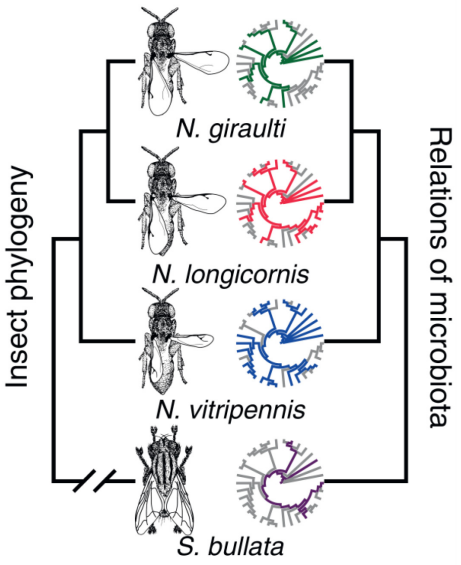

Figure 1.1 Example of the parallel relations between a phylogeny of insect species based on mitochondrial genes (the parasitoid species *Nasonia* and their host fly *Sarcophaga bullata*) and a dendrogram of the microbial community profiles. From Brucker and Bordenstein (2012a).

In addition to the high mortality recorded among *Nasonia* hybrids, larval melanization was also observed - which is a symptomatic characteristic of prominent immune responses in insects (Brucker and Bordenstein, 2012b). Additionally, hybrids possessed an imbalanced microbial community relative to parental controls, highlighted by the shift in the dominant bacterial taxa occupying the wasp's gut (Brucker and Bordenstein, 2013). These findings led the authors to believe that the lethality and the melanization of hybrids were a direct consequence of altered gut microbiomes. This hypothesis was validated through the rescuing of hybrid mortality by eliminating the resident microbial community using antibiotics (Brucker and Bordenstein, 2013). Additionally, re-infecting 'cured' hybrids restores normal (compromised) hybrid fitness by initiating the expression of innate immune genes, followed by some degree of melanization (Brucker and Bordenstein, 2013). Regarding

the molecular basis for the observed symbiotically-mediated hybrid lethality, the authors hypothesized that negative epistasis (mismatched gene-gene interactions) was taking place between the host's immune genes and the microbiome. This was supported through comparative transcriptomic analyses of the *Nasonia* hybrids, germ-free hybrids, and re-inoculated (previously germ-free) hybrids (Brucker and Bordenstein, 2013). Across all three groups, microbe-bearing hybrids (untreated and re-inoculated) exhibited a significant increase in transcript expression for innate immune genes relative to the germ-free treatments, suggesting a possible breakdown of a highly specific effector-triggered process that could contribute towards symbiont-mediated speciation.

While still in its formative stages, the microbiological contribution towards eukaryotic speciation is a rapidly expanding area within the field of symbiosis research and could complement how we approach the evolutionary dynamics of speciation.

1.5 HOMEOSTASIS OF SYMBIOSES

"*Symbiosis researchers, like romantic novelists, tend to focus on the routes by which partners come together and neglect the process by which partners coexist*"

- Angela Douglas, The Symbiotic Habit (2010)

And true to form, an entire chapter is passionately dedicated to the diversity of symbiont transmission routes and their ecological and evolutionary influences for both host and microbe (Chapter 2; Salem *et al*., submitted). Therefore, this section will highlight mechanisms that dictate the persistence of symbioses while drawing from classical examples that illustrate the stable integration of symbionts into the developmental biology, as well as ecology of their hosts.

The central theme to maintaining symbioses often relates to the host's ability to control the density and localization of its symbiont. Traditionally, that control is thought to operate on two fronts: (1) management of symbiont localization, and (2) restriction of symbiont growth and proliferation.

Examples of host control over the localization and density of its symbiotic partners are ubiquitous. Within *Acacia* trees, the hollow thorns (domatias) that house their protective ant partners are the only suitable material within the tree for nest building (Brouat *et al*., 2001). Concordantly, by controlling the quantity and size of the thorns, the tree can effectively manage the number of ants subsisting on its resources (Brouat *et al*., 2001). Similarly, the Hawaiian bobtail squid is able to manipulate the population density of its luminescent bacterial symbiont *Vibrio fischeri* in an attempt to align the partnership to its own bahavioral ecology (McFall-Ngai and Ruby, 1991; Kremer *et al*., 2013). *V. fischeri* is confined to specialized light organs in the squid's mantle, where in exchange for amino acids and sugars, the bacterium's luminescence conceals the squid's silhouette by matching light wavelengths emanating from the moon (McFall-Ngai and Ruby, 1991) – an adaptation presumed to nocturnally camouflage the squid from predators. While peaking at night, *V. fischeri*'s population densities, nonetheless, drop by nearly 90% at dawn through the active shedding of mantel tissue by the host (Ruby and Asato, 1993). *V. fischeri* is able to proliferate again throughout the day, however, this regulatory cycle is thought to benefit the host by limiting the nutritional costs of sustaining the partnership when it is not needed (i.e. during the day) (Ruby and Asato, 1993), as well as saturating

the immediate environment with the symbiont to facilitate uptake by juvenile squids (McFall-Ngai and Ruby, 1991) – a feature enabled by the *V. fischeri*'s capacity for subsisting outside the host.

Recent findings also involve the host's immune system in influencing the persistence and stability of host-associated microbiota. In *Drosophila melanogaster*, epithelial cells of the insect's midgut possess receptors that activate the immune deficiency (IMD) pathway, and in turn, mediate the expression of a multitude of antimicrobial peptides (AMPs) in response to pathogen exposure (Lemaitre and Hoffman, 2007). Similarly, the resident gut microbiota also induces IMD signaling; however, specialized transcription factors that inhibit the expression of AMPs are up-regulated (Ryu *et al*., 2008), suggesting that the insect's immune system is attuned to the remarkably complex community of microbes consistently colonizing the gut. Regarding the specificity of the antagonistic immune response, Roh and colleagues (2008) demonstrate that even when expressed, the AMPs do not exclusively repress the growth of resident bacteria; rather, they can contribute towards managing and regulating the composition and densities of the community. Here, AMPs can act to correct the relative ratios of bacterial species if altered (Roh *et al*., 2008). This is in line with a later study by Login and colleagues (2011) demonstrating that AMPs in weevils specifically target endosymbionts in bacteriocytes and regulate their growth rates through inhibition of cell division. Once the AMP-coding genes have been silenced with RNA interference, endosymbiont densities increased uncontrollably and poured out of the bacteriocytes and into other insect tissues (Login *et al*., 2011).

Further studies demonstrating increased recognition of beneficial microbes by the host's immune system point towards a greater capacity to immunologically sieve a selected consortium of microbes based on their molecular profiles. For example, in the association of tsetse flies with their vitamin-supplementing endosymbiont *Wigglesworthia glossinidia,* a peptidoglycan recognition protein with amidase activity (PGRP-LB) is specifically up-regulated in bacteriocytes housing the instracellular symbiont (Hu and Aksoy, 2006). Despite being a product of an activated IMD pathway, PGRP-LB nonetheless functions by degrading the bacterium's peptidoglycan ligand that initially triggered the immune response, thereby creating a cloaked façade concealing the magnitude of the monoclonal bacterial population persisting in the bacteriocytes, and contributing towards its adaptive persistence (Hu and Aksoy, 2006). While an example of how the responsiveness of an immune system can be modified to accommodate a beneficial symbiont, certain insect lineages have done without PGRPs and IMDs altogether (Gerardo *et al*., 2010). The evolutionary loss of both pathways in aphids, for example, is thought to be inflicted by the considerable selective pressures for maintaining the pivotal nutritional partnership with *Buchnera* (Gerardo *et al*., 2010).

In addition to highlighting that AMPs (and by extension, the IMD pathway) are selective in their antagonistic effects, these findings demonstrate that the innate immune system, much like the vertebrate adaptive immune system, can also promote the coexistence between the host and its resident microbial community. Therefore, it is emerging that - similar to parasites and pathogens - the resident microbial community plays a significant role in the evolution of the immune system in animals.

1.6 THE PYRRHOCORIDAE-ACTINOBACTERIA SYMBIOSIS

1.6.1 Hemiptera: Pyrrhocoridae

Pyrrhocorid bugs are part of the hemipteran superfamily Pyrrhocoroidea that also includes the Largidae. The family boasts an upward of 300 known species spanning many geographic locations across the Old and New World (Ahmed and Schaefer, 1987). The herbivorous members of this family are oligophagous but appear to have a clear preference for seeds of the Malvales plant order (Ahmed and Schaeffer, 1987; Kristenova *et al*., 2011). Within the Malvales, such preference is primarily restricted to the Malvaceae, Sterculiaceae, Bombacaceae and Tiliaceae families (Ahmed and Schaeffer, 1987; Kristenova *et al*., 2011). Despite aggregating on all parts of a malvales plant, the bugs, nonetheless, only utilize dry, ripened seeds for their nutrition, to which they seem attacted by some volatile substance (Ahmed and Schaeffer, 1987; Kristenova *et al*., 2011).

Among the better known representatives of firebugs are members of the *Dysdercus* genus (the so-called cotton stainers), and the European firebug, *Pyrrhocoris apterus*. The former are of agricultural and economic importance given a feeding preference for cotton crops (Ahmed and Schaefer, 1987), while the latter is an important model organism for the endocrinology and physiology of hemimetabolous insects (Socha, 1993). Members of the *Dysdercus* genus possess a cosmopolitan distribution mirroring that of cotton cultivars and the *Ceiba* cotton tree. They acquired their common name and pest status from the indelible fecal stains that they generate on cotton fibers while feeding. They have been shown to additionally harm cotton crops by inflicting significantly smaller bolls as a result of puncturing and feeding (Ahmed and Schaefer, 1987).

Pyrrhocorids exhibit a highly conspicuous aggregation behavior around Malvales plants, an adaptation thought to facilitate survivorship during nonfeeding periods by warning away predators through the striking aposematic colorations (Myers, 1927). Such behavior has also been suggested to help increase the volume of food ingested, since the collective enzymatic activity of numerous aggregating bugs can potentially expose more the seed content (if aggregations occur around the same seed) (Bongers and Eggermann, 1971), as it has been demonstrated for other pierce-and-flush insects (for example, stinkbugs of the Pentatomoidea superfamily) (Tachikawa and Schaefer, 1985).

1.6.2 *Coriobacterium glomerans*, the endosymbiont of pyrrhocorid bugs

Despite the ecological prevelance, economical importance, and model system distinction of firebugs, very little was known regarding the taxonomic and functional diversity of their microbial symbionts.

For *P. apterus*, early descriptions of its microbial gut associates revealed the presence of the extracellular symbiont *Coriobacterium glomerans* belonging to the actinobacterial family Coribacteriaceae (Haas and Konig, 1987; 1988). Microscopy-based approaches revealed that *C. glomerans* is capable of forming long chains (~150 μm) both along the epithelial layers of the insect's gut, as well as on plate (Haas and Konig, 1987; 1988). The anaerobic growth conditions required by *C. glomerans* led Haas and Konig (1988) to assume that the bacterium is not transmitted maternally via the egg surface, but rather horizontally through direct ingestion of feces from other firebug individuals. This assumption was supported by the authors' inability to detect the bacterium on the egg surfaces of *P. apterus* by cultivation-based methods.

More recently, a study by Kaltenpoth and colleagues (2009) aimed to supplement these findings with molecular techniques (PCR and FISH) in order to investigate the prevelance of *C. glomerans* infection within firebugs, and confirm their extracellular transmission route. Here, the symbionts were detected in the midgut section M3, the rectum, and to a lesser extent, in the hemolymph of female bugs. Greater diagnostic resolution offered by PCR methods also revealed the presence of *C. glomerans* on the eggs of *P. apterus*, suggesting that the bacterium can be transmitted vertically via the egg surface, thereby expanding on what has been described by Haas and Konig (1988). Behavioral observations detailing (1) females touching the eggs with their anus while secreting dark droplets, and (2) young nymphs probing the egg surface soon after hatching, provided support for this assertion (Kaltenpoth *et al*., 2009). Surface sterilization of the eggs through inundation in ethanol and bleach resulted in *Coriobacterium*-free (aposymbiontic) firebugs. Also, spreading firebug feces over the surface of sterlizied eggs reestablished infection, thereby confirming that egg surface contamination is an integral process for the establishment of the firebug-*Coriobacterium* symbiosis.

Therefore, if *C. glomerans* appears to be transmitted vertically from mother to offspring, and is consistently associated with firebugs throughout their development, then this inspires a range of additional questions. Specifically, does *C. glomerans* contribute beneficially towards host fitness, and if so, how? Additionally, is the association specific?

1.7 THESIS OUTLINE

Microbial symbionts can contribute significantly towards the nutrition of many insects. For example, the supply of essential amino acids by certain Gammaproteobacteria has been substantiated across several insect taxa including aphids (Douglas, 1998, 2009) and carpenter ants (Gil *et al*., 2003; Feldhaar *et al*., 2007). Several haemophagic insects rely on their gut microbes for the supply of B vitamins that are otherwise limiting in their blood meals, as demonstrated in tsetse flies (Nogg, 1981; Akman et al., 2002), bed bugs (Hosokawa *et al*., 2010) and triatomine bugs (Lake and Friend, 1968). Alternatively, symbiont-mediated detoxification has been demonstrated across a number of insect groups; most notably, broad-headed bugs of the *Riptortus* genus (Kikuchi *et al*., 2012).

Therefore, for this doctoral project, we aimed to (**1**) comprehensively characterize the microbial community associating with firebugs, and evaluate its overall stability across different geographical and ecological conditions, (**2**) assess if certain bacterial lineages (specifically *C. glomerans*) contribute beneficially towards host fitness, (**3**) elucidate the functional nature of these symbiotic contributions and (**4**) examine the immunological factors that facilitate the maintenance of the microbiota associating with this insect family. These four objectives were addressed throughout the seven chapters of this thesis.

In chapter 2 we comprehensively review and summarize the literature concerning the diversity and prevalence of extracellular symbiont transmission routes in insects. Throughout this chapter, we aim to delineate the evolutionary implications of symbiont transmission routes (intracellular vs. extracellular) relative to the transmission modes (vertical vs. horizontal), specifically when addressing the genomic and physiological consequences for both host and symbiont.

In chapter 3 we characterize the complex symbiotic gut community of the European firebug (*P. apterus*) with the aim of assessing the specificity of the microbiota to the host and evaluating the core microbial taxa that inhabit it. We also chart the continuity of the microbial community across different life stages of *P.apterus* in order to infer the symbiont population dynamics over the host's life cycle.

In chapter 4 we demonstrate the importance of the microbiota, specifically its actinobacterial constituents, towards the overall fitness and development of firebugs. In this chapter, we combine experimental manipulation with community-level analyses to ascribe the observed fitness effects to individual bacterial taxa of the insect's gut region. We additionally test the specificity of host-microbe associations through reciprocal cross-infections of microbial communities across two pyrrhocorid species.

Chapter 5 details the metabolic contributions of the symbiotic bacteria in cotton stainers. By utilizing a combination of transcriptomic analyses and bioassays that take advantage of a manipulable artificial diet, we elucidate that the contributions of Actinobacteria towards firebug fitness are nutritional in nature, specifically in the form of B vitamin supplementation and subsequently gain insights into the regulatory mechanisms mediating the exchange of nutritents between microbe and host.

Lastly, in chapter 6, we assess the immunological response of firebugs to symbiont perturbation. We do so by performing comparative transcriptomic analyses of genes expressed in the guts of aposymbiotic and control individuals and demonstrate a differential expression pattern for a range of anitimicorbial peptides that may play a role towards mediating the maintenance of a beneficial, conserved microbiota.

I discuss the implications of our findings with respect to the field of symbiosis in chapter 7.

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CHAPTER 2

AN OUT-OF-BODY EXPERIENCE: THE EXTRACELLULAR DIMENSION FOR THE TRANSMISSION OF MUTUALISTIC BACTERIA IN INSECTS

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2.1 ABSTRACT

Across animals and plants, numerous metabolic and defensive adaptations are a direct consequence of symbiotic associations with beneficial microbes. Explaining how these partnerships are maintained through evolutionary time remains one of the central challenges within the field of symbiosis research. While genome erosion and co-cladogenesis with the host are well-established features of symbionts exhibiting intracellular localization and transmission, the ecological and evolutionary consequences of an extracellular lifestyle have received little attention, despite a demonstrated prevalence and functional importance across many host taxa. The focus of this review is to discuss the implications of extracellular symbiont transmission mechanisms, using insect-bacteria symbioses as a model. The diverse routes by which extracellular symbionts are transmitted are unified by the common ability of the bacterium to survive outside of the insect host, thereby imposing different genomic, metabolic and morphological constraints than would be expected from a strictly intracellular lifestyle. Given the amenability of these systems to experimental manipulations, these symbioses serve as ideal models to explore the genomic, physiological, ecological, and evolutionary implications of intimate associations between hosts and their associated microbes.

"It's opener, out there, in the wide, open air"

— Dr. Seuss

2.2 INTRODUCTION

Through a variety of interactions, resident microorganisms have played a significant role in the origin and evolution of animals (McFall-Ngai *et al.,* 2013). Among animals, insects serve as excellent models to elucidate the functional importance of these interactions, since as a group, they engage in an unparalleled range of mutualisms with bacteria and fungi (Buchner, 1965). These associations have been demonstrated to benefit the insect most commonly through enhancements to metabolism, as well as through an assortment of defensive contributions aimed at protecting the host from pathogens, parasitoids, and predators (Moran, 2007; Douglas, 2009).

The remarkable diversity in form and function of insect-microbial interactions, however, can only be rivaled by the variety of symbiont transmission structures and behaviors that contribute towards the fixation, persistence and evolution of such partnerships (Buchner, 1965). In fact, for obligate mutualisms, successful symbiont transmission from one host generation to another constitutes an essential process, and as a result, specialized morphological, physiological, and behavioral adaptations have evolved in hosts as well as their bacterial symbionts to ensure the maintenance of these associations.

Some of the best-studied systems in insect-bacterial mutualisms have yielded extensive knowledge of intracellularly localized obligate symbionts. These mutualists can be transmitted in a number of ways during oogenesis or embryogenesis (Baumann, 2005), and the transmission mechanisms as well as the evolutionary implications of the intracellular lifestyle have been the focus of considerable attention. Many studies across different symbiotic associations provide evidence for host-symbiont co-cladogenesis and reductive genome evolution in the symbionts as a consequence of strict vertical transmission and reduced metabolic requirements that generally coincide with a permanent intracellular lifestyle (*e.g.* Baumann, 2005; Moran *et al.,* 2008).

Following a long-lasting focus on intracellular symbioses, research efforts of the past few decades have resulted in an enormous and steadily increasing body of knowledge on the diversity, function, and evolutionary history of extracellular symbionts in insects. Numerous studies indicate that specific extracellularly maintained and transmitted microbial symbionts play important roles across many of the major insect orders (Fig. 1; Supplementary Table 1). Given this wealth of recent data, it is now feasible to assess the fundamental ecological and evolutionary implications of these types of transmission routes for both host and microbe.

In this review, we build upon Paul Buchner's (1965) seminal and authoritative volume on the morphology of insect symbioses, and we tie this information to more recent studies based on experimental and molecular approaches. While providing an overview of extracellularly transmitted bacterial symbioses in insects, we discuss the evolutionary origin of such associations. We also emphasize the impact of transmission routes on the co-evolutionary trajectory of the symbioses and for the genome evolution of the symbionts. We focus primarily on symbiotic systems where (i) the bacterial partner has been molecularly characterized, (ii) a putative or demonstrated beneficial function

of the bacterial symbiont is proposed, and (iii) an extracellular transmission route is clearly described. We exclude symbioses involving fungal partners, since they have been recently reviewed by Gibson and Hunter (2010).

Box 1

Symbiosis:	Any long-lasting association between different species
Intracellular symbionts:	Symbionts localized within host cells (usually within the cytosol)
Extracellular symbionts:	Symbionts localized outside of host cells
Aposymbiosis:	Dissociation of a host from its symbiont
Vertical transmission:	Direct transfer of symbionts from parent to offspring
Horizontal transmission:	Indirect transfer of symbionts between con- or heterospecific host lineages
Co-cladogenesis:	Process of coinciding genetic divergence in host and symbiont lineages, usually due to vertical symbiont transmission, resulting in congruent phylogenies
Trophallaxis:	Social transfer of food among members of a community through mouth-to-mouth (stomodeal) or anus-to-mouth (proctodeal) feeding
Paratransgenesis:	Approach to control pathogens in vector populations through the introduction of exogenous genes via the vector's symbionts

2.3 OVERVIEW OF EXTRACELLULAR TRANSMISSION ROUTES OF INSECT SYMBIONTS

Despite broad functional and taxonomic diversity (Fig. 2; Supplementary Table 1), the vast majority of extracellularly transmitted symbionts can be unified by the ability of the bacterial partner to survive outside of its host for part, or all, of its lifetime. This feature markedly differentiates them from the majority of intracellular symbionts, where a free-living lifestyle is no longer possible, as is the case for *Buchnera* in aphids, and many other symbionts in insects feeding on nutritionally restricted diets like plant sap or vertebrate blood (Baumann, 2005; Douglas, 2009; Koga *et al.,* 2012).

Extracellular transmission routes for bacterial symbionts of insects include environmental determination, coprophagy, smearing of brood cell or egg surface, social acquisition, capsule transmission, or infection via milk gland secretions (Fig. 2; Supplementary Table 1). While most of these transmission routes are associated with vertical transmission of symbionts from parents to offspring, horizontal acquisition can occur regularly in systems with environmental uptake, coprophagy, and social transmission.

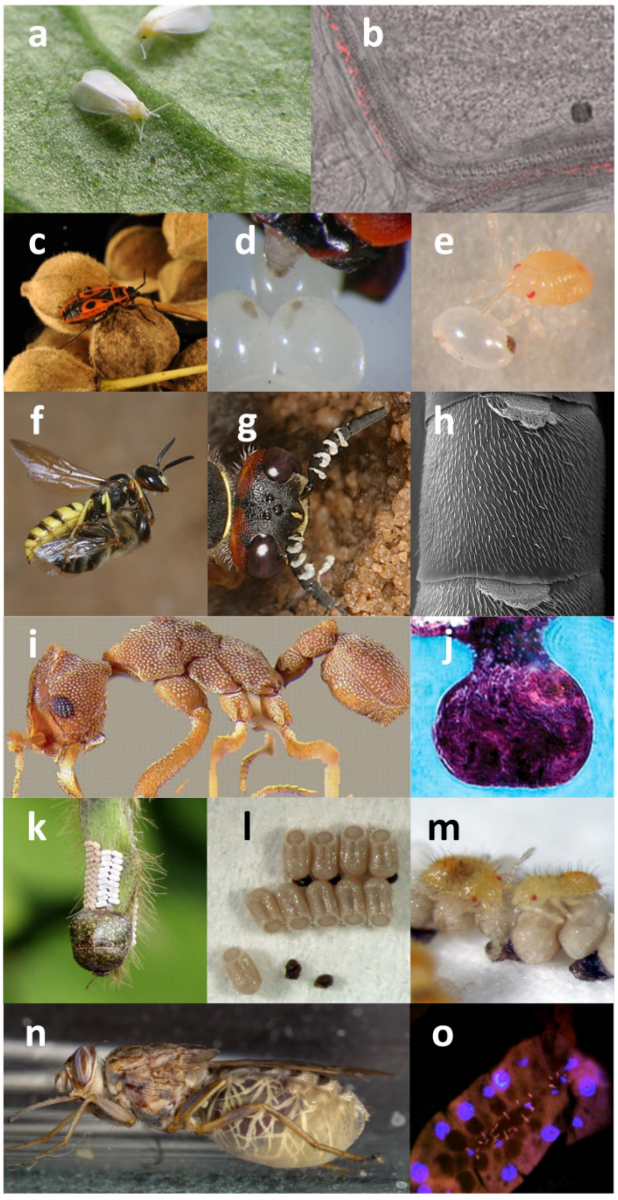

Figure 1. Transmission of *Rickettsia* among whiteflies (*Bemisia tabaci*) (**a**) involves the utilization of the insect's host plant (**b**). Transmission of beneficial gut symbionts in the European firebug (**c**) relies on anal secretions that are smeared over the egg surface following oviposition (**d**, **e**). Beewolves (**f**) cultivate and transmit the defensive symbiont '*Candidatus* Streptomyces philanthi' in specialized antennal gland reservoirs (**g**, **h**). Fungus-growing ants harbor defensive bacteria in specialized regions of their cuticle (**i**, **j**) and are transmitted via social behavior among nestmates. Beneath their egg mass, plataspid stinkbugs (**k**) deposit brown symbiont-bearing capsules (**l**) that are ingested by newly hatched nymphs (**m**) to initiate infection with a beneficial gut bacterium. A tsetse fly prior to larvipositing (**n**), as well as its nutritional mutualist *Wigglesworthia glossinidia* (shown in pink) in the milk glad lumen (**o**) where the bacteria are transmitted via milk secretions provided to the developing larva *in utero*.

2.3.1 Environmental determination

In animals, the acquisition of specific beneficial microbes from the environment is particularly prevalent in marine invertebrates including tubeworms, luminescent squids and sponges (Dubilier *et al.,* 2008; Nyholm and McFall-Ngai, 2004; Hentschel *et al.,* 2012). This can be partly attributed to protective benefits offered by aquatic environments against desiccation and UV-irradiation for free-living bacteria, as well as greater potential for long-range dispersal, which may facilitate acquisition by eukaryotic hosts. However, recent studies examining the microbial symbionts of broad-headed bugs and whiteflies demonstrated that terrestrial environments can also be a suitable source for the acquisition of beneficial microbes by insect hosts *de novo* every generation (Kikuchi *et al.,* 2007; Caspi-Fluger *et al.,* 2012).

Bean bugs (*Riptortus pedestris*), as well as many other stinkbugs within the Lygaeoidea and Coreoidea superfamilies, harbor environmentally acquired *Burkholderia* symbionts that localize primarily within crypts along their mid-gut section (Kikuchi *et al.,* 2007; 2011a). The environmental dimension of the symbionts' transmission route was first established following the inadvertent generation of developmentally regressed, aposymbiotic (symbiont-free) *R. pedestris* when the bugs were reared in sterile bottles. In fact, eggs laid in sterile laboratory settings by *Burkholderia*-infected individuals were also completely devoid of symbionts, strongly suggesting that the bugs acquired their free-living symbionts every generation from the environment, particularly the soil (Kikuchi *et al.,* 2007) – not unlike well-established plant-microbe partnerships involving rhizobia (Raymond *et al.,* 2004; Sullivan *et al.,* 1995). Similar to other systems that exhibit a "window of acquisition" during specific developmental stages (Nyholm and McFall-Ngai 2004), only juvenile insects in specific development stages can acquire symbionts (Kikuchi *et al.,* 2011b).

Among whiteflies (*Bemisia tabaci*), an environmental basis for the transmission of their widely occurring *Rickettsia* symbiont has also been described (Fig. 1a-b). However, in this system the transmission mechanism involves a direct utilization of the insect's host plant (Caspi-Fluger *et al.,* 2012). In *B. tabaci*, the α-proteobacterium has been described to greatly improve the overall fitness of infected individuals through enhancements to the insect's reproductive and developmental capacities (Himler *et al.,* 2011), which has contributed to the spread of the bacterium across natural populations of *Bemisia* at an unprecedented rate. The inconsistency in tree topologies of host and symbiont phylogenies suggests that the *Rickettsia* likely undergo substantial horizontal exchange between their insect hosts (Weinert *et al.,* 2009). Recent findings by Caspi-Fluger *et al.,* (2012) confirm this by demonstrating that infection frequencies of *Rickettsia* in *B. tabaci* hinges on the host plant, as demonstrated by the detection of the symbionts in the phloem of cotton, basil and black nightshade plants following feeding by an infected whitefly. Additionally, aposymbiotic individuals were successfully re-infected with the symbionts when allowed to feed on the same leaf (despite physical separation) as *Rickettsia*-infected *B. tabaci* (Caspi-Fluger *et al.,* 2012). These findings suggest that plants may serve as sinks for symbiont inoculants across a number of phloem-feeding insects, which, in turn, can have profound implications for the ecological and evolutionary success of the insect host.

2.3.2 Coprophagy

Acquisition of beneficial bacteria through conspecific probing of feces has been described as a predominant route of symbiont transmission for several Hemipteran (true bug) taxa (*e.g.*, Cydnidae, Coreidae, and Reduviidae) (Bourtzis and Miller, 2006). The symbionts usually reside in the insect gut, where they are shed alongside the gut lumen and excreted in feces (Hill *et al.,* 1976; Bourtzis and Miller, 2006). Symbiont acquisition by aposymbiotic individuals then requires direct contact with feces during or after excretion.

Interestingly, coprophagic symbiont transfer has been suggested to provide the opportunity for biological control of the reduviid bug *Rhodnius prolixus*, an important insect vector of the Chagas disease-causing parasite, *Trypanosoma cruzi* (Beard *et al.,* 2002). Despite near ubiquitous infection of adult *R. prolixus* in natural populations, newly hatched nymphs are aposymbiotic until they acquire their actinobacterial symbiont, *Rhodococcus rhodnii*, by probing conspecific feces (Hill *et al.,* 1976). The route of symbiont transfer, coupled with the bacterium's amenability for genetic transformation, could facilitate biological control via paratransgenesis, the introduction of exogenous genes via symbionts. (Beard *et al.,* 2002). Genes coding for specific trypanocidal compounds or antibodies targeting *T. cruzi's* major surface proteins have been successfully transformed into and expressed in the actinobacterial symbiont of *R. prolixus*, resulting in insects that are incapable of transmitting the parasite (Durvasula *et al.,* 1997). This highlights the potential for manipulation of extracellularly transmitted symbioses to decrease vector-borne diseases.

2.3.3 Social acquisition

Advanced social behavior in insects imposes different parameters for the transmission of microbial partners. For example, a central feature of many social and subsocial insects is the intimate interaction of conspecifics through behaviors such as trophallaxis, the transfer of food or other fluids through mouth-to-mouth (stomodeal) or anus-to-mouth (proctodeal) feeding (Vásquez *et al.,* 2012; Martinson *et al.,* 2012; Koch and Schmidt-Hempel, 2011). These behaviors can facilitate the exchange of microbes among nest members and thereby contribute to the maintenance of a beneficial microbiota. In fact, it has been speculated that the evolution of complex social forms could be reinforced, among other factors, by the convenience of acquiring beneficial microbes through recurring contact with conspecifics (Michael and Lombardo, 2007).

The extensively surveyed microbiota of honeybees (*Apis mellifera*) is composed of nine distinct bacterial strains that are consistent across different environments, geographic locations and host genotypes (Cox-Foster *et al.,* 2007; Martinson *et al.,* 2011; Mohr and Tebbe, 2006; Olofsson and Vasquez, 2008). Three common strains (*Gilliamella apicola*, *Snodgrassella alvi*, and *Lactobacillus kunkeei*) are extracellularly transferred to newly emerged workers via trophallaxis with older bees and/or exposure to the hive (Martinson *et al.,* 2011; 2012; Vasquez *et al.,* 2012).

Compared to honeybees, bumblebees (*Bombus* sp.) carry out trophallaxis at a lower frequency (Wilson, 1971). In this case, exposure of eclosing adults to feces from nestmates may constitute the main symbiont transmission route (Koch and Schmidt-Hempel, 2011). Molecular analyses carried out in three species of bumble bees (*B. sonorus, B. impatientis* and *Bombus* sp.) show that their microbial community is dominated by β-proteobacteria, including a highly abundant *Burkholderia* strain in

addition to the honeybee associates *S. alvi* and *G. apicola* (Martinson *et al.,* 2011; Koch *et al.,* 2013). Further examination of the transmission mechanisms that contribute towards the fixation of the dominant bacterial strains in this system revealed that *S. alvi* and *G. apicola* are transmitted vertically from the mother colony to daughter queens, and that social contact among nestmates following pupal emergence is required for intra-colony transmission (Koch *et al.,* 2013).

More broadly, recent examination of the gut microbiota across different bee species suggests that sociality plays an integral role for the maintenance of the distinctive microbial communities within the Apoidea superfamily (Cox-Foster *et al.,* 2007; Olofsson and Vasquez, 2008; Martinson *et al.,* 2011). While the majority of solitary bees examined by Martinson *et al.,* (2011) seem to be indiscriminately dominated by *Burkholderia* or *Wolbachia*, the social corbiculate clade (including *Bombus* and *Apis*) carry a largely conserved microbiota that may have co-evolved with the hosts as a byproduct of eusocial behavior.

Social transmission of symbionts has also been extensively described in ants, particularly among leaf-cutting ants (Attini: Formicidae). Here, the ants engage in a multipartite interaction to successfully fend off threats to their fungal gardens by the parasitic fungus *Escovopsis*, which can significantly compromise the ant colonies' overall fitness (Currie *et al.,* 1999, 2003, 2006).

Most attine ant genera extracellularly harbor Actinobacteria of the genus *Pseudonocardia* in specialized cuticular crypts (Currie *et al.,* 2006; Andersen *et al.,* 2013) (Fig. 1, i and j). These bacteria suppress growth of the parasite through production of antimicrobial compounds (Currie *et al.,* 1999; Oh *et al.,* 2009, Carr *et al.,* 2012). The presence of *Pseudonocardia* on foundress queens during their mating flight implies a vertical transmission route linking parent and offspring colonies (Currie *et al.,* 1999), and the singular association of each nest to individual *Pseudonocardia* strains further suggests that the symbionts proliferate among nest members via social behavior (Poulsen *et al.,* 2005; Andersen *et al.,* 2013). Comprehensive phylogenetic studies of both hosts and symbionts provide some support for host-symbiont specificity, while also demonstrating the possibility for horizontal exchange between colonies and direct uptake from the environment (Cafaro and Currie 2005; Mueller *et al.,* 2008; Cafaro *et al.,* 2011). In addition to *Pseudonocardia*, there is growing evidence that attine ants also associate with other antibiotic-producing actinomycete bacteria acquired from environmental sources (Kost *et al.,* 2007; Haeder *et al.,* 2009; Barke *et al.,* 2010; Seipke *et al.,* 2011).

As in Hymenoptera, transmission of beneficial bacteria in termites (Isoptera) is linked to sociality. Termites carry a complex microbiota consisting of Spirochaetes, Firmicutes, Bacteroidetes, Fibrobacteres and other taxa (Koehler *et al.,* 2012, Nalepa *et al.,* 2001; Hongoh 2010; Husseneder, 2010). The bacteria are located mainly on the epithelium, in the luminal fluid and, in the case of lower termites, also in extra- and intracellular associations with gut-inhabiting protists (Hongoh, 2010). Bacteria are transferred between nestmates by proctodeal trophallaxis, thereby resulting in neonates acquiring a concentrated microbial consortium known to enhance cellulolytic activity, nitrogen fixation and recycling of nitrogenous waste products (Nalepa *et al.,* 2001; Hongoh *et al.,* 2005; Hongoh, 2010; Husseneder, 2010; Koehler *et al.,* 2012).

2.3.4 Egg and oviposition site inoculation

Smearing bacteria over the surface of newly deposited eggs is one of the most commonly described routes of extracellular symbiont transfer in insects (Supplementary Table 1). Successful infection primarily depends on the ability of newly hatched nymphs or larvae to acquire their bacterial symbionts shortly after hatching, usually through active probing of egg or brood cell surfaces (Prado *et al.,* 2006; Kaltenpoth *et al.,* 2005; Hosokawa *et al.,* 2012).

Within the Hemiptera, numerous studies have reported on the role of egg smearing by the mother for successful transmission of beneficial microbes (Supplementary Table 1). For example, firebugs (Pyrrhocoridae) rely on excretion droplets that are later taken up from the egg surface by young nymphs soon after hatching for the successful transfer of the two co-occurring actinobacterial species *Coriobacterium glomerans* and *Gordonibacter* sp. (Kaltenpoth *et al.,* 2009; Sudakaran *et al.,* 2012; Salem *et al.,* 2013) (Fig. 1c-e). When prevented from symbiont acquisition through surface sterilization of newly laid eggs, resulting aposymbiotic individuals suffer retarded growth, higher mortality, and lower reproductive success (Salem *et al.,* 2013). Similarly, in stinkbugs of the family Acanthosomatidae, a γ-proteobacterial symbiont is harbored in cavities that are sealed off from the mid-gut main tract, as well as in a pair of lubricating organs associated with the female ovipositor (Kikuchi *et al.,* 2009). It is through these specialized organs that the symbionts are vertically transmitted via egg surface contamination. As for firebugs, aposymbiotic individuals (following egg surface sterilization) also suffer retarded growth, high mortality, and abnormal morphology.

In several fly species, introduction of bacteria into the oviposition site is a vital factor for effective transmission of their symbiotic bacteria. In the case of the Oriental fruit fly *Bactrocera dorsalis,* the dominant gut-associated bacteria, *Klebsiella oxytoca* and *Enterobacter sakazakii*, disperse from the female reproductive system and into the fruit where oviposition takes place (Shi *et al*, 2012). The closely related Mediterranean fruit fly *Ceratitis capitata*, which also uses fruits as an oviposition site, transmits symbiotic *K. oxytoca* as well as *Pectobacterium cypripedii* on the egg, either as a biofilm on the surface or occasionally inside the egg (Behar and Jurkevitch 2008). In both species, invasion of fruit by the symbiotic bacteria is pivotal since it facilitates bacterial proliferation and colonization within developing larvae.

Transmission of defensive *Streptomyces* symbionts of solitary digger wasps (*Philanthus* sp.) relies not on surface contamination of eggs but rather of brood cells where eggs are deposited (Kaltenpoth *et al.,* 2005; Goettler *et al.,* 2007) (Fig. 1, f – h). Prior to oviposition, female wasps secrete a symbiont-containing white substance from their antennal glands and onto the ceiling of brood cells. During cocoon spinning, larvae then take up the bacteria, which confer protection against pathogenic fungi through the provisioning of antibiotic substances on the cocoon (Kroiss *et al.,* 2010). Such defensive capabilities enable larvae to withstand many of the pathogenic threats that accompany their development during the hibernating stage in subterranean brood cells, where warm and humid conditions dominate (Kaltenpoth *et al.,* 2005).

2.3.5 Capsule transmission

Plataspid stinkbugs utilize one of the most specialized mechanisms for extracellular transmission at the oviposition site (Fukatsu and Hosokawa, 2002; Hosokawa *et al.,* 2005; 2006: 2007). In *Megacopta*

cribraria and *M. punctatissima*, adult females produce symbiont-enclosing "capsules", which they deposit among their newly laid egg masses to ensure the successful vertical transmission of their γ-proteobacterial mid-gut mutualist '*Candidatus* Ishikawaella capsulata' (Fig. 1, k – m) (Fukatsu and Hosokawa, 2002). Infection of newly hatched nymphs occurs upon oral ingestion of the capsule (Fukatsu and Hosokawa, 2002; Hosokawa *et al.,* 2005).

In addition to the adverse fitness effects associated with aposymbiosis (*e.g.* high juvenile mortality and slow development), capsule removal causes bugs to wander from the egg masses rather than resting in aggregation - the typical behavior following hatching (Hosokawa *et al.,* 2008). This suggests that insect behavior may be associated with increasing the likelihood of successful symbiont acquisition.

Symbiont acquisition by plataspids also impacts host plant utilization. *Megacopta punctatissima*, a pest species specializing on crop plants (*e.g.* soybean) in Asia owes its pest status to the genotype of its symbionts (Hosokawa *et al.,* 2007a). This was demonstrated when the symbionts of *M. punctatissima* and the labab-feeding stinkbug, *M. cribraria*, were exchanged across both species. While *M. cribraria* initially exhibited lower reproductive success when reared on a soybean-based diet with its native microbe, such trends were reversed once acquiring *M. punctatissima*'s symbiont (Hosokawa *et al.,* 2007a). Consistent with this finding, an invasive population of *M. cribraria* in North America, which is utilizing soybeans, has a symbiont population with an overall nucleotide and functional profile resembling that of the Asian pest-conferring symbionts in *M. punctatissima* (Brown *et al.,* 2013). These findings highlight that flexible symbiont acquisition mechanisms could facilitate interspecific symbiont switching and alterations of important traits such as host plant use.

2.3.6 Milk Gland Secretions

In tsetse flies (Diptera: Glossinidae), the nutritional endosymbiont *Wigglesworthia glossinidia* is transmitted via milk gland secretions, constituting a highly specialized adaptation to the insect's unique reproduction (Fig. 1, n and o) (Buchner, 1965; Attardo *et al.,* 2008; Rio *et al.,* 2012). The life cycle of tsetse flies involves egg and larval development *in utero*, during which nourishment is provided to the offspring via milk gland secretions until the third (and final) (Fig. 1n) developmental instar, when larvipositing takes place (Bequaert, 1956).

In adult tsetse flies, *W. glossinidia* is predominantly localized intracellularly in differentiated bacteriome-forming epithelial cells within a portion of the mid-gut (Aksoy, 1995), but a second population of *W. glossinidia* persists within the lumen of the milk glands (Attardo *et al.,* 2008) (Fig. 1o). Via milk gland secretions, the symbionts are extracellularly transmitted to the developing offspring in the mother's uterus (Buchner 1965; Attardo *et al.,* 2008). Thus, in contrast to all of the aforementioned systems, *Wigglesworthia* remains continuously housed within its insect host despite an extracellular transmission route.

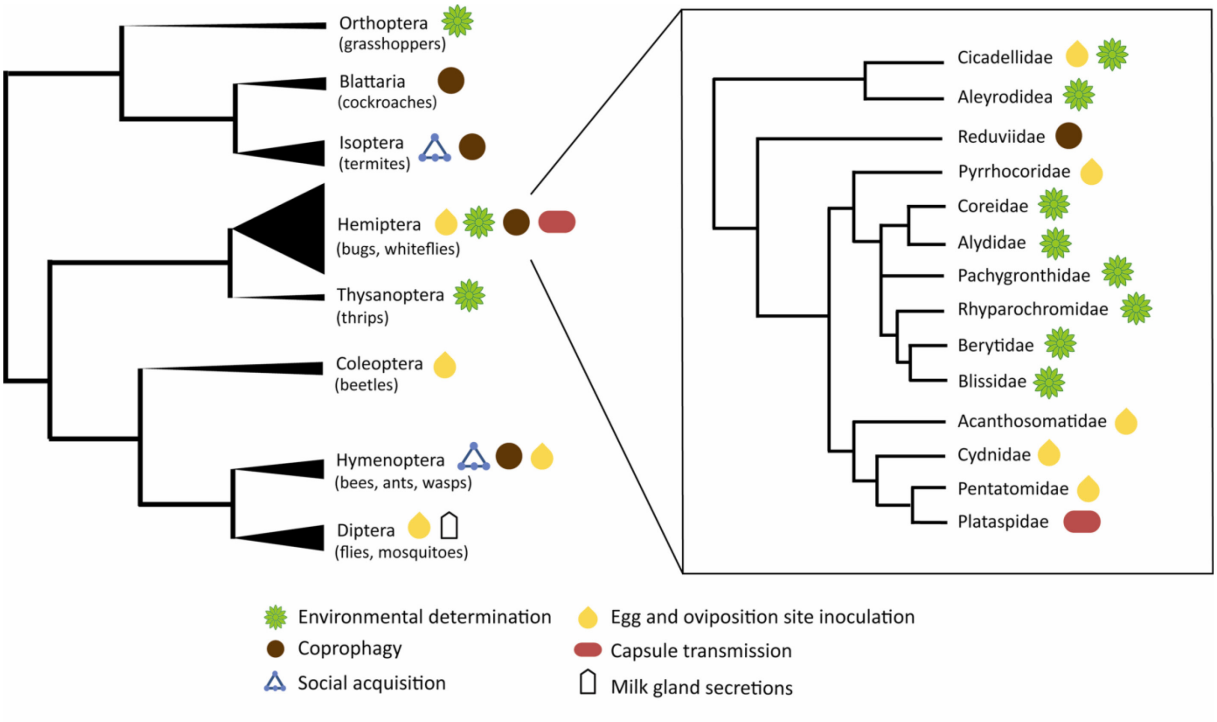

Figure 2. Cladogram depicting the diversity of insect orders with known extracellularly transmitted bacterial symbionts (as listed in Supplementary Table 1). Symbols indicate extracellular transmission routes. Terminal branch thickness is proportional to the number of families within the order known to exhibit extracellular transmission.

2.4 EVOLUTION OF MUTUALISTIC ASSOCIATIONS

Upon characterizing symbiont transmission mechanisms as adaptations to ensure the maintenance of mutualisms, it becomes imperative to understand the factors that initially contributed towards the emergence and fixation of the partnership, as well as the possible evolutionary transitions among transmission routes. Insects that rely on extracellular routes for the transmission of their beneficial microbes present us with excellent systems to address such topics, given the diversity in evolutionary states of the symbioses, ranging from facultative and horizontally acquired, to obligate and vertically transmitted.

The origin of bacterial mutualisms, however, remains one of the most elusive questions within the field of symbiosis (Szathmary and Smith, 1995). Traditionally, two hypotheses have been suggested for the initial evolution of microbial symbioses (Ewald, 1987). The first hypothesis suggests a parasitic ancestor for the microbial partner, where loss of virulence and improvement of mutualistic traits were selected for by vertical transmission and the coincident alignment of evolutionary fates for both host and microbe. This notion is supported by evidence for the evolutionary transition of *Sodalis glossinidius* from an entomopathogen to an intracellular secondary endosymbiont in tsetse flies (*Glossina* spp.) (Dale *et al.,* 2001), and the primary endosymbiont of weevils (Dale *et al.,* 2002). However, to our knowledge, examples for the transition from parasitism to mutualism involving extracellularly transmitted symbionts of insects are thus far lacking. The second hypothesis suggests that microbial mutualists instead evolved from environmental bacteria, where a free-living bacterial ancestor possessed traits that could offer immediate benefits to the host. Recent phylogenetic analyses

demonstrate the plausibility and occurrence of both scenarios (Sachs *et al.,* 2011) while emphasizing that evolution of bacterial mutualists from environmental strains appears to be more common.

For extracellularly transmitted symbionts, there are several systems consistent with environmentally acquired bacteria providing immediate benefits to their hosts upon establishment. For example, in *Rhizobium* symbioses with leguminous plants, the nitrogen fixing ability of the soil microbe, coupled with the gain of a core set of symbiosis loci as selected for by access to a metabolically stable environment (*i.e.* the host), enabled the establishment and maintenance of a cosmopolitan mutualistic partnership (Raymond *et al.,* 2004; Sullivan *et al.,* 1995). Analogous mutualisms that exemplify this rhetoric have also been described for insects. Within the bean bug's (*R. pedestris*) association with *Burkholderia*, recent findings by Kikuchi *et al.,* (2012) demonstrate that under certain conditions, the environment can select for the optimal symbiont; which, in turn, can inadvertently align the bilateral benefits of host and symbiont without the prerequisite of strict vertical transmission. Agricultural fields that have been heavily treated with the insecticide fenitrothion select for *Burkholderia* strains capable of degrading the compound (Kikuchi *et al.,* 2012). When reared on soybean seeds treated with fenitrothion, *R. pedestris* nymphs carrying insecticide-degrading *Burkholderia* exhibit significantly higher survival rates compared to bugs infected with the non-degrading strains (Kikuchi *et al.,* 2012).

In solitary beewolf wasps, phylogenetic analyses indicate that defensive *Streptomyces* symbionts have evolved from free-living ancestors (Kaltenpoth *et al.,* in press). Their capacity for producing antimicrobial compounds was probably originally adaptive because it provided a competitive advantage over other microorganisms in the soil environment. Upon acquisition by the beewolf and incorporation into the larval cocoon, the symbiont-mediated antimicrobial protection likely provided an immediate benefit to the host, so adaptations to maintain and nourish the symbionts were selectively favored.

For environmental acquisition of symbionts to be evolutionarily stable, the benefits of the transmission mechanism must outweigh one significant cost: the risk of not acquiring the symbiont. For example, for *R. pedestris* and several other bug species, failure to pick up specific bacterial symbionts during early stages of development significantly affects fitness (Kikuchi *et al.,* 2007). Similarly, the uptake of a suboptimal symbiont (*e.g.* a *Burkholderia* strain incapable of degrading insecticides) also poses risks for the host in terms of competitively subsisting on resources in a specific niche (*e.g.* insecticide-treated crops) (Kikuchi *et al.,* 2012). Interestingly, across the true bugs, there are multiple independent origins of *Burkholderia* symbioses, many, and possibly all, of which are presumed to be dependent on environmental acquisition (Kikuchi *et al.,* 2011a). These bug-*Burkholderia* partnerships are interspersed amongst other systems that utilize vertical transmission through both internal and external mechanisms. One possibility is that environmental acquisition is selected for in fluctuating environments when the symbiont genotype that is most optimal varies significantly across space or time. Thus, being able to switch partners, possibly to actively select the optimal partner or to allow potential partners to outcompete one another, may supersede the costs of risking the failure to obtain a partner at all.

This raises interesting questions relating to the initial stages of symbiont colonization; specifically, what are the mechanisms that mediate the recognition and uptake of the right symbiont, and how does the host select for these microbes while eliminating less beneficial ones?

Mechanisms could be behavioral, in which insects actively seek out symbionts with certain traits, or physiological, in which insects take in a diversity of microbes and then actively winnow down associations to a narrow few. There has been little exploration of the former, but there is increasing evidence for the latter. As previously discussed, *R. pedestris* possess remarkably efficient symbiont detection and uptake mechanisms, where a mere 80 *Burkholderia* cells in a gram of soil are sufficient for successful infection (Kikuchi and Yumoto, 2013). The efficient establishment of only a small subset of the diverse microbes that these bugs encounter in soil is mediated, at least in part, through complex anatomical modifications of the midgut, resulting in tight junctions capable of selectively "sieving" microbes (Ohbayashi *et al.,* unpublished). Such junctions only allow for bacteria with flagellar motility to pass through and colonize the specialized crypts (Ohbayashi *et al.,* unpublished), in a process analogous to the initiation of infection threads in the squid-*Vibrio* symbiosis (Nyholm and McFall-Ngai, 2004). These anatomical features seem to be paired with immunological processes that prevent growth of some bacteria and may tightly regulate growth of symbionts (Kim *et al.,* 2013a, b; Futashi *et al.,* 2013; Garcia *et al.,* 2014). Antimicrobial peptides isolated from the hemolymph of the *Burkholderia*-harboring coreoid *Alydus calcaratus,* for example, can suppress growth of some soil-dwelling gram negative bacteria (*e.g., Escherichia coli*), but not *Burkholderia* symbionts (Garcia *et al.,* 2014). Comparative transcriptomic analyses of the mid-guts of symbiont-containing and aposymbiotic *R. pedestris* revealed upregulation of cysteine-rich secretion proteins, which could inhibit growth of some bacteria and which could also function to regulate symbiont populations (Futahashi *et al.,* 2013). These findings are complemented by evidence suggesting that *Burkholderia* symbiont establishment and proliferation requires the bacteria to have several genes necessary for combating host-induced stress (Kim *et al.,* 2013a, b). This suggests that the host's anatomy and immune system may be under selection to suppress proliferation of non-symbionts while allowing for regulated growth of symbiont populations. *Burkholderia* may have become the primary symbionts because they possessed the necessary features to facilitate establishment (*e.g.,* motility, resistance to host antimicrobial activity) prior to and independent of any host-mediated selection.

Another factor that could favor the evolution of environmental acquisition would be the ubiquity of the microbe in the environment. Common occurrence of beneficial symbionts in the environment could relax selection to maintain vertical transmission mechanisms. To date, we have little data on the prevalence of beneficial symbionts in environmental reservoirs in systems where environmental acquisition is known (though see Garcia *et al.,* 2014, Kikuchi *et al.,* 2007; Kost *et al.,* 2007). Further insight into factors favoring the evolution of environmentally acquired mutualisms will require such environmental sampling as well as characterization of symbiosis mechanisms across groups of insects, such as has been started, to some extent, for the true bugs (Kikuchi *et al.,* 2011a).

2.5 EVOLUTIONARY TRANSITIONS AMONG TRANSMISSION ROUTES

Given the diverse mechanisms of extracellular transmission and their varying ecological and evolutionary implications, it is tempting to speculate on likely scenarios of evolutionary transitions between transmission routes. Considering that most symbiotic associations in insects appear to have evolved from free-living rather than pathogenic or commensal bacteria (Sachs *et al.,* 2011), environmental acquisition is likely the ancestral transmission route for many partnerships. However, due to the high selective pressures of endowing beneficial symbionts efficiently to the offspring, the

majority of specific mutualistic insect-bacteria interactions appear to transition to vertical or pseudo-vertical transmission routes over the course of evolution.

Extracellular nutritional symbionts are usually localized within the gut, and significant numbers of cells are often shed and excreted along with fecal matter (Bourtzis and Miller, 2006; Kaltenpoth *et al.,* 2009). Thus, transmission via feces (*i.e.,* coprophagy and proctodeal trophallaxis) constitutes a simple transitory step from environmental acquisition to vertical transmission as it does not require any specialized morphological adaptations of the host (Buchner, 1965). In taxa with social interactions between parents and offspring or within groups of related or unrelated conspecifics, direct transfer of feces by proctodeal trophallaxis also ensures symbiont transmission along with the provisioning of enzymes that may facilitate digestive processes in immature individuals (Nalepa *et al.,* 2001). Non-social insects without direct contact between symbiotic and aposymbiotic individuals, on the other hand, can increase the probability of successful transmission to the offspring by applying symbiont-containing feces to locations that have high chances of being frequented and probed by the hatching larvae (Buchner 1965). The egg surface is the most commonly used and reliable place of symbiont application and uptake (*e.g.* Buchner, 1965, Prado *et al.,* 2006; Salem *et al.,* 2013, Hosokawa *et al.,* 2012), but in special cases with locally confined developmental conditions, the brood cell surface can be equally suitable (Kaltenpoth *et al.,* 2005).

Coincident with, or subsequent to, an increased fidelity in vertical symbiont transmission, several insects with extracellular symbionts have evolved specialized structures to house and transmit symbionts to the offspring (*e.g.* Hosokawa *et al.,* 2012, Fukatsu and Hosokawa, 2002). These derived adaptations can be advantageous for the host, as they allow for enhanced control over the identity and number of symbiont cells allocated to offspring and thereby reduce the risks of co-transmitting potentially pathogenic microbes or provisioning either excessive or non-sufficient numbers of viable symbionts for successful colonization (Hosokawa *et al.,* 2007b). This is also consistent with theoretical predictions implicating the restriction of symbiont migration as a mechanism adopted by the host in order to reduce virulent tendencies arising from competition between heterospecific symbiont lineages (Frank, 1996).

Finally, the highest integration of host and symbiont occurs when symbiont transmission is internalized within the host's body. Starting out with an extracellular symbiosis, however, this can only be achieved through (i) a shift in the symbiont's lifestyle to intracellular maintenance and transmission to the developing oocyte (*e.g.*, *Buchnera* symbionts in aphids), or (ii) by internalizing parts of the juvenile development and maintaining extracellular transmission, as in tsetse flies (Balmand *et al.,* 2013).

2.6 IMPLICATIONS FOR SYMBIONT GENOME EVOLUTION

Obligate mutualisms can have a strong effect on the genome evolution of the bacterial partner. As exemplified by *Buchnera* and *Blochmannia* – the primary intracellular endosymbionts of pea aphids and carpenter ants, respectively – symbiont genomes can undergo strikingly convergent patterns of degradation and reduction (Baumannn, 2005; Feldhaar *et al.,* 2007; Moran *et al.,* 2009). These small genomes are coupled with extensive AT nucleotide enrichment and accelerated molecular evolution (Shigenobu *et al.,* 2000; McCutcheon and Moran, 2007). Such patterns of genome erosion and AT

bias have been reported for a multitude of intracellular primary symbionts of insects (Moran *et al.,* 2008), including the extracellularly transmitted, yet intracellularly localized *Wigglesworthia* mutualists of tsetse flies (Akman *et al.,* 2002). These features are presumed to be driven by gene loss resulting from a combination of strong genetic drift in small populations undergoing severe bottlenecks during transmission, and relaxed selection to no longer maintain genes necessary for an extracellular lifestyle.

As a result, many of the aforementioned genomic features became consequential hallmarks of an intracellular lifestyle within animal hosts (Moran and Wernegreen, 2000). Thus, it seemed unlikely that extracellularly transmitted and localized symbionts would undergo similar patterns of reductive genome evolution, considering that many of these microbes reside outside of the insect host for part, or all, of their life cycle, where they can readily undergo recombination to offset gene loss due to genetic drift, and generally need to retain a larger set of genes to survive in less stable environmental conditions and to move between various habitats (e.g, different host tissues and outside of hosts). To a certain extent this is true. For example, within *Wigglesworthia's* highly reduced genome (697 kb), the bacterium retains the ability to express a metabolically expensive flagellum (Akman *et al.,* 2002), suggesting that mobility remains an important feature for the symbiont, possibly to facilitate infection of the developing larva (Rio *et al.,* 2012). This is further supported by the specific up-regulation of *W. glossinidia*'s flagellar motility genes during the maternal transmission process, as well as during larval intrauterine development (Rio *et al.,* 2012). In contrast to strictly intracellular bacterial mutualists with similarly diminished genomes, flagellar retainment in *Wigglesworthia* serves as clear example of the stringent selective pressures exerted on extracellularly transmitted microbes to preserve features that are vital to their ecology outside of insect cells.

Studies examining genomic features of some extracellularly transmitted symbionts indicate that similar evolutionary processes can be exhibited in these symbionts as those restricted to a strict intracellular lifestyle (Hosokawa *et al.,* 2006; Kikuchi *et al.,* 2009: Nikoh *et al.,* 2011) (Fig. 3; Table 1).

Extracellularly transmitted symbionts of plataspid (capsule transmission) and acanthosomatid (egg smearing) stinkbugs possess highly reduced genomes estimated around ~ 0.8 and 0.9 Mb, respectively (Hosokawa *et al.,* 2006; Kikuchi *et al.,* 2009; Nikoh *et al.,* 2011). Furthermore, *Ishikawaella* isolated from *M. punctatissima* has other genomic features reminiscent of intracellular symbiotic bacteria, namely AT nucleotide bias and few mobile elements (Nikoh *et al.,* 2011). Examination of *Ishikawaella*'s metabolic potential reveals that despite significant gene loss, the bacterium retains the ability to synthesize almost all essential amino acids, in addition to some vitamins and cofactors (Nikoh *et al.,* 2011). This is consistent with the suggested benefit of the symbiont for *M. punctatissima*, which feeds on plant sap that is very poor in essential amino acids and certain vitamins.

Despite exhibiting similar patterns of reductive genome evolution, pairwise comparisons of gene profiles between *Ishikawaella* and a range of intracellular-localized symbionts revealed a number of important discrepancies that may reflect their different ecologies (Nikoh *et al.,* 2011). Most prominent was the complete retainment of genes involved in the TCA cycle, as well as many other genes underlying energy production and conversion. This was attributed to the more stringent metabolic requirements of an extracellular lifestyle, where access to metabolic intermediates in the host cytoplasm is not an option, unlike for many obligate intracellular symbionts. Additionally, *Ishikawaella* possesses a greater number of genes involved in the synthesis of amino acids and

cofactors than *Buchnera* (aphids), *Blochmannia* (ants), or *Wigglesworthia* (tsetse flies), a condition that implies a broader metabolic repertoire for supplementation, and/or a younger co-evolutionary history with its host.

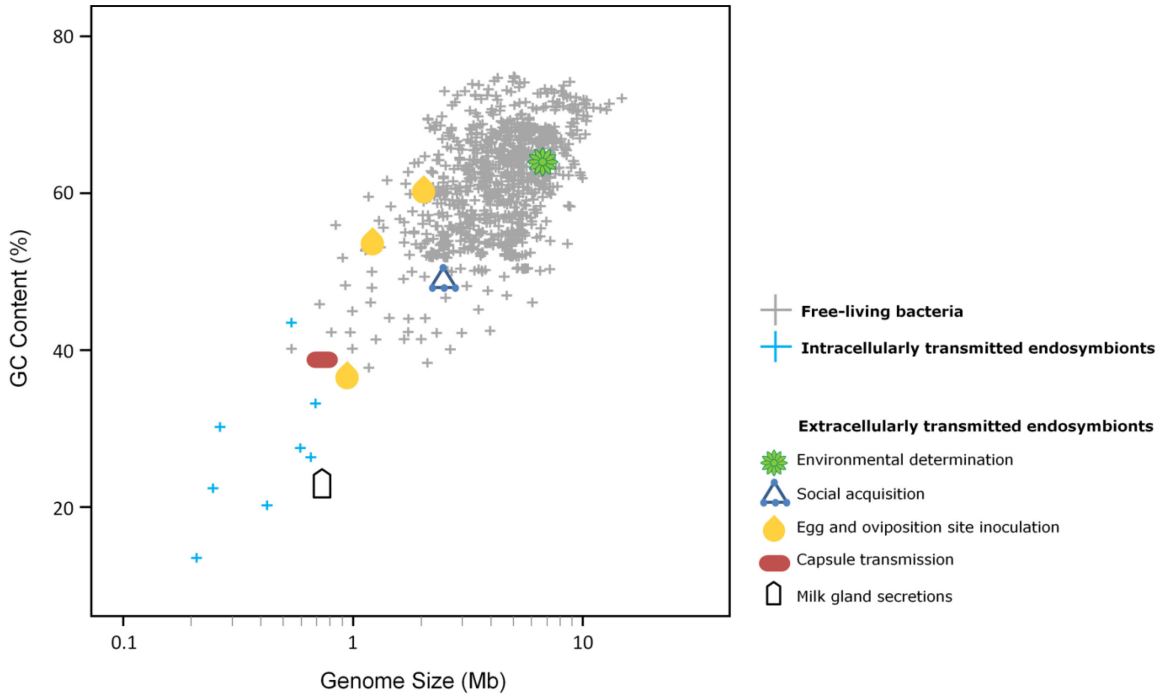

Figure 3. Relationship between genome size and GC content for intra- and extracellularly transmitted bacterial symbionts in insects (per Supplementary Table 2), as well as free-living taxa. Symbols indicate symbiont transmission route (intracellular vs. extracellular) and biotic condition (symbiotic vs. free-living).

The genomic features of these symbionts provide insight into the evolutionary forces driving genome reduction in obligate microbial mutualists (Hosokawa *et al.,* 2006; Kikuchi *et al.,* 2009: Nikoh *et al.,* 2011) by demonstrating that that these convergent traits are not strictly a consequence of an intracellular lifestyle but rather are more likely due to increased impact of genetic drift associated with a host-restricted lifestyle. This highlights small population sizes and strong bottlenecks promoted by spatial isolation, prior to and/or during transmission, as important factors for genome evolutionary patterns in heritable symbionts.

In line with these considerations are the large, GC-rich genomes of socially-transmitted and environmentally-acquired symbionts of termites and bean bugs, respectively (Fig. 3) (Graber *et al.,* 2004; Shibata *et al.,* 2013). For these microbes, exposure to other bacteria in the nest or soil possibly facilitates genetic recombination, thereby offsetting the possible degenerative consequences associated with genetic drift as a result of population bottlenecks during transmission; an integral feature complementing their physiological requirements during their extensive free-living phase.

These findings, coupled with analyses of a broad range of bacterial genomes demonstrating a clear inverse correlation linking genome size and the incidence of genetic drift (Kuo *et al.,* 2009) further

support the concept that reductive genome evolution can be associated with intracellularity but is not necessarily derived from it (Hokosawa *et al.,* 2006; Kikuchi *et al.,* 2009; Nikoh *et al.,* 2011).

Table 1. Evolutionary implications of symbiont transmission routes in insects.

Insect Host (Order: Family)	Bacterial Symbiont	Symbiont Localization	Transmission Route	Maternal Provisioning of Symbionts	Specialized Structures for Symbiont Cultivation or Transmission	Strict Host Symbiont Co-cladogenesis	Symbiont Genome Erosion	References
***Acyrthosiphon* spp. (Hemiptera: Aphididae)**	*Buchnera aphidicola*	Bacteriome	Intracellular: During embryogenesis	+	+	+	+	Shigenobu *et al.,* 2000; Baumann *et al.,* 2005
***Camponotus* spp. (Hymenoptera: Formicidae)**	*Blochmannia* spp.	Mid-gut epithelial cells	Intracellular: Infection of the ovaries	+	+	+	+	Feldhaar *et al.,* 2007
***Glossina* spp. (Diptera: Glossinidae)**	*Wigglesworthia glossinidia*	Bacteriome, milk glands	Extracellular: Milk gland secretions	+	+	+	+	Aksoy *et al.,* 1997; Akman *et al.,* 2002; Rio *et al.,* 2012
***Megacopta* spp. (Hemiptera: Plataspidae)**	*'Candidatus* Ishikawaella capsulata'	Mid-gut crypts	Extracellular: Symbiont capsule	+	+	+	+	Hosokawa *et al.,* 2006; Nikoh *et al.,* 2011
***Elasmostethus* spp. (Hemiptera: Acanthosmatidae)**	γ-Proteobacteria	Mid-gut crypts, 'lubricating organs'	Extracellular: Egg smearing	+	+	+	+	Kikuchi *et al.,* 2009
***Philanthus* spp. (Hymenoptera: Crabronidae)**	'*Candidatus* Streptomyces philanthi'	Antennal gland reservoirs	Extracellular: Brood cell smearing	+	+	+/-	Unexamined	Kaltenpoth *et al.,* 2005; in press
***Plautia* spp. (Hemiptera: Pentatomidae)**	*Erwinia* spp.	Mid-gut crypts	Extracellular: Egg smearing	+	-	-	Unexamined	Prado *et al.,* 2006; 2009
***Apis* spp. and *Bombus* spp. (Hymenoptera: Apidae)**	*Snodgrassella alvi, Gilliamella apicola*	Mid-gut lumen	Extracellular: Social transmission	+/-	-	-	Unexamined	Koch and Schmid-Hempel 2011, Koch *et al.,* 2013, Martinson *et al.,* 2012
***Riptortus pedestris* (Hemiptera: Alydidae)**	*Burkholderia* spp.	Mid-gut crypts	Extracellular: Environmental uptake	-	-	-	-	Kikuchi *et al.,* 2011a; Shibata *et al.,* 2013

2.7 HOST-SYMBIONT CO-EVOLUTIONARY DYNAMICS

Acquisition of complex traits and adaptations by insects to ensure that their progeny are endowed with beneficial microbes often results in symbiotic systems that are evolutionary stable and mutually obligatory. The high fidelity exhibited by these partnerships can be quantified (and visualized) through the congruent branching patterns of host and symbiont phylogenies in what is commonly referred to as co-cladogenesis (Moran *et al.,* 2008).

With co-cladogenesis as a measure for mutualism fidelity, inferring phylogenetic relationships between symbiotic bacteria relative to their insect hosts can provide insights into the evolutionary implications of different routes of symbiont transmission. Bugs of the Pentatomomorpha infraorder (*e.g.*, stinkbugs, shield bugs, etc.), whose bacterial partners colonize similar gut regions but utilize different transmission routes and modes to initiate infection (Fig. 4), provide a point of comparison for consequences of transmission routes on host-symbiont evolution. In members of this group that acquire beneficial bacteria via the environment (*e.g.* soil), there are numerous incongruencies between symbiont and host phylogenies (Kikuchi *et al.,* 2011a). Some host species harbor different symbiont genotypes, while others share a single identical symbiont (Kikuchi *et al.,* 2011a; Garcia *et al.,* 2014) (Fig. 4a). This lack of fidelity is driven by the bugs acquiring symbionts from a potentially diverse, shared environmental reservoir. Conversely, in instances where the gut symbionts are transferred directly from mother to progeny in a monoclonal manner, aided by symbiont-bearing structures and/or intimate behavioral responses, a remarkably convergent evolutionary history is often observed between host and microbe (Hosokawa *et al.,* 2006; 2012; Kikuchi *et al.,* 2009) (Fig. 4, b and c; Table 1).

For example, Acanthosomatidae shieldbugs and their symbionts, which are transmitted via egg smearing, exhibit near strict co-cladogenesis (Kikuchi *et al.,* 2009) (Fig. 4b), and among stinkbugs of the family Plataspidae, vertical transmission of their gut mutualist via symbiont capsules results in strict host-symbiont phylogenetic congruence (Hosokawa *et al.,* 2006) (Fig. 4c). Thus, despite consistent localization of symbionts in similar mid-gut environments within the host, differences in extracellular transmission mechanism alter patterns of co-cladogenesis and, more broadly, have consequences for co-evolution. Specifically, in cases where there is strict vertical transmission, bacterial and host fitness is strictly aligned even in the absence of intracellular maintenance and transmission. However, when symbionts are environmentally acquired, there is a loss in host-symbiont fidelity and reduction in the alignment of host and bacterial interests. In the former case, we can expect that host and symbionts are both evolving in response to one another. In the latter case, much of the bacteria's adaptation may be shaped by forces external to a given host.

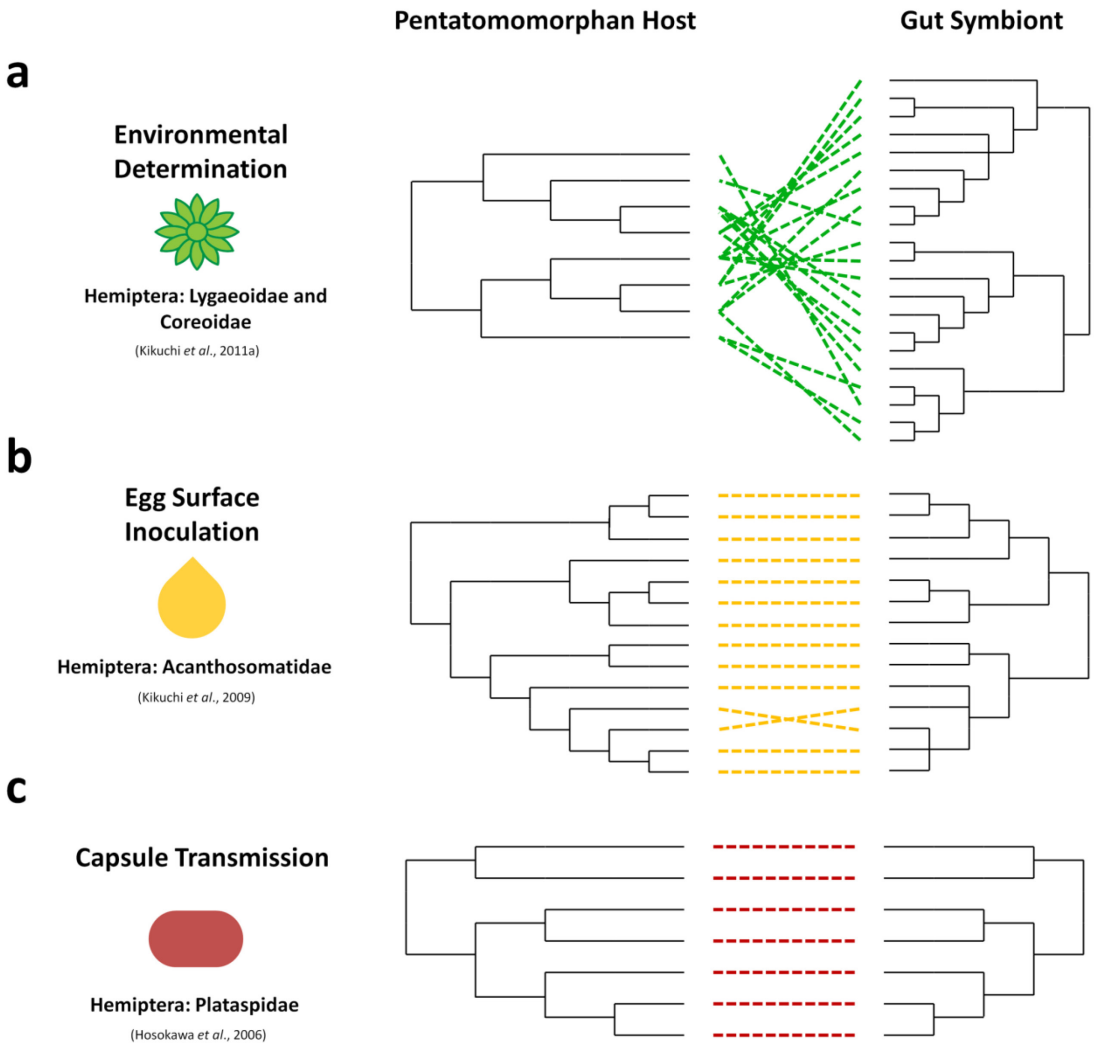

Figure 4. Comparisons of evolutionary relationships between pentatomoid bugs and their gut symbionts as it relates to the symbionts' extracellular transmission routes. These relationships were established for (**a**) Lygaeoidae and Coreoidae families and their environmentally acquired *Burkholderia* strains, as well as for the (**b**) Acanthosomatidae and (**c**) Plataspidae families relying on egg smearing and symbiont capsules, respectively, for the transmission of their γ-proteobacterial symbionts. The relationships among the Lygaeoidae/Coreoidae and their gut symbionts were established using mitochondrial COI gene sequences for the host (Yoshitomo Kikuchi, personal communication) and 16S rRNA gene sequences for the *Burkholderia* symbionts (Kikuchi *et al.,* 2009). Phylogenies for the Acanthosomatidae and Plataspidae families in relation to their gut microbes were adapted from Kikuchi *et al.,* (2009) and Hosokawa *et al.,* (2006), respectively.

2.8 CONCLUSIONS AND FUTURE PERSPECTIVES

In addition to providing insights into evolutionary aspects of symbiosis, insect-bacterial mutualisms that rely on extracellular mechanisms for symbiont transmission present excellent opportunities to elucidate functional aspects of the partnerships. Given a transiently aposymbiotic phase during the early stages of insect development, alongside the ability of the microbe to survive outside of the host's body for part of its lifetime - two conditions generally shared across the aforementioned systems - it is

in many cases experimentally feasible to physically separate both partners by disrupting the transmission cycle (*e.g.*, Hosokawa *et al.,* 2006, 2007; Prado *et al.,* 2006; Kikuchi *et al.,* 2009; Koch *et al.,* 2013; Salem *et al.,* 2013). Such experiments have been successfully employed to elucidate symbiont contributions towards host fitness, to assess host-symbiont specificity, and to detail the effects of symbiont replacement for host ecology (Hosokawa *et al.,* 2007; Salem *et al.,* 2013). They will undoubtedly provide additional insights into ecological aspects of insect symbioses.

Furthermore, the extracellular nature of the symbionts contributes to the likelihood that they can be cultured and genetically manipulated, which is not possible for most intracellular symbionts (but see Dale and Maudlin, 1999, Dale *et al.,* 2006). *In vitro* cultivation and manipulation can facilitate introduction of genetically modified symbionts into their insect hosts. Thereby, the importance of candidate symbiont genes for establishment or maintenance of a mutualistic association, as well as for the fitness benefits conferred to the host, can be directly assessed (Kim *et al.,* 2013a, b). Additionally, this strategy may prove valuable to manage agricultural pest species or disease vectors by modification of their symbionts (Beard *et al.,* 2002). Combining symbiont manipulation with targeted knock-down of host genes potentially involved in mediating symbiosis (Login *et al.,* 2011) will undoubtedly provide unprecedented opportunities to study host-symbiont molecular interactions and investigate the genomic and physiological underpinnings of insect-microbe symbioses.

Finally, as opposed to intracellular mutualists, which are almost exclusively known for invertebrates (but see Kerney *et al.,* 2011), extracellularly localized and transmitted symbionts are widespread in vertebrates and play an important role for human health (Funkhouser and Bordenstein, 2013). However, the complexity of vertebrate-associated microbial communities complicates experimental assessment of individual symbiont functions as well as host-microbiota molecular interactions. Insects, on the other hand, represent tractable model systems to investigate microbial contributions to host fitness, their interactions with the host immune system, and implications for health and disease. Thus, studies on insect-microbe symbioses can provide valuable insights to guide biomedical research in vertebrates by elucidating fundamental principles of the mechanistic basis and evolutionary dynamics of symbiotic interactions.

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Supplementary Table 1. Diversity of extracellularly transmitted bacterial mutualists in insects. Only those associations ae listed for which the molecular identification and/or characterization of the symbionts are available, and a description of the transmission route as well as at least a putative function is reported. Exemplary references are given for each symbiosis (not exhaustive).

Insect Host		Bacterial Symbiont		Transmission Route	(Putative)[a] Biological Function	References
Order	**Family - Genus/Species**	**Phylum**	**Genus/Species**			
Hemiptera	Plataspidae - Various species	γ-proteobacteria	*'Candidatus* Ishikawaella capsulata'	Capsule transmission	Nutrition[a]	Fukatsu and Hosokawa (2002) ; Hosokawa *et al.,* (2005); Hosokawa *et al.,* (2006); Nikoh *et al.,* (2011)
	Acanthosomatidae - Various species	γ-proteobacteria	*'Candidatus* Rosenkranzia clausaccus'	Egg smearing: gland secretion	Nutrition[a]	Kikuchi *et al.,* (2009)
	Parastrachiidae - *Parastrachia japonensis*	γ-proteobacteria	*'Candidatus* Benitsuchiphilus tojoi'	Egg smearing: gland secretion	Nutrition: Uric acid recycling[a]	Kashima *et al.,* (2006) Hosokawa *et al.,* (2012)
	Pentatomidae - *Plautia stali*	γ-proteobacteria	*Erwinia* sp.	Egg smearing: feces	Nutrition: vitamin supplementation[a]	Prado *et al.,* (2006)
	Pyrrhocoridae - Various species	Actinobacteria	*Coriobacterium glomerans* *Gordonibacter* sp.	Egg smearing: feces	Nutrition: B vitamin supplementation	Kaltenpoth *et al.,* (2009); Salem *et al.,* (2013)
	Lygaeoidea and Coreoidea Superfamilies - Various species	β-proteobacteria	*Burkholderia* sp.	Environmental determination	Nutrition[a], Insecticide resistance	Kikuchi *et al.,* (2007, 2011, 2012)
	Reduviidae - *Rhodnius prolixus*	Actinobacteria	*Rhodococcus rhodnii*	Coprophagy	Nutrition: B vitamin supplementation	Beard *et al.,* (2002)
	Aleyrodidae - *Bemisia tabaci*	α-proteobacteria	*Rickettsia* sp.	Environmental determination	Nutrition Reproductive enhancement	Caspi-Fluger *et al.,* 2011; Himler *et al.,* 2011
	Cicadellidae - *Scaphoideus titanus*[b]	α-proteobacteria	*Asaia* sp.	Egg smearing : bacterial colonies on egg surface Environmental determination	Nutrition, defense, mediation of gut homeostasis and microbial equilibrium[a]	Crotti *et al.,* (2009, 2010)
Thysanoptera	*Frankliniella occidentalis*	γ-proteobacteria	*Erwinia* sp.	Environmental determination	Unknown	De Vries *et al.,* (2001, 2004)

Coleoptera	Chrysomelidae - *Macroplea mutica,* *Macroplea appendiculata*	γ-proteobacteria	*'Candidatus* Macropleicola'	Egg smearing:bacterial mass droplets	Habitat colonization: secretion provision for cocoon building in wetlands	Kölsch *et al.,* (2009); Kleinschmidt *and* Kölsch (2011)
	Chrysomelidae - *Donacia marginata,* *Donacia semicuprea*	γ-proteobacteria	Unspecified	Egg smearing: bacterial mass droplets	Habitat colonization: secretion provision for cocoon building in wetlands	Kleinschmidt and Kölsch (2011)
	Staphylinidae - *Paederus sabaeus*	β-proteobacteria	*Pseudomonas* sp.	Egg smearing : unspecified	Defense: biosynthesis of defensive toxin	Kellner (2001, 2002)
Diptera	Tephritidae - *Bactrocera oleae*	γ-proteobacteria	*'Candidatus* Erwinia dacicola'	Egg smearing: gland secretion	Nutrition[a]: protein hydrolisis and amino acid provision	Capuzzo *et al.,* (2005); Estes *et al.,* (2009)
	Tephritidae - *Bactrocera dorsalis*	γ-proteobacteria	*Klebsiella oxytoca*	Egg smearing: gland secretion	Reproductive behavior: adult attractant	Shi *et al.,* (2012)
	Tephritidae - *Ceratitis capitata*	γ-proteobacteria	*Klebsiella oxytoca* *Pectobacterium cypripedii*	Egg smearing: biofilm on egg surface, potential fecal contamination	Nutrition: pectin degradation and nitrogen metabolism. Reproductive behavior: shortened mating latency in males	Behar *et al.,* (2008); Ben-Ami *et al.,* (2010)
	Muscidae - *Musca domestica*	γ-proteobacteria, Firmicutes	*Klebsiella oxytoca* *Bacillus cereus*	Egg smearing : unspecified	Defense: against competing fungi in substrate for larval nutrition. Nutrition: diet supplement for larvae Reproductive behavior: influence on oviposition decisions.	Lam *et al.,* (2009)

Diptera (Cont'd)	Culicidae - *Anopheles* spp	α-proteobacteria	*Asaia* sp.	Egg smearing: bacterial colonies on ovarian egg surface Environmental determination	Nutrition, defense, mediation of gut homeostasis and microbial equilibrium[a]	Favia *et al.,* (2007); Damiani *et al.,* (2010); Crotti *et al.,* (2009, 2010); Chouaia *et al.,* (2012)
	Culicidae - *Aedes aegypti*	α-proteobacteria	*Asaia* sp.	Egg smearing: unspecified Environmental determination	Nutrition, defense, mediation of gut homeostasis and microbial equilibrium[a]	Crotti *et al.,* (2009)
	Glossinidae - *Glossina* spp.	γ-proteobacteria	*Wigglesworthia glossinidia*	Milk gland secretions	Nutrition: B vitamin supplementation	Akman *et al.,* (2002); Attardo *et al.,*(2008); Rio *et al.,* (2012)
Hymenoptera	Crabronidae - *Philanthus* spp., *Trachypus* spp., *Philanthinus* spp.	Actinobacteria	*'Candidatus* Streptomyces philanthi'	Brood cell contamination: antennal gland secretion	Defense: Protection against detrimental fungi	Kaltenpoth *et al.,* (2005, 2010, 2012) Kroiss *et al.,* (2010)
	Formicidae - Various Attine species	Actinobacteria	*Pseudonocardia* sp.	Social acquisition	Defense: production of antifungal compound against cultivar pathogen (*Escovopsis* sp).	Currie *et al.,* (1999, 2006) Oh *et al.,* (2009)
	Apidae - *Apis* spp.	α-, β-, and γ-proteobacteria, Firmicutes	*Gilliamella apicola*, *Snodgrassella alvi* *Lactobacillus* sp., *Bifidobacterium* sp. and unspecified Acetobacteraceae	Social acquisition	Nutrition, Defense: pathogen inhibition	Martinson *et al.,* (2011, 2012) Vásquez *et al.,* (2012) Cox-Foster *et al.,* (2007) Mohr and Tebbe. (2006)
	Apidae - *Bombus* spp.	γ, β-Proteobacteria	*Burkholderia* *Gilliamella apicola*, *Snodgrassella alvi*	Social acquisition Coprophagy	Defense: protection against parasites	Martinson *et al.,* (2011); Koch and Schmid-Hempel. (2011)

Blattaria	Blattidae - *Shelfordella lateralis*	Firmicutes, Bacteroidetes, δ- proteobacteria	*Clostridium* sp., *Succinispira* sp. *Enterococcus* sp., *Erysipelothrix* sp.,	Coprophagy	Nutrition[a]	Schauer *et al.,* (2012)
	Polyphagidae - *Cryptocercus punctulatus*	Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes	*Treponema* sp., *Spirochaeta* sp., *others unspecified*	Coprophagy	Nutrition[a]	Berlanga *et al.,* (2009)
Isoptera	Rhinotermitidae - *Reticulitermes* spp. Kalotermitidae - *Cryptotermes* spp. *Neotermes castaneus* Termopsidae - *Zootermopsis angusticollis* (lower termites)	Spirochaetes, Firmicutes, Bacteroidetes, Proteobacteria	*Treponema* sp., *Clostridiales* spp., *Lactococcus* sp. *Enterococcus* sp. *Bacteroides* spp., *Desulfovibrio* sp.	Social acquisition	Nutrition: *N_2 fixation, acetogenesis, nitrogen recycling, cellulolytic activity.*	Husseneder (2010) Brune and Friedrich (2000) Graber *et al.,* (2004)
	Termitidae - *Microcerotermes* spp. *Nasutitermes* spp. (higher termites)	Spirochaetes, Firmicutes, Bacteroidetes, Fibrobacteres.	*Treponema* sp., *Clostridiales* sp., *Bacteroidales* sp.,	Social acquisition, Environmental determination	Nutrition: N_2 fixation, acetogenesis, nitrogen recycling, cellulolytic activity.	Hongoh *et al.,* (2005, 2010) Warnecke *et al.,* (2007)
Orthoptera	Acrididae - *Schistocerca gregaria*	γ-proteobacteria	*Pantoea, Enterococcus, Serratia, Klebsiella, Acinetobacter*	Environmental determination	Participation in pheromone biosynthesis	Dillon *et al.,* (2008, 2010)

[a] No conclusive evidence, but hypotheses or suggestive results are reported.

Supplementary Table 2. Genome size and GC content for intra- and extracellularly transmitted bacterial symbionts in insects (as illustrated in Figure 3).

Symbiont	Symbiont Localization	Symbiont Transmission Route	Size (Mb)	GC%	Reference
'Candidatus Zinderia insecticola'	Intracellular	Intracellular	0.21	13.5	McCutcheon and Moran (2010)
'Candidatus Sulcia muelleri GWSS'	Intracellular	Intracellular	0.25	22.4	McCutcheon and Moran (2007)
'Candidatus Uzinura diaspidicola'	Intracellular	Intracellular	0.26	30.2	Sabree *et al.,* (2013)
'Candidatus Moranella endobia'	Intracellular	Intracellular	0.54	43.5	McCutcheon and von Dohlen (2011)
'Candidatus Blattabacterium sp.'	Intracellular	Intracellular	0.59	27.5	Sabree *et al.,* (2011)
Buchnera aphidicola	Intracellular	Intracellular	0.66	26.4	Perez-Brocal *et al.,* (2006)
Baumannia cicadellinicola	Intracellular	Intracellular	0.69	33.2	Wu *et al.,* (2006)
Wigglesworthia glossinidia	Intra- and Extracellular	Extracellular (Milk gland)	0.70	22.5	Akman *et al.,* (2002)
'Candidatus Ishikawaella capsulata'	Extracellular	Extracellular (Symbiont capsule)	0.75	38.5	Nikoh *et al.,* (2011)
'Candidatus Rosenkranzia clausaccus'	Extracellular	Extracellular (Egg smearing)	0.94	37.6	Kikuchi *et al.,* (2009)
Gut symbiont of *Adomerus triguttulus*	Extracellular	Extracellular (Egg smearing)	1,22	53.6	Hosokawa *et al.,* (2012)
Coriobacterium glomerans	Extracellular	Extracellular (Egg smearing)	2.11	60.4	Stackelbrandt *et al.,* (2013)
Treponema azonutricium	Extracellular	Extracellular (Social acquisition)	3.91	50	Graber *et al.,* (2004)
Burkholderia sp. Strain RPE64	Extracellular	Extracellular (Environmental determination)	6.96	63.5	Shibata *et al.,* (2013)

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CHAPTER 3

GEOGRAPHIC AND ECOLOGICAL STABILITY OF THE SYMBIOTIC MID-GUT MICROBIOTA IN EUROPEAN FIREBUGS, *PYRRHOCORIS APTERUS* (HEMIPTERA, PYRRHOCORIDAE)

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3.1 ABSTRACT

Symbiotic bacteria often play an essential nutritional role for insects, thereby allowing them to exploit novel food sources and expand into otherwise inaccessible ecological niches. Although many insects are inhabited by complex microbial communities, most studies on insect mutualists so far have focused on single endosymbionts and their interactions with the host. Here we provide a comprehensive characterization of the gut microbiota of the red firebug (*Pyrrhocoris apterus,* Hemiptera, Pyrrhocoridae), a model organism for physiological and endocrinological research. A combination of several culture-independent techniques (454-pyrosequencing, quantitative PCR and cloning/sequencing) revealed a diverse community of likely transient bacterial taxa in the mid-gut regions M1, M2 and M4. However, the completely anoxic M3 region harbored a distinct microbiota consisting of facultative and obligate anaerobes including Actinobacteria *(Coriobacterium glomerans* and *Gordonibacter sp.*), Firmicutes (*Clostridium sp.* and *Lactococcus lactis),* and Proteobacteria (*Klebsiella sp.,* and a previously undescribed Rickettsiales bacterium). Characterization of the M3 microbiota in different life stages of *P. apterus* indicated that the symbiotic bacterial community is transmitted vertically and becomes well defined between the second and third nymphal instar, which coincides with the initiation of feeding. Comparing the midgut M3 microbial communities of *P. apterus* individuals from five different populations and after feeding on three different diets revealed that the community composition is qualitatively and quantitatively very stable, with the six predominant taxa being consistently abundant. Our findings suggest that the firebug mid-gut microbiota constitutes a functionally important and possibly coevolved symbiotic community.

3.2 INTRODUCTION

Symbiotic interactions with microorganisms play an important role for a broad range of plants and animals (Buchner 1965; Moran *et al.* 2008; Moya *et al.* 2008; Smith 1989). Insects harbour a particularly large diversity of symbiotic microorganisms (Buchner 1965; Moran *et al.* 2008), and in many cases the microbial cells outnumber the host's own cells (Dillon and Dillon 2004). Microbial symbionts have been shown to assist their insect hosts in various functions such as nutritional upgrading of the diet (Akman *et al.* 2002; Douglas 1998), detoxifying the ingested food material (Cardoza *et al.* 2006; Dowd 1989; Genta *et al.* 2006), providing defence against parasites or pathogens (Currie *et al.* 1999, 2003a,; Kaltenpoth 2009; Kaltenpoth *et al.* 2005; Kroiss *et al.* 2010; Oliver *et al.* 2003, 2009), mediating thermal tolerance to the host (Dunbar *et al.* 2007), and facilitating the exploitation of novel host plants (Tsuchida *et al.* 2011). These associations with bacteria can be vital for insects to invade otherwise inaccessible ecological niches (Feldhaar 2011).

Most studies on insect-symbiont interactions so far have focused on individual obligate endosymbionts that are typically harboured in specialised organs, so-called bacteriomes. However, many insects are inhabited by a complex microbial community (Dillon and Dillon 2004; Lysenko 1985), but functional analyses of complete communities are scarce. This is mainly due to problems in discriminating the 'core' indigenous microbial community from more transient microbes that were taken up with the food material as well as the difficulty to specifically manipulate the often very complex communities.

Until recently, detailed culture-independent surveys on insect-associated microbial communities could only be achieved by using polymerase chain reaction (PCR) amplification, cloning, and sequencing of the 16S ribosomal RNA gene, which is time-consuming and costly (Konstantinidis and Tiedje 2005; Wintzingerode *et al.* 1997). However, recent advances in high-throughput next generation sequencing technologies such as 454 pyrosequencing and Illumina sequencing provide more cost- and labour-efficient alternatives to comprehensively characterize complex microbial communities of environmental samples including insects (Metzker 2010; Sun *et al.* 2011).

The Heteroptera, also known as true bugs, comprise around 38,000 species worldwide, representing one of the most diverse hemimetabolous insect taxa (Henry 1997; Schaefer 1993; Schuh and Slater 1995). The infraorder Pentatomomorpha comprising over 12,500 insect species ("stinkbugs") are predominantly phytophagus species that exploit resources from roots to seeds of their host plants, with the exception of a few predacious and mycophagous species (Henry 1997; Schaefer 1993; Schuh and Slater 1995). It forms a monophyletic clade within the Heteroptera, consisting of five superfamilies Lygaeoidea, Coreoidea, Pyrrhocoroidea, Pentatomoidea, and Aradoidea (Schuh and Slater 1995). Most of the pentatomomorphan bugs have a specific symbiotic interaction with bacteria harboured in sacs or tubular outgrowths, called crypts or caeca, in a posterior region of the mid-gut (Buchner 1965; Fukatsu and Hosokawa 2002; Glasgow 1914; Hosokawa *et al.* 2010a; Kikuchi *et al.* 2011a; Miyamoto 1961; Prado and Almeida 2009). Most of these gut symbionts are vertically transmitted by post-hatching transmission mechanisms such as egg surface contamination, coprophagy, or the formation and deposition of special symbiont-containing capsules by the mother (Abe *et al.* 1995; Hosokawa *et al.* 2005; Kikuchi *et al.* 2009; Prado *et al.* 2006; Schorr 1957). In some cases, experimental elimination of the symbiotic bacteria has resulted in high mortality and reduced growth, indicating that the symbionts of these bugs play an important role for the fitness of the host insect (Abe *et al.* 1995; Fukatsu and Hosokawa 2002; Huber-Schneider 1957; Kikuchi *et al.* 2009; Muller 1956; Schorr 1957; Tada *et al.* 2011).

The Pyrrhocoridae are a family of around 300 species of terrestrial bugs, most of which feed on seeds of plants of the order Malvales. Among them, the red firebug (*Pyrrhocoris apterus)* is one of the most common and widespread Palaearctic species, which has been extensively studied in the fields of ecological, biochemical, physiological and endocrinological research (Socha 1993). Before reaching adulthood *P. apterus* passes through five nymphal instar stages. The development of the initial four instar stages takes approximately 14 days, and they remain between 7 to 10 days in the final instar stages (Socha 1993). Earlier observations suggest that the bugs do not start to feed until they have reached second instar (Puchkov 1974).

P. apterus predominantly utilizes dry ripe seeds of linden trees (*Tilia cordata* and *T. platyphyllos*) as a food source. However, during limited access to linden seeds, *P. apterus* has been shown to easily adapt to seeds of other Malvales (Kristenová *et al*. 2011) as well as host plants from different families (Socha 1993; Tischler 1959). Additionally, firebugs are opportunistic scavengers feeding occasionally on dead insects, and they even attack and consume freshly molted conspecifics (Henrici 1938; Southwood and Leston 1959).

The digestion of food material in pyrrhocorids takes between three to four days (Silva and Terra 1994). Previous studies in *Dysdercus peruvianus* and *P. apterus* have shown that the ingested food particles are retained in the M1 region for only about five hours, whereas the passage through M2 and M3 takes approximately 70 - 90 hours, before the ingested food quickly passes through the M4 and the hindgut (Kodrík *et al.* 2012; Silva and Terra 1994). Despite considerable interest in the digestive processes of *P. apterus*, little is known yet on the symbiotic microbial community that inhabits in the different gut regions and its possible contribution to the host's digestion. However, previous studies reported on a specific actinobacterial symbiont (*Coriobacterium glomerans)* that occupies the mid-gut section M3 (Haas and König 1987, 1988). *C. glomerans* is vertically transmitted to the offspring through egg smearing (Kaltenpoth *et al.* 2009) and appears to be essential for successful development and reproduction of the bugs (Salem *et al.*, submitted).

In the present study, we used a combination of several culture-independent techniques (454 pyrosequencing, cloning/sequencing, quantitative PCR and fluorescence *in-situ* hybridization) to comprehensively characterize the microbial community that inhabits different regions of the mid-gut of *P. apterus*. Furthermore, we analyzed changes in the microbial community in the course of the bugs' development and examined its compositional stability across different populations and upon different diets. The results allow us to draw conclusions on the intimacy of the host-symbiont association and to speculate on a possible coevolutionary history of *P. apterus* and its symbiotic mid-gut microbiota.

3.3 MATERIALS AND METHODS

3.3.1 Rearing conditions and sample collection

For the characterization of the microbial community composition across different gut regions and life stages, a laboratory culture of *P. apterus* was established with individuals collected in Jena, Germany. The bugs were reared in plastic containers (20 x 13 x 12 cm) at a constant temperature of 28˚C under long light conditions (16:8 h light/dark cycles) to keep the insects in reproductively active stage (Saunders 1983, 1987). Insects were supplied with linden seeds (*Tilia cordata* and *T. platyphyllos*) and water *ad libitum.* The linden seeds, water, and the soil provided in the rearing cages were autoclaved to minimize exposure to environmental microbes.

For the survey of the microbiota in the different gut regions of *P. apterus*, six adult individuals derived from the Jena population were killed by freezing at -20˚C for 1 h. Prior to dissection, the animals were immersed in 0.1% sodium dodecyl sulphate (SDS) and then rinsed with sterile de-ionized water in order to remove surface contaminants. The abdomen of the bug was incised on both sides to remove the dorsal cuticle, the gut was collected under sterile de-ionized water, and the different gut regions were separated and placed into individual Eppendorf tubes for DNA extraction.

In order to analyse changes in the microbial community during the development of *P. apterus*, a cohort of firebugs from six egg clutches from different females was established, and six replicates per life stage were collected and surface-sterilized as described above. For the early life stages (egg to 4th instar nymphs), several individuals were pooled for replicate DNA extractions, respectively (eggs: 6 individuals, 1st instar: 3 individuals, 2nd instar: 3 individuals, 3rd instar: 2 individuals, and 4th instar: 2 individuals) in order to ensure sufficient DNA extraction yields. In all cases, complete animals were used to extract genomic DNA.

To assess variation in the microbial community composition across different geographical locations, *P. apterus* specimens were collected from five different populations in Central Europe: Jena (n=6), Berlin (n=8), Regensburg (n=8), Würzburg (n=8) (all in Germany) and Maria Saal (n=5) (Austria). The M3 sections of the adults' mid-guts were obtained as described above, and DNA was extracted individually. To assess the effect of different diets on the mid-gut microbiota composition, *P. apterus* individuals derived from the Jena population were reared from egg to the adult stage exclusively on one of three different diets: i) linden seeds (*Tilia cordata* and *T. platyphyllos*), which represent their natural diet in the field, ii) sunflower seeds (*Helianthus annuus*), or iii) a carnivorous diet consisting of larvae of the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae), which had been killed by freezing at -20˚C for 1 h. For all three diet treatments, fresh food as well as sterile water was provided twice a week *ad libitum*. The DNA was extracted individually from the midgut M3 region of five specimens for each diet.

Individual extracts of P. apterus from each life stage, population and diet experiment were used for qPCR analyses, and one pooled DNA sample, respectively, from all bugs of each gut region, life stage, population and diet experiment was used for bacterial tag-encoded 454 FLX pyrosequencing (bTEFAP) of 16S rRNA amplicons. To analyze the transient microbial community that firebugs may take up from the diet, linden seeds (*Tilia cordata* and *T. platyphyllos*) were collected from the field (Beutenberg campus, Jena, Germany) and subjected to bTEFAP.

3.3.2 DNA extraction and amplification

Prior to DNA extraction, all samples were submerged in liquid nitrogen and crushed with sterile pestles. DNA was extracted using the MasterPure™ DNA Purification Kit (Epicentre Technologies) according to the manufacturer's instructions. An additional lysozyme incubation step (30 min at 37 °C; 4 µl of 100 mg/ml lysozyme, Sigma-Aldrich, USA) was included prior to proteinase K digestion to break up gram-positive bacterial cells. The successful extraction of the gut microbial DNA from *P. apterus* was verified by using PCR assays with general eubacterial 16S rRNA primers (fD1 and rP2) (Weisburg *et al.* 1991) (Table 1). Subsequently, the extracted DNA was used for 454 pyrosequencing and qPCR assays.

3.3.3 Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) and data analysis

BTEFAP was done by an external service provider (Research and Testing Laboratories, Lubbock, USA) using the 16S rRNA primers Gray28F and Gray519r (Table 1) (Ishak *et al.* 2011; Sun *et al.* 2011) with primers numbered according to their position in *Escherichia coli* 16S rDNA. Generation of the sequencing library was established through one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar high fidelity *Taq* polymerases (Qiagen). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Research and Testing Laboratory (RTL, Lubbock, TX, USA), based upon RTL protocols (http://www.researchandtesting.com). All low quality reads (quality cut-off = 25) and sequences < 200 bp were removed following sequencing, which left between 7,000 - 15,000 sequences per sample for subsequent analysis.

Processing of the high-quality reads was done using Qiime (Caporaso *et al.* 2010b). The sequences were clustered into operational taxonomic units (OTUs) using multiple OTU picking with cdhit (Li and Godzik 2006) and uclust (Edgar 2010) with 97% similarity cut-offs. For the analysis, the samples were grouped into four datasets namely gut regions, life stages, populations, and diets to ensure that OTUs were clustered consistently across samples by the OTU picking method. For each OTU, one representative sequence was extracted (the most abundant) and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast (Caporaso *et al.* 2010a), with the minimum sequence identity percent set to 75%. Taxonomy was assigned using RDP classifier (Wang *et al.* 2007), with a minimum confidence to record assignment set to 0.80. OTU tables were generated describing the occurrence of bacterial phylotypes within each sample (Tables S1-4). The tables were then manually curated by removing low-frequency reads (<0.5% in all samples) and by blasting the representative sequences against the NCBI and RDP databases. Based on the blast results, OTUs with the same genus-level assignments were combined for visualization of the results. The revised OTU table was used to construct heatmaps using the MultiExperiment Viewer (MeV) software (Saeed *et al.* 2003).

3.3.4 PCR amplification, cloning, and sequencing

To compare the microbial community composition as revealed by bTEFAP to the more conventional cloning/sequencing procedure, a pooled mid-gut M3 DNA sample from six *P. apterus* individuals of

the Jena population was used for PCR amplification using general eubacterial 16S rRNA primers (fD1 and rP2, see Table 1). PCR amplification was done using a UnoCycler (VWR International GmbH, Belgium) in a total reaction volume of 12.5 µL containing 1 µL of template DNA, 1xPCR buffer (20 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, and 0.01% Tween 20), 2.5 mM $MgCl_2$, 240 µM dNTPs, 0.8 µM of each primer, and 0.5 U of Taq DNA polymerase (VWR International GmbH, Belgium). Cycle parameters were: 3 min at 94˚C, followed by 35 cycles of 94˚C for 40 s, 68˚C for 40 s and 72˚C for 40 s, and a final extension step of 4 min at 72˚C. PCR products were cloned using the StrataClone PCR Cloning Kit (Agilent Technologies, USA) according to the manufacturer's instructions. Transformed *E. coli* cells were grown on LB agar containing 10 mg mL^{-1} ampicillin and appended with 2% 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) (Sigma Aldrich, Germany) for blue/white screening. Colony PCR was performed on randomly selected transformants with vector primers M13F and M13R (Table 1) using the above-mentioned reaction mix and cycling conditions except that an annealing temperature of 55°C was used. PCR products were checked for the expected size on a 1.5% agarose gel (130 V, 30 min) and purified using the peqGOLD MicroSpin Cycle Pure Kit (Peqlab Biotechnologies GmbH, Germany) prior to sequencing. In total, 179 clones were sequenced bidirectionally on an ABI 3730xl capillary DNA sequencer (Applied Biosystems, USA) using the M13 primers (Boutin-Ganache *et al.* 2001).

3.3.5 Molecular phylogenetic analysis

Nearly full-length 16S rRNA sequences (1.4kb, obtained from cloning/sequencing) of the six consistently detected microbial taxa in the *P. apterus* M3 mid-gut region were aligned to reference sequences obtained from the Ribosomal Database Project (RDP) using the ClustalW algorithm implemented in MEGA5 (Tamura *et al.* 2011; Thompson *et al.* 1994). Phylogenetic trees were computed using maximum likelihood (Tamura-Nei model, G+I rate variation) with 500 bootstrap replicates in MEGA5 (Tamura *et al.* 2011).

3.3.6 Quantitative PCR

Specific primers for quantitative PCR (qPCR) were designed using Primer3 (http://primer3.sourceforge.net/) for the six most consistently found and abundant bacterial taxa in the M3 region of the mid-gut (*Coriobacterium glomerans*, *Gordonibacter sp.*, *Clostridium sp.*, unknown Rickettsiales *sp.*, *Lactococcus lactis* and *Klebsiella sp.)* based on an alignment of the representative set of sequence data for all OTUs available from the cloning/sequencing and 454 pyrosequencing. PCR conditions for each primer pair were optimized using gradient PCRs with a pooled *P. apterus* gut sample (Jena) as a template (Table 1). The specificity of the primers was verified *in silico* against the RDP database (Maidak *et al.* 2001) and *in vitro* by sequencing the optimized PCR product directly without prior cloning. If the sequence matched the expected OTU, the primer pair was assumed to specifically amplify the target OTU within the *P. apterus* gut. Additionally, specificity was monitored via qPCRs by including a melting curve step at the end to ensure that amplicons were the same across samples for each primer assay.

Quantitative PCRs for individual bacterial symbionts were performed on a RotorgeneQ cycler (Qiagen, Germany) in final reaction volumes of 25 µL containing 1 µL of template DNA (usually a

1:10 dilution of the original DNA extract), 2.5 µL of each primer (10 µM), and 12.5 µL of SYBR Green Mix (Rotor-Gene SYBR Green kit, Qiagen). Standard curves were established by using 10^{-8} – 10^{-2} ng of specific PCR product as templates for the qPCR. A NanoDropTM1000 spectrophotometer (Peqlab Biotechnology Limited, Germany) was used to measure DNA concentrations for the templates of the standard curve. Six different replicates of the standard concentrations for each bacterial taxon were used to calculate a correction factor to alleviate any errors of the template standard curve (e.g. inaccuracies of the DNA concentration measurements). PCR conditions were as follows: 95˚C for 5 min, followed by 35 cycles of 60˚C for 30 s, 72˚C for 20 s, and 95˚C for 15 s; then a melting curve analysis was performed by increasing the temperature from 60˚C to 95˚C within 20 min. Based on the standard curves, the 16S copy number could be calculated for each individual bug from the qPCR threshold values (Ct) by the absolute quantification method (Lee *et al.* 2006, 2008), taking the dilution factor and the absolute volume of DNA extract into account. The absolute 16S copy numbers were logcontrast-transformed (Aitchison 1986) and then subjected to discriminant analysis (SPSS) to test for quantitative differences in the microbial community composition across different populations or diets.

Table 1: Primers and probes used for the characterization (PCR, cloning, sequencing), quantification (qPCR), and localization (FISH) of bacterial taxa in the mid-gut of *P. apterus*. All primers target the bacterial 16S rRNA gene. Use: (1) general amplification of gut bacteria, (2) cloning/sequencing, (3) 454 sequencing, (4) qPCR, and (5) FISH.

Primer	Primer sequence (5´ - 3´)	Fwd/ Rev	5´mod	Target	Use	Reference
FD1	AGAGTTTGATCCTGGCTCAG	Fwd.		Eubacteria	1	Weisburg et al. 1991
RP2	ACGGCTACCTTGTTACGACTT	Rev.		Eubacteria	1	Weisburg et al. 1991
M13F	CAGGAAACAGCTATGAC	Fwd.		Eubacteria	2	Boutin-Ganache et al. 2001
M13R	GTAAAACGACGGCCAG	Rev.		Eubacteria	2	Boutin-Ganache et al. 2001
Gray28F	GAGTTTGATCNTGGCTCAG	Fwd		Eubacteria	3	Ishak et al. 2011
Gray519R	GTNTTACNGCGGCKGCTG	Rev		Eubacteria	3	Ishak et al. 2011
Corio_DSM20642_91F	TGACCAACCTGCCCTGCGCT	Fwd.		*Coriobacterium*	4	This study
Corio_300rev	CCCGTAGGAGTCTGGGCCG	Rev.		*Coriobacterium*	4	This study
Egg_1079fwd	CACTGCTGCCTCCCGTAGGAGT	Fwd		*Gordonibacter*	4	This study
Egg_1253Rev	CATACCTCACCTGGGGTGTGTGG	Rev		*Gordonibacter*	4	This study
Clostridium_1050-fwd	CTCGTGTCGTGAGATGTTGG	Fwd		*Clostridium*	4	This study
Clostridium_1248-rev	GCTCCTTTGCTTCCCTTTGT	Rev		*Clostridium*	4	This study
Proteobac_16s_fwd	GTGGCAAACGGGTGAGTAAT	Fwd		unknown Rickettsiales	4	This study
Proteobac_16s_Rev	GAAGTCTGGGCCGTATCTCA	Rev		unknown Rickettsiales	4	This study
Lactococcus_975-fwd	CGCTCGGGACCTACGTATTA	Fwd		*Lactococcus*	4	This study
Lactococcus_1175-rev	GCAGCAGTAGGGAATCTTCG	Rev		*Lactococcus*	4	This study
Klebsiella_250-fwd	CAGCCACACTGGAACTGAGA	fwd.		*Klebsiella*	4	This study
Klebsiella_453-rev	GTTAGCCGGTGCTTCTTCTG	Rev.		*Klebsiella*	4	This study
EUB338-Cy5	GCTGCCTCCCGTAGGAGT	Rev.	Cy5	*Eubacteria*	5	Amann et al. 1990
Cor653-Cy3	CCCTCCCMTACCGGACCC	Rev.	Cy3	*Coriobacterium*	5	Kaltenpoth et al. 2009
Egg583-Cy3	GAGGCTTCGCTTAGGCAACC	Rev.	Cy3	*Gordonibacter*	5	This study
Proteo-Cy3	ATTACTCACCCGTTTGCCAC	Rev.	Cy3	unknown Rickettsiales	5	This study
Clost -Cy3	TACCAACTCCCATGGTGTGA	Rev.	Cy3	*Clostridium*	5	This study
Lact_Cy3	GCTCCCTACATCTAGCAC	Rev.	Cy3	*Lactococcus*	5	Ercolini et al. 2003
Kleb_Cy3	TCTCAGTTCCAGTGTGGCTG	Rev.	Cy3	*Klebsiella*	5	This study

3.3.7 Fluorescence in-situ hybridization (FISH)

To localize the dominant symbionts of *P. apterus*, FISH was performed using sections of the M3 portion of the mid-gut. The M3 was fixed in 70% ethanol, dehydrated in acetone, and then embedded in cold polymerizing resin (Technovit 8100, Germany) according to the manufacturer's instructions. Sections of 4 mm thickness were prepared with a diamond knife on a Microm HM 355 S microtome (Thermo Scientific, Germany). FISH on the embedded M3 mid-gut tissue sections was carried out

using the specific probes *Cor*653, *Egg*583, *Proteo*, *Clost, Lact*, *Kleb* (all Cy3 probes), in combination with the general eubacterial probe EUB338-Cy5 (Amann *et al.* 1990) and DAPI (Table 1). Some of the probes were the same as one of the primers used for diagnostic qPCR (unknown *Rickettsiales* sp. and *Klebsiella sp.*); the others were designed based on the sequencing data available from the 454 pyrosequencing and cloning/sequencing (*Gordonibacter* sp. and *Clostridium sp.*). Sequences of probes for *Coriobacterium* sp. and *Lactococcus lactis* were derived from the literature (Ercolini *et al.* 2003; Kaltenpoth *et al.* 2009). The specificity of all probes was tested by using cultures of other bacterial taxa (*Escherichia coli*, *Bacillus subtilis* and *Pseudomonas fluorescens)* on eight-field microscope slides as negative controls, and a mid-gut (M3) suspension from an adult *P. apterus* as a positive control. Hybridization with probes was achieved as described (Kaltenpoth *et al.* 2009, 2012), and localization of bacterial taxa was recorded using an Axioimager Z1 fluorescence microscope (Carl Zeiss, Germany).

3.3.8 Microelectrode measurements

Freshly extracted guts of adult *P. apterus* individuals were placed on top of a 2 mm layer of 1.5% agarose in a micro-chamber and covered with 2 mm of 0.5% agarose (Brune *et al.* 1995). The microelectrode was positioned using a manual micromanipulator (Unisense, Denmark). The current was measured with a picoammeter (model 1201; Diamond-General, Ann Arbor, USA) connected to a strip chart recorder. An oxygen microelectrode (Unisense, Denmark) with a tip diameter of 20-30 µm was calibrated before each experiment by using water saturated with air (21% O_2) or 100% N_2 (0% O_2), respectively. This set-up was used to radially measure oxygen concentrations of six replicates in the M3 region of the mid-gut using a step increment of 50 µm. A pH microelectrode (Unisense, Denmark) with a tip diameter of 20-30 µm was calibrated with standard buffers at pH 4.0, 7.0 and 10.0 before each experiment. The pH was recorded in three replicates in the mid-section of different gut regions (M1, M2, M3, and M4). All measurements were performed at ambient temperature (22 ± 1˚C).

3.4 RESULTS

3.4.1 Bacterial communities in different gut regions of *P. apterus*

The microbiota of different mid-gut regions (M1, M2, M3, and M4) of *P. apterus* were characterised using 454 pyrosequencing (Fig. 1). After quality trimming, a total of 52,357 bacterial 16S rRNA sequences were obtained, which were binned into a total of 78 OTUs after removing singletons and OTUs below 0.5% abundance (Table S1). The most dominant bacterial taxa present in the M1 and M2 were Alphaproteobacteria (*Brevundimonas sp.*, *Rhizobium sp.*, *Caulobacter sp.,* and *Ensifer sp.*), and Gammaproteobacteria (*Pseudomonas sp.* and *Hafnia sp.*). The M3 portion exhibited a unique bacterial community, which was dominated by Actinobacteria *(Coriobacterium glomerans* and *Gordonibacter sp.*), Alphaproteobacteria (Rickettsiales sp), and Firmicutes (*Clostridium sp.*) (Fig. 1 and 2). The microbiota of M4 was highly diverse and consisted of all major taxa found in the previous gut sections, i.e. Alphaproteobacteria (*Brevundimonas sp.* and *Rhizobium sp.*), Gammaproteobacteria (*Pseudomonas sp.*), Firmicutes (*Clostridium sp.* and *Lactococcus lactis*) and Actinobacteria (*Coriobacterium glomerans* and *Gordonibacter sp.*). Although female *P. apterus* have gastric caeca associated with the M4 region (Buchner 1965), no symbiotic bacteria could be detected in these

structures by direct microscopic observations or FISH using general eubacterial probes (data not shown).

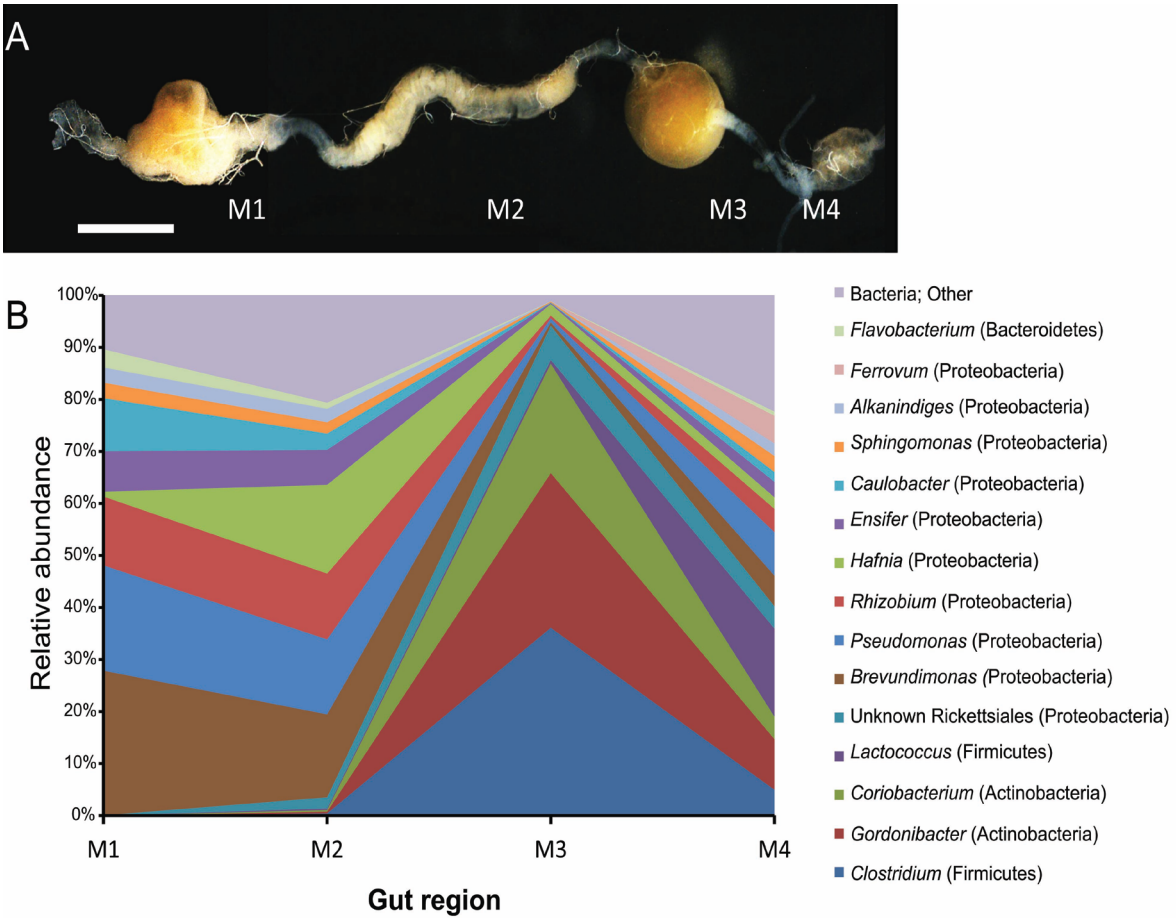

Figure 1. Bacterial community in different mid-gut regions of *P. apterus*. (**A**) Overview over the different parts of the digestive tract of *P. apterus* (M1-M4) (scale: 2mm). (**B**) Frequency of bacterial taxa in the four different regions of the mid-gut represented in a relative area graph as revealed by bTEFAP (52,357 16S rRNA reads in total) of a pooled sample of six adult individuals from Jena, Germany.

The major taxa in the M3 region were each represented by several closely related OTUs of different abundances (Table S2). Based on the available data, we cannot tell whether this observed microdiversity reflects true biological diversity or was caused by sequencing artefacts. However, for all of the individual analyses (gut regions, developmental stages, different populations and diets, respectively), the variation in OTU abundances was consistent across samples, with the same major OTU(s) representing the dominant bacterial genera. Thus, we decided to limit the further description of the results as well as the discussion to genus-level patterns, in order to facilitate an understanding of our general findings. It should be noted, however, that our conclusions based on genus-level classifications are identical with those based on OTU level analyses, and all OTU tables (including all OTUs with abundances >0.5% in at least one of the samples) are available as supplementary tables (see Tables S1-S4).

The microbiota profile of complete adult bugs (both males and females) was very similar to the one of the isolated M3 mid-gut region (Fig. 3), indicating that the bacterial community present in the M3 by far outnumbers all other microbes present in *P. apterus*. Hence, we focused on the M3 region of the mid-gut to analyse in more detail whether the bug-associated microbiota changes across different life stages, populations, and diets.

The composition of the M3 microbiota obtained from the cloning/sequencing analysis was qualitatively similar to the 454 pyrosequencing data with the dominant phylotypes (*Coriobacterium glomerans*, *Gordonibacter sp.* and *Clostridium sp.*) all being detected, but there were considerable differences between both datasets in relative abundances of microbial taxa (Fig. S1).

3.4.2 Phylogenetic placement of *P. apterus* midgut symbionts

Based on near full-length 16S rRNA sequences obtained from the cloning/sequencing approach, a maximum likelihood tree including representative sequences of in- and outgroup taxa was reconstructed (Fig. 2). The *Coriobacterium* sequence was closely related to the type strain of *C. glomerans* that had been previously isolated from the intestinal tract of *P. apterus* by Haas and König (1987) and remains to date the only validly described species in the genus. The other actinobacterial taxon identified in the firebug gut (i.e. *Gordonibacter sp.)* shows only about 92% similarity to the 16S rRNA gene sequence of its closest relative *Gordonibacter pamelaeae* (Würdemann *et al.* 2009), and 90-91% similarity to species in the genus *Eggerthella*. Both *Coriobacterium glomerans* and *Gordonibacter sp*. (Fig. 2) belong to the family Coriobacteriaceae (Actinobacteria), a group of anaerobic bacteria that is known to occur in the human intestine (Holdeman *et al.* 1976) and as opportunistic pathogens in human oral infections (Downes *et al.* 2001; Nakazawa *et al.* 1999; Poco *et al.* 1996). But to our knowledge these bacterial taxa have not yet been reported from insects outside the Pyrrhocoridae family.

Firmicutes that were detected in the firebug´s gut as well as the gamma-proteobacterial symbionts are closely related to cultured strains (96% similarity to *Clostridium hathewayi*, 100% to *Lactococcus lactis*, and 100% to *Klebsiella pneumoniae*, respectively, Fig. 2). However, the alpha-proteobacterial taxon is only distantly related to any other known strain within the order Rickettsiales, with the closest relatives being an uncharacterized symbiont of the flea *Oropsylla hirsuta* (90% similarity, Jones *et al.* 2008), and *Holospora obtusa*, an intracellular symbiont of *Paramecium* (79% similarity, Amann *et al*. 1991). In general, the order Rickettsiales comprises mostly intracellular bacteria such as *Wolbachia* and *Rickettsia,* which are common reproductive parasites and pathogens of insects as well as other animals including humans (Hilgenboecker *et al.* 2008; Hosokawa *et al.* 2010b; Rousset *et al.* 1992). However, mutualistic interactions with *Wolbachia* and Rickettsia are known to occur in nematodes, leeches and bed bugs (Hosokawa *et al.* 2010b; Kikuchi *et al*. 2002; Taylor *et al.* 2005).

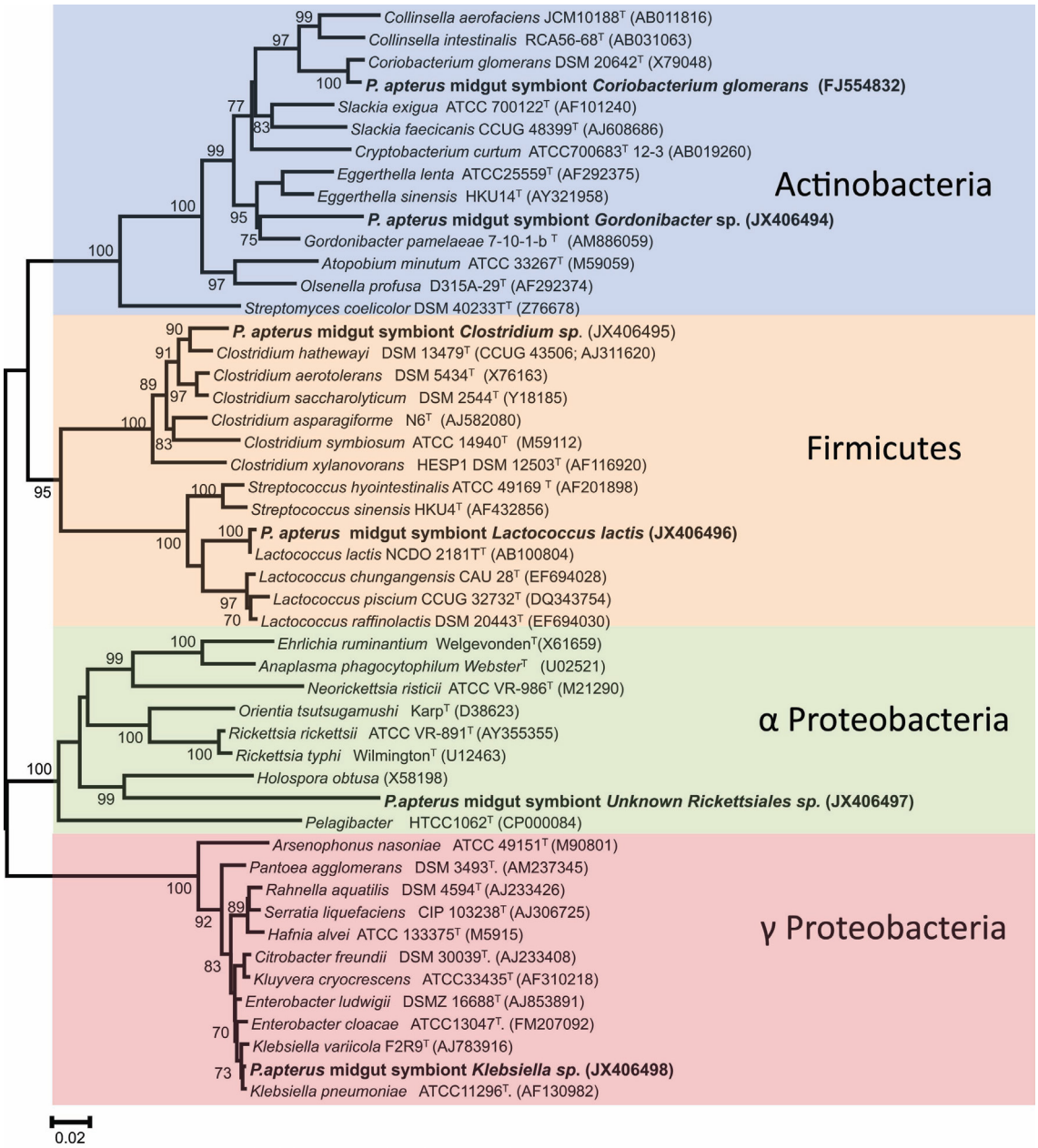

Figure 2. Phylogenetic position of symbiotic *Coriobacterium glomerans, Gordonibacter sp, Unknown Rickettsiales, Clostridium sp., Lactococcus sp,* and *Klebsiella sp.* from the *Pyrrhocoris apterus* mid-gut M3 region. Maximum likelihood tree constructed on the basis of 1.4 kbp of 16S rRNA gene sequences. Bootstrap values (in percent) were obtained from a search with 500 replicates. Strain and accession numbers are given behind the species names. Type strains are indicated by superscript T.

3.4.3 Ontogenetic changes of the *P. apterus* microbiota

Using qPCR and 16S rRNA data (90,899 16S rRNA sequences obtained by 454 pyrosequencing, clustered into 176 OTUs), we analysed the relative abundances of bacterial taxa across the different developmental stages of *P. apterus* in order to assess the symbionts' population dynamics within the insect host. The most abundant taxa *Clostridium sp.*, *Coriobacterium glomerans, Gordonibacter sp.,*

as well as the next three most abundant bacterial taxa i.e. the unknown Rickettsiales species, *Lactococcus lactis,* and *Klebsiell*a *sp.* were detected in or on *P. apterus* eggs, suggesting that they are vertically transmitted from mother to offspring (Fig. 3, Table S2). Although the gut microbiota was present in low abundances from the egg stage to the second instar, the microbial community of *P. apterus* that characterizes adult individuals was quantitatively established during the 2nd or 3rd instar and remained largely unchanged until *P. apterus* reached its adult stage (Fig. 3).

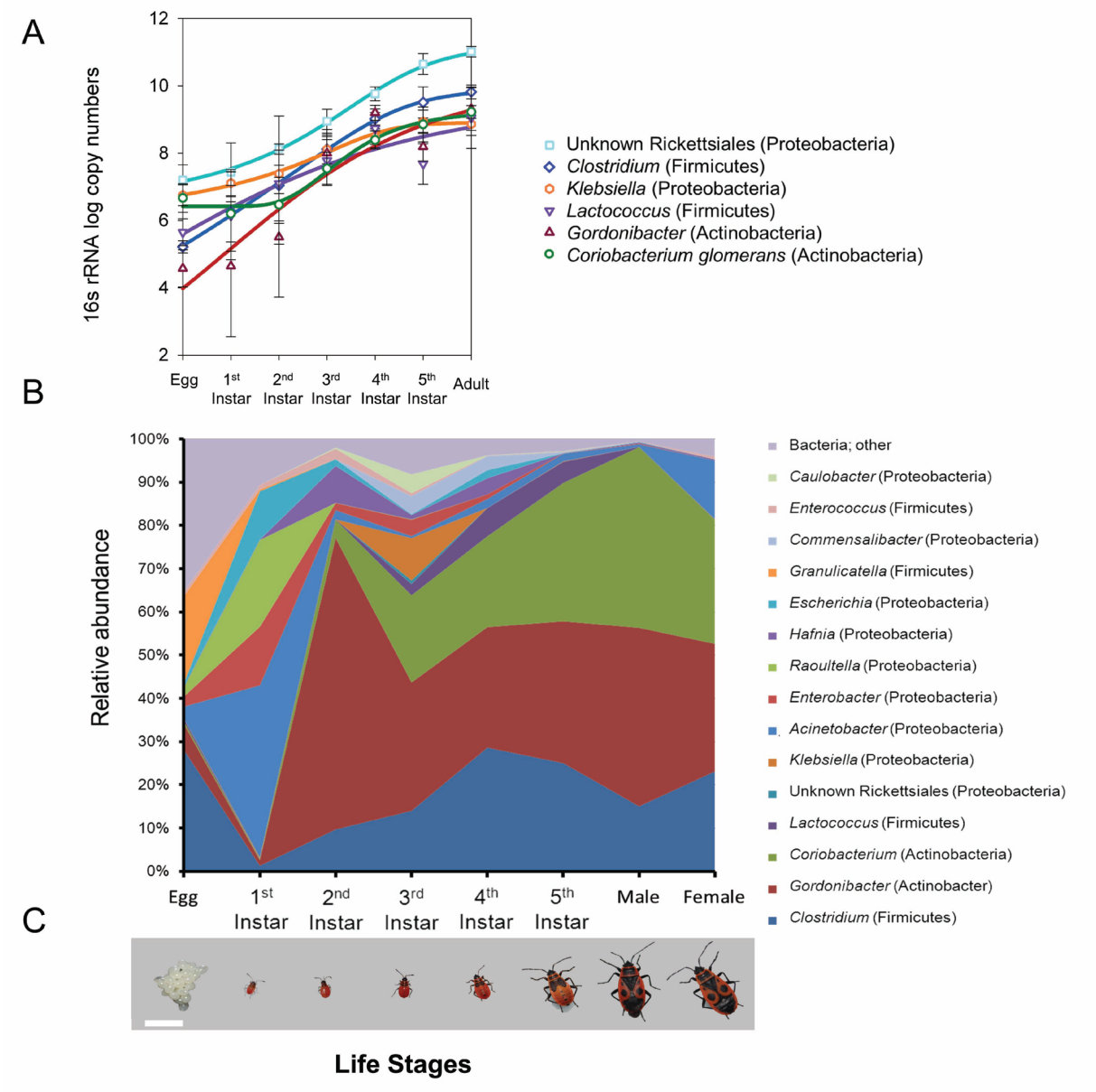

Figure 3. Bacterial community composition of *P. apterus* during its different life stages. (**A**) Ontogenetic change of the six major bacterial taxa across different life stages of *P. apterus* as revealed by qPCR using six replicates per life stage, with sigmoidal curve fitting (five parameters). (**B**) Relative abundance of bacterial taxa across different life stages of *P. apterus* (90,899 sequences in total) represented in a relative area graph of a pooled sample of six replicates per life stage. (**C**) Images of different life stages of *P. apterus*. Scale: 5mm.

3.4.4 Inter-population differences in the mid-gut microbiota of *P. apterus*

After quality trimming, a total of 83,174 bacterial 16S rRNA sequences and 103 OTUs were obtained from pooled samples of five different populations in Central Europe (Table S3). A quantitative and qualitative comparison of the gut microbiota revealed only minor differences across populations. The most abundant taxa *Clostridium sp.*, *Coriobacterium glomerans, Gordonibacter sp.,* and the next three most abundant bacterial taxa (i.e. unknown Rickettsiales sp., *Lactococcus lactis,* and *Klebsiell*a *sp.*) were consistently present across all populations (Fig. 4A,B). QPCR analysis showed that all six bacterial taxa were present in similar abundances across all populations (Fig. 4B). Discriminant analysis of the 16S log copy numbers obtained from qPCRs showed slight, but significant quantitative differences in the microbial communities derived from Central European bug populations (Wilk's λ =0.337, χ^2 = 36.5, n = 45, P = 0.049) (Fig. 4C).

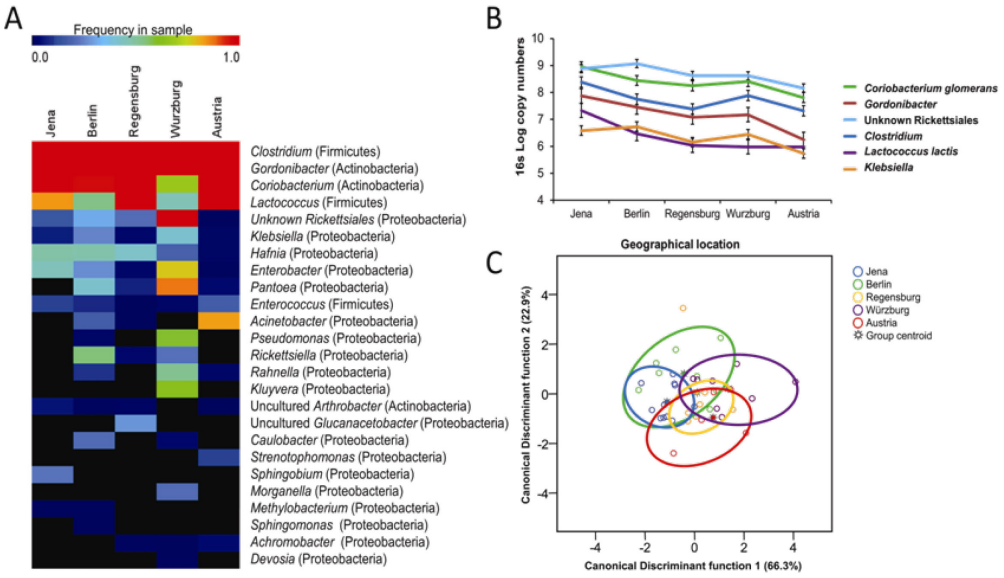

Figure 4. Gut bacterial community composition of *P. apterus* collected from five different geographical locations. (**A**) Relative abundance of gut bacterial taxa from 454 pyrosequencing of 16S rRNA amplicons (83,174 reads in total), represented as a heat map based on the log-transformed values, with warm colors indicating higher and cold colors lower abundance. (**B**) Infection rate and abundance of the six most dominant bacterial taxa (*Coriobacterium glomerans, Gordonibacter sp.,* unknown Rickettsiales, *Clostridium sp., Lactococcus sp.* and *Klebsiella sp.*) as revealed by diagnostic qPCR of multiple specimens for each population of *P. apterus* (Jena (n=6), Berlin (n=8), Regensburg (n=8), Würzburg (n=8) (all in Germany) and Maria Saal (n=5) (Austria)). Lines represent means, error bars denote standard errors. (**C**) Multivariate discriminant analysis of the microbial community of *P. apterus* from five geographic localities based on the qPCR data from (B) (Wilk's λ =0.337, χ^2 = 36.5, n = 45, *P* = 0.049).

3.4.5 Effect of different diets on the mid-gut microbiota of *P. apterus*

The mid-gut microbiota of experimental *P. apterus* populations fed exclusively on one of three different diets (linden seeds, sunflower seeds, and beewolf larvae, respectively) were analysed by 454 pyrosequencing and qPCR. The 454 pyrosequencing data (46,423 bacterial 16S rRNA sequences and 75 OTUs after quality trimming) revealed that the microbiota of *P. apterus* reared on the three

different diets were similar, with *Clostridium* sp., *Coriobacterium glomerans* and *Gordonibacter sp.* being abundant in all three populations (Fig. 5A, Table S4). However, bugs fed on linden seeds and beewolves, respectively, showed unusually high abundances of gamma-Proteobacteria in the M3 region. This was especially surprising in the linden seed treatment, as Proteobacteria were much less common in the other experimental bugs from the same population and food source (see sections on the microbial community of different gut regions and populations).

Although the Rickettsiales *sp.*, *Klebsiella sp.* and *Lactococcus lactis* were not detected in the 454 pyrosequencing dataset, they were consistently found by qPCR of the same samples. The results of the qPCR analysis indicated that the six most dominant bacterial taxa were present in similar abundances across the different diet treatments, thereby indicating that the community composition of these taxa was not affected by the diet of the host (Fig. 5B). Discriminant analysis confirmed that there was no significant difference in the relative abundances of the six major bacterial taxa across diets (Wilk's $\lambda = 0.543$, $\chi^2 = 7.029$, $n = 45$, $P = 0.134$) (Fig. 5C).

3.4.6 Transient microbes from ingested food material

The microbiota of the preferred food source of *P. apterus* (linden seeds) was analyzed by 454 pyrosequencing (Table S5) to assess the possible influence of transient microbes from the ingested food material in the symbiotic gut microbial community. After quality trimming, a total of 12,017 16S rRNA sequences were obtained. Around 47% of the sequences originated from chloroplasts; these sequences were excluded from further analysis. Some of the most abundant bacterial taxa identified were Gammaproteobacteria (*Pseudomonas sp.*, *Stenotrophomonas sp.*), Alphaproeobacteria (*Sphingomonas sp.*, *Methylobacterium sp.*), and Actinobacteria (*Microbacterium sp.*, *Rhodococcus sp.*). Several bacterial taxa that could be detected in linden seed samples were also constituents of the microbiota of M1, M2, and M4 mid-gut regions, notably *Brevundimonas sp.*, *Rhizobium sp.*, *Pseudomonas sp.*, and *Caulobacter sp.* (Table S5).

Interestingly, *Wolbachia sp*. was one of the bacterial taxa identified in the *P. apterus* individuals reared on beewolf larvae as the sole food source (Fig. 5A). As beewolves are commonly infected with *Wolbachia* (Kaltenpoth 2006), it is likely that the sequences found in *P. apterus* represent transient bacteria or DNA residues acquired from the food source. Both the occurrence of linden seed-associated bacteria in the mid-gut of bugs feeding on these seeds and the discovery of *Wolbachia* in beewolf-fed bugs highlight the possible confounding effect of transient microbes on the apparent composition of gut microbial communities. This effect should be especially problematic in studies analyzing microbial gut communities based on low numbers of field-collected samples that were exposed to diverse and unknown microbial communities.

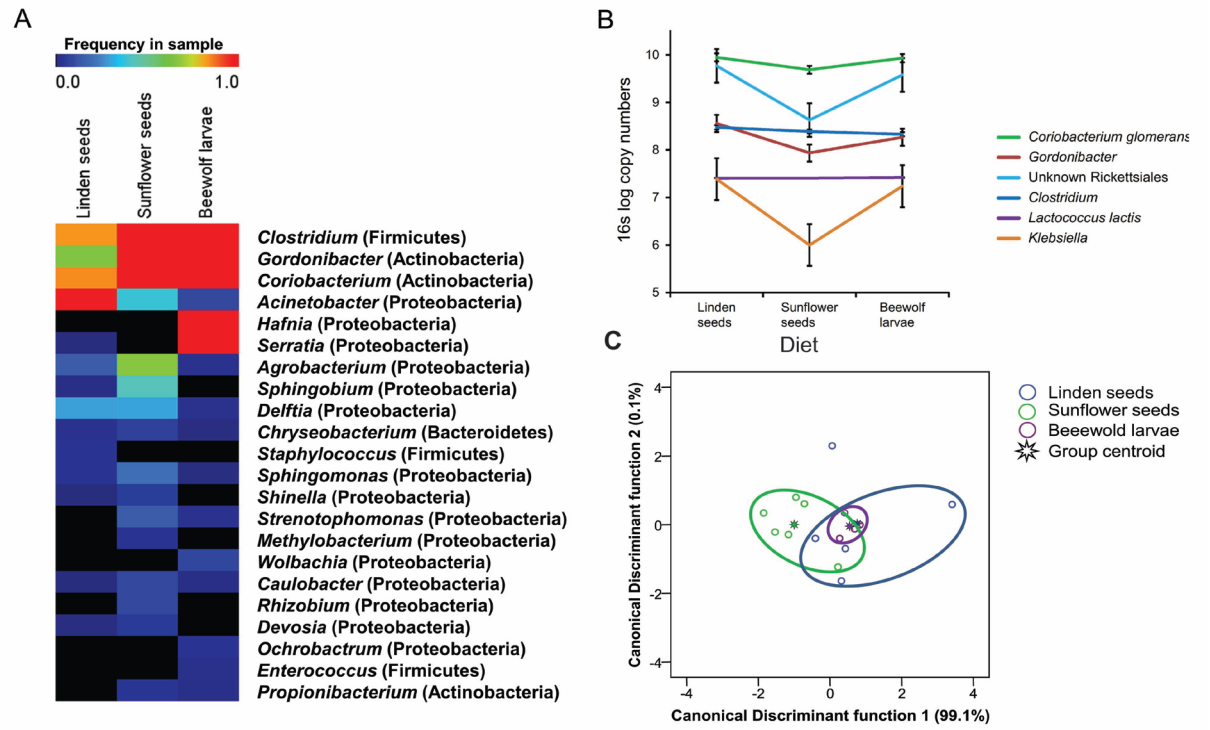

Figure 5. Gut bacterial community composition of *P. apterus* reared on three different diets. (**A**) Frequency of gut bacterial taxa from 454 pyrosequencing data (46,423 sequences in total) represented as a heat map based on the log-transformed values, with warm colors indicating higher and cold colors lower abundance. (**B**) Infection rate and abundance of the six most dominant bacterial taxa (*Coriobacterium glomerans, Gordonibacter sp.,* Unknown Rickettsiales, *Clostridium sp., Lactococcus* sp., and *Klebsiella sp.*) as revealed by diagnostic qPCR of five replicates for each different diet. Lines represent means, error bars denote standard errors. (**C**) Multivariate discriminant analysis of the microbial community of *P. apterus* reared on three different diets based on the qPCR data from (B) (Wilk's $\lambda = 0.543$, $X^2_{45} = 7.029$, $P = 0.134$, 66.7% of cases classified correctly).

3.4.7 Localisation of microbial symbionts

The numerically most dominant bacterial taxa found in the M3 mid-gut portion of *P. apterus* (*Clostridium sp.*, *Gordonibacter sp.*, *Coriobacterium glomerans*, unknown Rickettsiales *sp.*, *Lactococcus lactis* and *Klebsiella sp.)* were localized in gut sections using FISH. *C. glomerans* and *Gordonibacter* sp. were present in the gut lumen and on the epithelial walls, with *C. glomerans* being predominantly localized towards the anterior end of the M3. The unknown Rickettsiales *sp.*, *Klebsiella sp.*, *Lactococcus lactis* and *Clostridium* sp. were present in the gut lumen, with *Lactococcus lactis* and *Clostridium* sp. being particularly prevalent in the anterior region of the M3 (Fig. S2).

3.4.8 Microelectrode measurements

Radial oxygen measurements in the M3 region of the *P. apterus* mid-gut indicated that the conditions were completely anoxic throughout the M3 (Fig. S3). The pH measurement in the mid-gut of *P. apterus* revealed slightly acidic conditions throughout the mid-gut. The M3 region of the mid-gut had a pH of 5.8, while the other regions of the mid-gut (M1, M2, and M4) showed a pH of 5.4. The acidic

pH conditions in the mid-gut region are required for the effective functioning of the digestive enzymes (Kodrík *et al.* 2012; Silva and Terra 1994).

3.5 DISCUSSION

3.5.1 Composition of the *P. apterus* gut microbiota

In this study, we comprehensively characterized the microbiota inhabiting the mid-gut of *P. apterus* using a combination of different culture-independent techniques. The M1 and M2 mid-gut regions showed similar microbial profiles that were dominated by Alpha- and Gammaproteobacteria (Fig. 1). Many of the bacterial taxa were also found in the microbiota of the bug's food source (linden seeds), suggesting that the bacterial taxa detected in the M1 and M2 region were taken up with the food. The microbiota of the M4 mid-gut region shared bacterial taxa with all preceding mid-gut regions (Fig. 1). The presence of such a diverse bacterial community in M4 is likely due to the passing of bacteria from the other gut regions with the digested food material to the rectum for excretion. However, the M3 region was characterized by a distinct microbiota compared to the microbiota present in the other mid-gut regions and the food source, and predominantly harboured *Coriobacterium glomerans*, *Gordonibacter sp., Clostridium sp.,* as well as *Lactococcus lactis,* an unknown Rickettsiales species, and *Klebsiella sp.* (Fig. 1 and 2). Furthermore, the microbial profile of the M3 mid-gut region resembled the microbiota of the whole adult insect (male and female) (Fig. 3), indicating that the symbionts residing in the M3 greatly outnumber any other bacterial taxa occurring in other parts of the bug. Interestingly, *Dysdercus fasciatus*, another species within the Pyrrhocoridae, harbours a mid-gut M3 microbiota similar to *P. apterus*, with the most abundant bacterial taxa being *Coriobacterium glomerans*, *Gordonibacter sp*., and *Clostridium sp*. (Kaltenpoth et al. 2009; Salem *et al.* submitted).

By contrast, other pentatomomorphan bugs are associated with proteobacterial symbionts that inhabit specialized structures such as gastric caeca or crypt regions in the posterior region of the midgut (Buchner 1965; Glasgow 1914; Kikuchi *et al.* 2011a). As in pyrrhocorid bugs (Salem et al., submitted), the symbionts are often essential for successful growth and reproduction of the host (Abe *et al.* 1995; Fukatsu and Hosokawa 2002; Huber-Schneider 1957; Kikuchi *et al.* 2009; Muller 1956; Schorr 1957; Tada *et al.* 2011). In the superfamily Pentatomoidea, most of the species are associated with one of several distinct lineages of γ-Proteobacteria that are vertically transmitted (Fukatsu and Hosokawa 2002; Kikuchi *et al.* 2009; Prado *et al.* 2006; Prado and Almeida 2009). Bugs of the superfamily Coreoidea and several families of the Lygaeoidea, on the other hand, harbour *Burkolderia* (β-Proteobacteria) symbionts in the mid-gut crypts, and it has been shown for some broad-headed bugs (Alydidae) that these symbionts are acquired *de novo* from the environment in every host generation (Kikuchi *et al.* 2011a). However, other families of Lygaeoidea have secondarily lost the crypt-inhabiting symbionts and evolved bacteriomes housing a distinct clade of γ-Proteobacteria (Kuechler *et al*. 2012; Matsuura *et al*. 2012).

In contrast, no such bacteriomes could be detected in *P. apterus* and the gastric caeca constitute small and little developed invaginations that are present only in females and appear to be devoid of any bacteria (Buchner 1965, this study). Hence, our results show that the symbiotic microbiota of Pyrrhocoridae bugs namely *P. apterus* and *D, fasciatus* is markedly different from bugs in other superfamilies within the infraorder Pentamomorpha (Fukatsu and Hosokawa 2002; Hosokawa *et al.*

2010a; Kikuchi *et al.* 2011a; Prado and Almeida 2009), with regard to both the localization of the symbionts in the M3 region of the mid-gut and the composition, consisting predominantly of Actinobacteria and Firmicutes as well as γ- and α-Proteobacteria. This indicates that the microbiota represented in the mid-gut M3 region of *P. apterus* could be specific to bugs in the superfamily Pyrrhocoroidea, and the relationship between gut microbes and pyrrhocorid bugs may represent an ancient and possibly coevolved symbiotic community.

3.5.2 Transmission and establishment of the microbial mid-gut community in *P. apterus*

In order to gain a better understanding of the transmission route and establishment of the symbiotic community within *P. apterus*, we characterized the microbiota in different life stages of the bug. In many species of stinkbugs, the hosts are highly dependent on their microbial partners, and experimental removal of the symbionts has been shown to result in retarded growth and/or high mortality (Abe *et al.* 1995; Fukatsu and Hosokawa 2002; Huber-Schneider 1957; Kikuchi *et al.* 2009; Muller 1956; Schorr 1957; Tada *et al.* 2011). To ensure infection of their offspring with the symbiotic microbes, most of these bugs evolved a vertical transmission route via egg smearing, coprophagy, or the deposition of symbiont-containing capsules (Abe *et al.* 1995; Hosokawa *et al.* 2005; Kikuchi *et al.* 2009; Prado *et al.* 2006; Schorr 1957). In the case of *P.apterus*, the presence of the six most dominant gut bacterial taxa already within the eggs or on the surface indicates that they are likely transmitted vertically from mother to offspring, which has been shown previously for *C. glomerans* (Kaltenpoth *et al.* 2009).

A high diversity of bacterial taxa was detected in the nymphs until the second instar, before the symbiotic bacterial community became well-defined between the second and the third larval instar stage. This is in accordance with direct observations of the feeding behaviour in *P. apterus* nymphs, as the nymphs start feeding on linden seeds only in the 2nd instar stage (Puchkov 1974). Similarly successful establishment of the symbiont in the second instar has been observed in the bean bug (*Riptortus pedestris*) (Kikuchi *et al.* 2011b). Even though in this case the symbiont is acquired horizontally from the environment, acquisition and establishment of the symbionts in later instars were much less effective and could lead to fitness costs for the host (Kikuchi *et al.* 2007; Kikuchi *et al.* 2011b). As newborn nymphs possess a high amount of yolk in their gut and can develop into the second instar without feeding, nutritional symbionts are probably not essential during this developmental period (Kikuchi *et al.* 2011b; Leal *et al.* 1995). Although the symbionts are already present in first instar *P. apterus*, they do not increase to significant abundances until the larva have reached second/third instar. Thus, the microbial community only becomes well-defined upon the initiation of feeding on the herbivorous diet, when the symbionts probably begin to play an important role in supplementing limiting nutrients to the bug (Salem et al., submitted).

3.5.3 Ecological stability of the *P. apterus* gut microbiota

The insect gut is constantly exposed to different diets and to transient microbes that could significantly affect the composition of the indigenous microbiota. This has been evident in several systems such as in mosquitos (*Aedes albopictus* and *A. aegypticus*, Zouache *et al.* 2011) and chestnut weevils (*Curculio sikkimensis*) (Toju and Fukatsu 2011), where the microbial communities vary with the

geographical location and other ecological parameters. Similarly, in European corn borer moths the bacterial community structure differed between lab-reared and wild type hosts (Belda *et al.* 2011). In cockroaches, the supply with an artificial diet that was low in protein and high in fibre content resulted in predictable alterations in the microbial gut community and concomitant changes in gut physiology, with a decline of streptococcal and lactobacillus symbionts from the foregut resulting in a decrease in the production of lactate and acetate (Kane and Breznak 1991). Similarly, the hindgut microbiota of crickets changed significantly with the diet, which resulted in a reduction of hydrogen and carbon dioxide production (Santo Domingo *et al.* 1998).

Our results indicate that the gut microbiota of *P. apterus* is both qualitatively and quantitatively remarkably stable across different populations and diets. Although there were some diet-associated changes in the bacterial community, the six dominant microbial strains were consistently abundant. This ecological stability of the microbial community implies functional importance of the dominant taxa, which is supported at least for the actinobacterial symbionts by experimental manipulation of the microbiota and subsequent fitness assays (Salem et al. submitted). Over evolutionary timescales, functionally relevant and vertically transmitted microbial symbionts are expected to co-evolve with their host, resulting in a congruence of microbial communities on higher taxonomic levels. Such patterns with shared microbial core communities across different species have recently been suggested for ants (Anderson *et al.* 2012), honey bees and bumble bees (Martinson *et al.* 2011) as well as termites and some closely related cockroaches (Schauer *et al.* 2012). Future studies on other Pyrrhocoridae species will allow for an identification of long-term associated microbial symbionts with this family of bugs and reveal the evolutionary history of this association.

3.5.4 Comparison of methods for the characterization of microbial communities

Comparative analyses of the 454 pyrosequencing and the qPCR data of different populations, diets, and life stages of *P. apterus* showed differences in the relative abundances of the dominant bacterial taxa in qPCR and 454 pyrosequencing, indicating that relative abundances obtained by 454 amplicon sequencing alone have to be considered with caution when analyzing the composition of unknown microbial communities (Amend *et al.* 2010; Zhou *et al.* 2011). In addition, some of the dominant bacterial taxa that were consistently present in the M3 region of the mid-gut were not always detected by bTEFAP 454 pyrosequencing, although their presence in the gut could be demonstrated by qPCR. Furthermore, the analysis of the 454 pyrosequencing data revealed multiple OTUs representing each of the most dominant bacterial taxa. Even though this pattern was consistent across different samples and may therefore reflect true biological microdiversity within the gut of *P. apterus*, we cannot exclude the possibility of sequencing artefacts and noise causing an overestimation of bacterial OTUs (Gilles *et al.* 2011; Quince *et al.* 2011), so further analyses are necessary to establish strain-level associations and symbiont microdiversity within *P. apterus*. Thus, even though bTEFAP is an advanced high throughput technique that allows for rapid, cost-effective, and detailed analyses of complex microbial communities, it suffers from similar flaws as the traditional cloning and sequencing approach (PCR biases and other possible confounding factors, as well as a higher rate of sequencing errors), and the results therefore have to be considered with caution (Amend *et al.* 2010; Zhou *et al.* 2011). The combination of several molecular techniques (bTEFAP, cloning/sequencing, diagnostic PCR) is advisable for an accurate characterization of microbial communities.

3.5.5 Putative function of the *P. apterus* mid-gut microbiota

Symbiotic gut microbes can assist their host to subsist on suboptimal diets by increasing its digestion efficiency, detoxifying plant allelochemicals, or by providing digestive enzymes or limiting nutrients (Douglas 1992). Plant material is often low in nitrogen, essential amino acids, B vitamins and sterols (Douglas 1998). However, many herbivorous insects are associated with symbiotic microbes that possess the metabolic abilities to synthesize these compounds and thereby enable the insects to exploit otherwise inaccessible food sources (Douglas 1992; Jones 1984). In several insect taxa, e.g. in crickets, termites and cockroaches, the gut microbiota are involved in breaking down the ingested polysaccharides, notably lignocellulose, the most abundant biological polymer on earth, and fermenting the resulting monosaccharide mixture into short chain fatty acids (Bäckhed *et al.* 2005; Brune and Friedrich 2000; Kaufman and Klug 1991; Nalepa *et al.* 2001). In this mutualistic relationship, the host gains carbon and energy, while the microbes are provided with a rich source of glycan, a protected anoxic environment, as well as a reliable transmission into the next generation of host insects.

Pyrrhocorid bugs preferentially feed on seeds of Malvales plants, which are generally avoided by other phytophagus insects due to the detrimental effects of their phytochemical defenses such as gossypol and cyclopropenoic fatty acids, which can interfere with the digestion of food materials, cause retarded growth, and lead to sterility (Abou-Donia 1976; Allen *et al.* 1967; Kristenová *et al*. 2011). Our comparative analysis of the different gut regions and the microbiota of the ingested food material suggest that specific symbiotic bacteria are restricted to the M3 region, where the food particles also remain for the longest period of time (Kodrík *et al.* 2012; Silva and Terra 1994). Previous studies have shown that the experimental removal of the microbiota by sterilizing the egg surface had a strong negative effect on the fitness of *P. apterus*, as the aposymbionts showed an increased mortality as well as delayed development in comparison to individuals with the native microbiota (Kaltenpoth et al. 2009, Salem *et al.*, submitted).

The mid-gut M3 of *P. apterus* is a completely anoxic environment ideal for fermentation (Fig. S3), and consequently, the resident bacterial taxa are either facultative or obligate anaerobes. Thus, the bacterial taxa residing in the M3 region could play an important role for the insect by degrading complex dietary components, providing nutrient supplementation, or detoxifying noxious chemicals (e.g. cyclopropenoic fatty acids or gossypol) in the diet. Targeted manipulation experiments of the bacterial community in bugs fed on different food sources indicated that the differences in growth rate and survival between aposymbiotic and symbiotic individuals are likely due to a nutritional contribution of the actinobacterial symbionts rather than the detoxification of plant secondary metabolites (Salem et al., submitted). Thus, the symbionts may enable their insect hosts (Pyrrhocoridae) to exploit and diversify in a specific ecological niche (Malvales plants) that is inaccessible to many other insects.

As several members of the Pyrrhocoridae are pests of economically important crops such as cotton (*Gossypium hirsutum*) and okra (*Abelmoschus esculentus)*, elucidating the role of the gut microbiota of pyrrhocorid bugs could provide valuable information to be used in biocontrol. Furthermore, since the vast majority of eukaryote-bacteria symbioses likely constitute complex multipartite rather than one-host/one-symbiont interactions (Ferrari and Vavre 2011), pyrrhocorid bugs represent one of the few established and experimentally amenable systems so far that can be used to address fundamental

questions on the functional roles and the interactions of multiple bacterial symbionts within an insect host.

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3.8 SUPPLEMENT

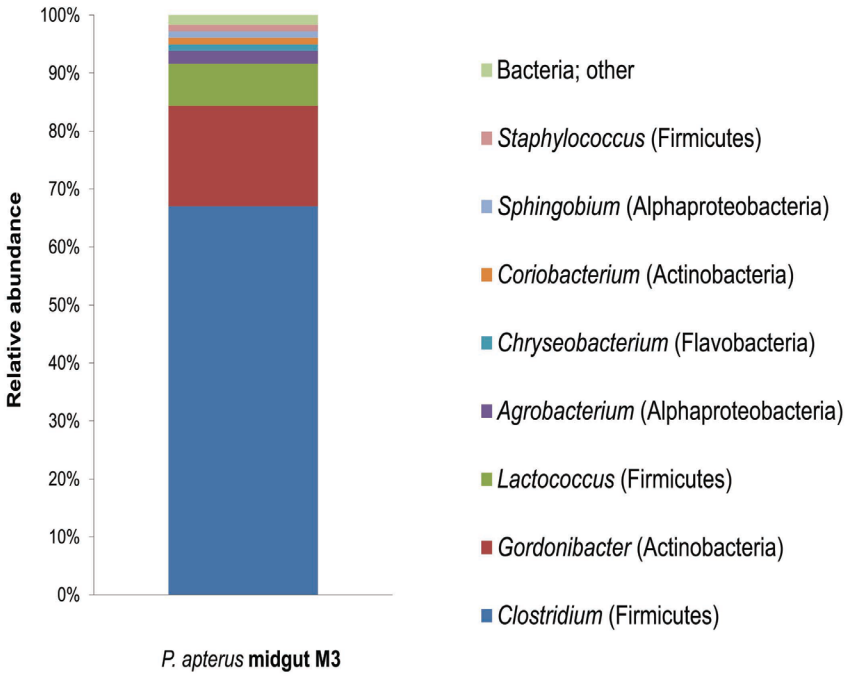

Fig. S1 Composition of the gut microbial community of *Pyrrhocoris apterus* as characterised by using PCR amplification with general eubacterial primers followed by cloning and capillary sequencing [based on 179 near full-length 16S rRNA gene sequences (1.4 kbp)].

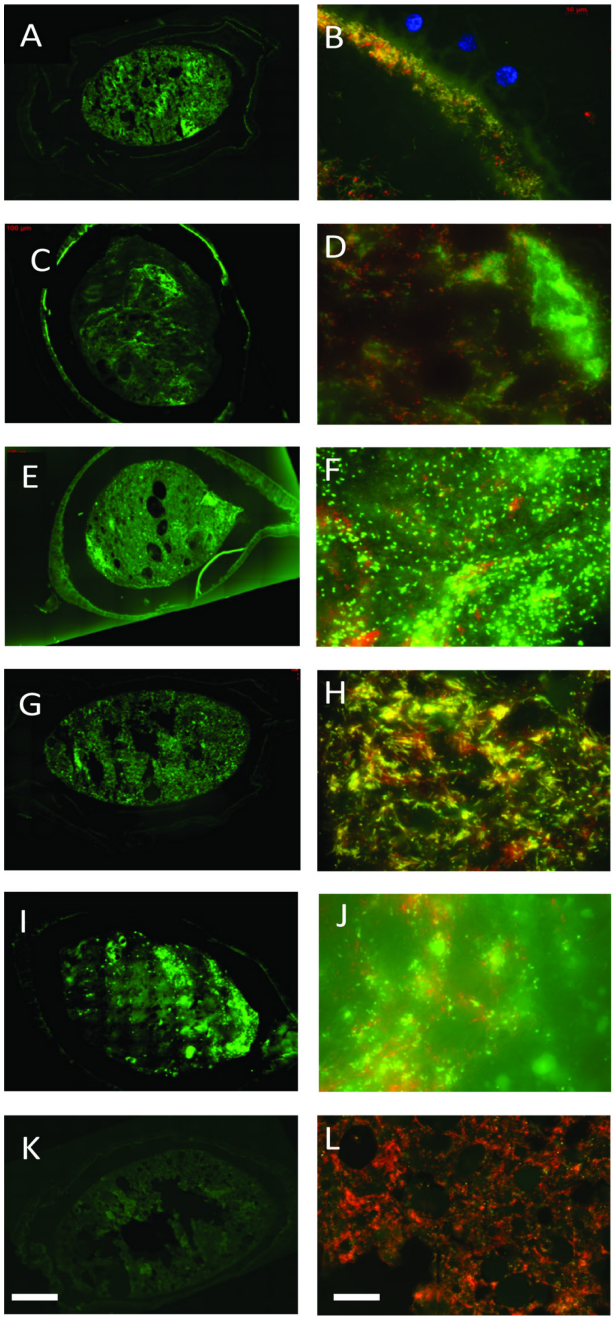

Fig. S2 Fluorescence *in situ* hybridization of complete mid-gut M3 sections (left panel) and close-up views of bacterial cells (right panel) after staining with specific probes (green) for *Coriobacterium glomerans* (A, B) *Gordonibacter* sp. (C, D), *Clostridium* sp. (E, F), Rickettsiales sp., (G, H), *Lactococcus lactis* (I, J), and *Klebsiella* sp. (K, L) as well as the general eubacterial probe EUB338 (red) and DAPI (blue). For the complete mid-gut pictures (left panel), only the green channel was included in the picture. Scale bars: left panel: 100 µm, right panel: 10 µm.

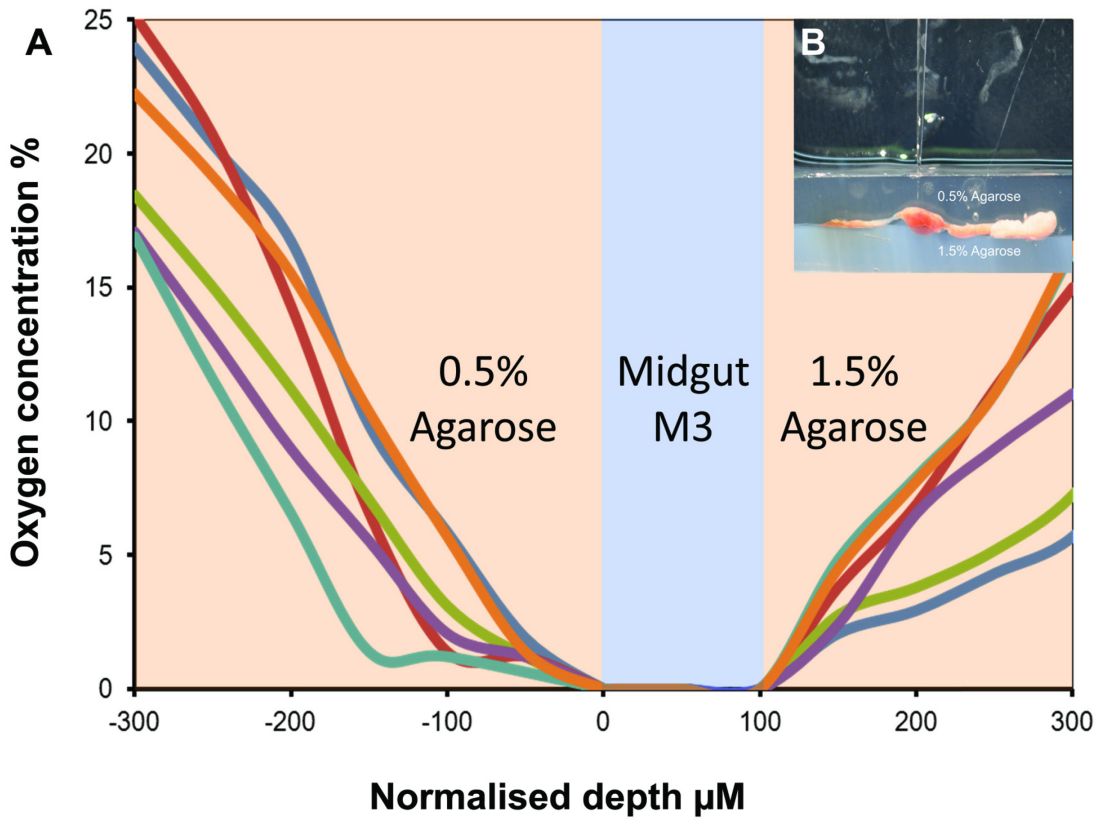

Fig. S3 Oxygen content in the mid-gut M3 region of *Pyrrhocoris apterus*. (A) Radial oxygen measurement in the mid-gut M3 region of six different *P. apterus* individuals. The step increments were 50 µm. (B) Image of the microelectrode set-up used for the oxygen measurement in the mid-gut M3 region.

CHAPTER 4

ACTINOBACTERIA AS ESSENTIAL SYMBIONTS IN FIREBUGS AND COTTON STAINERS (HEMIPTERA, PYRRHOCORIDAE)

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4.1 ABSTRACT

Actinobacteria engage in defensive symbioses with several insect taxa, but reports of nutritional contributions to their hosts have been exceptionally rare. Cotton stainers (*Dysdercus fasciatus*) and red firebugs (*Pyrrhocoris apterus*) (both Hemiptera, Pyrrhocoridae) harbor the actinobacterial symbionts *Coriobacterium glomerans* and *Gordonibacter* sp.as well as Firmicutes (*Clostridium* sp. and *Lactococcus* sp.) and Proteobacteria (*Klebsiella* sp. and a Rickettsiales bacterium) in the M3 region of their mid-gut. We combined experimental manipulation with community-level analyses to elucidate the function of the gut symbionts in both pyrrhocorid species. Elimination of symbionts by egg-surface sterilization resulted in significantly higher mortality and reduced growth rates, indicating that the microbial community plays an important role for host nutrition. Fitness of symbiont-deprived bugs could be completely restored by re-infection with the original microbiota, while reciprocal cross-infections of microbial communities across both pyrrhocorid species only partially rescued fitness, demonstrating a high degree of host-symbiont specificity. Community-level analyses by quantitative PCRs targeting the dominant bacterial strains allowed us to link the observed fitness effects to the abundance of the two actinobacterial symbionts. The nutritional mutualism with Actinobacteria may have enabled pyrrhocorid bugs to exploit Malvales seeds as a food source and thereby possibly allowed them to occupy and diversify in this ecological niche.

4.2 INTRODUCTION

Insects, the most abundant animal class on earth, engage in a remarkable diversity of symbiotic associations involving microbial partners (Buchner, 1965). Many of these partnerships benefit the insect host by improvements to the metabolism, physiology and catabolic capacity through nutritional supplementation or the degradation of complex dietary compounds (Douglas, 1998; 2009; Moran, 2002). Additionally, an increasing number of defensive associations are being described where the symbionts protect their respective hosts and/or the host's food resources from parasites, pathogens or parasitoids (Currie *et al*., 1999; Kellner, 2002; Oliver *et al*., 2003; Kaltenpoth *et al*., 2005; Scarborough *et al*., 2005; Kroiss *et al*., 2010).

Members of the bacterial phylum Actinobacteria are especially prevalent as defensive symbionts due to their ecological and physiological prerequisites, including the ability to utilize a diverse range of nutritional resources and a remarkable versatility in producing secondary metabolites with antibiotic properties (Kaltenpoth, 2009). In contrast, direct evidence for nutritional mutualisms involving this bacterial group have been limited to vectors of the Chagas disease (*Rhodnius prolixus*) and their vitamin-supplementing *Rhodococcus* endosymbionts (Durvasula *et al*., 2008). Nonetheless, an increasing number of studies suggested Actinobacteria to be involved in the nutrition of a range of invertebrates. In scarab beetles of the genus *Pachnoda,* a number of bacterial strains with hemicellulolytic capabilities were isolated from the hindgut, including *Promicromonospora pachnodae*, an actinobacterial species capable of producing a range of xylanases and endoglucanases – two enzyme families involved in cellulose degradation (Cazemier *et al*. 1999, 2003; Andert *et al*. 2010). Additional studies have also demonstrated the occurrence of Actinobacteria across different termite species. However, evidence for their nutritional contributions alongside indications of species-specific associations remains to be provided (Bignell *et al*., 1991; Shinzato *et al*. 2007). In this study, we present evidence for a highly specific partnership involving members of the Pyrrhocoridae insect family and two actinobacterial symbionts.

Within the Pyrrhocoridae, an oligophagous family of bugs, the best known members are cotton strainers of the genus *Dysdercus*, which are serious pests of cotton, and the red firebug (*Pyrrhocoris apterus*), an important model organism for endocrinology and physiology of hemimetabolous insects (Socha, 1993). Cotton stainers exhibit a cosmopolitan distribution mirroring that of the cotton cultivars, with each continent having its own group of species (Pearson, 1958; Ahmad and Schaefer, 1987). *Dysdercus* species harm cotton crops through the indelible staining of the cotton fiber resulting from the excrements of the bug or the accidental processing of insect-bearing bolls, as well as through the emanation of seed juices as a result of puncturing and feeding. Additionally, feeding by puncturing young cotton bolls usually results in the reduction of boll size (Pearson, 1958). *P. apterus* also specializes on seeds of the plant order Malvales, particularly dry seeds of linden trees (*Tilia cordata* and *T. platyphyllos*) (Socha, 1993; Kristenova *et al*., 2011). However, some studies also reported on the exploitation of seeds of other plants groups within and – to a lesser extent – beyond this plant order, as well as on the occasional feeding of firebugs on dead or weakened arthropods (Ahmad and Schaefer, 1987; Kershaw and Kirkaldy, 1908; Kristenova *et al*., 2011).

Previous studies investigating the microbial community of *P. apterus* revealed the presence of extracellular gut symbionts (*Coriobacterium glomerans*) belonging to the actinobacterial family Coriobacteriacae within the digestive tract of the insects, particularly in the M3 section (Haas and Konig, 1987; Kaltenpoth *et al*., 2009). Further characterization of the whole microbiota of *P. apterus*

using bacterial 16S rRNA amplicon pyrosequencing yielded an additional actinobacterial strain belonging to the Coriobacteriacae family (*Gordonibacter* sp.) alongside a range of other facultative and obligate anaerobes such as *Clostridium* sp., an undescribed Rickettsiales bacterium, *Klebsiella* sp. and *Lactococcus* sp. (Sudakaran *et al*., in press; Fig. 1). The consistency of the symbiotic microbial community across geographical localities and different food sources in *P. apterus* suggests that the complex microbiota might have co-evolved with the hosts over millions of years, and that they contribute significantly to the fitness of the insect (Sudakaran *et al*., in press).

Across different insect lineages, the mode of transmission of symbionts from one generation to another is highly variable. The majority of insects harboring intracellular primary symbionts rely on transovarial transmission modes where the infection originates inside of the female hosts during the early stages of oogenesis or embryogenesis (Buchner, 1965; Douglas, 1998; Schroder *et al*., 1996; Sauer *et al*., 2002; Nordon, 2006). In Hemiptera, however, post-hatch transmission is the most common mechanism of transfer, with symbiont acquisition resulting from ingestion of adult fecal droplets (Beard *et al*., 2002), through probing of symbiont containing capsules deposited close to the egg clutch (Fukatsu and Hosokawa, 2002; Hosokawa *et al*., 2005; 2006), or by egg-surface contamination and subsequent probing and uptake of the symbionts by the nymphs (larvae) during the early developmental stages (Prado *et al*., 2006). The latter route of symbiont transfer has also been described for Pyrrhocoridae, and surface sterilization of the egg surface resulted in symbiont-free individuals (Kaltenpoth *et al*., 2009).

In this study, by taking advantage of the transmission mechanism of the symbionts, we experimentally tested the significant contribution of the microbial community towards the fitness of *D. fasciatus* and *P. apterus.* We utilized a quantitative, community-level analysis aimed at identifying strains that contribute directly to the overall fitness of their host. Furthermore, we assessed the specificity of this partnership in the two pyrrhocorid species by reciprocal exchange of symbionts.

4.3 RESULTS

4.3.1 Midgut microbiota of *D. fasciatus*

The midgut microbiota of *D. fasciatus* was analysed using 454 pyrosequencing of bacterial 16S rRNA amplicons. The sequencing data (10,026 sequences) revealed *Clostridium* sp. and *Lactococcus lactis* (Firmicutes), *C. glomerans* and *Gordonibacter* sp., (Actiobacteria), as well as several Gammaproteobacteria as the major microbial taxa in the gut of *D. fasciatus* (Fig. S1, Table S1). While the two Actinobacteria and the two Firmicutes are shared with *P. apterus* (Sudakaran et al. in press), the Rickettsiales bacterium and *Klebsiella* sp, which are present in high frequencies in *P. apterus*, were not detected by 454 in *D. fasciatus*. Using diagnostic qPCR assays with primer pairs designed based on the *P. apterus* symbiont sequences, however, the two strains were consistently found in similar abundances across both bug species, indicating that the absence of these strains in the 454 dataset reflects a technical bias rather than true absence from *D. fasciatus*. In the case of *Klebsiella* sp., the inherently low taxonomic resolution of bTEFAP due to the short read lengths may cause the differences in the classification within Enterobacteriaceae between the *P. apterus* and the *D. fasciatus* symbionts, especially because this bacterial group contains many species (and even genera) with 16S rRNA similarities of more than 97% (our OTU clustering threshold similarity).

4.3.2 Success of symbiont manipulation procedure

Egg surface sterilization successfully eliminated *C. glomerans* in aposymbiotic treatments across both pyrrhocorid species as confirmed by *C. glomerans*-specific diagnostic PCRs (data not shown). In addition, the reinstitution of the microbial community using mid-gut suspensions from conspecific as well as heterospecific individuals confirmed that the nymphs readily accepted symbionts resulting from the experimental smearing of their egg surfaces.

Symbiont abundance estimates from quantitative PCR analyses of corresponding treatments for the two pyrrhocorid species (log-transformed) revealed that the core microbial communities of adult *P. apterus* and *D. fasciatus* were similarly influenced by prior surface sterilization of the eggs. Consistently affected were *C. glomerans* and *Gordonibacter* sp. abundances, with significant reductions in symbiont population sizes in the aposymbiotic treatments (ANOVA, $P < 0.05$) (Fig. 1 and 2). For both symbionts across the two host species, qPCR amplification in the aposymbiotic individuals was indistinguishable from background levels, and melting curve analyses suggested non-target amplification in late cycles (Fig. 1 and 2). Thus, the reported copy numbers likely represent overestimations of the two actinobacterial taxa, and aposymbiotic bugs may in fact be completely devoid of these symbionts.

The abundance of *Clostridium* sp. and *Lactococcus* sp. cells was significantly reduced by surface sterilization of the eggs only in *D. fasciatus* (ANOVA, $P < 0.05$) (Fig. 1), but not in *P. apterus* (Fig. 2). Uniformly unaffected by the egg sterilization procedures were the *Klebsiella* sp. and Rickettsiales populations, since their abundances were constant across treatments in both pyrrhocorid species (Fig. 4 and 5).

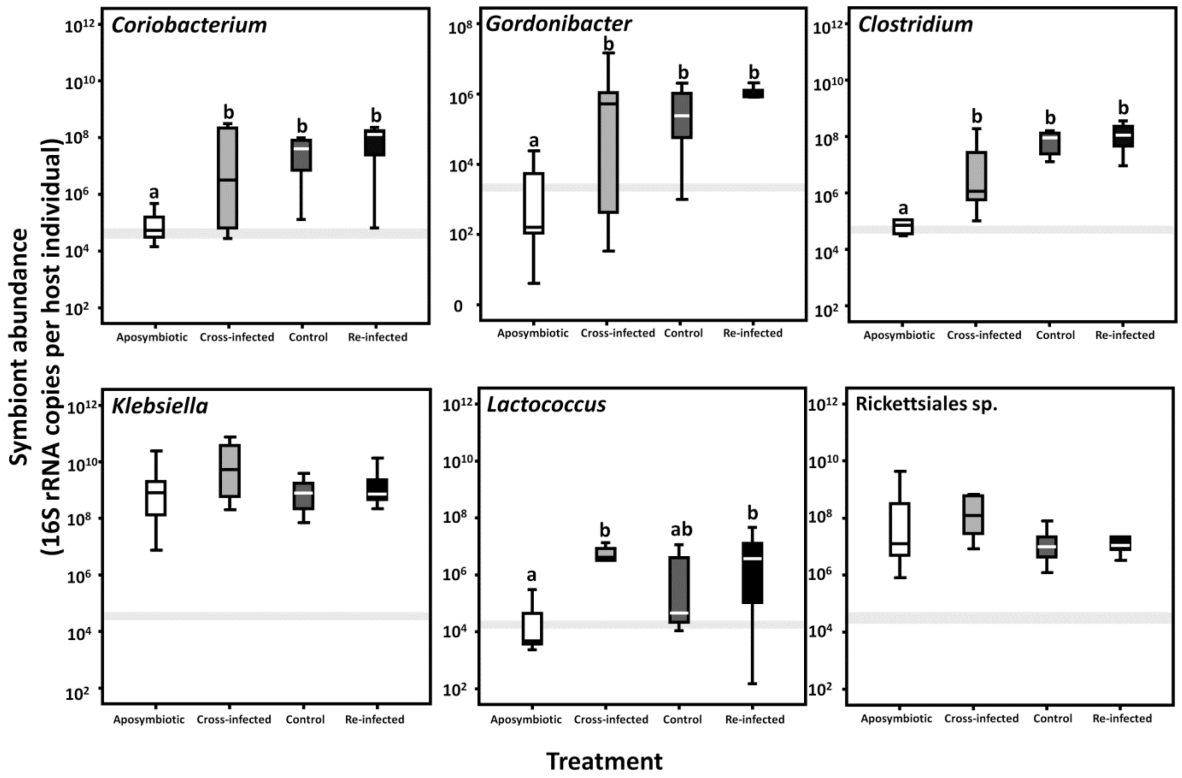

Figure 1. Quantitative PCR analyses of the six dominant microbial strains across the four experimental treatments for *D. fasciatus*. Symbiont numbers represent estimated 16S rRNA gene copy numbers obtained from qPCR assays. Shading of boxes signifies the experimental treatment. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Grey bands represent the range of unspecific background amplification for the negative controls. Different letters above boxes indicate significant differences in copy numbers (repeated-measures anova, $P<0.05$).

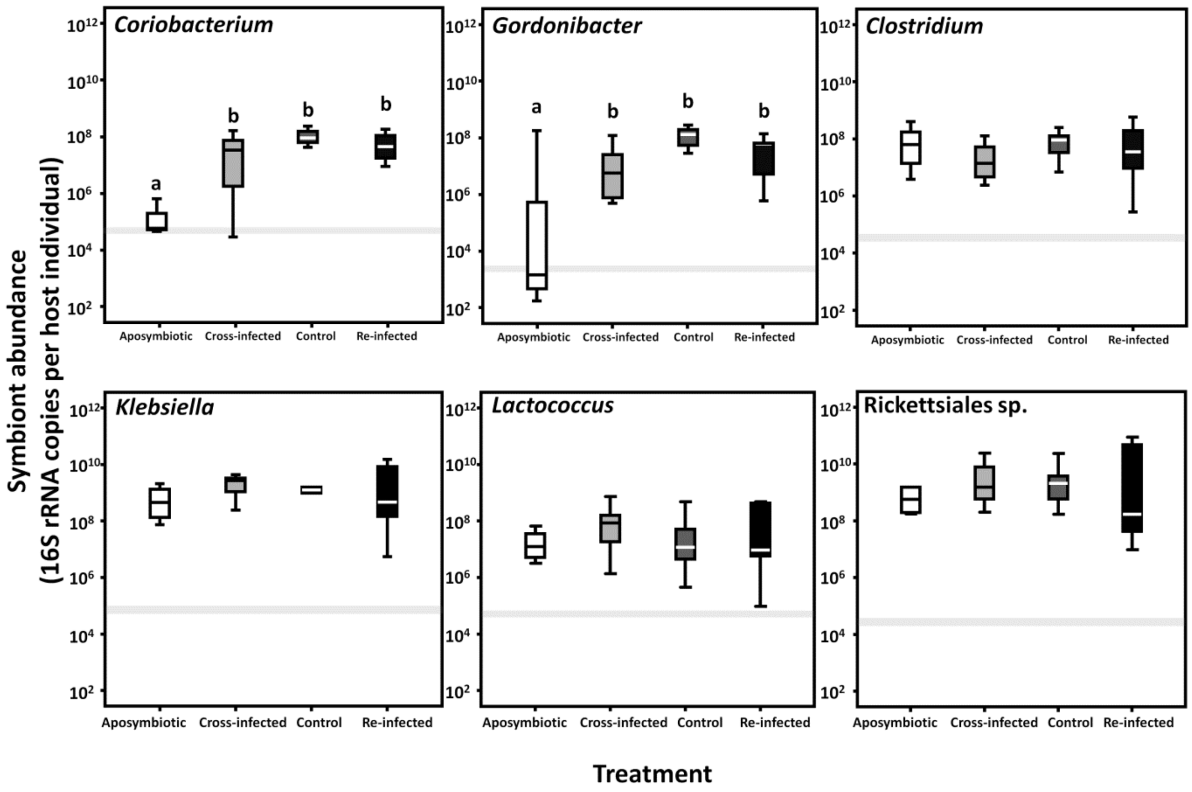

Figure 2. Quantitative PCR analyses of the six dominant microbial strains across the four experimental treatments for *P. apterus*. Symbiont numbers represent estimated 16S rRNA gene copy numbers obtained from qPCR assays. Shading of boxes signifies the experimental treatment. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Grey bands represent the range of unspecific background amplification for the negative controls. Different letters above boxes indicate significant differences in copy numbers (repeated-measures anova, $P<0.05$).

4.3.3 Fitness of *P. apterus* and *D. fasciatus* following symbiont manipulation

Aposymbiotic individuals across the two species of pyrrhocorids were found to suffer significantly higher mortality relative to the control treatments (Friedman test, $P < 0.01$ for both pairwise comparisons) (Fig. 3 A and B). Re-infection using mid-gut suspensions from conspecific individuals entirely rescued the adverse fitness effects caused by symbiont elimination. However, cross infection using mid-gut suspensions from heterospecific hosts did not significantly enhance chances to survive until adulthood compared to aposymbiotic treatments (Fig. 3 A and B). The developmental time until adulthood was also found to be negatively influenced by symbiont elimination and exchange (Fig. 3 C) in *D. fasciatus* (Friedman test, $P < 0.05$). Similar trends were also observed for the *P.apterus* aposymbiotic treatment, but the effects were not significant (Fig. 3 D).

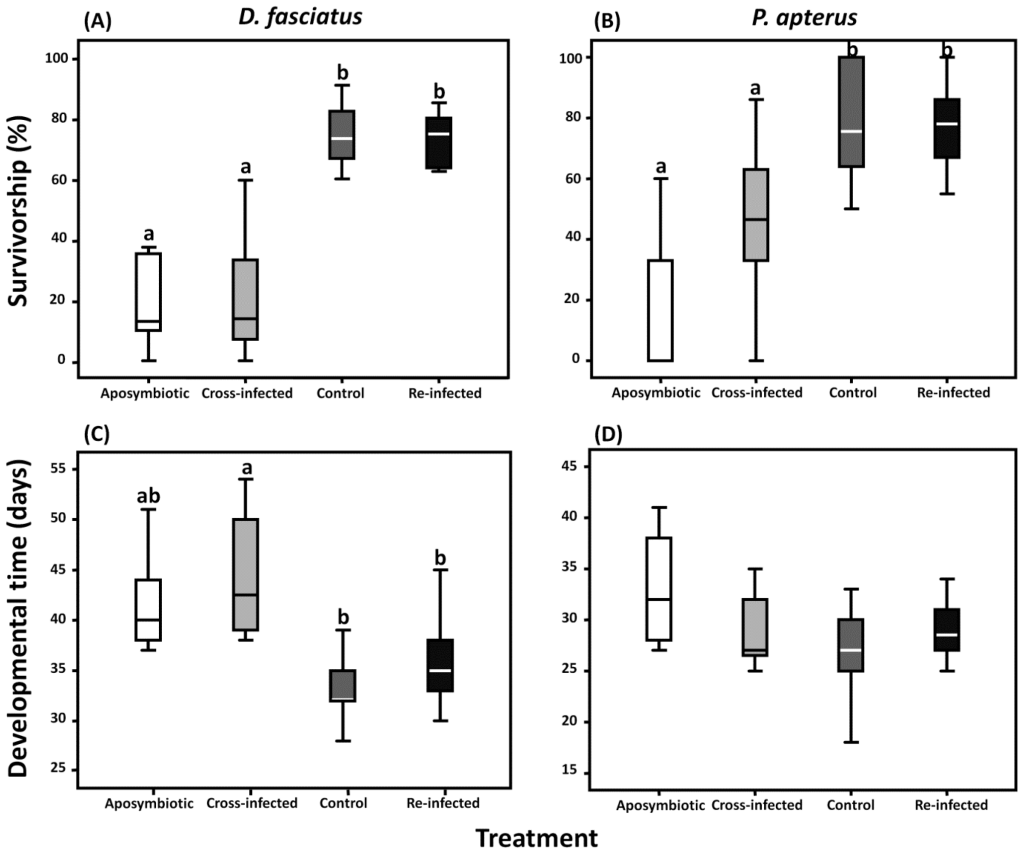

Figure 3. Fitness of aposymbiotic, symbiotic (control and re-infected) and cross-infected individuals of *D. fasciatus* (left panel) and *P. apterus* (right panel). **A** and **B**. Survivorship from egg hatching to adulthood. **C** and **D**. Nymphal development time until adult stage (days). Shading of boxes denotes the experimental treatment. Lines represent medians, boxes comprise the 25–75 percentile, and whiskers denote the range. Different letters above boxes indicate significant differences (Friedman test, $P<0.05$).

4.3.4 Reproductive success of adult *D. fasciatus* females following symbiont manipulation

On average, mating initiated across all symbiont-containing groups (control, re-infected and cross-infected) 3-7 days following the moult into adulthood. Behavioral assays revealed a complete lack of copulation among adults of the aposymbiotic treatment, which ultimately resulted in a lack of ovipositing and, thus, a significantly reduced reproductive success compared to the other treatments (ANOVA, $P < 0.05$) (Fig. 4 A). Aposymbiotic individuals were also observed to be less active, and the males were less aggressive in their mating pursuits.

For the control, re-infected, and cross-infected treatments, mated females laid on average 3.4 egg clutches throughout their lifespan, with the average reproductive output of 127-192 eggs. There were no differences in reproductive success across the three symbiont-containing treatments (Fig. 4 B) (ANOVA, $P > 0.05$).

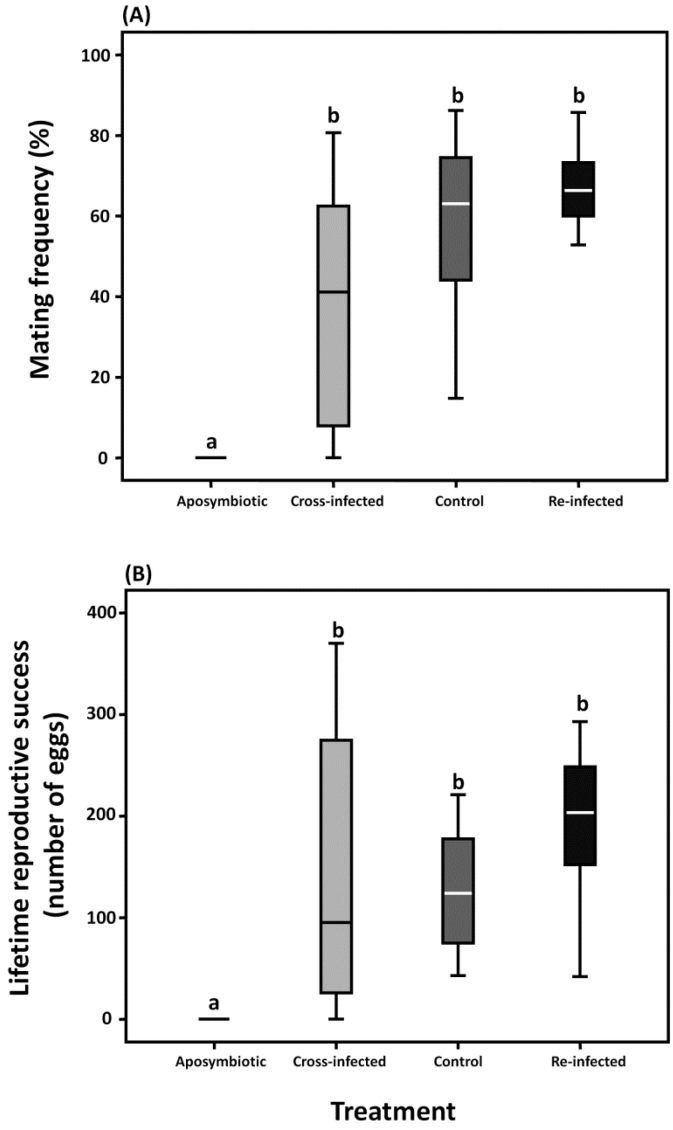

Figure 4. Reproductive success of aposymbiotic, symbiotic (control and re-infected) and cross-infected individuals of *D. fasciatus*. **A**. Mating frequencies. **B**. Lifetime reproductive success. Shading of boxes denotes the experimental treatment. Lines represent medians, boxes comprise 25–75 percentile, and whiskers denote the range. Different letters above boxes indicate significant differences (anova, $P<0.05$).

4.3.5 Correlations linking individual strain abundances to fitness

Abundance estimates for each of the six dominant strains across treatments were correlated with mortality of the respective replicate treatment groups to make inferences regarding the species-specific contributions of the symbionts towards the enhancement of host fitness. These analyses revealed positive correlations linking survivorship to higher frequencies of *C. glomerans* and *Gordonibacter* sp. (Spearman, $P < 0.05$) for both *P. apterus* and *D. fasciatus* (Fig. 5). While we are confident that the possible overestimation of actinobacterial abundances in aposymbiotic treatments constitutes a possible source of error that is conservative with regard to the hypothesis tested, we repeated the correlation analyses under the assumption that the aposymbiotic individuals were completely devoid of Actinobacteria as suggested by the melting curve analyses of the qPCRs. Hence, for this second analysis, we substituted the abundances of symbionts that yielded deviating melting curves in the qPCR assays and showed similar threshold cycle values as the negative controls with 0. The results

remained the same, with abundances of *C. glomerans* and *Gordonibacter* sp. being significantly correlated with host fitness for both *P. apterus* and *D. fasciatus* (Spearman, $P < 0.05$). In addition to the Actinobacteria, *Clostridium* sp. abundances were positively correlated with survivorship in *D. fasciatus (*Spearman*,* $P < 0.001$) (Fig. S2), but not for *P. apterus* ($P = 0.677$) (Fig. S3). No significant correlations between survival and strain abundance were detected for any of the other strains (Spearman, $P > 0.05$ for all correlations, Fig. S2 and S3).

Mortality breakdown per developmental stage among aposymbiotic individuals of *P. apterus* indicated that mortality is highest between the 2nd and 4th instars (Fig. 6 A), which correlates directly to the host developmental stages where, according to quantitative PCR analyses by Sudakaran *et al.* (in press), the gut microbial community (including the Coriobacteriacae symbionts) exhibits the highest growth rates (Fig. 6 B).

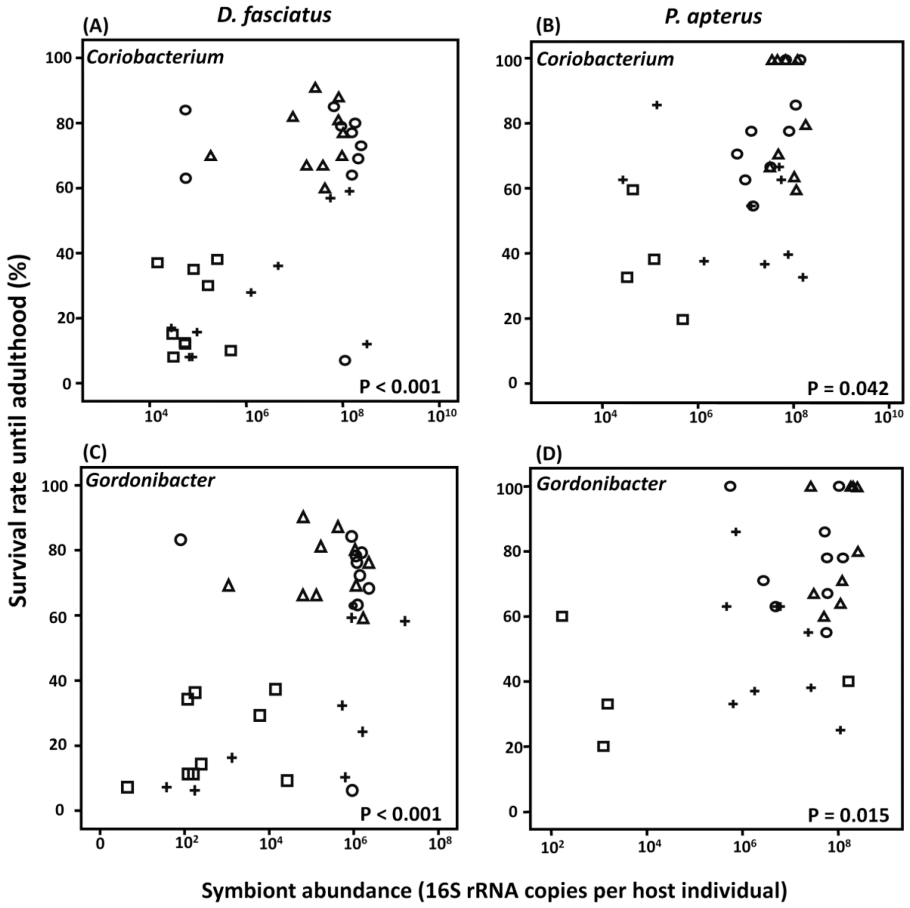

Figure 5. Correlations of survival rate and Coriobacteriacae abundances for *D. fasciatus* (A, C) and *P. apterus* (B, D) across treatments. Survival (%) relates to the survival rate of replicate treatments from egg hatching to adulthood. Coloration of samples signifies the experimental treatment each individual was ascribed to (square = aposymbiotic, cross = cross infected, triangle = untreated control, circle = re-infected). Lines denote significant correlations of symbiont copy number and survival rate (Spearman, $P < 0.05$).

4.3.6 Fitness of *D. fasciatus* fed on sunflower seeds

To assess the possible effects of specific toxic components in the linden seed diet on the fitness of symbiotic and aposymbiotic bugs, we performed an additional experiment with sunflower seeds that are devoid of the toxic compounds often present in plants of the order Malvales. As on the linden seed diet, however, aposymbiotic individuals of *D. fasciatus* that were fed exclusively on sunflower seeds were found to suffer significantly higher mortality relative to the control treatment (Wilcoxon signed ranks test, $P < 0.05$) (Fig. 7 A). Additionally, developmental time until adulthood was significantly increased after symbiont elimination in *D. fasciatus* (Friedman test, $P < 0.05$, Fig. 7 B). Interestingly, both aposymbiotic and symbiotic sunflower-fed bugs exhibited higher mortality and longer developmental times than their linden seed-fed counterparts, respectively (Fig. 3 and Fig. 7).

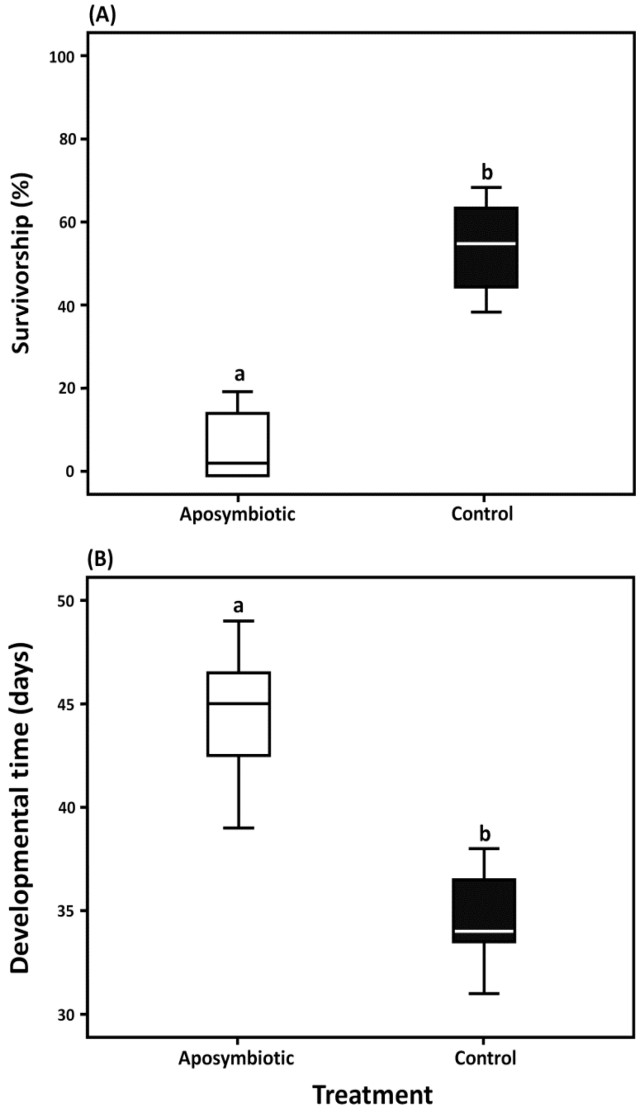

Figure 6. Fitness of aposymbitic and control groups for D. fasciatus when fed on sunflower seeds. (**A**) Survival rate from egg hatching to adulthood. (**B**) Nymphal development time until adult stage (days). Boxes represent the 25-75 percentile, the middle bands represent the median, and the whiskers denote the range. Different letters above boxes indicate significant differences (Wilcoxon test, $P < 0.05$).

4.4 DISCUSSION

The ubiquity of Actinobacteria in the environment, coupled with their capacity towards producing substances with antimicrobial compounds has probably predisposed them to engage in defensive symbioses with soil-dwelling insects (Kaltenpoth, 2009). In contrast, direct evidence for their involvement in nutritionally-based partnerships has been relatively scarce (but see Lake and Friend, 1967; 1968). Here we report on the occurrence of two actinobacterial taxa (*C. glomerans* and *Gordonibacter* sp.) as essential nutritional symbionts in pyrrhocorid bugs.

Fitness assays demonstrate that symbiont elimination via surface sterilization of the egg surface significantly reduces host fitness and reproductive output of both pyrrhocorid species used in this study, which is consistent with the putative function of the symbionts for the nutritional upgrading of the host. The ability to restore normal fitness to the resulting nymphs from previously sterilized eggs by reapplying native microbial suspensions ensures that the method of symbiont elimination was not responsible for the adverse effects observed for aposymbiotic individuals (Fig. 3). Consistent with these findings is the breakdown of aposymbiotic treatment mortality per developmental stage. These analyses point towards the 2nd, 3rd and 4th instars as the stages where individuals from this treatment suffer the greatest reduction in fitness (Fig. 6 A). It is during those stages that the Coriobacteriacae symbiont populations experience the highest growth rate in untreated control individuals (Fig. 6 B), further confirming the strong correlation between actinobacterial presence and fitness effects. This is congruent with a previous study addressing the contributions of the actinomycete *Rhodococcus rhodnii* towards its triatomine host (*Rhodnius prolixus*) where aposymbiotic individuals suffered the greatest mortality during the 3rd and 4th instars (Lake and Friend, 1968).

The combined analysis of the 454 pyrosequencing and qPCR revealed that the core microbial community, namely Actinobacteria (*C. glomerans* and *Gordonibacter* sp.), Firmicutes (*Clostridium* sp., and *Lactococcus lactis*) and Proteobacteria (an uncultivated bacteria associated with the Rickettsiales, and *Klebsiella* sp.) is shared across both pyrrhocorid hosts (Fig. S1). The symbiont manipulation and exchange procedure utilized in our study consistently influenced the microbial community present within pyrrhocorid bugs. For both bug species, we observed significant variation between aposymbiotic and control treatments for *Gordonibacter* sp. and *C. glomerans* frequencies, suggesting that that the egg surface sterilization procedure was especially effective in ridding pyrrhocorids from their Coriobacteriacae symbionts (Fig. 1 and 2). More direct evidence for strain-specific contributions to host fitness was provided by the correlative analyses linking estimated strain abundances for emerging adults to the survivorship of the replicate treatment that the individual belonged to. These correlations singled out *Gordonibacter* sp. and *C. glomerans* as essential symbionts given their consistently significant correlations across the two pyrrhocorid species (Fig. 5). This indicates that one or both actinobacterial strains are important for the fitness of pyrrhocorid bugs. However, until strain-specific reinfection procedures are performed, we cannot disentangle the individual contributions of *C. glomerans* and *Gordonibacter* sp. towards host fitness. Despite observing a significant positive correlation between the frequency of *Clostridium* sp. and survivorship in *D. fasciatus*, their mutualistic potential was undermined by their high abundance values in the aposymbiotic treatments of *P. apterus* (Fig. S2 and S3). Additionally, no such tends were observed for *Lactococcus* sp. in either pyrrhocorid species despite an apparent fluctuation in symbiont abundances in *D. fasciatus* as a result of the surface sterilization procedure (Fig. S2). Possibly, the infection with *Clostridium* sp. and *Lactococcus* sp. may be beneficial for pyrrhocorids, but not essential for growth

and development of the host. The consistency of both proteobacterial species (*Klebsiella* sp. and the undescribed Rickettsiales bacterium) across all treatments suggests that those symbionts are not influenced by the surface sterilization procedure (Fig. 1 and 2), possibly due to a transovarial transmission route where the infection of the eggs can originate inside of the female host during the early stages of oogenesis. Due to our experimental approach targeting only bacteria that are transmitted via the egg surface, we cannot make any inferences here about the influence of these strains on host fitness.

High mortality rates observed for pyrrhocorid bugs that were cross-infected with microbial suspensions from heterospecific individuals indicate that the fitness benefits conferred to the insect host are governed by a high degree of specificity. Interestingly, quantitative PCR measurements of cross-infected individual indicate that the acquired number of Coriobacteriacae symbiont cells (1.4×10^7 cells) were comparable to those present in control and re-infected treatments (Fig 1 and 2). Thus, host-symbiont interactions rather than lower symbiont titers appear to be responsible for the fitness reductions in cross-infected bugs. This is contrary to the experimental exchange of obligate gut symbiotic bacteria of the stinkbugs *Megacopta punctatissima* and *Megacopta cribraria*, where symbionts from heterospecific hosts were found to fully restore fitness irrespective of the receiving insect host species (under the condition that the insects were fed on optimal host plants) (Hosokawa *et al*., 2007). The high degree of relatedness of *M. punctatissima* and *M. cribraria* can account for the variation in symbiont specificity observed in Hosokawa *et al*. 2007, where symbiont exchange was conducted on an inter-species level as opposed to the inter-generic scale of this study.

Reports on the feeding biology of Pyrrhocoridae have indicated a clear preference for ripened seeds of plants belonging to the angiosperm order Malvales (including cotton) (Socha, 1993; Ahmad and Schaefer, 1987). However, some studies have also pointed towards limited intra- and interspecific carnivorous behavior where the bugs have been found to utilize their stylets to attack slower, weaker prey or recently deceased arthropods (Ahmad and Schaefer, 1987; Kershaw and Kirkaldy, 1908). Therefore, unlike symbiotic relationships in insects persisting exclusively on nutritionally deficient diets (e.g. blood or sap-feeding insects) - but similar to the *Blochmania*-harboring carpenter ants (Feldhaar *et al*., 2007) – pyrrhocorids, to a certain degree, appear to supplement their specialized food source of Malvales seeds with a nutritionally rich carnivorous diet. This raises the question regarding the putative function of the Coriobacteriacae symbionts in this family.

Generally, bacterial symbionts can enable insect hosts to exploit specialized food sources by supplying additional nutrients that are limited in their diet, assisting in the degradation of complex plant tissue, or by providing a detoxifying function against the plant's secondary compounds (Douglas, 2009). Cotton plants and a range of other Malvaceae species contain high concentrations of gossypol, a phenolic aldehyde that acts as an inhibitor for several dehydrogenase enzymes (Abou-Donia, 1976; Reeves and Valle, 1923), as well as malvalic and sterculic acid, which are cyclopropenoic fatty acids that have been demonstrated to inhibit the desaturation of stearic acid in animals fed on cotton-seed derivatives (Allen *et al*., 1967). However, pyrrhocorid bugs as well as some other herbivorous insects appear to have evolved a mechanism to cope with the toxic plant compounds. We hypothesized that the actinobacterial symbionts of Pyrrhocoridae may aid in the detoxification of gossypol or other toxic components of Malvales seeds. However, our current findings do not support this hypothesis, since aposymbiotic individuals exhibited high mortality even when fed on a gossypol and cyclopropenoic acid-free diet composed of sunflower seeds (Fig. 7). Thus, the primary role of the symbionts appears to be a more general nutritional function rather than the detoxification of noxious secondary plant

compounds. Whitsitt (1933) demonstrated that cottonseed meals offer prohibitively low amounts of vitamins, especially riboflavin, to be utilized exclusively as a food source. Based on these considerations and the results of the present study, it seems likely that the main function of the Coriobacteriaceae symbionts lies either in the degradation of complex plant compounds (e.g. cellulose) or the supplementation of the diet with limiting vitamins. However, until we assess the importance of the gammaproteobacterial symbionts (*Klebsiella* sp. and the Rickettsiales bacterium) and Firmicutes (*Clostridium* sp. and *Lactococcus* sp.), and disentangle the individual contributions of *C. glomerans* and *Gordonibacter* sp. through strain specific reinfections, we cannot rule out detoxification as a putative additional function of the microbial community.

Members of the genus *Dysdercus* are of serious importance given their worldwide distribution spanning every major cotton-producing continent, and the irreversible damage that they confer to this economically important crop plant. Elucidating the contribution of the resident symbiotic community to the fitness of pyrrhocorid bugs and the fundamental mechanisms by which these benefits are conferred not only expands our understanding of the ecology of a serious agricultural pest, but may also provide novel leads for biological control by manipulation of the host's microbiota. To elucidate the exact functions of *C. glomerans* and *Gordonibacter* sp. towards their hosts, additional approaches examining the metabolism, physiology and genomic signatures of these symbionts are required.

While most studies investigating insect-bacterial symbioses are focused on elucidating the contributions of a single microbial symbiont towards its host (Douglas, 1998; 2009; Moran, 2002), the majority of insects, including the Pyrrhocoridae insect family, are inhabited by a complex gut microbial community (Buchner, 1965; Sudakaran *et al*., in press; Dillon and Dillon, 2004; Ferrari and Vavre, 2011). Despite their abundance and ecological importance, functional analyses detailing the fitness contributions of symbionts in multipartite interactions have been scarce to date. Thus, we believe that our quantitative community-level approach for investigating the fitness contributions of several bacterial strains towards a single host presents a powerful tool that can be broadly utilized to gain important insights into functional roles of individual microbial taxa in symbiotic systems that involve multiple partners.

4.5 EXPERIMENTAL PROCEDURES

4.5.1 Insect sampling and rearing

Adult specimens of *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae) were collected from the vicinity of linden trees (*Tilia cordata* and *Tilia platyphyllos*) in Jena, Germany. The insects were reared in plastic containers (20×35×22 cm) at a constant temperature of 28°C and long light regimes (16h/8h light/dark cycles) to prevent the insects from entering into diapausal states. Bugs were provided with previously autoclaved water and crushed dry linden seeds (*T. cordata* and *T. platyphyllos*). *Dysdercus fasciatus* were acquired from a laboratory culture maintained at the University of Würzburg, Germany, which had been originally collected from the Comoé National Park, Côte d'Ivoire. The insects were reared under the same conditions as described for *P. apterus.*

4.5.2 Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) and data analysis

Prior to DNA extraction, six complete *D. fasciatus* adults (three males and three females) were submerged in liquid nitrogen and crushed with sterile pestles. DNA was extracted using the MasterPure^{TM} DNA Purification Kit (Epicentre Technologies) according to the manufacturer's instructions. BTEFAP was done using an external service provider (Research and Testing Laboratories, Lubbock, USA) with 16S rRNA primers Gray28F and Gray519R (Ishak *et al.* 2011; Sun *et al.* 2011). A sequencing library was generated through one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar high fidelity *Taq* polymerases (Qiagen). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Research and Testing Laboratory (RTL, Lubbock, TX, USA, http://www.researchandtesting.com). All low quality reads (quality cut-off = 25) and sequences < 200 bp were removed following sequencing, which left 10,026 sequences for analysis. The raw reads (sff files) were deposited in the short read archive (SRA) of NCBI under accession number SRA058953.

Analysis of the high-quality reads was conducted by using QIIME (Caporaso *et al.* 2010b). Cdhit (Li and Godzik 2006) and Uclust (Edgar 2010) with 97% similarity cut-offs was employed in Multiple OTU picking to cluster the sequences into operational taxonomic units (OTUs). The most abundant sequences was chosen as representative sequence was for each OTU picked and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast (Caporaso *et al.* 2010a), with the minimum sequence identity percent set to 75%. RDP classifier was used for taxonomy assignment (Wang *et al.* 2007), with a minimum confidence to record assignment set to 0.80. OTU tables was generated describing the occurrence of bacterial phylotypes within the sample. The table was then manually curated by removing low-frequency reads (<0.1% in all samples) and through database comparisons of the representative sequences with the NCBI and RDP databases. Based on the BLASTn results, OTUs with the same genus-level assignments were combined for visualization of the results. The revised OTU table was used to construct heatmaps using the MultiExperiment Viewer (MeV) software (Saeed *et al.* 2003).

4.5.3 Experimental manipulation of the microbial gut community

To avoid pseudoreplication, ten egg clutches (>35 eggs each) from different females of *P. apterus* and *D. fasciatus,* respectively, were harvested five days after ovioposition and kept separately. Each clutch was randomly separated into four different experimental treatments: (1) aposymbiotc, (2) re-infected with native microbial community, (3) cross-infected with heterospecific microbial community, (4) untreated control. Aposymbiotic individuals were generated by surface sterilization of eggs following the procedure describe by Prado *et al*. [28]. Briefly, eggs were submerged in bleach (12% NaOCl) for 45 seconds, followed by a five minute 95% ethanol treatment which was subsequently washed off thoroughly with sterile H_2O. Reapplication of the symbiotic microbial community was accomplished by spreading a suspension of the crushed M3 gut region of a conspecific or a heterospecific individual over previously sterilized eggs. Future contamination of the experimental treatments with environmental bacteria was reduced by using autoclaved food (linden seeds) and water throughout the course of the study.

To investigate the possibility of symbiont-mediated detoxification of plant secondary compounds associated with the linden seeds, eight additional egg clutches from different *D. fasciatus* females were

harvested and separated into two different experimental treatments: (1) aposymbiotic, and (2) untreated control. Both treatments were reared as described above, but the newly hatched nymphs were supplemented with autoclaved sunflower seeds instead of linden seeds.

4.5.4 Fitness measurements

Individuals across all experimental treatments were observed on a daily basis for the assessment of fitness effects across the different groups. Growth rate (nymphal stage) and survival until adulthood (%) were recorded. A replicate treatment group of bugs was defined to have completed a nymphal stage when 50% of the nymphs had successfully molted into the following developmental stage.

To quantify the reproductive success of emerging adult *D. fasciatus* females, eight females and eight males were collected across the replicates of each treatment, respectively. Due to the high mortality in the aposymbiotic group, only six males could be allocated to the eight aposymbiotic females. For each female, we measured the copulation frequencies and the total number of eggs laid throughout its lifespan (lifetime reproductive success). Mating frequencies were determined by assessing the percent of days an individual was found in a mating pair. All egg masses laid by each of the females were collected, and the number of eggs was counted throughout the lifespan of the bug.

4.5.5 DNA extraction and PCR screening for *C. glomerans*

A single emerging adult individual from every experimental treatment for both species was subjected to DNA extraction three days after adult emergence (Kaltenpoth *et al*., 2009). Males and females were used indiscriminately, as they have been shown previously to harbor identical microbial communities in the mid-gut (Sudakaran *et al*., in press). To validate that the surface sterilization and reinfection procedures were successful, primers specific for *C. glomerans* (Kaltenpoth *et al*., 2009) were utilized to screen for the symbiont using diagnostic PCR reactions (Table 1). To account for any failures during DNA extraction, additional PCR screens targeting the host 18S rRNA genes of *P. apterus* and *D. fasciatus* were performed (Li *et al*., 2005), and negative samples were discarded from further analysis (Table 1). PCR amplifications were conducted on a VWR® Gradient Thermocycler (VWR, Radnor, PA, USA) using 12.5 µl reactions, including 1 µl of DNA template, 1×PCR buffer (20 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, and 0.01% Tween 20), 2.5 mM $MgCl_2$, 240 mM dNTPs, 0.8 µM of each primer, and 0.5 µl of Taq DNA polymerase (VWR, Radnor, PA, USA). The following cycling parameters were used: 3 min at 94ºC, followed by 32 cycles of 94ºC for 40 s, 68/66ºC for 1 min (Cor/Pyr primers, respectively), and 72ºC for 1 min, and a final extension time of 4 min at 72ºC.

Table 1. Diagnostic primers used for the specific detection of *C. glomerans* in pyrrhocorids and for positive control amplification of host DNA.

Primer	Primer sequence (5′–>3′)	Orientation	Target group	Reference
Cor_2F	GGTAGCCGGGTTGAGAGACC	Fwd.	*C. glomerans*	Kaltenpoth *et al*., 2009
Cor_1R	ACCCTCCCMTACCGGACCC	Rev.		
Pyr18S_2F	GGGAGGTAGTGACAAAAAATAACG	Fwd.	Pyrrhocoridae	Sudakaran *et al*., in press
Pyr18S_4R	GTTAGAACTAGGGCGGTATCTG	Rev.		

4.5.6 Quantitative PCR

Quantitative PCRs (qPCRs) for the six dominant bacterial strains within pyrrhocorids (Sudakaran *et al*., in press) were conducted across the four experimental treatments of *P. apterus* and *D. fasciatus* using a RotorGene®-Q cycler (Qiagen, Hilden, Germany), with the same individual DNA extracts used for the *C. glomerans* screen. The final reaction volume of 25 µl included the following components: 1 µl of DNA template, 2.5 µl of each primer (10 µM), 6.5 µl of autoclaved distilled H_2O, and 12.5 µl of SYBR Green Mix (Qiagen, Hilden, Germany). The primers used were specific for the 16S rRNA genes of *C. glomerans, Gordonibacter* sp., *Clostridium* sp., the undescribed Rickettsiales bacterium, *Klebsiella* sp. and *Lactococcus lactus* (Table 2) (Sudakaran *et al*., in press). Verification of primer specificity was conducted *in silico* by comparison with reference sequences of all bacterial taxa in *P. apterus* (Sudakaran *et al*., in press). Additionally, PCR products of all strain-specific PCRs were sequenced without prior cloning from mid-gut samples of *P. apterus* (Sudakaran *et al*., in press) and *D. fasciatus* (this study) to confirm primer specificity *in vitro*. Conditions for qPCR were optimized using a VWR® Gradient Thermocycler (VWR, Radnor, PA, USA) at various annealing temperatures (60-68 °C). Standard curves (10-fold dilution series from 1 ng/µl to 10^{-6} ng/µl) were generated using purified PCR products for all six primers after measuring the PCR products using a NanoDropTM1000 spectrophotometer (Peqlab). The following cycling parameters were used: 95°C for 10 min., followed by 45 cycles of 68ºC for 30 s, 72ºC for 20 s, and 95ºC for 15 s. Subsequently, a melting curve analysis was conducted by increasing the temperature from 60ºC to 95ºC within 20 min. Six replicates of one of the standard concentrations were used, for each primer pair and concentration, for the configuration and calibration of the standard curve. The resulting averages were then utilized to correct for possible errors in the DNA concentration measurments. Based on the standard curve, absolute copy numbers of specific 16S templates were calculated according to Lee *et al.* (Lee *et al*., 2006; 2008).

Table 2. Bacterial 16S rRNA primers used for the strain-specific qPCR assays.

Primer	Primer sequence (5′–>3′)	Orientation	Target group	Reference
Cor_2F	GGTAGCCGGGTTGAGAGACC	Fwd.	*C. glomerans*	Kaltenpoth *et al*., 2009
Cor_1R	ACCCTCCCMTACCGGACCC	Rev.		
fD1 (=27F)	AGAGTTTGATCCTGGCTCAG	Fwd.	*Gordonibacter* sp.	Weisburg *et al*., 1991 this study
Egg_1R	CCGGAGCTTCTTCTGCAGGT	Rev.		
Proteobac_16s_fwd	GTGGCAAACGGGTGAGTAAT	Fwd.	Undescribed Rickettsiales species	Sudakaran *et al*., in press
Proteobac_16s_rev	GAAGTCTGGGCCGTATCTCA	Rev.		
Klebsiella_250-fwd	CAGCCACACTGGAACTGAGA	Fwd.	*Klebsiella* sp.	Sudakaran *et al*., in press
Klebsiella_453-rev	GTTAGCCGGTGCTTCTTCTG	Rev.		
Clostridium_1050-fwd	CTCGTGTCGTGAGATGTTGG	Fwd.	*Clostridium* sp.	Sudakaran *et al*., in press
Clostridium_1248-rev	GCTCCTTTGCTTCCCTTTGT	Rev.		
Lactococcus_975-fwd	CGCTCGGGACCTACGTATTA	Fwd.	*Lactococcus lactus*	Sudakaran *et al*., in press
Lactococcus_1175-rev	GCAGCAGTAGGGAATCTTCG	Rev.		

4.5.7 Statistical analysis

For both Pyrrhocoridae species, growth and survival rates until adulthood were compared across the four experimental treatments using Friedman tests with Wilcoxon-Wilcox posthoc measures for the analysis of dependent samples (with replicates across treatments paired via individual egg clutches), using the statistical software BiAS 7.40 (Epsilon-Verlag; Hochheim-Darmstadt, Germany). To compare symbiont 16S copy numbers estimated in the qPCRs, and reproductive success of emerging females (only *D. fasciatus*), ANOVA was used as implemented in the SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA).Growth and survival rates of the two *D. fasciatus* treatments (aposymbiotic and untreated control) reared on sunflower seeds were compared using Wilcoxon signed ranks test using the BiAS 7.40. Non-parametric bivariate correlations (Spearman) between symbiont abundance (of the representative individual subjected to qPCR) and treatment mortality within each replicate were performed for each symbiont strain in order to infer the individual contributions of the strains towards the host's overall fitness and development (SPSS 17.0).

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4.8 SUPPLEMENT

Table S1. Absolute and relative (in percent) sequence abundance of bacterial OTUs in *D. fasciatus*.

Bacterial taxa	Sequence abundance	Relative abundance
Clostridium (Firmicutes)	1998	19.9
Gordonibacter (Actinobacteria)	1011	10.1
Coriobacterium (Actinobacteria)	711	7.09
Lactococcus (Firmicutes)	2085	20.8
Enterobacter (Proteobacteria)	1455	14.5
Stenotrophomonas (Proteobacteria)	983	9.80
Kluyvera (Proteobacteria)	720	7.18
Lactobacillus (Firmicutes)	699	6.97
Commensalibacter (Proteobacteria)	71	0.70
Unknown Actinomcyetes	50	0.49
Azospirillum (Proteobacteria)	46	0.46
Agrobacterium (Proteobacteria)	39	0.39
Brevundimonas (Proteobacteria)	34	0.34
Methylobacterium (Proteobacteria)	34	0.34
Sphingomonas (Proteobacteria)	25	0.25
Enterococcus (Firmicutes)	24	0.24
Variovorax (Proteobacteria)	14	0.14
Bartonella (Proteobacteria)	13	0.13
Propionibacterium (Actinobacteria)	13	0.13
Hafnia (Proteobacteria)	1	0.01
Total	**10026**	**100**

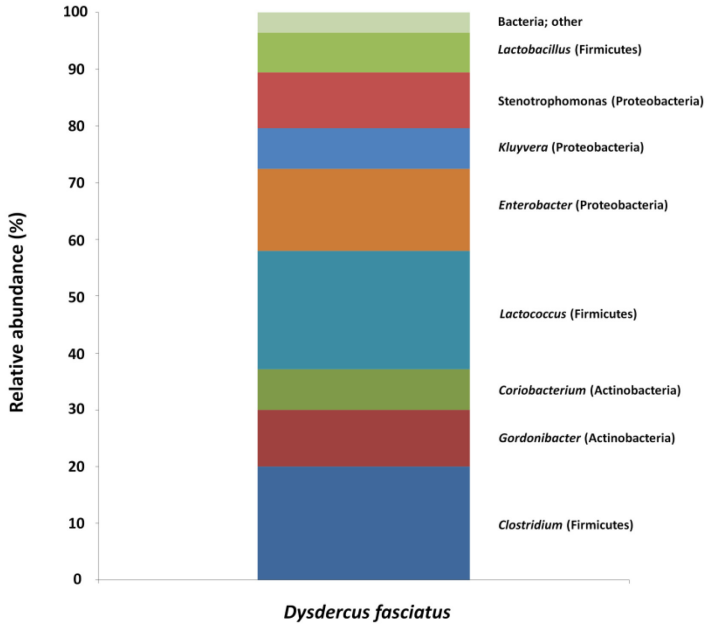

Figure S1. Bacterial community of *P. apterus* and *D. fasciatus*. Relative abundance of gut bacterial taxa (genus-level) from 454 pyrosequencing of 16S rRNA amplicons (10,026 reads in total) of a pooled sample of six adult individuals.

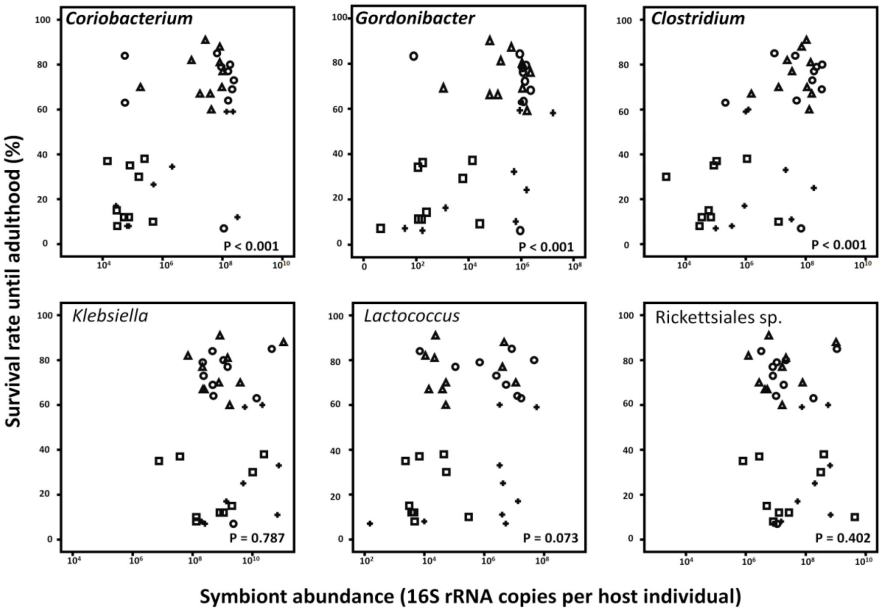

Figure S2. Correlations of survival rate and individual symbiont abundances for *D. fasciatus* across treatments. Survival (%) relates to the survival rate of replicate treatments from egg hatching to adulthood. Shapes of data points signifies the experimental treatment each individual was ascribed to (square = aposymbiotic, cross = cross infected, triangle = untreated control, circle = re-infected). Bold subtitles denote significant correlations of symbiont copy numbers and survival rates (Spearman, $P < 0.05$).

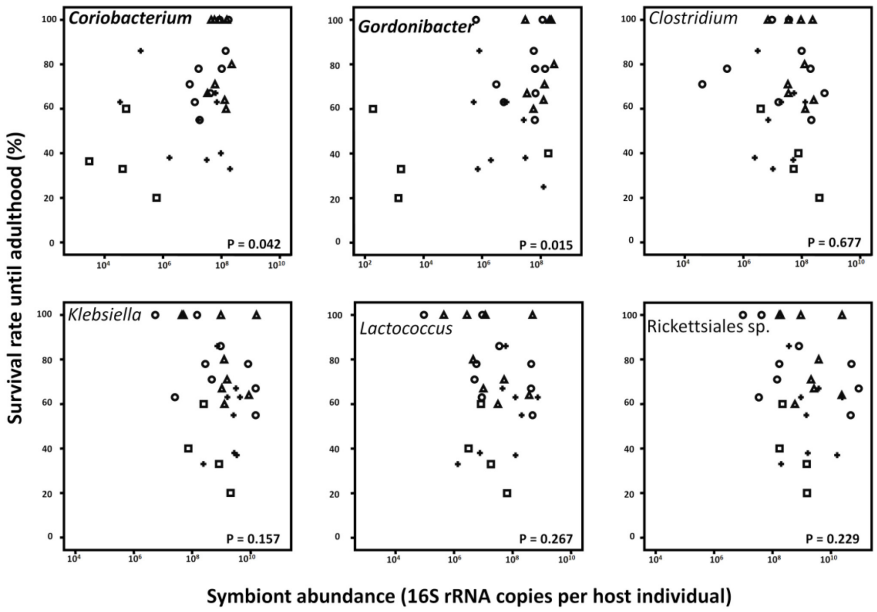

Figure S3. Correlations of survival rate and individual symbiont abundances for *P. apterus* across treatments. Survival (%) relates to the survival rate of replicate treatments from egg hatching to adulthood. Shapes of data points signifies the experimental treatment each individual was ascribed to (square = aposymbiotic, cross = cross infected, triangle = untreated control, circle = re-infected). Bold subtitles denote significant correlations of symbiont copy numbers and survival rates (Spearman, $P < 0.05$).

CHAPTER 5

VITAMIN SUPPLEMENTATION BY GUT SYMBIONTS ENSURES METABOLIC HOMEOSTASIS IN AN INSECT HOST

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5.1 ABSTRACT

Many animals are dependent on intestinal microbes for nutrition. However, our understanding of how the host regulates its metabolism in response to beneficial symbionts remains limited. Here we elucidate the functional importance of the African cotton stainer's (*Dysdercus fasciatus*) association with two actinobacterial gut symbionts and subsequently examine the insect's transcriptional response following symbiont elimination. Genomic analyses and bioassays demonstrate the symbionts' contribution towards host fitness through the supplementation of B vitamins. Concordantly, comparative transcriptomic analyses reveal a differential up-regulation of genes involved in import and processing of B vitamins in aposymbiotic bugs; an expression pattern that is indicative of vitamin deficiency in animals. Normal expression levels of these genes, however, can be restored by either artificial supplementation of B vitamins into the insect's diet or reinfection with actinobacterial symbionts. Furthermore, the functional characterization of the differentially expressed thiamine transporter 2 (*THTR2*) through heterologous expression in *Xenopus laevis* oocytes confirms its role in cellular uptake of vitamin B1. Taken together, our findings demonstrate that – despite an extracellular localization – beneficial gut microbes can be integral to an insect's metabolic homeostasis, reminiscent of bacteriome-localized intracellular mutualists.

[#] = Equal contributions

5.2 INTRODUCTION

Insects derive a range of nutritional benefits from their microbial and fungal partners. These can include the supply of essential nutrients, as well as aiding in the detoxification and digestion of ingested food (Douglas, 2009). As a result, many nutritional partnerships are considered to have played a significant role in the ecological diversification of insects by mediating the host's subsistence on novel, and often nutritionally deficient, food sources (Moran, 2007).

Like other animals, insects lack the metabolic pathways for the synthesis of several vital nutritional compounds, including the ten essential amino acids, as well as most vitamins (Moran, 2007; Payne and Loomis, 2006). Hence, insects can achieve complete nourishment only by acquiring the missing nutrients either from ingested food sources, or through symbiotically-mediated supplementation involving microbial partners. Aphids, for example, are capable of exploiting plant sap (deficient in essential amino acids as well as some vitamins) largely due to the supplementing capacities of their obligate symbiont *Buchnera aphidicola* (Douglas, 2009). Similarly, in their subsistence on B vitamin-deficient vertebrate blood, bed bugs (*Cimex lectularius*) and tsetse flies (*Glossina spp*.) rely on intracellular symbionts for complete development and successful reproduction (Hosokawa *et al*., 2010; Akman *et al*., 2002). In bed bugs, rescuing of aposymbionts' (symbiont-deprived) fitness was achieved through the oral supplementation of B vitamins (Hosokawa *et al*., 2010). For polyphagous insects, the metabolic burdens of acquiring these essential nutrients solely through their food sources are less evident; however, findings by Feldhaar and colleagues (Feldhaar *et al*., 2007) in carpenter ants (*Camponotus floridanus*) demonstrated the impact of symbiont-mediated nutritional upgrading towards enhancing the competitive ability of the insect host relative to symbiont-lacking individuals.

In bugs of the family Pyrrhocoridae (Hemiptera), recent characterization of the microbial community revealed a consistent and conserved microbiota, with the notable co-occurrence of two actinobacterial taxa belonging to the Coriobacteriaceae family (*Coriobacterium glomerans* and *Gordonibacter* sp.) (Sudakaran *et al*., 2012; Salem *et al*., 2013). Similar to other heteropteran insects (Fukatsu, 2002; Kikuchi *et al*., 2007), firebugs rely on an extracellular post-hatch mechanism for the vertical transmission of their actinobacterial symbionts. This involves the deposition of symbiont-containing droplets by adult females over newly laid eggs, and the subsequent probing and uptake of the symbionts by the hatched nymphs (Kaltenpoth *et al*., 2009). In the European firebug (*Pyrrhocoris apterus*) and the African cotton stainer (*Dysdercus fasciatus*) (both Pyrrhocoridae), egg surface sterilization results in symbiont-deprived individuals (aposymbionts) (Kaltenpoth *et al*., 2009) that, relative to their symbiotic counterparts, exhibit slower growth rates, higher mortality and lower reproductive success (Salem *et al*., 2013), suggesting an essential function of the actinobacterial symbionts towards their insect hosts. Re-infection of previously sterilized eggs with the Coriobacteriaceae symbionts (via gut suspensions) completely rescues firebug survivorship and reproductive potential, thereby demonstrating that the adverse effects associated with aposymbiosis cannot be attributed to the egg surface sterilization procedure (Salem *et al*., 2013).

Dysdercus species, like many pyrrhocorids, are specialized feeders that exhibit a strong preference for seeds of the plant order Malvales, particularly cottonseeds (Ahmad and Schaeffer, 1987). Given the rich protein content of many Malvales seeds, the putative nutritional provisions by the symbionts have been suggested to lie not in the supply of essential amino acids – as with aphids and other sap-feeding insects – but rather in the supplementation of B vitamins (Sudakaran *et al*., 2007; Salem *et al*., 2013),

which were described as limiting in many seed-based diets, including those of the Malvales plant order (e.g. cotton) (Whitsitt, 1933).

In this study, we used an integrated approach to study both host and symbiont contributions towards vitamin production, transport, and processing in cotton stainers (*D. fasciatus*), in order to gain comprehensive insights into the metabolic integration of extracellular gut symbionts in an insect host. To this aim, we first investigate the possible nutritional role of the Coriobacteriaceae symbionts towards *D. fasciatus* using a defined artificial diet that allows for omission of specific nutrients. Through bioassays that combine symbiont elimination with direct manipulations of the host's diet, we test the hypothesis that the actinobacterial symbionts provide *D. fasciatus* with B vitamins. These experiments are complemented by genomic analyses of *C. glomerans*' potential for vitamin production. Furthermore, we examine the effect of symbiont elimination on the mid-gut transcriptome of cotton stainers, specifically with respect to the expression of B vitamin transporters and downstream processing genes, as well as genes involved in the host's response to metabolic stress, with the expectation that these candidate genes would be differentially regulated in case of vitamin deficiency. Our findings provide a detailed description for symbiotic contributions of the Coriobacteriaceae towards pyrrhocorid bugs, and shed light on a highly dependent and surprisingly integrated nutritional partnership featuring extracellularly localized gut symbionts.

5.3 RESULTS

5.3.1 Success of symbiont elimination procedure

In order to manipulate symbiont infection status, *D. fasciatus* eggs were surface-sterilized by ethanol and bleach treatment (Salem *et al*., 2013). This procedure consistently resulted in aposymbiotic firebugs that were free of both Coriobacteriaceae symbionts, as confirmed by *C. glomerans*- and *Gordonibacter*-specific diagnostic PCRs. Conversely, symbiotic bugs tested positive for both bacterial species (data not shown).

5.3.2 Fitness of symbiotic and aposymbiotic *D. fasciatus* on different variations of an artificial diet

To investigate the possibility of symbiont-mediated supplementation of B vitamins, we examined the survivorship of aposymbiotic and symbiotic bugs reared on two variations of an artificial diet: (i) aposymbiotic bugs reared on a B vitamin deficient diet, (ii) aposymbiotic bugs reared on a complete diet (iii) symbiotic bugs reared on a B vitamin deficient diet, and (iv) symbiotic bugs reared on a complete diet. When reared on a complete artificial diet containing all B vitamins in sufficient quantities, firebug survivorship until adulthood was consistently high, irrespective of symbiont presence (Fig. 1A). Equivalent survivorship rates were observed for symbiotic bugs reared on a B vitamin deficient diet (Fig. 1A). However, aposymbiotic bugs reared on a vitamin deficient diet suffered significantly higher mortality compared to all other treatments (Friedman test, $P = 0.012$, with Wilcoxon-Wilcox posthoc measures, $P < 0.05$) (Fig. 1A). While similar trends were also observed for the developmental rates across treatments, with vitamin omission from the artificial diet increasing the

time needed to reach adulthood of aposymbiotic individuals, the differences were not significant (Friedman test, P = 0.129) (Fig. 1B).

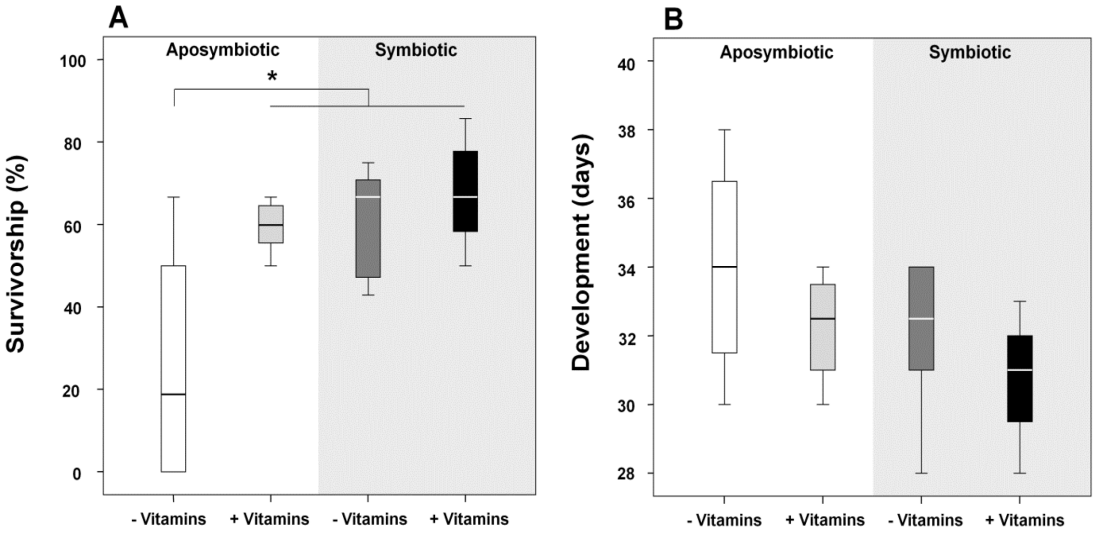

Figure 1. Fitness of control and aposymbiotic *D. fasciatus* on an artificial diet with and without B vitamins, respectively. (**A**, **B**) Survivorship and developmental time from egg hatching to adulthood, respectively. Shading of boxes signifies the experimental treatments. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Significant differences (Friedman test with Wilcoxon-Wilcox posthoc measures, $P < 0.05$) are marked by an asterisk.

5.3.3 Midgut transcriptome of *D. fasciatus* reared on the natural diet of cottonseeds

To examine the metabolic response to symbiont elimination under natural feeding conditions, we conducted a comparative transcriptome analysis of the mid-guts of aposymbiotic and symbiotic *D. fasciatus* that had been reared on their natural diet of cottonseeds (Fig. S1).

Among the 55,222 assembled contigs, 20,174 received a BLAST annotation (Fig. S1, Table S1). The majority of those annotations were based on hits from the bean bug (*Riptortus pedestris*) and the pea aphid (*Acyrthosiphon pisum*), which represent the closest relatives of *D. fasciatus* with sequenced genomes available. From the BLAST results, 3,362 contigs were assigned to functional categories according to gene ontology (GO) (Fig. S1). However, we focus here on gene candidates involved in the transport and processing of B vitamins, a heat shock protein for the assessment of a general stress response, as well as a glucose transporter for comparing the expression pattern of the vitamin transporters with an unrelated nutritional transporter (that has also been described as a stress indicator) (Piroli *et al*., 2004).

In total, 11 transcripts of proteins putatively involved in the intracellular activation (Fig. 2A) and transport (Fig. 2B) of B vitamins were detected in the *D. fasciatus* midgut transcriptome. For transport genes, these included the thiamine transporter 2 (*THTR2*) and a proton-coupled folate transporter (*PCFT*) for the epithelial transport of thiamine and folate, respectively. Additionally, an extracellular thiamine alkaline phosphatase (*ALKP*) gene was annotated and described to putatively catalyze the

transphosphorylation of thiamin (B1) to thiamin monophosphate, thereby facilitating its epithelial transport. Assessing relative expression levels across individuals by quantitative PCR (normalized to the expression of the 60S ribosomal protein L13a), all three genes were found to be significantly up-regulated in the aposymbiotic treatment as compared to symbiont-containing control individuals (Fig. 2B).

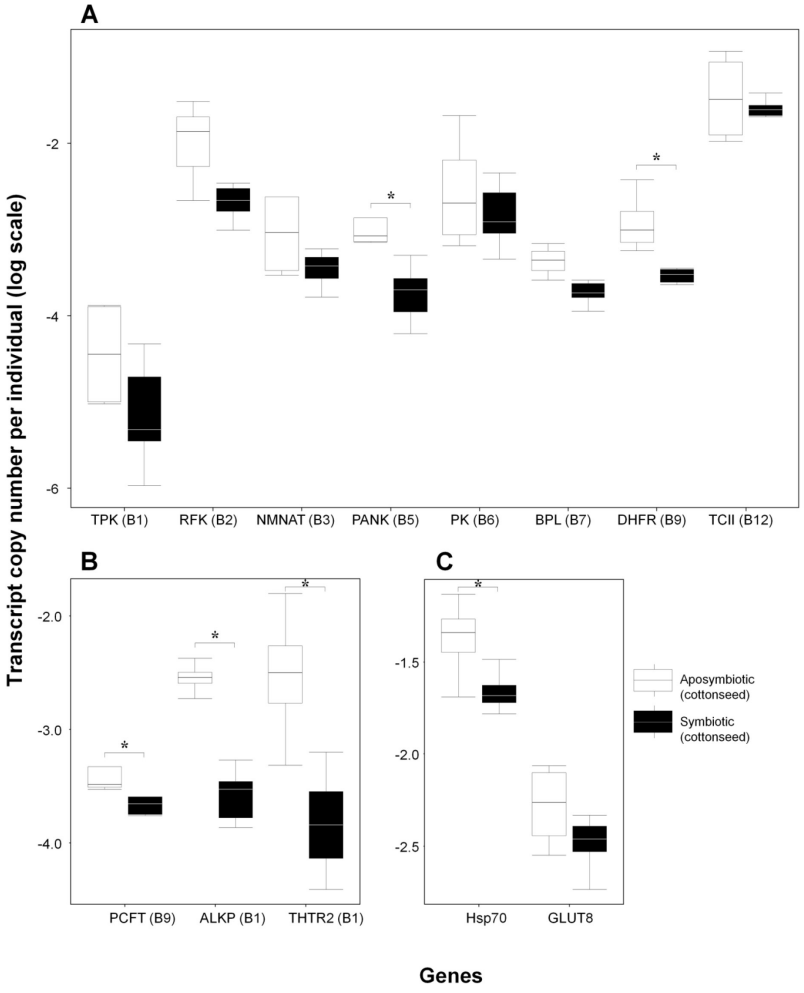

Figure 2. Differential expression of candidate host genes by qPCR for symbiotic and aposymbiotic *D. fasciatus* reared on cottonseeds.

(**A**) Intracellular B vitamin processing genes: thiamine pyrophosphokinase (*TPK*), riboflavin kinase (*RFK*), nicotinamide mononucleotide adenylyltransferase (*NMNAT*), pantothenate kinase (*PANK*), pyridoxal kinase (*PK*), biotin-protein lyase (*BPL*), dihydrofolate reductase (*DHFR*), and transcobalamine 2 (*TCII*).
(**B**) B vitamin transport and extracellular processing genes: thiamine transporter 2 (*THTR2*), proton-coupled folate transporter (*PCFT*) and thiamine alkaline phosphatase (*ALKP*).
(**C**) A stress indicator; heat shock protein (*Hsp*) 70, and a glucose transporter (*GLUT8*). Shading of boxes signifies the experimental treatments (see legend). Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Significant differences were assessed based on the normalized expression in reference to the 60S ribosomal protein L13a with Mann-Whitney U-tests. Differentially expressed transcripts at $P < 0.05$ are marked by *****.

Furthermore, transcripts of eight intracellular enzymes involved in the activation of all B vitamins were detected (Fig. 2A), including thiamine pyrophosphokinase (*TPK*) for vitamin B1, riboflavin kinase (*RFK*) for vitamin B2, nicotinamide mononucleotide adenylyltransferase (*NMNAT*) for vitamin B3, pantothenate kinase (*PANK*) for vitamin B5, pyridoxal kinase (PK) for vitamin B6, biotin-protein lyase (*BPL*) for vitamin B7, dihydrofolate reductase (*DHFR*) for vitamin B9, and transcobalamine 2 (*TCII*) for vitamin B12. Similar to the aforementioned transport genes, *PANK* and *DHFR* were differentially up-regulated in the aposymbiotic treatment relative to symbiotic bugs (Fig. 2A). While similar trends were also observed for *TPK*, *RFK*, *NMNAT*, *PK*, *BPL* and *TCII* (Fig. 2A), the effects were not significant.

A stress response was additionally ascribed to aposymbiotic bugs due to the up-regulation of many stress-related genes (Table S1) – including the highly expressed heat shock protein (*Hsp*) 70 – relative to the symbiotic treatment (Fig. 2C). While *Hsp70* had been previously characterized in firebugs by Kostal and Tollarova-Borovanska (Kostal and Tollarova-Borovanska, 2009) under conditions of temperature stress, in other animal groups, the elevated expression of *Hsp70* has also been reported under conditions of nutrient limitation (Ribeil *et al*., 2007). Given that symbiotic and aposymbiotic bugs were reared under the same constant temperatures, a climate-induced stress response can be ruled out.

5.3.4 Vitamin transport and processing gene expression for symbiotic and aposymbiotic *D. fasciatus* reared on complete and vitamin-deficient artificial diets

Quantitative PCRs (qPCRs) for the candidate host genes involved in B vitamin metabolism were conducted across the four experimental treatments of aposymbiotic and symbiotic bugs reared on a complete and vitamin-deficient artificial diet to examine if the up-regulation of vitamin-transport and -processing genes (Fig. 2) substantiates under controlled conditions with vitamin availability as the only nutritional difference between dietary treatments.

For most B vitamin related genes annotated in this study, a consistent differential expression pattern was observed across the four experimental treatments of (i) symbiotic bugs reared on a complete diet, (ii) symbiotic bugs reared on a vitamin deficient diet, (iii) aposymbiotic bugs reared on a complete diet, and (iv) aposymbiotic bugs reared on a vitamin deficient diet. Based on quantitative PCR analyses, aposymbiotic bugs reared on a vitamin deficient diet consistently and significantly exhibited higher relative expression levels of vitamin-transport and –processing genes than aposymbiotic bugs on a vitamin-deficient diet or symbiotic *D. fasciatus* reared on either complete or vitamin-deficient diets. This was the case for 10 out of the 11 predicted B vitamin related genes (Fig. 3A and B), the heat shock protein (*Hsp70*), as well as the glucose transporter (*GLUT8*) (Fig. 3C). While the same trend was observed for the proton-coupled folate transporter (*PCFT*), the differences were not significant.

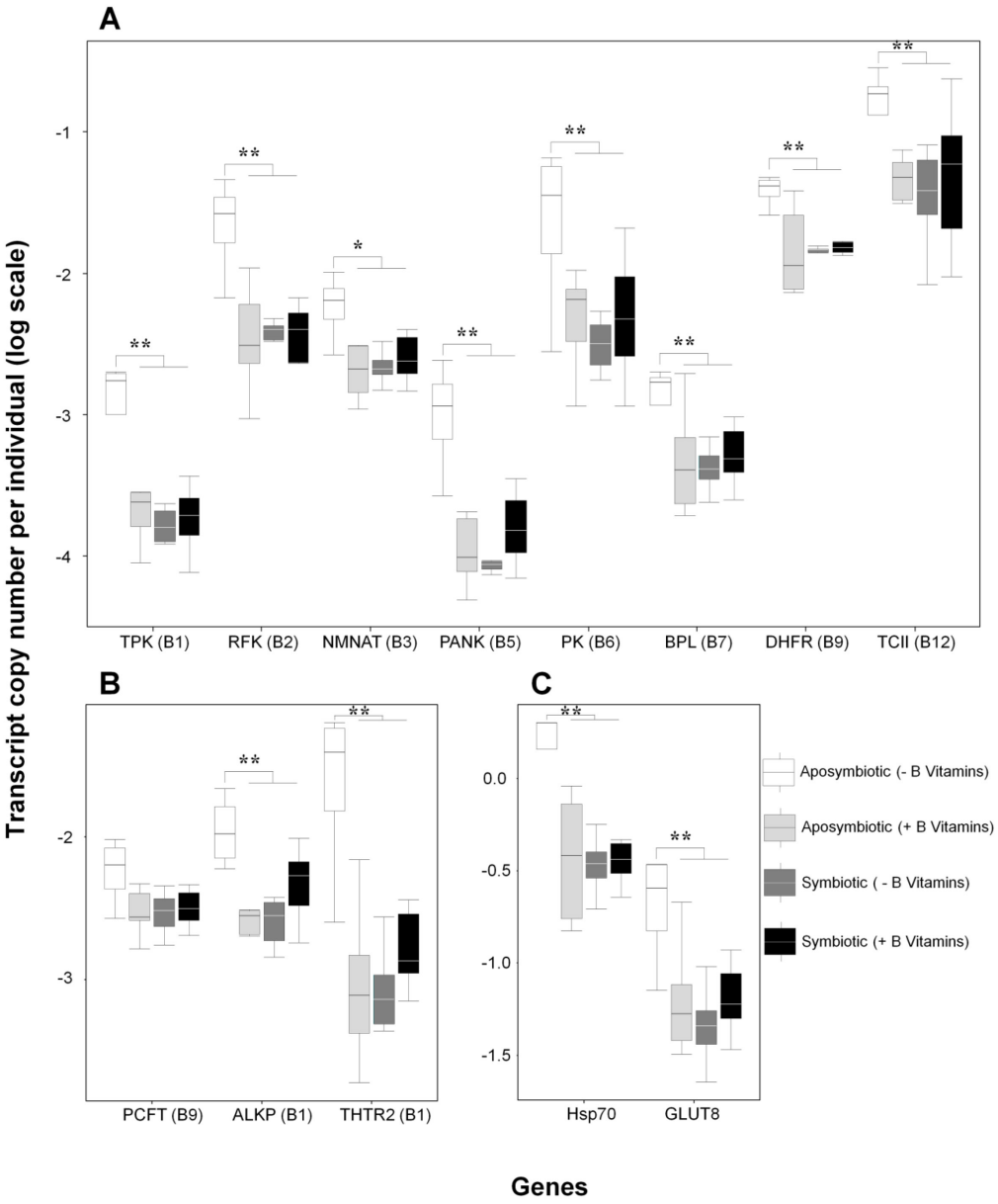

Figure 3. Differential expression of candidate host genes by qPCR for symbiotic and aposymbiotic *D. fasciatus* reared on a complete or vitamin deficient artificial diet.
(**A**) Intracellular B vitamin processing genes: *TPK*, *RFK*, *NMNAT*, *PANK*, *PK*, *BPL*, *DHFR*, *TCII*.
(**B**) B vitamin transport and extracellular processing genes: *THTR2*, *PCFT* and *ALKP*.
(**C**) A stress indicator *Hsp70*, and a glucose transporter *GLUT8*. For enzyme abbreviations see Fig. 2. Shading of boxes signifies the experimental treatments (see legend). Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Significant differences were assessed based on the normalized expression in reference to the 60S ribosomal protein L13a with the Kruskal-Wallis test and Dunn posthoc tests. Differentially expressed transcripts at $P < 0.05$ and $P < 0.01$ are marked by * and **, respectively.

5.3.5 Functional characterization of the transport potential for *THTR2*

Of the two B vitamin transporters detected in *D. fasciatus*' transcriptome (*PCFT* and *THTR2*), only *THTR2*'s complete sequence could be completely assembled from the retrieved transcripts, and in turn, functionally analyzed by heterologous expression in *Xenopus laevis* oocytes. The functional assessment of *THTR2* through radiotracer experiments revealed that the transporter is in fact capable of transporting thiamine (B1). This was demonstrated through the significant uptake of labeled thiamine in frog oocytes injected with *THTR2* cRNA relative to oocytes injected with cRNA for the human glucose transporter 1 (h*GLUT1*), or water ($P < 0.001$, ANOVA; Fig. 4A). Conversely, no discernible transport activity of labeled deoxyglucose could be reported for oocytes expressing *THTR2* relative to h*GLUT1*-injected oocytes ($P < 0.001$, ANOVA; Fig. 4B). The specific and elevated transport activity reported for thiamin or deoxyglucose in oocytes injected with *THTR2* or h*GLUT1* cRNA, respectively, demonstrates that the injection procedure itself was not responsible for the uptake of radiolabelled compounds.

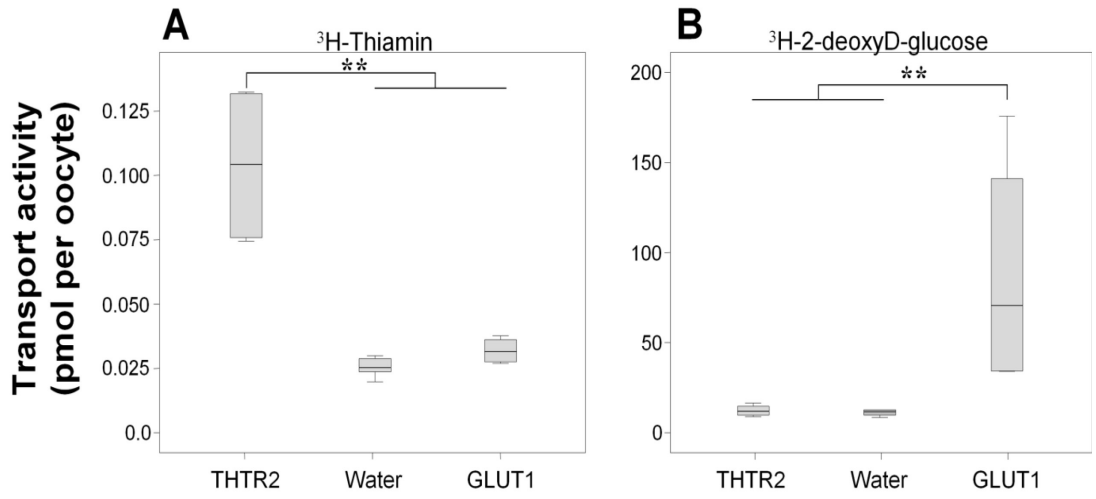

Figure 4. THTR2 mediated uptake of (**A**) 50 pM Thiamin and (**B**) 100 µM 2-deoxyD-glucose into *Xenopus* oocytes. Transport activity was determined by quantifying the radiolabeled substrate uptake in oocytes injected with THTR2-cRNA, water, or hGLUT1-cRNA. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Significant differences at $P < 0.01$ are marked by ****** (ANOVA)**.**

5.3.6 *C. glomerans*' genomic potential for B vitamin supplementation

From the recently sequenced genome of *C. glomerans* (Stackebrandt *et al*., 2013), we report on the presence of pathways for the biosynthesis of five B vitamins (Fig. 5). For thiamine (B1), three distinct pathways leading to thiamine phosphate were discovered, indicating that *C. glomerans* is capable of synthesizing the compound under different conditions. The gene coding for thiamine pyrophosphokinase (*TPK*) - the enzyme responsible for catalyzing the cleavage of the final phosphate group to form thiamine (B1) - is nonetheless missing from the genome. However, the host itself possesses the *TPK* gene (Fig. 2 and 3), thereby providing a metabolic complement to *C. glomerans*'

three synthesis pathways for thiamine phosphate. For riboflavin (B2), genes encoding its conversion to flavin adenine dinucleotide (FAD) were detected, and the pathway for nicotinamide (B3) contained all the necessary genes for its conversion from nicotinate. For the complete biosynthesis of pantothenate (B5), two separate pathways were discovered from aspartate and valine. For the aspartate pathway, all necessary genes were accounted for, while for the valine pathway, only ketopantoate reductase (*KPR*) could not be annotated. Lastly, the pathway leading to the biosynthesis of folic acid (B9) from purine was nearly complete, except for the gene *FolQ*. However, *FolQ* is absent from the genomes of many bacteria that are known to produce folic acid (de Cercy-Lagard *et al*., 2007), therefore it has been proposed that alternate (but in most cases currently unknown) genes can substitute for this gene.

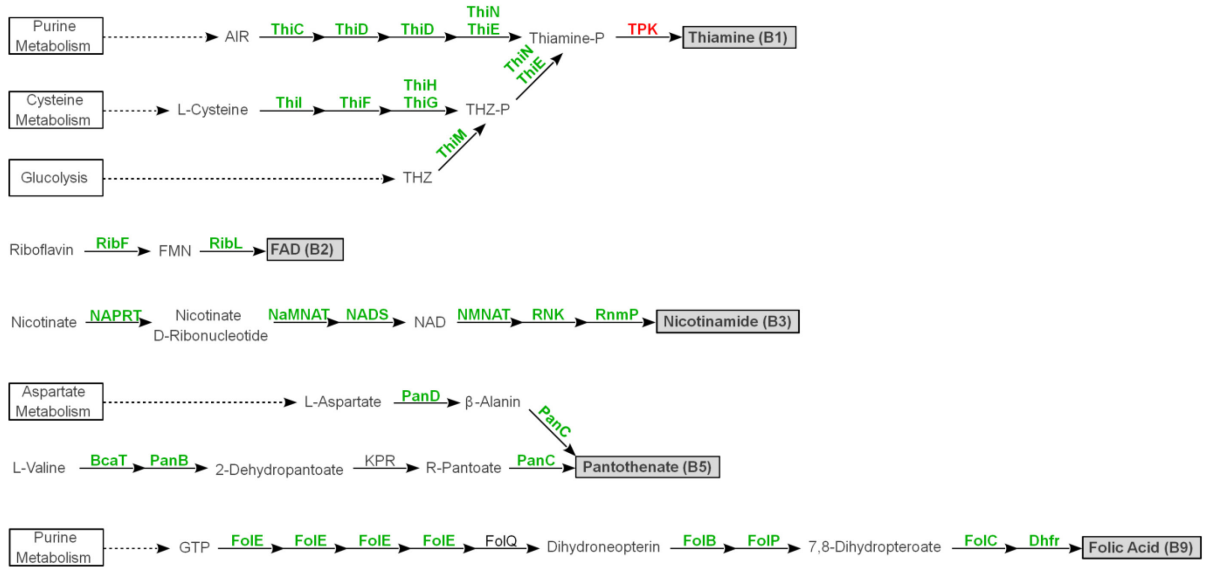

Figure 5. Metabolic pathways for B vitamin biosynthesis in the genome of *C. glomerans*. Each arrow represents one step in the biosynthetic pathway. Enzyme names in green font indicate that a candidate gene for this step was detected in the annotated genome sequence. Enzyme names in red indicate that a candidate gene for this step was detected in the host's genome (see Fig. 2A and 3A).

For *Gordonibacter* sp., the absence of a sequenced genome (or that of a closely related species) and its refractoriness to *in vitro* cultivation prevented us from making inferences regarding its genomic potential for B vitamin synthesis.

5.4 DISCUSSION

In animals, the ability to synthesize many vitamins, particularly B vitamins, has been lost and replaced with uptake mechanisms aimed at deriving these compounds from plants or microorganisms through complex multipartite interactions. Insects, depending on their feeding ecology, require vitamins of the B complex in varying amounts (Fraenkel and Blewett, 1943a), and early work by Fraenkel and Blewett (1943b) demonstrated that the variation in the vitamin requirements of similarly oligophagous

insects can often be attributed to the presence (or absence) of vitamin-supplementing intracellular symbionts.

In contrast to their performance on Malvales seeds (Salem *et al*., 2013), aposymbiotic *D. fasciatus* that were exclusively fed on a complete artificial diet were found to exhibit near identical survivorship values relative to symbiotic individuals. Upon omission of B vitamins from the artificial diet, however, survivorship of aposymbiotic individuals decreased significantly compared to the symbiotic treatment on the same diet, as well as compared to symbiotic and aposymbiotic firebugs fed on a complete artificial diet (Fig. 1), thereby implicating the Coriobacteriaceae symbionts in supplementing these compounds to the host. The case for symbiont-mediated nutritional supplementation in firebugs is further supported by the maintenance of many genes involved in the metabolism of B vitamins as predicted by *C. glomerans*' recently sequenced genome (Stackelbrandt *et al*., 2013), including pathways for the synthesis of thiamine (B1), flavin adenine dinucleotide (FAD) (B2), nicotinamide (B3), pantothenate (B5), and folic acid (B9) (Fig. 5).

In an effort to understand the host's response to this nutritional partnership, we assessed how symbiont elimination affects gene expression in the midgut of *D. fasciatus* when the bugs are reared on their natural diet of cottonseeds, as well as on complete and vitamin-deficient artificial diets. We specifically considered the expression patterns of host genes involved in the B vitamin metabolism. We initially observed that symbiont elimination in cottonseed-fed firebugs generally induces the up-regulation of genes involved in the transport and processing of B vitamins (Fig. 2; A and B). This pattern of differential gene expression was even more evident in aposymbiotic firebugs reared on an artificial diet deficient in B vitamins (Fig. 3; A and B). We propose that the variation in the degree of differential expression across both sets of experiments can be attributed to the presence of some (albeit limiting) amounts of B vitamins in cottonseeds compared to the vitamin-deficient artificial diet.

The gene expression patterns reported for *D. fasciatus* in this study are consistent with previous findings in mammals and yeast highlighting the negative regulatory control of genes involved in the transport and activation of B vitamins, including thiamine, riboflavin, nicotinamide, panthothenate, pyradoxin, biotin, folate and B12 (Hohmann and Meacock, 1998; Abbas and Sibirny, 2011; Said et al., 1994; Ink and Henderson, 1984; Said *et al*., 2007; Zhao *et al*., 2011). For example, the expression of thiamine-related uptake and processing genes is up-regulated under conditions of thiamine deficiency (Hohmann and Meacockm 1998; Rajgopal and Zhao, 2001). Regulation in this system is mediated by transcriptional activators whose binding appears to be dependent on the vitamin's absence, as evidenced by the transcriptional repression of thiamine transporter genes following the supplementation of the vitamin to a previously deficient cell culture (Rajgopal and Zhao, 2001). Similarly, the mouse homolog of the proton-coupled folate transporter (*PCFT*) investigated in this study was also shown to undergo similar patterns of regulation, where a 13-fold increase in expression was recorded in the small intestine of mice fed on a folate-deficient diet relative to mice reared on a complete diet (Qui *et al*., 2007). This is also the case for intracellular processing and activation genes. For example, pantothenate kinase (*PANK*), the enzyme catalyzing the phosphorylation of pantothenate (B5) to form phosphopantothenate, is regulated through a two-step negative feedback inhibition, both by pantothenate, as well as the downstream product acetyl-CoA (Jackowski and Rock, 1981).

The elevated expression of *Hsp70* and several other stress-related genes in cottonseed-fed aposymbiotic firebugs suggests that symbiont elimination also induces a general stress response in the host (Fig. 2C), possibly as a product of nutrient limitation. This hypothesis was confirmed by the

differential up-regulation of *Hsp70* in aposymbiotic firebugs reared on a vitamin deficient diet. The stress response could be rescued by artificial B vitamin supplementation, or by the presence of the actinobacterial symbionts (Fig. 3C), demonstrating that only in the absence of the Coriobacteriaceae is vitamin omission stressful for firebugs. Concordantly, the differential expression pattern for the glucose transporter *GLUT8* indicates that the host responds to vitamin limitation by the general up-regulation of other non-vitamin-related transporters (Fig. 3C). Collectively, these findings demonstrate that while symbiont elimination initiates a general nutritional stress response in firebugs, the trigger is nonetheless specific to the deficiency in B vitamins.

Haematophagous (blood-feeding) insects like the assassin bug *Rhodnius prolixus*, the tsetse fly *Glossina morsitans,* and the bedbug *Cimex lectularius* have thus far presented us with excellent systems that illustrate the host's nutritional dependency on beneficial microbes for the supply of B vitamins that are consistently lacking in their vertebrate blood meals (Beard *et al*., 2002; Akman *et al*., 2002; Hosokawa *et al*., 2010). Symbiont-mediated B vitamin supplementation has also been demonstrated in non-haematophagous insects, such as the anobiid beetles *Lasioderma serricorne* and *Stegobium paniceum*, which specialize on a range of oilseeds, cereals and dried fruit (Blewett and Fraenkel, 1944).In both *L. serricorne* and *S. paniceum,* the beetles were found to rely on the B vitamin-producing capacities of their intracellular fungal partners, since – similar to this study – symbiont elimination only affected beetles that were reared on an artificial diet deficient in these compounds (Blewett and Fraenkel, 1944). While the exact mode for deriving B vitamins from the gut symbionts in firebugs is currently unknown, preliminary findings suggests that the active lysis of symbiont cells by the host plays an important role.

To date, most reports of symbiont-mediated vitamin supplementation based on artificial diet experiments were met with concerns due to the possible presence of trace amounts of vitamins in the "deficient" diets (Douglas, 2009). While this could also be argued for the present study, we provide additional evidence for symbiont-mediated vitamin supplementation by demonstrating a B vitamin starvation response in the host; one that can be specifically rescued either through the supplementation of B vitamins in the diet or by re-establishing the symbiosis with the Coriobacteriaceae symbionts.

In addition to the capacity for dietary upgrading of pyrrhocorids, the contributions of the symbionts may extend towards offsetting some of the detrimental effects of toxins present in seeds of the Malvales plant order, particularly cyclopropenoic fatty acids (CPFAs). These fatty acids are almost exclusively found in Malvales seeds – the preferred food source of pyrrhocorid bugs – and have a demonstrated ability for inhibiting the desaturation of stearic acid into oleic acid (Allen *et al*., 1967). This results in slower growth rates and higher mortality across a number of animal groups, including insects (Phelps *et al*., 1965). However, upon supplementing these CPFA-containing diets with a range of B vitamins including pyridoxine, pantothenate and biotin, many of the adverse fitness effects observed, especially high juvenile mortality, were reversed in rats (Schneider *et al*., 1968). Thus, the acquisition of vitamin-supplementing symbionts by pyrrhocorid bugs may have represented a key evolutionary innovation that allowed the bugs to exploit the nutritionally challenging (vitamin-deficient and CPFA-containing) diet of Malvales seeds.

In contrast to the aforementioned associations involving putative vitamin-supplementing symbionts that are housed intracellularly in specialized bacteriocytes, firebugs derive their vitamins from extracellular symbionts residing in their midgut lumen. In bacteriocyte-based mutualisms, the complementary partnerships are highly integrated from a metabolic standpoint (Douglas, 2006).

Aphids – in their reliance on the essential amino acids provided by their primary endosymbiont *Buchnera* – differentially regulate gene expression to accommodate and facilitate the beneficial mutualism (Nakabachi *et al*., 2005). This includes the up-regulation of host genes involved in the transport of essential amino acids in *Buchnera*-harboring bacteriocytes compared to the rest of the aphid's body. Additionally, bacteriocytes also exhibit an upregulation of genes involved in the synthesis of non-essential amino acids (Nakabachi *et al*., 2005), an expression pattern that is consistent with the hypothesis that *Buchnera* derives these compounds from the host, given the extensive loss affecting the bacterium's ability to produce non-essential amino acids (Nakabachi *et al*., 2005). Thus, despite the extracellular symbiont localization in cotton stainers, the differential expression of genes involved in the transport and processing of symbiont-provided nutritional compounds is reminiscent of bacteriome-associated intracellular symbioses, indicating a high degree of intimacy in the Pyrrhocoridae-Coriobacteriaceae association.

Our results offer novel insights into the metabolic integration between an animal host and its gut microbes. We demonstrate that despite an extracellular localization in *D. fasciatus*' midgut, the Coriobacteriaceae symbionts influence a range of metabolic pathways in firebugs including the differential expression of genes related to the transport and processing of B vitamins, thereby highlighting an important interface for the exchange of symbiont-provided nutritional supplements. Such findings enhance our understanding of the mechanisms involved in symbiont-mediated nutritional supplementation between animals and their extracellularly localized gut symbionts.

5.5 MATERIALS AND METHODS

5.5.1 Insect sampling and rearing

Live specimens of *Dysdercus fasciatus* were originally collected from the Comoé National Park, Cote d'Ivoire, but have since been bred in the laboratory at the University of Würzburg, Germany, and a sub-culture was later established at the Max Planck Institute for Chemical Ecology, Jena, Germany. The insects were reared in plastic containers (20 × 35 × 22 cm) at a constant temperature of 28ºC and exposed to long light regimes (16h/8h light/dark cycles). The bugs were provided *ad libitum* with previously autoclaved water and dry cotton seeds (*Gossypium hirsutum)* or different variations of the artificial diet (see below).

5.5.2 Development and manipulation of a defined artificial diet

The artificial diet was modified for our study system from Panizzi and colleagues (2000). A single batch was produced for every experimental treatment and used throughout the course of the study to ensure consistency. Composition of the nutritionally complete diet is listed in Table S2. A second variation of this diet was developed with the complete omission of B vitamins, i.e. thiamine (B1), riboflavin (B2), nicotinamide (B3), calcium pantothenate (B5), pyridoxine (B6), biotin (B7), folic acid (B9), and vitamin B12. Both diets were stored at -20°C, and small cubes (1 cm^3) were thawed and supplied every other day to cages of the various experimental treatments.

5.5.3 Experimental setup

The generation of aposymbiotic bugs involved the sterilization of egg surfaces following the procedure utilized in Salem *et al*. (2013). Briefly, the eggs were submerged in ethanol for 5 minutes, followed by bleach (12% NaOCl) for 45 seconds. Residual bleach was removed by washing in sterile autoclaved water. To investigate symbiont-mediated supplementation of B vitamins, eight egg clutches (~35 eggs each) from different females of *D. fasciatus* were harvested three days following oviposition and kept separately. We then split each of the collected egg clutches into four experimental treatments: (i) aposymbiotic bugs reared on a vitamin deficient diet (aposymbiotic -B vitamins), (ii) aposymbiotic bugs reared on a complete diet (aposymbiotic +B vitamins), (iii) symbiotic bugs reared on a vitamin deficient diet (symbiotic -B vitamins), and (iv) symbiotic bugs reared on a complete diet (symbiotic +B vitamins). The four treatments were provided with autoclaved water and the two variations of the artificial diet *ad libitum*. To examine the differential pattern of host gene expression in response to symbiont elimination on the natural diet of cotton seeds, five additional egg clutches of *D. fasciatus* were harvested, and each clutch was separated into two experimental treatments: (i) symbiotic, and (ii) aposymbiotic bugs reared on cotton seeds.

5.5.4 Fitness measurements

Individuals across all experimental treatments were observed on a daily basis for the assessment of fitness effects across the different groups. For the bugs reared on the different variations of the artificial diet, growth rate (days until molting to the next stage) and survival until adulthood (%) were recorded (Salem *et al*., 2013).

5.5.5 RNA extraction and reverse transcription

Three days following adult emergence, a single individual was collected from every experimental treatment replicate, and through dissection, its midgut region (M1-M4) was harvested. Once dissected, the midgut region was stabilized in RNA*later* solution (Qiagen) and stored at −20°C. Total RNA isolations were performed using the RNeasy Micro Kit (Qiagen) following the manufacturer′s guidelines. Integrity and quality of the RNA samples were determined using the RNA 6000 Nano LabChip kit (Agilent Technologies) on an Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions. cDNA was then generated according to the manufacturer's guidelines (QuantiTect Reverse Transcription kit, Qiagen). To account for possible shortcomings during the RNA extraction procedure and to test for the success of the symbiont elimination procedure, diagnostic PCR screens targeting the host's 18S rRNA gene and the Coriobacteriaceae symbionts' 16S rRNA genes were conducted using the generated cDNA according to procedures described in Salem *et al*. (8).

5.5.6 Illumina-based transcriptome sequencing and quantitative PCRs

RNA was extracted from dissected whole midgut regions (M1-M4) of five symbiotic and aposymbiotic bugs, respectively, fed on their natural diet of cottonseeds, resulting in two pooled

samples. Transcriptome sequencing of poly-A enriched mRNAs was done commercially (Fasteris; http://www.fasteris.com) on the HiSeq™ 2000 Sequencing System from Illumina (http://www.illumina.com/ webcite), utilizing the paired read 100 bp technology. For details on assembly and annotation procedures see Supplementary Methods.

Quantitative PCRs (qPCRs) for the candidate host genes involved in B vitamin metabolism were conducted across (i) aposymbiotic and symbiotic bugs reared on their natural diet of cotton seeds (to confirm the transcriptome sequencing results), and (ii) across the four experimental treatments of aposymbiotic and symbiotic bugs reared on a complete and vitamin-deficient artificial diet to examine if such patterns substantiate under controlled conditions with vitamin availability as the only nutritional difference between dietary treatments. Primers were designed based on the candidate gene sequences available from the transcriptome, checked for specificity *in vitro* using capillary sequencing of amplified PCR products (Table S3), and used for quantitative PCRs on a RotorGene®-Q cycler (Qiagen, Hilden, Germany) (see Supplementary Methods).

5.5.7 Functional characterization of transporter activity using *X. laevis* oocytes

THTR2 and hGLUT1 (positive control) cRNA were generated by *in vitro* transcription using mMESSAGE mMACHINE kit (Ambion Inc). *X. laevis* oocytes were provided by Stefan Heinemann (University of Jena). 100–125 ng cRNA was injected per oocyte, and RNase-free water was used as a negative control. All oocytes were maintained at 17.5 °C in modified Barth's saline (MBS, in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaC1_2$, TRIS-HCL, pH 7.4) with 10 µg ml^{-1} penicillin, 10 µg ml^{-1} streptomycin and 4 µg ml^{-1} cefuroxim solution for 3 days.

Functional zero-trans-influx studies were carried out using radiolabeled thiamine hydrochloride and deoxy-glucose as substrates in *X. laevis* oocytes. Three days after cRNA injection, groups of 5-10 oocytes were transferred into tubes containing 200 µl Barth´s saline and thiamin (4 µCi ml^{-1} thiamine-[^{3}H]-hydrochloride; American Radiolabelled Chemicals), or 2-deoxy-D-glucose (2 µCi ml^{-1} 2-Deoxy-D-[1-^{3}H]-glucose; Amersham Biosciences). After incubation at room temperature, cells were transferred to ice-cold Barth´s saline, washed three times, solubilized with 100 µl 1% (w/v) SDS, and measured individually (five replicates).

5.5.8 B vitamin pathways in the genome of *C. glomerans*

To examine the metabolic potential of *C. glomerans* for B vitamin supplementation, we analyzed the genomic information pathways using the symbiont's recently sequenced genome (17) (NC_015389). This was accomplished using the pathway analysis platform available in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al*., 2012). Complete pathways with all necessary genes were recorded; however, a number of missing genes were additionally supplemented by manual curation. In this process, each missing gene was searched in closely related bacterial species for potential homologs. The homologs were then searched with blastn and blastx (using an e-value cutoff score of 10^{-5}) against a custom created database of all predicted genes in the genome of *C. glomerans*. The additional genes discovered by this method were then included in the respective pathways.

5.5.9 Statistical analysis

Growth and survival rates until adulthood across the four experimental treatments of symbiotic and aposymbiotic groups fed on a complete and vitamin deficient diets were compared using Friedman tests with Wilcoxon-Wilcox post-hoc measures using BiAS 7.40 (Epsilon-Verlag; Hochheim-Darmstadt, Germany). To assess differential expression patterns of host genes from aposymbiotic and symbiotic bugs reared on cottonseeds, as well as on the two variations of the artificial diet, gene copy numbers estimated from qPCRs were first normalized against the 60S ribosomal protein L13a (Table S3), then compared using the Mann-Whitney U-test and Kruskal-Wallis tests with Dunn posthoc tests, respectively, to asses levels of significance ($P < 0.05$) across treatments. To compare the differential uptake in labeled compounds in *X. laevis* oocytes, ANOVA was used as implemented in the SPSS 17.0 software package (SPSS, Chicago, IL, USA).

5.6 ACKNOWLEDGMENTS

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5.8 SUPPLEMENTARY METHODS

5.8.1 Illumina-based transcriptome sequencing and library construction

Sequencing was conducted using RNA extractions from dissected whole midgut regions (M1-M4) of five symbiotic and aposymbiotic bugs, respectively, fed on their natural diet of cottonseeds, resulting in two pooled samples. Prior to sequencing, the extracted RNA was exposed to a poly-A enrichment strategy. RNA sequencing was performed by a commercial service provider (Fasteris; http://www.fasteris.com) using 5 µg total RNA (per sample) on the HiSeq™ 2000 Sequencing System from Illumina (http://www.illumina.com/ webcite), utilizing the paired read 100 bp technology. The reads were trimmed according to the sequencing quality test using Timmomatic (Lohse *et al*., 2012). The leading and trailing bases of each read were cut off if the quality values were below the default threshold. Additionally, reads were discarded if they were shorter than 30 base pairs.

Following quality checks, the trimmed reads were assembled *de novo* into contigs using Trinity (Grabherr *et al*., 2011). The minimal contig length was set to 200 and the *k-mer* length to 25 base pairs. The read libraries of symbiotic and aposymbiotic bugs were pooled and assembled into a single backbone, respectively. After the assembly with Trinity, the contigs were clustered by CD-HIT EST according to their sequence similarity to remove potential duplicates. Sequences with more than 99% sequence similarity to other contigs were subsequently collapsed. For the assignment of expression values to each constructed transcript in the respective library, the original reads were mapped back to the respective backbone assembly using the algorithm Bowtie2 (Langmead *et al*., 2012). The generated output was processed using SAMtools (Huang *et al*., 2010) to create BAM files and asses the coverage depth as the number of reads mapped to each transcript. The files were parsed with the custom script nucdepth.R using the R package Rsamtools (Delhomme *et al*., 2012) for further analysis. The correction for biases due to the different depths of sequencing across treatments and due to different transcript sizes were addressed using the RPKM (reads per kilobase of transcript per million of mapped reads) transformation to obtain estimates of relative expression levels. Homology searches (BLASTx and BLASTn) of unique sequences and functional annotation were determined using the BLAST2GO software suite v2.4.1 (http://www.blast2go.de).

5.8.2 Validation of host gene expression with quantitative PCR

Quantitative PCRs (qPCRs) for the candidate host genes involved in B vitamin metabolism were conducted across (i) aposymbiotic and symbiotic bugs reared on their natural diet of cotton seeds (to confirm the transcriptome sequencing results), and (ii) across the four experimental treatments of aposymbiotic and symbiotic bugs reared on a complete and vitamin-deficient artificial diet to examine if such patterns substantiate under controlled conditions with vitamin availability as the only nutritional difference between dietary treatments. Primers were designed based on the candidate gene sequences available from the transcriptome, and checked for specificity *in vitro* using capillary sequencing of amplified PCR products (Table S2).

The qPCR reactions were performed using a RotorGene®-Q cycler (Qiagen, Hilden, Germany), with the same individual cDNA extracts used for the diagnostic PCR screens. The final reaction volume of 25 µl included the following components: 1 µl of cDNA template, 2.5 µl of each primer (10 µM), 6.5 µl of autoclaved distilled H_2O, and 12.5 µl of SYBR Green Mix (Qiagen, Hilden, Germany).

Conditions for qPCR were optimized using a VWR® Gradient Thermocycler (VWR, Radnor, PA, USA) at various annealing temperatures (60-68 °C). Standard curves for absolute quantification in the qPCR (10-fold dilution series from 1 ng/µl to 10^{-6} ng/µl) were generated using purified PCR products for all primer pairs after measuring the PCR product concentrations using a NanoDropTM1000 spectrophotometer (Peqlab). The following cycling parameters were used: 95°C for 10 min., followed by 45 cycles of 68ºC for 30 s, 72ºC for 20 s, and 95ºC for 15 s. Subsequently, a melting curve analysis was conducted by increasing the temperature from 60ºC to 95ºC within 20 min. Six replicates of one of the standard concentrations were used, for each primer pair and concentration, for the configuration and calibration of the standard curve. The resulting averages were then utilized to correct for possible errors in the DNA concentration measurements. Based on the standard curve, absolute copy numbers were calculated.

5.9 SUPPLEMENTARY REFERENCES

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Table S1: Summary of all stress-related ioforms detected in the transcriptome of symbiotic and aposymbiotic *D. fasciatus* reared on cottonseeds. Transcripts with Aposymbiotic/Symbiotic fold change > 2 are marked with an asterisk.

Seq. Name	Seq. Description	Symbiotic (RPKM)	Aposymbiotic (RPKM)	Fold Aposymbiotic/Symbiotic
Dfas-25287	Heat shock protein 70	432.19	1,162.36	2.69*
Dfas-38373	Stress-activated protein kinase jnk	2.40	2.51	1.05
Dfas-54540	Heat shock protein 90	107.91	227.00	2.10*
Dfas-38957	Heat shock protein 70	27.80	14.23	0.51
Dfas-28415	Heat shock protein 70	3.27	4.21	1.28
Dfas-40833	Heat shock protein 90	135.47	381.08	2.81*
Dfas-08437	Related to glyoxal oxidase precursor	2980.39	3231.26	1.08
Dfas-41199	Related to glyoxal oxidase precursor	171.70	338.03	1.97
Dfas-11475	Related to glyoxal oxidase precursor	211.52	412.40	1.95
Dfas-19692	Peroxiredoxin 6	733.49	668.13	0.91
Dfas-10108	Ccaat enhancer-binding	59.72	63.70	1.07
Dfas-47994	Superoxide dismutase 2	16.61	17.92	1.08
Dfas-48940	Superoxide dismutase	220.71	165.31	0.75
Dfas-23888	Superoxide dismutase	0.87	2.75	3.15*
Dfas-22754	Superoxide dismutase	2.40	3.39	1.41
Dfas-52644	Mitochondrial manganese superoxide dismutase	36.37	43.21	1.19
Dfas-39352	Tryparedoxin peroxidase	0.40	0	N/A
Dfas-12213	Peroxiredoxin- mitochondrial-like	161.79	122.79	0.76
Dfas-01237	Peroxiredoxin 4	0.82	2.21	2.70*
Dfas-36450	Thioredoxin peroxidase 2-like	0.31	2.10	6.75*
Dfas-39210	Thioredoxin family trp26	5.07	6.73	1.33
Dfas-38112	Venom allergen 5-like	0.33	3.38	10.13*
Dfas-35010	Trypsin-like protease	1.96	0	N/A
Dfas-31914	Glucose transporter type 1-like	0.94	1.33	1.41
Dfas-19251	Solute carrier family facilitated glucose transporter member 8	25.94	63.95	2.47*

Table S2. Composition of the artificial diet. To generate the vitamin-deficient diet, the B vitamin solution was substituted with water.

Component	amount
soybean protein	15.0 g
potato starch	7.5 g
dextrose	7.5 g
sucrose	2.5 g
cellulose	12.5 g
B vitamin stock solution	15.0 ml
thiamine (**B1**) (0.25 g)	
riboflavin (**B2**) (0.5 g)	
nicotinamide (**B3**) (1 g)	
calcium pantothenate (**B5**) (1 g)	
pyradoxine (**B6**) (0.25 g)	
biotin (**B7**) (0.02 ml)	
folic acid (**B9**) (0.25 g)	
cobalamin (**B12**) (1g)	
water (*ad* 1000 ml)	
soybean oil	20 ml
wheat germ	10 g
water	25 ml

Table S3: Summary of all primer pairs utilized in this study.

Primer	Primer Sequence	Orientation	Target Gene
TPK fwd	TCTTTCCGAAGGATTTGGTG	Fwd.	Thiamine pyrophosphokinase (TPK)
TPK rev	TTTCGGGACAAATTCGAGAG	Rev.	
RFK fwd	GATGGGCGAAACTTGAAGAA	Fwd.	Riboflavin kinase (RFK)
RFK rev	AGGGTTCCAACCAACACTCA	Rev.	
NMNAT fwd	CTGGGAATGCAGTCAGGAAA	Fwd.	Nicotinamide mononucleotide adenylyltransferase (NMNAT)
NMNAT rev	TGGCTCGTCATTCTCATTCA	Rev.	
PANK fwd	GGAGACAGCACAAGCTGGAC	Fwd.	Pantothenate kinase (PANK)
PANK rev	ACCGCTCTACCCATTCCTTC	Rev.	
PK fwd	ATAGCGCTCCATGCTTCATC	Fwd.	Pyridoxal kinase (PK)
PK rev	GATCCGGTAATGGGTGACAA	Rev.	
BPL fwd	GGATGTGCTACGTTTTCTCTCC	Fwd.	Biotin-protein lyase (BPL)
BPL rev	TAAGGCCTCAAGTCCGTGTT	Rev.	
DHFR fwd	GAGTGTCTGGATAATCGGAGGA	Fwd.	Dihydrofolate reductase (DHFR)
DHFR rev	TCTTCTTGGACTTCCGTTGG	Rev.	
TCII fwd	CTTTAAGAAGCCGGCAACAG	Fwd.	Transcobalamine 2 (TCII)
TCII rev	TTGACAGGCATAAGGGTCGT	Rev.	
ALKP fwd	GACATATGCGGCGAACAAAC	Rev.	Thiamine alkaline phosphatase (ALKP)
ALKP rev	GTCGGGCCTCTTGTTTAAGG	Fwd.	
THTR2 fwd	GCTTCGACAAGTCCATTCCA	Rev.	Thiamine transporter 2 (THTR2)
THTR2 rev	GATGTTCTGGTGGGCGTTAG	Fwd.	
PCFT fwd	AGACGAGGCAAACTGTTCCA	Rev.	Proton-coupled folate transporter (PCFT)
PCFT rev	GGCGTCTTCTCTGTGCTGTT	Fwd.	
GLUT8 fwd	AGGGTGGAAGGTTTGCTTCT	Fwd.	Glucose transporter (GLUT8)
GLUT8 rev	GAAAGCCCTAATGGTGCTGA	Rev.	
Hsp70 fwd	GGATGCCGGTACAATTTCTG	Fwd.	Heat shock protein (Hsp) 70
Hsp70 rev	GGTTCCACCACCAAGATCAA	Rev.	
RPL13A fwd	CGAGGATAAGACGGAACTTGG	Rev.	60S ribosomal protein L13a
RPL13A rev	CATGAAGGCTATGGGTCTGG	Fwd.	

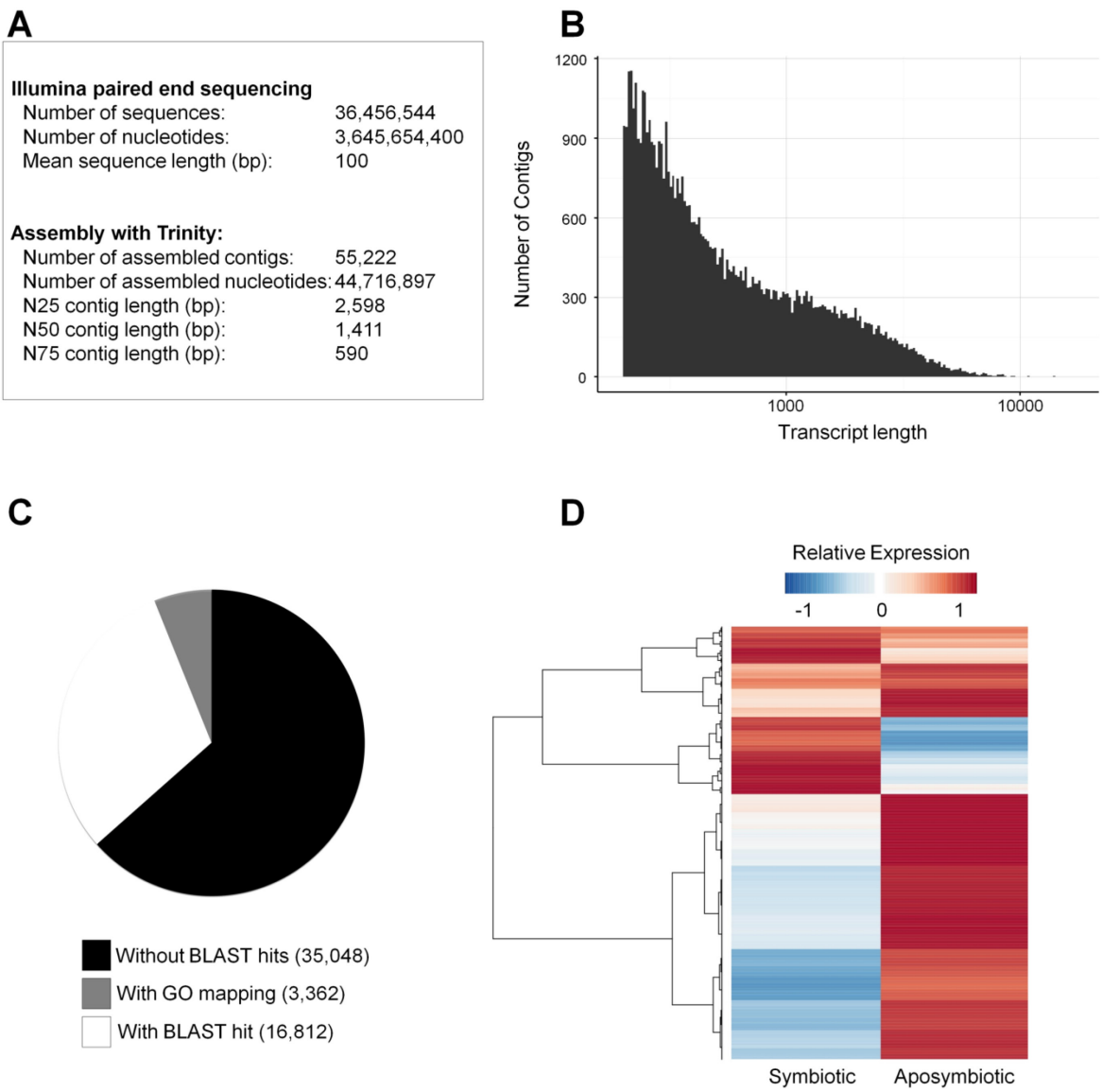

Figure S1. Midgut transcriptome of cottonseed-fed symbiotic and aposymbiotic *D. fasciatus*. (**A**) Illumina transcriptome sequencing and assembly statistics. (**B**) Histogram of contig lengths as assembled by Trinity. (**C**) Summary of the BLAST and GO annotation results. (**D**) Heatmap of the normalized expression (RPKM) of all differentially expressed transcripts. For each row, the expression was centered according to the mean and standard deviation. The row dendrogram represents the Pearson correlation of all genes.

CHAPTER 6

TRANSCRIPTOMIC IMMUNE RESPONSE OF THE FIREBUG *DYSDERCUS FASCIATUS* TO EXPERIMENTAL ELIMINATION OF VITAMIN-SUPPLEMENTING INTESTINAL SYMBIONTS

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6.1 ABSTRACT

The acquisition and vertical transmission of bacterial symbionts plays an important role in insect evolution and ecology. However, the molecular mechanisms underlying the stable maintenance and control of mutualistic bacteria remain poorly understood. The cotton stainer *Dysdercus fasciatus* harbours the actinobacterial symbionts *Coriobacterium glomerans* and *Gordonibacter* sp. in its midgut. The symbionts supplement limiting B vitamins and thereby significantly contribute to the host's fitness. In this study, we experimentally disrupted the symbionts' vertical transmission route and performed comparative transcriptomic analyses of genes expressed in the gut of aposymbiotic (symbiont-free) and control individuals to study the host immune response in presence and absence of the mutualists. Annotation of assembled cDNA reads identified a considerable number of genes involved in the innate immune system, including different protein isoforms of several immune effector proteins (specifically i-type lysozyme, defensin, hemiptericin, and pyrrhocoricin), suggesting the possibility for a highly differentiated response towards the complex resident microbial community. Gene expression analyses revealed a constitutive expression of transcripts involved in signal transduction of the main insect immune pathways, but differential expression of certain antimicrobial peptide genes. Specifically, qPCRs confirmed the significant down-regulation of c-type lysozyme and up-regulation of hemiptericin in aposymbiotic individuals. The high expression of c-type lysozyme in symbiont-containing bugs may serve to lyse symbiont cells and thereby harvest B-vitamins that are necessary for subsistence on the deficient diet of Malvales seeds. Our findings suggest a sophisticated host response to perturbation of the symbiotic gut microbiota, indicating that the innate immune system not only plays an important role in combating pathogens, but also serves as a communication interface between host and symbionts.

6.2 INTRODUCTION

One of the reasons for the ecological success of insects is the acquisition and vertical transmission of bacterial symbionts (Salem et al, submitted). These mutualists can aid in the digestion of complex dietary polymers (Breznak *et al*., 1994), offer nutritional supplementation (Moran and Degnan, 2006) or act as a defensive barrier against intruding pathogens (Kaltenpoth *et al*., 2005). In particular, intestinal associations with microorganisms have been shown to be beneficial for the host by the provision of nutrients important for growth (Nakabachi *et al*., 2005). Controlling and maintaining these populations of intestinal microorganisms is a challenging task for the host: While certain microorganisms are beneficial, others are detrimental and can lead to fitness disadvantages. Moreover, under certain conditions even microbes which are otherwise beneficial can cause damage (Login *et al*., 2011). Therefore, the host needs a regulatory system to keep symbiont populations in line. This fine-tuned regulation is realized by the innate immune system, which serves as a communication interface between host and symbiont to guide and modulate cooperative behaviour (Muraille, 2013).

In insects, the humoral immune response is triggered primarily by pattern-recognition proteins, which identify specific microbial surface compounds, e.g. peptidoglycans (Dziarski, 2004). After this recognition step, several immune pathways can be induced. Signal transduction via the Toll, Imd and JAK/STAT pathways lead to the expression of immune effector proteins such as antimicrobial peptides (AMP), which act directly against the invading microorganisms (Lehrer and Ganz, 1999). In addition to this, phenoloxidase activation by serine proteases can also lead to an adaptive response by encapsulation of foreign cells (Soderhall and Cerenius, 1998).

Transcriptomic studies on the molecular nature of the insect immune response towards beneficial symbionts have been confined to very few model systems (Futahashi *et al*., 2013). These studies show the differential expression of antimicrobial peptides in response to symbiont perturbation. In particular, c-type lysozyme and defensin-like genes were upregulated in the stinkbug *Riptortus pedestris*, when bacterial symbionts localized in midgut crypts were experimentally removed to create aposymbiotic individuals (Futahashi *et al*., 2013). However, studies based on nutritionally relevant gut symbionts without a specialized localization are still lacking, partially because insect models which allow the targeted experimental removal of intestinal microorganisms are scarce.

Within the Pyrrhocoridae (Hemiptera), characterization of the intestinal microbial community revealed a consistent and conserved microbiota, with the co-occurrence of two actinobacterial taxa belonging to the family Coriobacteriaceae (*Coriobacterium glomerans* and *Gordonibacter* sp.) (Sudakaran *et al*., 2012; Salem *et al*., 2013). Similar to other hemipteran insects (Fukatsu and Hosokawa, 2002; Kikuchi *et al*., 2007), firebugs rely on an extracellular post-hatch mechanism for the vertical transmission of their actinobacterial symbionts by the deposition of bacteria-containing fecal droplets by adult females over newly laid eggs, with the subsequent probing and uptake of the symbionts by the hatched nymphs (Kaltenpoth *et al*., 2009). In the European firebug (*Pyrrhocoris apterus*) and the African cotton stainer (*Dysdercus fasciatus*) (both Pyrrhocoridae), sterilization of the egg surface results in aposymbiotic individuals, which lack the actinobacterial symbionts (Kaltenpoth *et al*., 2009). Such individuals show slower growth rates, higher mortality and lower reproductive success (Salem *et al*., 2013), which indicates an essential function of the actinobacterial symbionts towards their insect host. Furthermore, a recent study demonstrated that this essential function can be attributed to the nutritional provisioning of B-vitamins to the host, and transcriptomic analyses revealed a tight integration of the symbionts into the host's metabolism (Salem *et al.*, submitted).

In this study, we investigated the immune response of *D. fasciatus* with respect to symbiont perturbation by a comparative transcriptomic analysis of midgut samples from aposymbiotic and symbiotic individuals. We accompany the expression analysis with qPCR validations and phylogenetic analyses of key genes involved in the response to symbiosis such as hemipticin and defensin. The results of this study provide first insights into the host immune factors involved in the maintenance of this complex and specific extracellular gut microbiota.

6.3 MATERIALS AND METHODS

6.3.1 Insect rearing and ethics statement

Live specimens of *Dysdercus fasciatus* were obtained from a laboratory culture at the University of Würzburg, Germany, and reared at the Max Planck Institute for Chemical Ecology, Jena, Germany. No specific permits were required for rearing, as *D. fasciatus* is not an endangered or protected species. The insects were reared in plastic containers (20 × 35 × 22 cm) at a constant temperature of 28ºC and exposed to long light regimes (16h/8h light/dark cycles). The bugs were provided *ad libitum* with previously autoclaved water and dry cotton seeds (*Gossypium barbadense)*.

6.3.2 Experimental elimination of the Coriobacteriaceae symbionts

The generation of aposymbiotic bugs involved the sterilization of egg surfaces following the procedure utilized (Salem *et al*., 2013). Briefly, three day old eggs were submerged in ethanol for 5 minutes, followed by bleach (12% NaOCl) for 45 seconds. Subsequently, residual bleach was removed by washing several times in water. Untreated eggs served as symbiont-containing controls, as it was previously shown that the egg surface sterilization procedure itself does not adversely affect host fitness (Salem *et al*., 2013). To examine the differential pattern of host gene expression in response to symbiont elimination on the natural diet of cotton seeds, five egg clutches of *D. fasciatus* were harvested, and each clutch was separated into two experimental treatments and reared on cotton seeds: (i) symbiotic (untreated), and (ii) aposymbiotic (egg surface sterilized). This design was chosen to reduce the potential influence of genetic variability among host egg clutches and allowed the use of paired statistical tests for analysis.

6.3.3 RNA extraction and reverse transcription

Three days following adult emergence, a single individual was collected from every experimental treatment replicate, and through dissection, its midgut region (M1-M4) was harvested. Once dissected, the midgut region was stabilized in RNA*later* solution (Qiagen) and stored at −20°C. Total RNA isolations were performed using the RNeasy Micro Kit (Qiagen) following the manufacturer′s guidelines. Integrity and quality of the RNA samples were determined using the RNA 6000 Nano LabChip kit (Agilent Technologies) on an Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions. cDNA was then generated with the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's guidelines. To account for possible

shortcomings during RNA extraction and reverse transcription, diagnostic PCR screens targeting the host's 18S rRNA gene, and the Coriobacteriaceae symbionts' 16S rRNA genes were conducted using the generated cDNA according to procedures described previously (Salem *et al*., 2013).

6.3.4 Transcriptomic sequencing and library construction

Sequencing was conducted using RNA extractions from dissected whole midgut regions (M1-M4) of symbiotic and aposymbiotic bugs fed on their natural diet of cottonseeds. RNA from five individuals (one per egg clutch) of each treatment was pooled, resulting in two samples.

Prior to sequencing, the extracted RNA was exposed to rRNA depletion to minimize the amount of ribosomal RNA in the final samples (Analytik Jena, Jena, Germany). Additionally, a poly-A enrichment strategy was used to separately enrich for eukaryotic and prokaryotic RNA, respectively (Analytik Jena, Jena, Germany). RNA sequencing of the resulting four samples (poly-A enriched and depleted fractions of symbiotic and aposymbiotic bugs, respectively) was performed by a commercial service provider (Fasteris; http://www.fasteris.com) using 5 µg total RNA (per sample) on the HiSeq™ 2000 Sequencing System from Illumina (http://www.illumina.com/ webcite), utilizing the paired read 100 bp technology. The sequence data generated in this study have been deposited at the European Nucleotide Archive in the Short Read Archive database under study accession number PRJEB6171. The complete study can be directly accessed under http://www.ebi.ac.uk/ena/data/view/PRJEB6171.

The paired-end reads were trimmed according to the sequencing quality test using Timmomatic (v0.30) (Lohs *et al*., 2012). The leading and trailing bases of each read were cut off if the quality values were below the default threshold. Additionally, reads were discarded if they were shorter than 30 base pairs after trimming. Following quality checks, the trimmed reads were assembled *de novo* into contigs using Trinity (r2012-10-05) (Grabherr *et al*., 2011). The minimal contig length was set to 200 and the *k-mer* length to 25 base pairs. The read libraries of the poly-A depleted and enriched treatments were separately pooled and assembled into backbones, respectively. After the assembly with Trinity, the contigs were clustered by CD-HIT EST (v4.5.7) (Huang *et al*., 2010) according to their sequence similarity to remove potential duplicates. Sequences with more than 99% sequence similarity to other contigs were subsequently collapsed. For the assignment of expression values to each constructed transcript in the respective library, the original reads were mapped back to the respective backbone assembly using Bowtie2 (v2.0.3) (Langmead and Salzberg, 2012). The generated output was processed using SAMtools (v0.1.18) (Li *et al*., 2009) to create BAM files and asses the coverage depth as the number of reads mapped to each transcript using the R package Rsamtools (v1.14.1).

The expression information was normalized using the RPKM (reads per kilobase of transcript per million of mapped reads) transformation to obtain estimates of relative expression levels. Homology searches (BLASTx and BLASTn) of unique contig sequences and functional annotation by gene ontology terms (GO; http://www.geneontology.org) were conducted with an E-value cutoff of 10^{-10} using the BLAST2GO software suite v2.4.1 (http://www.blast2go.de).

Transcripts with annotations related to the innate immune response were extracted using specific GO terms. Additionally, sequences representing antimicrobial peptides were detected, using a bi-

directional local BLAST search (E-value cutoff of 10^{-5}) with a custom database consisting of publicly available (GenBank) antimicrobial peptide sequences from insects (see Supplementary Material).

6.3.5 Phylogenetic analyses of selected transcripts

Phylogenetic analyses were performed with the software tool MEGA5 (Tamura *et al*., 2011) for selected transcripts with the same annotation. The coding regions of the nucleotide sequences was first determined and translated into protein sequences using Augustus (v2.7) (Stanke and Morgenstern, 2005). The protein sequences were subsequently aligned with Muscle 8 (Edgar, 2004). Maximum likelihood trees were then constructed with 500 bootstrap replicates.

6.3.6 Validation of host gene expression by quantitative PCR

Quantitative PCRs (qPCRs) for the candidate host immune response genes were conducted across the same RNA samples from aposymbiotic and symbiotic bugs used for sequencing to confirm the transcriptome sequencing results. Primers were designed based on the candidate gene sequences available from the transcriptome and checked for specificity *in vitro* using capillary sequencing of amplified PCR products. The qPCR reactions were performed using a RotorGene®-Q cycler (Qiagen, Hilden, Germany), with the same individual cDNA extracts used for the diagnostic PCR screens. The final reaction volume of 25 µl included the following components: 1 µl of cDNA template, 2.5 µl of each primer (10 µM), 6.5 µl of autoclaved distilled H_2O, and 12.5 µl of SYBR Green Mix (Qiagen, Hilden, Germany).

Conditions for qPCR were optimized using a VWR® Gradient Thermocycler (VWR, Radnor, PA, USA) at various annealing temperatures (60-68 °C). Standard curves for absolute quantification in the qPCR (10-fold dilution series from 1 ng/µl to 10^{-6} ng/µl) were generated using purified PCR products for all primer pairs after measuring the PCR product concentrations using a NanoDropTM1000 spectrophotometer (Peqlab, Erlangen, Germany). For qPCR, the following cycling parameters were used: 95°C for 10 min, followed by 45 cycles of 68ºC for 30 s, 72ºC for 20 s, and 95ºC for 15 s. Subsequently, a melting curve analysis was conducted by increasing the temperature from 60ºC to 95ºC within 20 min. Six replicates of one of the standard concentrations were used, for each primer pair and concentration, for the configuration and calibration of the standard curve. The resulting averages were then utilized to correct for possible errors in the DNA concentration measurements. Based on the standard curve, absolute transcript copy numbers were calculated according to (Lee *et al*., 2008).

6.3.7 Statistical analysis

The absolute expression determined by qPCR was normalized with the expression of a housekeeping gene (60S ribosomal protein L13a). The normalized expression levels in the aposymbiotic and symbiotic treatments were then compared using the Wilcoxon signed rank test to assess levels of significance ($P < 0.05$).

6.4 RESULTS

6.4.1 Success of symbiont elimination

Sterilization of egg surfaces resulted in aposymbiotic firebugs that were free of both Coriobacteriaceae symbionts, as confirmed by *C. glomerans*- and *Gordonibacter*-specific diagnostic PCRs. Conversely, symbiotic bugs tested positive for both bacterial species (data not shown).

6.4.2 Bacterial transcripts in the midgut of *D. fasciatus*

In total, RNA sequencing of the poly-A depleted treatments yielded 38,207,274 reads with a combined length of 3.8 Gbp. After trimming, backbone assembly and subsequent clustering of similar sequences resulted in 89,807 contigs with a N50 length of 955 base pairs. Despite the poly-A separation strategy, only a small subset of bacterial transcripts (1,651 contigs) were recovered and annotated in the poly-A depleted fraction (Fig. 1), which, in turn, failed to provide a coherent overview of the microbiota's metabolic and cellular processes in *D. fasciatus'* midgut. However, the transcriptional signal was still indicative of the taxonomic diversity of metabolically active bacterial species within the midgut of symbiotic and aposymbiotic firebugs.

Overall, the expression patterns between aposymbiotic and symbiotic bugs were highly similar with minor differences in Lactobacillales, Rickettsiales and Rhizobiales. The strongest difference was found in relation to transcripts belonging to the Coriobacteriaceae symbionts (*C. glomerans* and *Gordonibacter sp.*). As expected and in contrast to the high expression patterns observed in symbiotic bugs, no Coriobacteriaceae transcripts were retrieved from the aposymbiotic treatment.

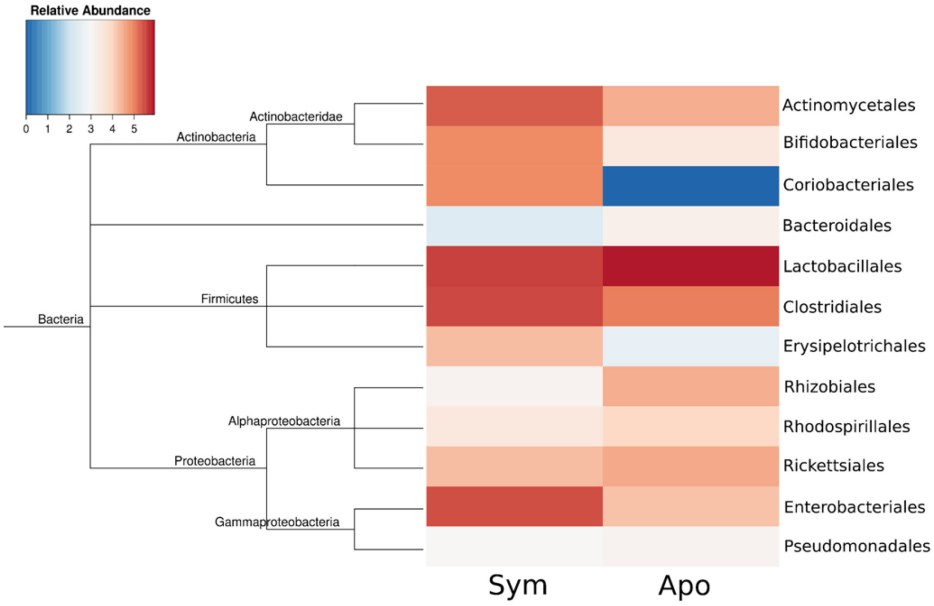

Figure 1: Heatmap of the sum of mapped reads (logarithmic scale) on bacterial transcripts annotated as the depicted taxonomic groups. The tree on the left represents the phylogenetic relationships of the bacteria based on the NCBI database.

6.4.3 Immune system-related transcripts in the midgut transcriptome of *D. fasciatus*

In total, RNA sequencing of the poly-A enriched treatments yielded 36,456,544 reads with a combined length of 3.6 Gbp. After trimming, backbone assembly and subsequent clustering of similar sequences resulted in 55,222 contigs with a N50 length of 1,411 base pairs. Of those contigs, 20,174 received an annotation via BLAST homology. The majority of the annotations were based on hits from *Riptortus pedestris* and *Acyrthosiphon pisum*, the bean bug and pea aphid, respectively. From the BLAST results, 3,362 contigs were assigned to functional categories according to gene ontology (GO), most of which were determined to be involved in cellular and metabolic processes and biological regulation.

Here we focus on gene candidates involved in immune-system processes of the insect host. Four genes putatively responsible for recognition, and 20 genes involved in signal transduction in the major insect immune pathways Toll, Imd, JAK/STAT, and phenoloxidase were recovered (Table 1, Suppl. Fig. 1). With nine annotated transcripts, the Toll pathway constituted the most comprehensive set, whereas the Imd and JAK/STAT pathways contained significantly fewer annotated transcripts (both four transcripts). For the phenoloxidase pathway, three transcripts were assigned as pro-phenoloxidase (Dfas-51289), serine protease easter-like (Dfas-14165), and mature phenoloxidase (Dfas-45148).

Table 1: Annotation and normalized expression values (RPKM) of transcripts putatively involved in recognition and signalling of the innate immune response. Differentially expressed genes (>2fold) are marked with asterisks.

		Normalized expression (RPKM)		Fold change
Sequence ID	**Annotation**	**Symbiotic**	**Aposymbiotic**	**(Sym / Apo)**
Recognition				
Dfas-12970	Cohesin subunit sa-1-like	1.39	2.50	0.56
Dfas-25404	PGRP s2-like	0.39	0.13	2.96*
Dfas-27487	Hemolectin cg7002-pa	0.00	0.33	-
Dfas-45194	Galectin partial	1.47	2.12	0.69
Toll Pathway				
Dfas-02931	Ecsit_drome ame	4.06	3.76	1.08
Dfas-49270	Myd88 protein	47.44	48.31	0.98
Dfas-17230	Serpin 38f	7.22	15.29	0.47*
Dfas-03438	Partial spaetzle precursor	10.27	12.48	0.82
Dfas-17931	Protein spaetzle	2.59	4.52	0.57
Dfas-01522	Protein toll	0.70	0.24	2.96*
Dfas-16185	Cactus	10.89	42.43	0.26*
Dfas-36948	Dorsal 1c	0.78	0.79	0.99
Dfas-25024	Mapkk kinase dsor1	2.33	4.84	0.48*
IMD pathway				
Dfas-48512	Nf kappa b inhibitor	1.60	0.36	4.44*
Dfas-03678	Tp53-regulated inhibitor	4.05	5.62	0.72
Dfas-30553	Tak1-associated binding protein	1.45	1.42	1.02
Dfas-48614	Elongator complex protein 1	0.86	2.26	0.38*
JAK/STAT pathway				
Dfas-28681	Tyrosine-protein kinase jak2	0.00	0.36	-
Dfas-39587	Tyrosine-protein kinase hopscotch	0.86	0.44	1.97
Dfas-35575	Cytokine receptor	3.15	0.32	0.32*
Dfas-43095	Signal transducing adapter molecule	15.34	20.14	0.76
Phenoloxidase pathway				
Dfas-51289	Pro-phenol oxidase subunit 2	0.58	0.79	0.74
Dfas-45148	Phenoloxidase subunit a3-like	0.42	0.84	0.49*
Dfas-14165	Serine protease easter-like	6.85	7.68	0.89
Dfas-26420	Melanization-related protein	0.00	1.21	-
Dfas-50961	Limulus clotting factor c	0.19	2.07	0.09*

The expression of transcripts presumably involved in recognition and signaling generally showed relatively low expression levels based on the normalized RPKM values (Table 1). The low expression pattern of these transcripts nonetheless points towards an up-regulation (Fold>2) in aposymbiotic individuals (Table 1), with the exception of the transcripts annotated as protein toll (Dfas-01522), nf-kappa b inhibitor (Dfas-48512) and peptidoglycan recognition protein s2 (Dfas-25404), which were up-regulated (Fold>2) in symbiotic bugs. Most of the transcripts potentially involved in signaling showed no differential expression at all.

6.4.4 Antimicrobial peptide transcripts

Among the immune effector genes, several AMP types with potential isoforms were detected (Table 2): 11 transcripts were annotated as hemipticin (Dfas-21661, Dfas-00011, Dfas-50935, Dfas-16990,

Dfas-33450, Dfas-43693, Dfas-01725, Dfas-21700, Dfas-46208, Dfas-03942, Dfas-25640), three as defensin-like genes (Dfas-02709, Dfas-51099, Dfas-33854), two as pyrrhocoricin (Dfas-00911, Dfas-33105) and two as c-type (Dfas-30397, Dfas-07111) and i-type lysozyme (Dfas-45802, Dfas-30083), respectively. The immune effector genes generally showed much higher expression levels than genes putatively involved in immune signaling and microbe recognition (Table 1 and 2). Comparison of AMP expression between symbiotic and aposybiotic bugs revealed that most of the detected transcripts were differentially expressed (Fold>2), with some genes being up- and others down-regulated in response to symbiont elimination (Table 2). Among the transcripts up-regulated in aposymbionts were i-type lysozyme, hemipticin, and defensin, whereas pyrrhocoricin and c-type lysozyme were down-regulated in symbiont-deprived bugs. Within each AMP, the expression of the isoforms was relatively consistent, with only minor variations in the RPKM values.

Table 2: Annotation and normalized expression values (RPKM) of putative antimicrobial peptide transcripts. Differentially expressed genes (>2fold) are marked with asterisks.

		Normalized expression (RPKM)		Fold change
Sequence ID	**Annotation**	**Symbiotic**	**Aposymbiotic**	**(Sym / Apo)**
Dfas-45802	Lysozyme i-type	1.27	2.57	0.49*
Dfas-30083	Lysozyme i-type	0.84	2.38	0.35*
Dfas-30397	Lysozyme c-type	649.66	130.97	4.96*
Dfas-21661	Hemipticin	1.86	2.52	0.74
Dfas-00011	Hemipticin	0.82	7.02	0.12*
Dfas-50935	Hemipticin	0.89	1.81	0.49*
Dfas-16990	Hemipticin	0.39	5.09	0.08*
Dfas-33450	Hemipticin	1.20	7.00	0.17*
Dfas-43693	Hemipticin	0.00	0.28	0.28*
Dfas-01725	Hemipticin	1.60	8.25	0.19*
Dfas-21700	Hemipticin	1.24	12.18	0.10*
Dfas-46208	Hemipticin	0.68	6.23	0.11*
Dfas-03942	Hemipticin	1.00	3.37	0.30*
Dfas-25640	Hemipticin	3.08	5.28	0.58
Dfas-02709	Defensin 4	2.40	1.62	1.48
Dfas-51099	Defensin a	0.86	1.45	0.59
Dfas-33854	Defensin-2 precursor	1.10	3.23	0.34*
Dfas-00911	Pyrrhocoricin	36.77	19.81	1.86
Dfas-33105	Pyrrhocoricin	19.91	8.62	2.31*

6.4.5 Expression validation of antimicrobial peptides with qPCR

To validate the observed normalized expression patterns of c- and i-type lysozyme, defensin and hemipticin (Table 2), quantitative PCRs were performed (Fig. 2). The same expression patterns were observed for hemipticin and c-type lysozyme compared to the RNAseq expression analysis. The expression of hemipticin showed a significant up-regulation in aposymbiotic individuals (Wilcoxon signed rank test, $p<0.01$), whereas the gene for c-type lysozyme was significantly down-regulated in aposymbiotic bugs (Wilcoxon signed rank test, $p<0.05$). For defensin and i-type lysozyme, no significant differences could be detected in the qPCRs.

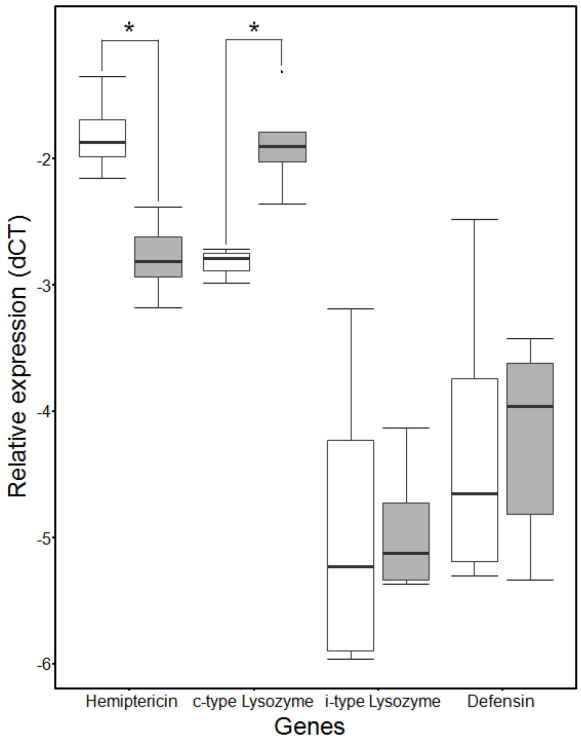

Figure 2: Relative expression of antimicrobial peptides based on qPCR experiments. Expression values were calculated as the dCT based on the expression of a housekeeping gene (60S ribosomal protein L13a). Grey boxes represent expression values of symbiotic bugs, open boxes expression values of aposymbionts. Significant differential expression is indicated with an asterisk (Wilcoxon signed rank tests, $p<0.05$).

6.4.6 Phylogenetic analysis of transcripts encoding antimicrobial peptides

To study the micro-diversity of sequences annotated as defensin and hemiptericin, phylogenetic analyses were performed (Fig. 3). For both genes, sequence alignments revealed various amino acid substitutions across the different isoforms (Suppl. Fig. 2 and 3). Defensin-related transcripts showed two distinct isoforms, one of which clustered with a published sequence for *P. apterus* (Fig. 3A). For hemiptericin, phylogenetic analysis revealed three distinct isoforms (Fig. 3B), one of which clustered together with sequences of *P. apterus* obtained from the GenBank database. The transcripts annotated as c-type lysozyme (Table 2) had one isoform, which was highly similar to a *R. pedestris* sequence (Suppl. Fig. 4).

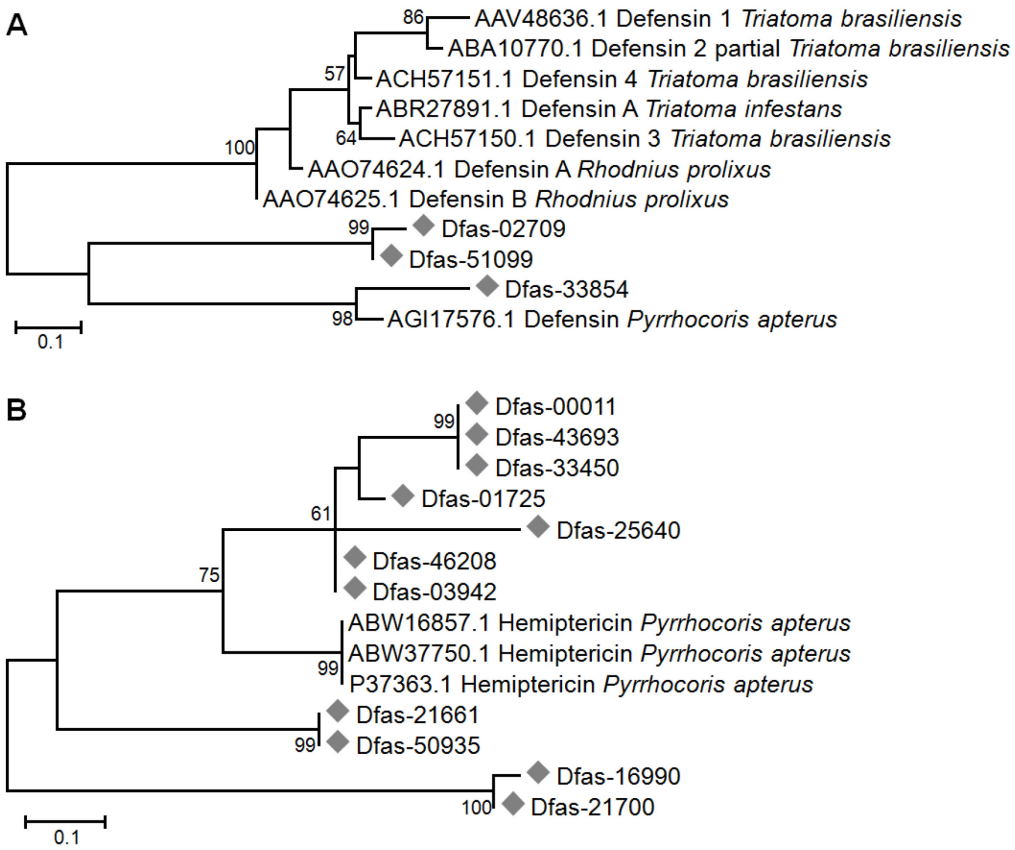

Figure 3: Maximum likelihood trees of defensin (A) and hemipticin (B) transcripts. Sequences recovered in this study are marked with a grey diamond. Bootstrap values above 50% are given at the nodes (based on 500 replicates).

6.5 Discussion

In this study, we conducted a comparative transcriptomic analysis of midgut samples from symbiont-containing and aposymbiotic individuals of the cotton stainer, *D. fasciatus*. We identified a number of differentially expressed transcripts involved in the immune response of the host following symbiont elimination. These results provide first insights into the molecular interactions between cotton stainers and their extracellular intestinal symbionts. In the following paragraphs, we will discuss the putative biological roles of the immune related genes identified in this study and the implications of their differential expression upon perturbation of the microbial midgut community in *D. fasciatus*.

6.5.1 Effect of symbiont perturbation on the gut microbiota

The few collected transcripts of bacterial taxa (Fig. 1) were insufficient to provide a comprehensive view of the microbial gene expression. However, they did provide significant insight into the taxonomic composition of metabolically active bacterial symbionts in the midgut of *D. fasciatus*. Our analysis revealed a microbial community that is largely consistent with earlier results characterizing the gut microbiota of firebugs, with high abundances of Actinobacteria, Firmicutes, and Proteobacteria (Sudakaran *et al.*, 2012; Salem *et al.*, 2013). Most importantly, bacteria of the family

Coriobacteriaceae were abundant in symbiotic bugs, but completely absent from aposymbiotic individuals. These results augment previous findings (Salem *et al*., 2013) demonstrating that the egg surface sterilization is specific towards ridding firebugs from their association with *C. glomerans* and *Gordonibacter* sp.

Concerning the distribution of other microbial taxa, only minor quantitative differences between aposymbiotic and symbiotic bugs were detected (Fig. 1). This indicates that even though a major group of mutualistic bacteria were removed from the intestinal ecosystem, the host was still able to maintain a specific composition of the remaining microbiota, either by providing a selective environment or by regulation through the immune system. The minor differences in gene expression of transcripts belonging to Lactobacillales, Rickettsiales and Rhizobiales might be in this respect indicative for a compensatory mechanism from the host side.

6.5.2 Constitutive expression of immune signaling pathways

In our study we identified a subset of the known pattern recognition proteins (Table 1) involved in the sensing of foreign organisms (Dziarski, 2004). Among those was one short type peptidoglycan recognition protein. In *Drosophila* species, short class peptidoglycan recognition proteins are responsible for the detection of gram-positive bacteria and subsequent induction of immune pathways (Bischoff *et al*., 2004). Thus, this protein may be involved in the recognition of the mutualistic Coriobacteriaceae in *D. fasciatus*, which is consistent with the up-regulation in symbiotic as compared to aposymbiotic bugs (Table 1).

Among the immune-related transcripts detected in this study (Table 1, Suppl. Fig. 1), all four major insect immune pathways were recovered (Bischoff *et al*., 2004). The detected Toll pathway provides the most comprehensive view, with annotated transcripts spanning the signal transduction cascade from precursor protein *spaetzle* to the transcription factors *dorsal* and *cactus* (Anderson *et al*., 2000). In contrast, the detected genes involved in the Imd pathway provide only a fragmented view of this signaling cascade. The scarcity of annotated Imd-pathway genes is likely due to the absence of many Imd genes in the pea aphid *Acyrthosiphon pisum* (Gerardo *et al*., 2010), which is the closest organism to the Pyrrhocoridae with a sequenced genome available, and thus, with reliable homology information. Across the four major pathways (Toll, Imd, JAK/STAT, phenoloxidase), gene expression was generally low. Furthermore, no distinct regulation patterns could be detected, with only few genes being differentially expressed (>2fold) between symbiotic and aposymbiotic individuals. These observations are in line with other studies, demonstrating the constitutive expression of immune signaling pathway genes (Hussain *et al*., 2013).

6.5.3 Expression patterns of immune effectors depict differentiated biological roles

In contrast to the low and constitutive expression of pathway-internal genes, the transcripts of the end products of signal transduction, the immune effector genes, show generally higher expression levels that differ between symbiotic and aposymbiotic bugs (Table 2). Considering that antimicrobial peptides (AMPs) are the front line of defense against microbial pathogens (Lehrer and Ganz, 1999), this indicates a highly specific as well as regulated immune response towards symbiont perturbation.

The expression patterns across the detected antimicrobial peptides differed considerably (Table 2). In general, two groups could be differentiated: i) Transcripts down-regulated in aposymbiotic bugs comprised c-type lysozyme and pyrrhocoricin, whereas ii) defensin, hemiptericin and i-type lysozyme were up-regulated upon symbiont elimination. Validation of expression patterns via qPCR (Fig. 2) confirmed the differential expression for c-type lysozyme and hemiptericin, but not for defensin and i-type lysozyme across the studied individuals. Therefore, c-type lysozyme and hemiptericin were considered strong candidates for an important role in responding towards perturbation of the microbial midgut community.

The high expression of c-type lysozyme and pyrrhocoricin in symbiotic bugs might serve to regulate the association with the pivotal Coriobacteriaceae symbionts (Fig. 2). In weevils of the genus *Sitophilus*, the expression of a single antimicrobial peptide gene (coleoptericin A [*colA*]) was found to be important in controlling population dynamics of mutualistic bacteria, which overreplicated in the host, when *colA* was knocked down with RNA interference (Login *et al*., 2011). The same might be true in Pyrrhocoridae species, which may require c-type lysozyme to control the abundance of the intestinal symbionts in order to maintain the host-symbiont equilibrium. This equilibrium may be particularly important for gut ecosystems, since at a higher microbial growth rate the host might compete with its symbionts for important nutrients rather than engage in a mutualistic relationship.

Furthermore, considering the nutritional relevance of the symbiotic Coriobacteriaceae (Salem *et al*., submitted) up-regulation of c-type lysozyme might cause the release of nutrients via lysis of the bacterial cells (Salem *et al.*, submitted). In *Drosophilla melanogaster,* lysozyme expression plays an important role in the digestion of intestinal bacteria (Daffre *et al*., 1994), indicating a similar pattern as observed in this study (Fig. 2). This harvesting mechanism is conceptually different from other insect symbiosis such as the detoxification mutualism in the related species *R. pedestris* (Kikuchi *et al*., 2012). Here, c-type lysozyme is up-regulated in aposymbiotic individuals (Futahashi *et al*., 2013), likely representing a mechanism to counteract infestations by pathogens, rather than the release of nutrients from mutualists.

In contrast to c-type lysozyme and pyrrhocoricin, hemiptericin was significantly up-regulated in aposymbiotic individuals, and defensins as well as i-type lysozyme showed tendencies towards higher expression in aposymbiotic bugs. As in other insects, hemiptericin is known to be important in *P. apterus* for the response to foreign microorganisms (Cociancich *et al*., 1994). The up-regulation upon symbiont elimination in *D. fasciatus* might therefore constitute a compensatory mechanism to control the remaining gut microbiota, which might otherwise overreplicate in the ecological niche freed by the absence of the Coriobacteriaceae.

6.5.4 Isoforms of antimicrobial peptides indicate a global response to the gut microbiota

Interestingly, several isoforms of hemiptericins and defensins were detected (Fig. 3), which may allow for a fine-tuned and dynamic interaction with the intestinal community, since the different isoforms have divergent sequences (Suppl. Fig. 2 and 3) and may thus act against different microbial taxa. This is particularly relevant in cotton stainers, as in addition to the Coriobacteriaceae symbionts, they consistently harbor several bacterial taxa in the Firmicutes and Proteobacteria (Fig. 1), which could be differentially regulated in their abundance by individual AMPs. The presence of AMP isoforms has

also been described for other insects (Futahashi *et al*., 2013; Vogel *et al*., 2014)), but their role and adaptive significance in immunity remains unknown.

6.6 CONCLUSIONS AND PERSPECTIVE

Using a comparative transcriptomics approach, our study revealed a differentiated immune response towards the elimination of a specific group of extracellular gut symbionts. This response includes both up- and down-regulation of certain antimicrobial peptides of the host, respectively, which may serve to maintain a stable equilibrium of the complex microbiota in the host's gut and could additionally be involved in harvesting nutrients through lysis of symbiont cells. While the transcriptomic approach provides a global view into gene expression patterns in the cotton stainer's gut, targeted knock-down of individual immune effector genes by RNA interference (Kostal *et al*., 2009) is necessary to provide functional insights into the interactions between the microbial symbionts and the host immune system.

6.7 ACKNOWLEDGMENTS

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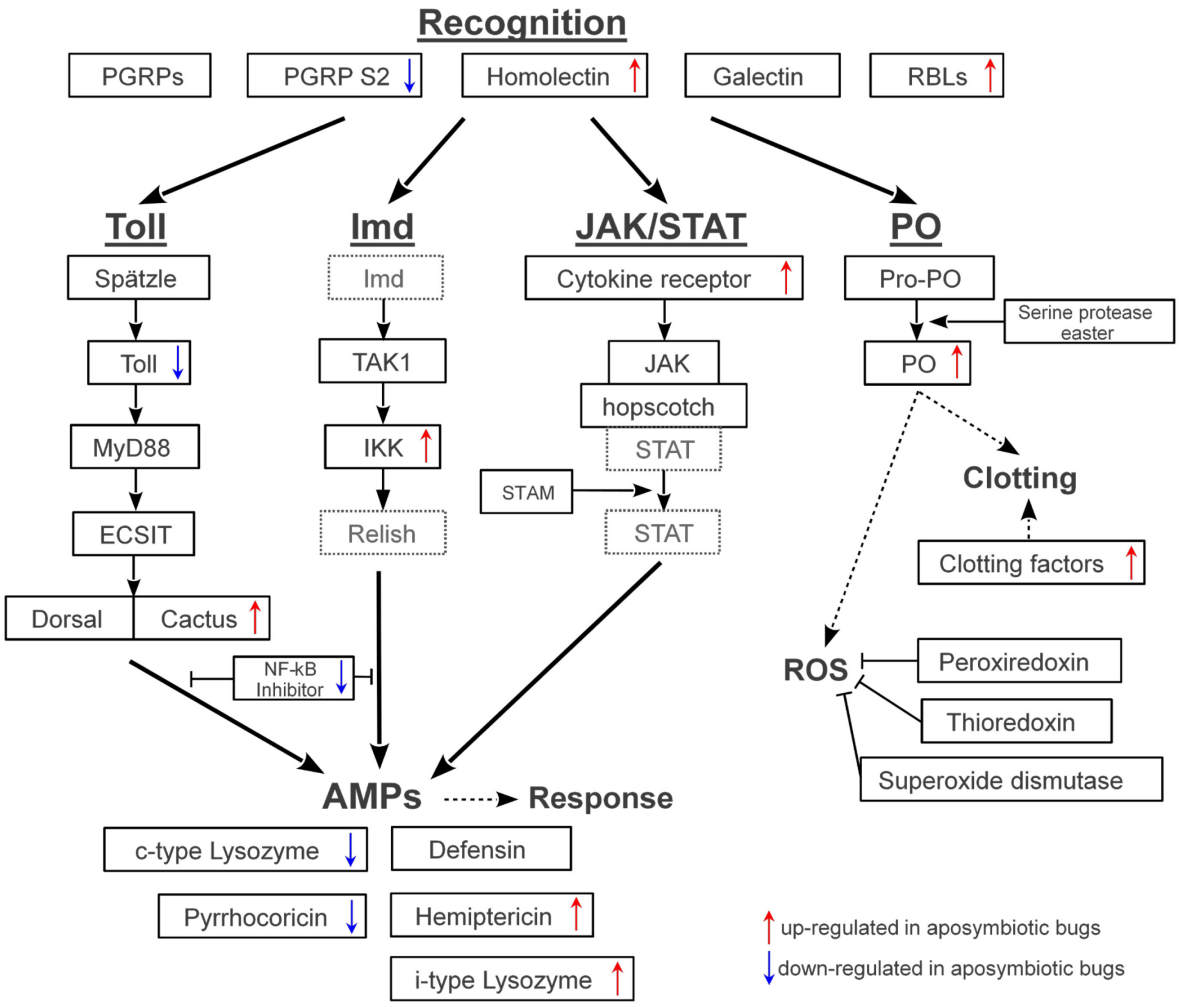
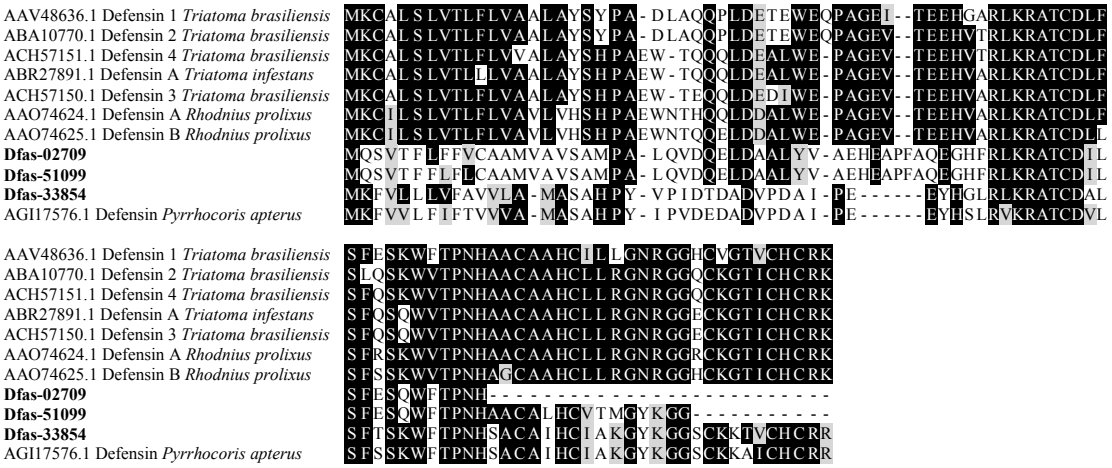
6.9 SUPPLEMENT



Supplementary Figure 1: Overview of detected immune response related transcripts with their respective regulation pattern (>2fold). Red arrows denote up-regulation, blue arrows down-regulation upon symbiont elimination. Components in dotted boxes were not detected in the transcriptome.



Supplementary Figure 2: Multiple sequence alignment of translated defensin transcripts detected in this study (bold), in comparison with publicly available sequences.

CHAPTER 7

GENERAL DISCUSSION

7.1 THE INSECT GUT AS AN ECOLOGICAL NICHE: DETERMINANT FACTORS FOR MICROBIAL COLONIZATION

Numerous studies have thus far illustrated the pivotal role gut microorganisms have played in the diversification of insects (Engel and Moran, 2013). These relationships have been integral towards the host's subsistence on nutritionally imbalanced diets, protection from parasites and pathogens, as well as enhancing the insect's reproductive potential.

Following the recent surge of studies, aided by advancements in sequencing technologies, we are now only beginning to comprehend the complexity of these associations, as well as the factors contributing to how gut microbial communities are organized and maintained across this metazoan class.

As an adaptation to different feeding ecologies, the digestive tracts of insects have undergone a range of modifications marking the radiations of the various lineages. The basic structure of the digestive tract, however, remains conserved and the various compartments can largely be assigned to one of three primary regions: foregut, midgut, and hindgut (Chapman *et al*., 2013). The foregut and hindgut are derived from the embryonic ectoderm; the former functions to separate ingested food particles for temporary storage, while the latter serves to receive feces, set up fermentation chambers and, to a lesser extent, absorb important nutrients.

In most insects, the principal location for digestion and absorption, however, is the midgut, which – unlike the foregut and hindgut – is derived from endodermal tissue (Chapman *et al*., 2013). It is in the midgut that the greatest diversity and abundance of microorganisms often occurs, in part due to the rich source of digested food that becomes readily available. Within the midgut, bacteria are prevented from directly colonizing the midgut through a thin envelope called the peritrophic membrane; a porous matrix capable of sieving important nutrients and enzymes to and from the midgut without allowing bacterial passage (Lehane, 1997; Edwards and Jacobs-Lorena, 2000). The membrane also provides protection from mechanical damage by food particles.

Despite the nutritional benefits of such a niche, microbial colonization of insect guts can be hampered by a number of factors. Most notably, insects molt throughout development, and as a result, shed the exoskeletal lining of both the foregut and hindgut, alongside the associated microbial communities (Chapman *et al*., 2013). The shedding of the peritrophic matrix similarly impacts the midgut's microbiota, albeit less severely in presence of crypts or invaginations that can allow for microbial persistence (Lehane, 1997).

Successful microbial colonization is also dependant on a range of physiochemical conditions in the lumen across the different gut regions, specifically pH and oxygen concentrations (Engel and Moran, 2013). Gut lumens undergo substantial regulation in their pH, which often deviates from what is encountered intracellularly, as well as in the hemolymph (normally around 7). The high pH of lepidopteran guts, for example, has been linked to feeding on leaves rich in tannin, where alkaline conditions improve the binding efficiency of digestive enzymes acting on the polyphenolic compound (Berenbaum, 1980). The alkaline guts of lepidopteran larvae (pH 8-12) are thought to select for a small subset of microbial lineages that are capable of successfully subsisting in an otherwise extreme

environment (since the pH growth optimum of most bacteria is around 6-7) (Berenbaum, 1980). This is also the case with some soil-feeding termites where the extreme alkalinity in some compartments of the guts was found to preferentially support the growth of alkaline-tolerant bacterial lineages, namely Firmicutes, Clostridium and Planctomycetes (Köhler *et al*., 2008; Bignell, 2010). While thought to function as a derived anoxic fermentor, the termite hindgut also possesses sharp oxygen and hydrogen gradients that have been demonstrated to be maintained by the different resident bacterial communities (Brune, 2006).

On the other hand, insect guts possessing complex microbial communities can sometimes undergo severe changes in their abiotic conditions depending on the taxonomic and metabolic composition of their gut symbionts. In many instances this shift in abiotic conditions depends largely on the buffering capability of the gut contents relative to the size and degree of metabolic activity of the resident microbiota (Dillon and Dillon, 2004). Hindguts of scarab beetles (*Pachnoda ephippiata*) owe their lower pH values (relative to the foregut and midgut) to the fermentation products (e.g. acetate, formate and lactate) generated by the greater microbial densities inhabiting that region of the gut (Lemke *et al*., 2003; Cazemier *et al*., 1997), highlighting the physiological influence of the symbionts on their ecological niche and vice versa.

Bacterial species constitute the vast majority of microbial constituents in most insects (Engel and Moran, 2013), however, microbial community structure in insect guts varies greatly in terms of density as well as localization. While numerous insects (including bees, kissing bugs, crickets, etc.) have been found to harbor around 10^9 bacterial cells in their guts (Dillion and Dillion, 2004); others like locusts, or the common fruit fly (*Drosophila melanogaster*) associate with significantly lower symbiont titers hovering around 10^5 bacterial cells (Dillon *et al*., 2005; Wong *et al*., 2014). In contrast, bacterial densities within the guts of plant sap-feeding insects are significantly lower than other insect groups. Aphids and other sharpshooters, for example, harbor very few taxa (Cheung and Purcell, 1993; Douglas, 1988). Instead, they possess intracellularly localized bacteria that are predominantly housed in specialized organs (i.e. bacteriomes) and take over important nutritional functions (Baumann, 2005).

Two important factors contributing to high ratios of bacterial gut symbionts relative to the host's body mass are: a) gut lengths, and b) textural complexity (Engel and Moran, 2013). Short and narrow guts often correlate with smaller bacterial communities, both in species abundance and diversity – as has been demonstrated in *Drosophila*, mosquitoes and aphids. On the other hand, termites – which possess highly compartmentalized guts – boast some of the highest ratios of bacteria/host cell ratios of any insect group (Cazemier *et al*., 1997).

In addition to the physiochemical properties of the gut, behavioral and feeding ecologies of the insect host can significantly contribute to the maintenance (via vertical or horizontal transmission) of distinct and possibly adaptive microbial communities. Unlike mammals and birds, most insects do not provide parental care following oviposition, and as a result, lack direct opportunities for conspecific transmission of gut symbionts (Funkhouser and Bordenstein, 2013) and as a result, many insects are characterized by a transient and taxonomically variable microbial community that is determined by the environmental availability of microbial symbionts (Engel and Moran, 2013). However, among Corbiculate bees, the social clades of *Bombus* and *Apis* were found to possess highly conserved gut microbial communities composed of specific phylotypes (*Gilliamella apicola*, *Snodgrassella alvi*, and *Lactobacillus kunkeei*) that were consistent across different environments and geographic locations (Cox-Foster *et al*., 2007; Martinson *et al*., 2011; Koch and Schmidt-Hempel, 2011; Koch *et al*., 2013). Here, eusocial behavior has been implicated as an important factor for the conserved nature of this

microbiota, since solitary bees within the Apoidea superfamily seemed to be indiscriminately dominated by an inconsistent consortium of microbes (Martinson *et al*., 2011).

7.1.1 Stability of the European firebug's microbiota across space and time

Similar to termites and social corbiculate bees, firebugs form conserved associations with specific bacterial lineages (Chapter 3; Sudakaran *et al*., 2012). Comprehensive characterization of the European firebug's (*P. apterus*) midgut microbiota revealed a community that is highly transient in its anterior (M1 and M2) and posterior portions (M4), but highly conserved and consistent in the middle region (M3) (Chapter 3; Sudakaran *et al*., 2012). The overlapping microbial composition between the M1 and M2 midgut regions and the insect's food source (linden seeds) implies a transitory community that undergoes nominal regulation by the host (Chapter 3; Sudakaran *et al*., 2012). The M3 region of the midgut, however, harbors a distinct microbiota that was shared neither with the food source nor the other midgut sections. That community is highlighted by the presence of *Coriobacterium glomerans*, *Gordonibacter* spp., *Clostridium* sp., *Lactococcus lactis*, *Klebsiella* sp., as well as an unknown Rickettsiales species. Unlike numerous pentatomomorphan bugs (Chapter 2; Salem *et al*., submitted), firebugs do not possess specialized gut crypts or caeca that promote symbiotic associations with proteobacterial symbionts. For example, pentatomoid bugs engage in highly obligate partnerships with singular γ-proteobacterial species (Hosokawa *et al*., 2006; 2007; Kikuchi *et al*., 2009). These symbionts are often transmitted vertically from mother to offspring, and have been demonstrated to significantly influence host fitness (Hosokawa *et al*., 2006; 2007; Kikuchi *et al*., 2009).

The overlap in microbial profiles between the M3 region and the microbiota of whole adult bugs suggests that this region of the gut is representative of the overall microbial diversity in *P. apterus*, and that its resident microbes vastly outnumber those harbored elsewhere in the insect's body (Chapter 3; Sudakaran *et al*., 2012). The unique physiochemical conditions of the M3 portion of the midgut likely contribute to the distinct taxonomic composition of that gut region. Unlike the other midgut compartments, the M3 is completely anaerobic and is slightly more acidic (with a pH value of 5.4) (Chapter 3; Sudakaran *et al*., 2012). While unsubstantiated, it is conceivable that these conditions can contribute towards selectively sieving transient bacterial constituents, while maintaining a core set of beneficial microbes that are capable of growing in anaerobic and slightly acidic conditions.

Gut microbial communities in many insect groups can exhibit significant variation (Coleman *et al.*, 2012). Such variation can be attributed to changes in the host's geographical range, or feeding ecologies that introduce an assortment of novel – albeit transient – bacterial species that can significantly alter the insect's resident gut microbiota.

For example, characterization of host-associated bacteria across two species of wild mosquitoes in Madagascar revealed that the overall diversity differed significantly according to habitat type (Zouache *et al*., 2011). Additionally, the microbiota diversity and composition was dependent on the sex of the host, as well as the extent of a population's geographical range (and its consortium of plants and mammals that the mosquitoes can utilize for food), where the greater the range, the greater the diversity in bacterial associates found (Zouache *et al*., 2011). Similarly, the common fruit fly (*Drosophila melanogaster*) maintains a highly capricious microbiota that can be fundamentally altered both in species diversity and individual strain abundance depending on the growth media the insects were provided (Sharon *et al*., 2010). The altered microbiota, in turn, would contribute towards the

fly's mating preference – flies reared on a molasses food source preferred to mate with individuals reared on the same diet, instead of, for example, flies reared on a starch medium.

In sharp contrast, the gut microbiota of *P. apterus* was found to be highly stable irrespective of where the bugs were sampled, or following which diet they were supplied (Chapter 3; Sudakaran *et al*., 2012). Comparative examination of the *P. apterus*' microbial community across five different populations in Germany and Austria, using both quantitative and qualitative approaches, revealed only limited variation, and that the six aforementioned core taxa remained as the most abundant bacterial species. Similarly, the rearing of firebugs on one of three herbivorous or carnivorous diets did not affect the relative abundances of the core microbiota (Chapter 3; Sudakaran *et al*., 2012).

The ecological stability of *P. apterus*' gut microbial community, coupled with the demonstrated vertical transmission and overall functional importance of its Coriobacteriaceae symbionts (Kaltenpoth *et al*., 2009; Salem *et al*., 2013), lend credence to the hypothesis that the community might have co-evolved with its insect host (Sudakaran *et al*., in preperation), as has been previously demonstrated for a number of insect groups, including ants (Anderson *et al*., 2012), social bees (Martinson *et al*., 2011), and termites (Schauer *et al*., 2012).

7.2 TRANSMISSION OF BENEFICIAL GUT MICROBES IN INSECTS

Given the subsistence of many insects on nutritionally imbalanced diets (e.g. plant sap, vertebrate blood, or wood), their dependence on the metabolic provisions of their gut microbes constitutes a substantial component of their ecology and evolution. As a result, it would be expected that the insect host would evolve mechanisms to ensure the faithful transfer of symbionts to its offspring.

Complex microbial communities are maintained across insect generations through a multitude of transmission mechanisms. As discussed in Chapter 2, the mode of symbiont transfer (vertical vs. horizontal) contributes significantly towards the evolution of host and microbe. Horizontally acquired bacterial mutualists often engage in highly promiscuous associations with the host, ones that are prone to inter- and intraspecific host-switching. On the other hand, various insect lineages associate with highly integrated microbes. To ensure the continuum of the association, the host invests in a vertical route for symbiont transfer as mediated by a range of morphological and/or behavioral adaptations to ensure a successful endowment of symbionts to their progeny.

Insects utilize numerous strategies that vary in their mode of transfer, as well as their degree of intimacy to ensure the continuity of beneficial associations with gut microbes (Buchner, 1965; Chapter 2). These can include *de novo* acquisition of bacteria from the environment (Kikuchi *et al.,* 2007), smearing of egg/brood surfaces (Prado *et al*., 2006; Kikuchi et al., 2009; Hosokawa *et al.*, 2012), social transmission among eusocial insects (Currie *et al*., 2003; Hongoh, 2010; Martinsen *et al*., 2012), provisioning of symbiont-bearing capsules (Hosokawa *et al*., 2006), via milk glad secretions to developing larvae (Aksoy, 1995), as well as transovarially to the developing eggs or embryos (Baumann, 2005).

7.2.1 The egg surface as an interface for symbiont transfer in firebugs

Egg surface contamination serves as a common route for symbiont transfer among insects of the order Hemiptera (Chapter 2). Across this group, numerous lineages have adopted a range of adaptations to

facilitate the process of symbiont transfer, including modifications to the insect's anatomy (Kikuchi *et al*., 2009) as well as behavior (Hosokawa *et al*., 2012).

In firebugs, we highlight that the egg surface serves as an important interface for the transmission of the Coriobacteriaceae constituents of the insect's conserved microbiota (Kaltenpoth *et al*., 2009; Salem *et al*., 2013). Following the sterilization of egg surfaces of *P. apterus* and D. *fasciatus*, quantitative PCRs targeting the six dominant bacterial strains within the insects' mid-gut revealed that the procedure is specific towards ridding firebugs from their Coriobacteriaceae symbionts in a manner that does not significantly affect the overall abundances of the remaining taxa (Chapter 4; Salem *et al*., 2013). Eggs that have been surface sterilized resulted in bugs that are aposymbiotic for *C. glomerans* as well as *Gordonibacter* sp. across both bug species (Salem *et al*., 2013). Therefore, the remaining core taxa may be transmitted either intracellularlly (during oogenesis or embryogenesis), or are also transmitted via the egg surface (but are resistant to the sterilization procedure).

This was further supported through comparative transcriptomic analysis examining the mid-guts of aposymbiotic and symbiotic firebugs (Chapter 7; Bauer *et al*., in preparation). Following a poly-A depletion treatment, the annotated bacterial transcripts revealed the taxonomic diversity of metabolically active bacterial species (Chapter 7; Bauer *et al*., in preparation) – one that is consistent with previous descriptions using culture independent techniques (454 pyrosequencing, quantitative PCR and cloning/sequencing) (Chapter 3; Sudakaran *et al*., 2012). Overall, the expression patterns between aposymbiotic and symbiotic bugs were highly similar, except in relation to transcripts ascribed to the Coriobacteriaceae family (*C. glomerans* and *Gordonibacter sp*.). In contrast to the high expression patterns observed in symbiotic bugs, no Coriobacteriaceae transcripts were retrieved from the aposymbiotic treatment (Chapter 7; Bauer *et al*., in preparation).

Additionally, successful re-infection of previously sterilized eggs with the Coriobacteriaceae symbionts by spreading mid-gut suspensions over the egg surfaces (Salem *et al*., 2013) supported behavioral observations by Kaltenpoth and colleagues (2009) linking the active probing of the egg surface by newly hatched nymphs as an adaptation to initiate infection. This is consistent with similar findings by explicitly linking symbiont presence in the vicinity of eggs as a mediating factor in the aggregating behavior exhibited by the hatchlings of the plataspid stinkbug *Megacopta punctatissima* during the first few days following emergence (Hosokawa *et al*., 2008).

7.2.2 Effect of symbiont elimination on firebug survivorship and reproductive output

With respect to survivorship and lifetime reproductive output, Coriobacteriaceae presence plays a significant role for successful host development. As demonstrated across *P. apterus* and *D. fasciatus*, aposymbiotic individuals suffered higher mortality and slower development during the nymphal stages leading to adulthood (Chapter 4; Salem *et al*., 2013). Behavioral observations of symbiotic and aposymbiotic firebugs also revealed that symbiont elimination contributes to a regression in adult mating frequency. These observations correlate to the lack of ovipositing among adults of the aposymbiotic treatment, and their significantly lower reproductive output (measured in the number of eggs laid over a female's lifetime) when compared to the symbiotic treatment (Chapter 4; Salem *et al*., 2013). The ability to reinstitute the symbiotic condition, and thereby rescue developmental and reproductive potential of nymphs emanating from previously sterilized eggs by reapplying the microbial community over the egg surface demonstrates that the method of symbiont elimination was

not responsible for the adverse fitness effects associated with aposymbiosis (Chapter 4; Salem *et al*., 2013).

These results are consistent with prior findings, particularly among study systems detailing the importance and specificity of gut bacteria towards hemipterans of the pentatomoid superfamily (i.e. stinkbugs, shieldbugs, etc.). For example, among the stinkbug families Acanthosomatidae and Parastrachiidae, elimination of their respective egg-smeared, monoclonal communities of γ-proteobacterial gut symbionts through egg surface sterilization results in retarded growth, high mortality, and abnormal morphology; suggesting a significant role for the symbionts towards the host's development (Kikuchi *et al*., 2009; Hosokawa *et al*., 2012). Similarly, for plataspid stinkbugs (*Megacopta punctatissima* and *Megacopta cribraria*), the separation of newly laid eggs from the maternally provisioned, and highly derived, symbiont-bearing capsules also results in aposymbiotic bugs that exhibit lower survivorship, longer developmental times and paler colorations relative to their symbiotic counterparts (Hosokawa *et al*., 2006). However, functional characterization of the exact symbiotic contributions across these systems is currently lacking.

7.2.3 Specificity of the firebug-Coriobacteriaceae association

Among plataspid stinkbugs, the genotypic signature of the symbiont contributes significantly to the host's ecology, particularly as it relates to the range of plants that the insect can subsist on (Hosokawa *et al.* 2007 a and b). This aspect of the association offered a great deal of insight into the role of gut microbes towards defining the host's ecological niche.

In addition to the aforementioned fitness benefits associated with *Megacopta* species possessing their gut symbionts, a later study discovered that the γ-proteobacterial symbionts play an important role for the stinkbugs' host plant adaptation capacities in Japan. *M. punctatissima*, which is a pest species specializing on crop plants (e.g. soybean) was found to owe its pest status to the genotype of its symbionts (Hosokawa *et al.* 2007a). This was demonstrated when the symbionts of *M. punctatissima* and the legume-feeding stinkbug, *M. cribraria*, were exchanged across both species. While *M. cribraria* initially exhibited lower reproductive success when reared on a soybean-based diet with its native microbe, such trends were reversed once acquiring *M. punctatissima*'s symbiont (Hosokawa *et al*., 2007b), indicating that the genotype of the *Ishikawaella* symbionts contributes significantly towards host plant colonization success among plataspid stinkbugs.

Similarly, cross infection of firebugs with gut microbes emanating from heterospecific pyrrhocorids negatively affects the host's chances to survive until adulthood. In both *P. apterus* and *D. fasciatus*, cross-infected bugs suffered equally poor survivorship and developmental rates as the aposymbiotic treatment relative to the untreated control, as well as bugs re-infected with their native microbial community (Chapter 4; Salem e*t al.*, 2013). The high Coriobacteriaceae symbiont titres in the cross-infected treatments suggest that despite successful symbiont colonization, the fitness benefits conferred to the host are nonetheless governed by a high degree of specificity.

Additionally, host plant preferences did not have a discernable influence over the observed shortcomings in fitness since *D. fasciatus* (normally specializing on cotton seeds) performs equally poorly when infected with *P. apterus*'s microbes despite being reared on linden seeds (the preferred food source of *P. apterus*) (Chapter 4; Salem e*t al.*, 2013). The degree of relatedness of *M.*

punctatissima and *M. cribraria* may account for the lower degree of specificity observed by Hosokawa *et al.* (2007b), where symbionts were exchanged across hosts on an interspecies level as opposed to the intergeneric scale differentiating *D. fasciatus* and *P. apterus*.

7.3 SYMBIOTIC CONTRIBUTIONS OF GUT MICROBES IN INSECTS

The microbial gut communities of insects have been implicated in a range of beneficial functions towards their hosts, including nutritional supplementation, mediation of developmental processes, immunological priming, as well as pathogen and/or parasite resistance (Engel and Moran, 2013). In the following sections, I aim to summarize some of the better studied insect-microbe systems where the functional contributions of the gut bacteria have been elucidated, in an effort to integrate our findings within the research field of insect symbiosis.

7.3.1 Protection through association: heritable gut bacteria as mediators of colonization resistance against parasites and pathogens

Commensal or beneficial gut bacteria can contribute significantly towards increasing host resistance against parasite invasion. Such protection can be conferred through nutrient competition (Bartlett, 1979; Ivanov *et al*., 2009), niche occupation (Endt *et al*., 2010), antibiotic/toxin production (Vollaard and Clasener, 1994) and/or immune priming (Stecher and Hardt, 2011) against gut pathogens, in what is collectively referred to as colonization resistance.

For example, the resident microbiota of bumble bees has recently been shown to confer significant protective benefits against the widespread, but highly specialized trypanosomatid pathogen *Crithidia bombi* (Koch and Schmid-Hempel, 2011). Here, following the separation of young pupae from social contact with nestmates, the bees developed to adulthood in the absence of their resident microbial community. Prior to exposure to *C. bombi*, these aposymbiotic bees were then provided either with feces from symbiotic conspecifics or with sterile sugar water. Bees that were fed feces from nest mates restablished a gut microbial profile similar to bees raised with contact to the colony. Additionally, individuals belonging to the re-infected treatment were found to harbor few *C. bombi* cells following exposure to the pathogen. Conversely, aposymbiotic bees only provisioned with sugar water were highly infected with the virulent gut parasite (Koch and Schmid-Hempel, 2011).

Similarly, using the desert locust as a model, Dillion and colleagues (2005) demonstrate that an inverse correlation exists between the complexity of the gut microbiota and the degree of pathogen infection rate and virulence. This is in line with theoretical predictions outlining that species-rich communities are more resistant to pathogen invasion, in part due to the greater competitive costs imposed on the pathogen for niche colonization and nutrition (Ivanov *et al*., 2009), in addition to increasing the immunological competence of the host through a range of priming mechanisms (Ayres and Schneider, 2009; Chambers *et al*., 2012). For the latter, the best-studied example comes from the tripartite association featuring mosquitoes (*Anopheles* spp.), their gut microbiota, and the protozoan parasite *Plasmodium falciparum* (Pumpuni *et al*., 1993; Gonzalez-Ceron *et al*., 2003; Cirimotich *et al*., 2011a). Following antibiotic treatment, germ-free *Anopheles gambiae* and *A. stephensis* were far more susceptible to *P. falciparum* than their symbiotic counterparts (Beier *et al*., 1994; Dong *et al*., 2009;

Meister *et al*., 2009). Concordantly, co-culturing *P. falciparum* with Gram-negative bacterial species isolated from the *A. stephensis*' gut further demonstrated the antagonistic effect of the microbiota towards the gut parasite. In addition to inhibiting *P. falciparum* growth, several antiplasmodial effector proteins were detected to be upregulated in symbiotic *Anopheles*, thereby suggesting that the bacteria can induce the immune response from the host to indirectly control the propagation of *P. falciparum* by spurring immune effector production (Cirimotich *et al*., 2011b).

7.3.2 Nutritional contributions by gut bacteria

Comprehensive characterization of beneficial bacterial contributions towards host nutrition has been conducted across a multitude of insect lineages, with roles ranging from the supplementation of essential nutrients (e.g. vitamins), aiding in the digestion of complex dietary compounds, or the detoxification of toxins associated with their intake of food (Douglas, 2009). These associations vary in complexity of bacterial taxa involved, integrative propensity linking host and symbiont(s), as well as long-term mutualistic stability.

Bacteria as detoxifying agents for insects

Herbivores often contend with the risk of ingesting plant secondary compounds that are potentially hazardous. How animals offset the deleterious effects of these compounds has been a central, driving question within the field of chemical ecology (Meinwald and Eisner, 2008). Traditionally, such protection has been demonstrated to involve the detoxification and/or sequestration of the toxic compounds via enzymatic reactions that are endogenous to the insect host. However, more recently, a study by Kikuchi and colleagues (2012) has implicated the gut microbial associates of bean bugs (*Riptorus pedestis*) as mediators of the detoxification process.

R. pedestis associates with environmentally-acquired bacterial symbionts belonging to the *Burkholderia* genus (Kikuchi *et al*., 2007). The environmental acquisition of these microbes requires the presence of *R. pedestis* in the proximity of infected soil, where a mere 80 bacterial cells in a gram of soil can initiate infection (Kikuchi and Yumoto, 2013). Recent findings have shown that agricultural fields that have been heavily treated with the insecticide fenitrothion selected for a greater proportion of *Burkholderia* strains that are capable of degrading the compound, compared to non-treated fields (Kikuchi *et al.,* 2012). Once introduced to insecticide-enriched soils, 90% of bean bugs successfully established symbiosis with the fenitrothion-degrading *Burkholderia* strains, and subsequent isolation of bacteria from the symbiotic organ of these insects showed significant fenitrothion-degrading activities (Kikuchi *et al.,* 2012). When reared on soybean seeds treated with fenitrothion, *R. pedestris* nymphs carrying insecticide-degrading *Burkholderia* exhibited significantly higher survival rates compared to bugs infected with the non-degrading strains (Kikuchi *et al.,* 2012).

Symbiotically-mediated nutrient provisioning

While diverse in their range of diets, insects are nonetheless uniform in their nutritional requirements (Dadd, 1985). Within the spectrum of herbivores, carnivores and generalist scavengers, the capacity to subsist on certain diet is often dependent on traits that are not endogenous to the insect, as has been

demonstrated through the range nutritional supplements provided by symbiotic microbes (Douglas, 2009).

For example, within the field of symbiosis research, sap-feeding insects and their intracellular endosymbionts serve as hallmark examples detailing how the provisioning of important nutrients by beneficial bacteria can enable the host to exploit oligotrophic niches (Baumann, 2005). The nutritional role of these microbes has been most carefully studied in the pea aphid (*Acyrthosiphon pisum*) and its primary intracellular symbiont *Buchnera aphidicola* (reviewed in Douglas, 2006). Here, substantial experimental and genomic evidence points towards the biosynthetic capacity of the bacterial partners in producing and supplying essential amino acids and some vitamins to the host, thereby offsetting the nutritional handicap associated with the aphid's monophagous subsistence on plant sap (which is highly deficient in these compounds) (Douglas, 2006). Intracellular endosymbionts have also been implicated in the nutritional upgrading of omnivorous insects subsisting on eutrophic diets. For example, among carpenter ants of the genus *Camponotus*, genomic and experimental studies have demonstrated a nutritional function for the insect's obligate endosymbiont *Blochmannia floridanus*, particularly as it relates to the supplementation of essential amino acids, as well as mediation of the nitrogen recycling capacities of the host (Feldhaar *et al*., 2007).

In addition to studies featuring nutritionally beneficial intracellular bacterial mutualists, extracellular gut microbes have also been found to contribute significantly towards host nutrition (Engel and Moran, 2013). For example, in their subsistence on nutritionally-poor, decaying plant materials, termites rely on specialized gut microorganisms to mediate the digestion of lignocelluloses, serving a rich source of carbon. Despite massive variation in complexity across the symbiotic associations within lower (wood-feeders) and higher (wood-, litter-, soil-, and grass-feeders) termites (Inward *et al*., 2007; Lo *et al*., 2007), great parallels have been drawn illustrating the convergent patterns in the evolution of mutualistic traits; specifically relating to symbiont-mediated digestion of lignocelluloses and subsequent production of acetate – a rich carbon source (Breznak and Switzer, 1986; Warnecke *et al.*, 2007).

While lignocellulose digestion in lower termites is accomplished primarily by protists belonging to the genera *Parabasalia* and *Preaxostyla* (Inoue *et al*., 1997; Nakashima *et al*. 2002), higher termites lack these protists and instead rely on the cellulolytic activity of bacteria within specific gut segments (Warnecke et al., 2007; Köhler *et al*., 2012). For the former, it has been shown that the protists enclose wood particles into vacuoles where the fermentation of cellulose to acetate presumably takes place, while for the latter, extensive cellulolytic activity was detected in the highly alkaline posterior proctodeal segments of the termite's hidgut (Tokuda *et al.*, 2005; Tokuda and Watanabe, 2007). Extensive metagenomic and proteomic analyses of these gut regions in higher termites further revealed a high abundance of bacterial genes and proteins involved in cellulose degradation (Warnecke *et al*., 2007; Burnum *et al*., 2010).

7.3.3 The Coriobacteriaceae as essential contributors to the B vitamin metabolism of firebugs

The ability to synthesize B vitamins, like essential amino acids, has been lost in animals. Instead, metazoans achieve complete nourishment by acquiring these vital compounds either directly from ingested food sources, or symbiotically through associations with bacterial or fungal partners (Dadd, 1985).

In firebugs, the high mortality exhibited by aposymbiotic individuals relative to the symbiotic treatment can be rescued by rearing the bugs on a nutritionally rich artificial diet (Chapter 5; Salem *et al*., submitted). However, upon omitting B vitamins from the diet, aposymbiotic mortality rates revert to the high levels (Chapter 5; Salem *et al*., submitted) observed when the bugs were reared on Malvales seeds (Chapter 4; Salem *et al*., 2013), strongly suggesting that the Coriobacteriaceae symbionts supplement these compounds to the host. The case for symbiont-mediated B vitamin supplementation in firebugs is further supported by the maintenance of many genes involved in the production of B vitamins in *C. glomerans*' recently sequenced genome (Stackebrandt *et al*., 2013), including pathways for the synthesis of thiamine (B1), flavin adenine dinucleotide (FAD) (B2), nicotinamide (B3), pantothenate (B5), and folic acid (B9) (Chapter 5; Salem *et al*., submitted).

Traditionally, studies outlining symbiont-mediated provisioning of B vitamins in animals have focused on hosts exhibiting a haematophagous feeding ecology, given that vertebrate blood is deficient in these nutrients. For example, a number of studies have demonstrated that bed bugs (*Cimex lectularius*) and tsetse flies (*Glossina spp*.) rely on B vitamin-supplementing intracellular symbionts for complete development and successful reproduction (Hosokawa *et al,* 2010; Nogge, 1976). In both systems, high mortality and low fecundity exhibited by aposymbiotic individuals can be reversed through the oral supplementation of B vitamins into their blood meal (Hosokawa *et al*., 2010; Nogge, 1976). In tsetse flies, subsequent genomic analysis of the bacterial symbiont *Wigglesworthia* further supported the expectation that the bacterium plays an important role towards upgrading the B vitamin metabolism of the host (Akman *et al*., 2002). Here, functional annotation of the bacterium's highly reduced genome (0.7Mb) revealed that despite the extensive gene loss affecting *Wigglesworthia*'s biosynthetic capacities, it nonetheless retained complete pathways for the synthesis of thiamine (B1), riboflavin and FAD (B2), nicotinamide (B3), pantothenate (B5), pyridoxine (B6), biotin (B7) and folate (B9) (Akman *et al*., 2002).

While descriptions of symbiont-mediated B vitamin supplementation have been comparatively scarce in herbivorous insects, Fraenkel and Blewett (1943) nonetheless demonstrate that anobiid beetles – which specialize on a range of seed-based diets – rely on the B vitamin-producing capabilities of their intracellular fungal partners since symbiont elimination only negatively affects beetles that were reared on artificial diets deficient in these compounds. Similarly, using *Drosophila melanogaster* as their model, Wong *et al*. (2014) demonstrate that the insect's highly capricious gut microbiota seemingly contributes towards sustaining the insect host on diets that are deficient in various B vitamins.

However, many of the aforementioned reports for symbiont-mediated B-vitamin supplementation have thus far been met with concerns due to the possible presence of trace amounts of vitamins in the "deficient" diets (but see Hosokawa *et al*., 2010), as well as the physiologically intrusive methods utilized for symbiont elimination (Douglas 2009). For the former, experiments conducted by Fraenkel and Blewett (1944) as well as Wong *et al*. (2014) contained trace amounts of B vitamins that may have been unequally distributed across experimental treatments. For the latter, detractors highlight that the foundational bioassays for symbiont-mediated B vitamin supplementation in tsetse flies are based entirely on experiments where the presence of *Wigglesworthia* has been manipulated following the administration of lysozyme (Nogge, 1976). Such criticism is in fact warranted given the nonspecific damage that lysozyme can inflict on the insect host (Douglas, 2009).

In our exploration of the contributions of the Coriobacteriaceae symbionts towards upgrading the B vitamin metabolism of firebugs, we believe to have circumvented these concerns empirically in two respects. First, the sterilization of egg surfaces is demonstrated as a "clean" method for symbiont manipulation, given that it does not induce nonspecific damage to the host's physiology (Chapter 4; Salem *et al*., 2013). Second, while it it's also applicable that our artificial diet contained trace amounts of B vitamins – given the inclusion of wheat germ as an ingredient – we nonetheless complemented our bioassays with transcriptomic analyses that specifically outline the host's metabolic response following symbiont elimination (Chapter 5; Salem *et al*., submitted). Here, aposymbiotic *D. fasciatus* reared on their natural diet of cottonseeds exhibited the differential up-regulation of genes involved in the import and processing of B vitamins, an expression pattern that has been previously described in animals exhibiting B vitamin starvation (e.g. Qiu *et al*., 2008; Rajgopal *et al*., 2001). While this expression pattern substantiates when aposymbionts were reared on an artificial diet where B vitamins are omitted, the inclusion of these compounds into the diet, or reinfection of firebugs with the actinobacterial symbionts restores normal expression of these genes, as well as host survivorship (Chapter 5; Salem *et al*., submitted). Collectively, these findings highlight a specific B vitamin starvation response in firebugs following symbiont elimination, one that can be averted in the presence of a B vitamin source – either symbiotically (through association with the Coriobacteriaceae symbionts), or artificially (through supplementation of B vitamins into the host's artificial diet).

7.3.4 Host immune response to nutritionally beneficial symbionts

Following a wealth of studies outlining the diversity in form and function of microbial gut associates in insects (Engel and Moran, 2013), it became increasingly clear that the immune system plays a central role towards facilitating the persistence of these complex communities (Lemaitre and Hoffman, 2007). A central question here, however, relates to the host's immunological ability to differentiate between beneficial or commensal gut bacteria and pathogenic microorganisms, specifically as we consider the production of effector proteins, for example, antimicrobial peptides (AMPs) (Lemaitre and Hoffman, 2007; Chambers and Schneider, 2012).

While AMP production during induced immune responses is traditionally considered a classical immunological resistance mechanism to parasites and pathogens, recent studies have demonstrated a range of feedback loops mediating host responses towards resident gut microorganisms (Lemaitre and Hoffman, 2007). AMP production can be induced through a range of signaling cascades, including the Toll, IMD and JAK/STAT pathways (Lemaitre *et al*., 1995; 1996). Characterization of the immune response in the gut of *D. melanogaster* revealed that the IMD is predominantly active in the gut (Chambers and Schneider, 2012) as shown through the binding of different variants of bacterial peptidoglycan (PGN) to a range of epithelial transporters belonging to the peptidoglycan recognition protein (PGRP) family. However, while native gut microbiota in flies can induce IMD signaling in the epithelial cells, the downstream expression of AMPs does not follow suite (Ryu *et al*., 2008). Inhibition of AMP production was found to be reliant on expression of the homeobox gene *caudal* that is responsive to PGNs from native microbes, as evidenced by the upregulation of AMP production when the gene is silenced. If the composition of the microbial community is altered through the introduction of foreign microbes, *caudal* expression is repressed and AMPs are expressed to restore a microbial community that is dominated by native species (Ryu *et al*., 2008). Similarly, immunological regulation in the gut of *D. melanogaster* can be dependent on the expression of other sensing

receptors, for example PGRP-LE (Bosco-Drayon *et al*., 2012). While PGRP-LE has been implicated in the induction of an immune response to potentially pathogenic microbes, it has also been shown to sustain immunological tolerance to the native microbiota by inducing the expression of a range of PGN-cleaving proteins that can selectively mask the overall abundance of bacteria in the gut, thereby repressing systematic immune responses induced by PGN originating from native gut microbes (Bosco-Drayon *et al*., 2012).

In firebugs, transcriptome sequencing of the mid-gut revealed a number of PGRPs putatively involved in the sensing of foreign microbes (Chapter 6; Bauer *et al*., in preparation), including a short-type PGRP that has been previously described in *D. melanogaster* to be implicated in the detection of gram-positive bacteria, and subsequent induction of immune pathways (Bischoff *et al*., 2006). Additionally, immune-related transcripts for all four signal cascades were recovered, including genes for the Toll, IMD and JAK/STAT pathways (Chapter 6; Bauer *et al*., in preparation). Of all pathways, the best characterized in our system was the Toll pathway, with annotated transcripts spanning the signal transduction cascade from precursor proteins to the transcription factors (Chapter 6; Bauer *et al*., in preparation). In contrast, the detected genes involved in the IMD pathway provided only a limited overview of this signaling cascade – likely due to the absence of many IMD genes in the pea aphid *Acyrthosiphon pisum* (Richards *et al*., 2010), the closest organism to the Pyrrhocoridae with a sequenced genome available, and thus, with reliable homology information. Across the four major pathways (Toll, Imd, JAK/STAT, phenoloxidase), limited expression and regulatory patterns could be detected for firebugs that were symbiotic or aposymbiotic for the Coriobacteriaceae symbionts (Chapter 6; Bauer *et al*., in preparation), consistent with previous studies highlighting the conserved constitutive expression patterns of immune signaling pathway genes (Hussain *et al.*, 2013).

Conversely, comparative analysis of the end products of the signal transduction pathways, the immune effector genes, revealed substantial and significant patterns of differential expression between symbiotic and aposymbiotic firebugs (Chapter 6; Bauer *et al*., in preparation), indicating that the host's control of the microbiota involve the end products of the immunological pathways. Here, hemiptericin was found to be significantly up-regulated in firebugs that were aposymbiotic for the Coriobacteriaceae symbionts relative to the symbiotic treatment. Hemiptericin has been previously described in *P. apterus* in response to exposure to foreign microorganisms (Cociancich *et al.*, 1994) and the observed up-regulation upon symbiont elimination might therefore represent an immunological means to control the proliferation of foreign bacterial strains, which might otherwise overreplicate in the ecological niche freed by *C. glomerans* and *Gordonibacter* spp. (Chapter 6; Bauer *et al*., in preparation).

On the other hand, the reverse expression pattern held true for c-type lysozyme (Chapter 6; Bauer *et al*., in preparation). Considering the nutritional relevance of the symbiotic Coriobacteriaceae to the B vitamin metabolism of firebugs (Chapter 5; Salem *et al*., submitted) up-regulation of c-type lysozyme may facilitate the release of B vitamins via lysis of the bacterial cells. This is consistent with previous findings in *D. melanogaster* where lysozyme activity in the midgut has been described as a pivotal step towards deriving important nutrients from the insect's microbial constituents (Daffre *et al*., 1994).

7.4 CONCLUDING REMARKS

Our findings highlight that firebugs possess a highly conserved microbiota that is dominated by a few taxa, including two Coriobacteriaceae species (*C. glomerans* and *Gordonibacter* spp.) that are transmitted vertically via the egg surface. The ecological and geographical stability of the microbiota further implied possible coevolution between host and microbe(s) and a functionally important role of the gut bacteria.

Elimination of the Coriobacteriaceae symbionts following egg surface sterilization resulted in slower growth rates and higher mortality in firebugs, and further bioassays indicated the gut microbes contribute towards host fitness through nutritional supplementation of B vitamins – an assertion further supported by *C. glomerans'* genomic potential for B vitamin synthesis.

Concordantly, comparative transcriptomic analyses of firebugs reared on their natural diet of cotton seeds reveal a differential up-regulation of genes involved in import and processing of B vitamins in aposymbiotic bugs; an expression pattern that is indicative of vitamin deficiency in animals. Our ability to restore normal expression patterns for these genes in firebugs either by reestablishing the symbiosis with the Coriobacteriaceae or through artificial supplementation of B vitamins into their artificial diet suggests that the nutritional contributions of gut symbionts significantly contributes towards maintaining the metabolic homeostasis of the insect host – not unlike highly derived mutualisms featuring integrated bacterial symbionts that are intracellularly housed in specialized structures (e.g. bacteriocytes).

Throughout this thesis, we aim to augment our understanding of how stable microbial gut communities in animals, specifically insects, are maintained and transferred from one host generation to another, in addition to detailing the functional importance of these associations, particularly as it relates to the nutritional upgrading of the insect host's metabolism. Collectively, we demonstrate that despite an extracellular localization, gut bacteria can nonetheless partake in highly integrated partnerships with their animal hosts.

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CHAPTER 8

SUMMARY

The microbial gut communities of insects have repeatedly been shown to significantly contribute towards the host's fitness through a range of beneficial functions that can include nutritional supplementation, mediation of developmental processes, immunological priming, as well as pathogen and/or parasite resistance. For this doctoral thesis, we investigated the stability, specificity and functional importance of bacterial gut associates of firebugs, primarily using *Pyrrhcoris apterus* and *Dysdercus fasciatus* as our model systems.

Comprehensive characterization of the symbiotic gut community of *P. apterus* and *D. fasciatus* revealed a consistent, core community that is largely dominated by facultative and obligate anaerobes including Actinobacteria *(Coriobacterium glomerans* and *Gordonibacter sp.*), Firmicutes (*Clostridium sp.* and *Lactococcus lactis),* and Proteobacteria (*Klebsiella sp.,* and a previously undescribed Rickettsiales bacterium). Examination of the microbiota across different life stages and populations of firebugs revealed that the community composition is qualitatively and quantitatively constant, with the six predominant taxa consistently abundant.

Towards elucidating the functional importance of the microbiota, we combined experimental manipulation with community-level analyses to ascribe the observed fitness effects to individual bacterial taxa of the insect's gut region. Elimination of symbionts by egg-surface sterilization resulted in significantly higher mortality and reduced growth rates in firebugs. Community-level analyses by quantitative PCRs targeting the dominant bacterial strains allowed us to tie the observed effects specifically to the absence of the two actinobacterial symbionts, *C. glomerans* and *Gordonibacter* sp.).

In order to detail the metabolic contributions of the actinobacterial symbionts in firebugs, we utilized a number of approaches, including transcriptomic and genomic analyses, as well as bioassays that take advantage of a manipulable artificial diet. Findings from artificial diet experiments demonstrate that the symbionts contribute towards their host through the provisioning of B-vitamins; a condition further supported by the discovery of complete pathways for the biosynthesis of thiamine (B1), riboflavin (B2), nicotinamide (B3), pantothenate (B5) and folic acid (B9) in the sequenced genome of *C. glomerans*. In agreement, comparative transcriptomic analyses revealed a differential up-regulation of genes involved in import and processing of B vitamins in aposymbiotic bugs; an expression pattern that is reflective of vitamin deficiency in animals. Our ability to restore normal expression patterns of these genes by either supplementing B vitamins into the insect's artificial diet, or through reinfection with the actinobacterial symbionts strongly support the proposed role of the actionbacterial symbionts towards upgrading the B vitamin metabolism of the firebug host.

Differential upregulation of immunological host genes – specifically c-type lysozyme – in symbiotic firebugs relative to the aposymbiotic treatment suggests that the bugs may serve to lyse symbiont cells and thereby harvest B-vitamins that are necessary for subsistence on the deficient diet of Malvales seeds. Such findings contribute to our knowledge of how the immune system is not purely utilized in response to parasites and pathogens, but instead can be harnessed to facilitate associations with beneficial microorganisms.

CHAPTER 9

ZUSAMMENFASSUNG

Der Verdauungsapparat vieler Insekten ist reich an mikrobiellen Gemeinschaften, deren positive Effekte auf die Fitness der Wirte vielfach gezeigt wurden. Unter anderem stellen diese Gemeinschaften den Insekten zusätzliche Nährstoffe zur Verfügung, beeinflussen Entwicklungsprozesse, stärken das Immunsystem durch Vorbereitung (‚priming') auf zukünftige Infektionen und können ihre Wirte resistent gegenüber Pathogenen und Parasiten machen. Im Rahmen dieses Ph.D.-Projektes wurden die Stabilität, die Spezifität, und die Funktion von symbiontischen Bakterien bei Feuerwanzen untersucht. Als Modellsysteme dienten dabei hauptsächlich die beiden Arten *Pyrrhcoris apterus* und *Dysdercus fasciatus*.

Unsere umfassenden Charakterisierungen zeigten eine konsistente Bakteriengemeinschaft im Verdauungssystems von *P. apterus* und *D. fasciatus,* die hauptsächlich von fakultativ und obligat anaeroben Actinobakterien *(Coriobacterium glomerans* und *Gordonibacter sp.*), Firmicutes (*Clostridium sp.* und *Lactococcus lactis)* und Proteobakterien (*Klebsiella sp.* und unbeschriebene Rickettsiales Bakterien) dominiert wird. Verschieden Populationen und Entwicklungsstadien unterscheiden sich dabei weder in der qualitativen noch in der quantitativen Zusammensetzung dieser sechs Taxa.

Um die Einflüsse einzelner Bakterientaxa auf die Fitness der Feuerwanzen zu untersuchen kombinierten wir Beschreibungen der Bakteriengemeinschaften des Verdauungssystems mit experimentellen Manipulationen. Die Elimination von Symbionten durch Oberflächensterilisation von Eiern führte zu geringeren Wachstumsraten und einer erhöhten Sterberate der Feuerwanzen. Diese Effekte sind auf die Eliminierung der zwei symbiontischen Actinobakterien *C. glomerans* und *Gordonibacter* sp, zurückzuführen, wie wir durch quantitative PCR Methoden zeigen konnten.

Wir verwendeten verschiedene Methoden – von Manipulationen des künstlichen Nährmediums bis zu Transkriptom- und Genom-Analysen – die es uns ermöglichten den Beitrag der symbiontischen Actinobakterien zum Metabolismus der Feuerwanzen zu entschlüsseln. Manipulationen des Nährmediums zeigten, dass die Symbionten ihre Wirte mit B-Vitaminen versorgen. Diese Ergebnisse konnten durch die Entschlüsselung der Stoffwechselwege für die Herstellung von Thiamin (B1), Riboflavin (B2), Nicotinamiden (B3), Pantothensäure (B5) and Folsäure (B9) im sequenzierten Genom von *C. glomerans* bestätigt werden. Vergleichende Analysen der Transkriptome zeigten ausserdem in von Symbionten befreiten Wanzen eine Hochregulierung von Genen die am Import und der Prozessierung von B-Vitaminen beteiligt sind, was einen Vitaminmangel anzeigt. Die normalen Expressionsmuster konnten entweder durch künstliche Vitaminzugaben oder durch Reinfektion mit den Actinobakterien wiederhergestellt werden, was unsere Annahme untermauert, dass die symbiontischen Bakterien den Feuerwanzen Vitamine bereitstellen.

Unterschiede in der Hochregulierung verschiedener am Immunsystem beteiligter Gene (besonders Gene für Lysozyme des C-Typus) bei Feuerwanzen mit Symbionten im Vergleich zu Feuerwanzen ohne Symbionten lassen vermuten, dass Wanzen ihre Symbionten verdauen und sich dadurch mit B-Vitaminen versorgen, die in deren Nahrung der Wanzen – den Samen von Malvengewächsen – fehlen. Diese Ergebnisse zeigen, dass das Immunsystem von Insekten nicht ausschliesslich auf Parasiten und

Pathogene Einfluss nimmt, sondern auch an Interaktionen mit mutualistischen Mikroorganismen beteiligt sein kann.

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Actinobacteria as essential symbionts for firebugs and cotton stainers (Hemiptera: Pyrrhocoridae).
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Schussler E, Rowland F, Distel C, Bauman J, Keppler M, Kawarasaki K, McCarthy M, Glover A and **Salem H** | 2011
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College Science Teaching. 40: 32-35

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Graduate research fellowship, Max Planck Society | 2010
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Host-associated Microbiota Workshop, Basel, Switzerland

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EIGENSTÄNDIGKEITSERKLÄRUNG

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

_____

Hassan Salem

Jena, den 13. Mai, 2014