Investigation of host-symbiont-parasite interactions using the African cotton stainer insect (*Dysdercus fasciatus*)

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by M.Sc. Thomas Ogao Onchuru

Born on 25.07.1986, Kenya

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Supervisors:

1)

2)

Date of oral examination:

"Although nature needs thousands or millions of years to create a new species, man needs only a few dozen years to destroy one." — Victor Scheffer

Summary

Symbiotic associations are ubiquitous in nature and can extend along a dynamic continuum from mutualism to parasitism. In mutualism, the interacting organisms benefit from each other while in parasitism, one partner benefits and the other is harmed. Here, I studied mutualism and parasitism in the African cotton stainer insect (*Dysdercus fasciatus*) mid-gut using a combination of molecular, microbiological, and microscopic techniques as well as experimental manipulation. For millions of years, this insect has maintained a mutualistic relationship with its gut bacterial community that is dominated by *Coriobacterium glomerans* and *Gordonibacter* sp. (Actinobacteria), *Hungatella* sp. and *Lactococcus lactis* (Firmicutes), and *Klebsiella* sp. (Proteobacteria) bacterial symbionts that supplement the insect's cotton seed diet with limiting B-vitamins. Additionally, their guts are often infested with *Leptomonas pyrrhocoris*, a generalized monoxenous trypanosomatid parasite, that co-localizes with the bacterial symbionts within the mid-gut. Because of their importance, the bacterial symbionts are transmitted to the offspring vertically (via egg surface smearing and probing) and horizontally (via coprophagic behavior). In this thesis, I investigated the risk associated with bacterial symbiont transmission, the role of gut bacterial symbionts in protecting the host against *L. pyrrhocoris* infections, and molecular interactions between the bacterial symbionts and the insect's immunity. I revealed that transmission of the bacterial symbionts entails a significant risk of co-transmitting *L. pyrrhocoris* parasite that hitch-hikes on the symbiont transmission routes. Further, I showed that successful transmission of *L. pyrrhocoris* with the bacterial symbionts results in low parasite titre infections characterized by prolonged nymphal developmental times and a slight alteration of the gut microbiota composition. However, in the absence of the bacterial symbionts, the infections are characterized by significantly higher titers of this parasite which can invade the hemolymph resulting in an uncontrolled replication and ultimately the death of the host. Colonization of the insect's peritrophic matrix along the gut wall by symbiotic bacteria likely acts as a barrier blocking parasite attachment, multiplication and entry into the peritrophic matrix and hemolymph. Importantly, I showed that the gut bacterial symbionts remain insensitive to the cotton stainer antimicrobial immune effectors once established in the mid-guts of second and third instar nymphs. This suggests that close associations of beneficial bacterial symbionts with their hosts can result in the evolution of mechanisms ensuring that symbionts remain resistant to host immunological responses, which may be important for the evolutionary stability of beneficial animal-microbe symbiotic associations.

Zusammenfassung

Symbiotische Assoziationen sind in der Natur weit verbreitet und lassen sich entlang eines Kontinuums von Mutualismus bis Parasitismus einordnen. In Mutualismen profitieren beide Partner voneinander während in Parasitismen der eine Partners Nutzen auf Kosten des anderen aus der Interaktion zieht. In dieser Arbeit habe ich mutialistische und parasitische Interaktionen der im Mitteldarm der Afrikanischen Baumwollwanze (*Dystercus fasciatus*) unter Nutzung molekularer, mikrobiologischer und mikroskopischer Methoden sowie experimenteller Manipulation des Systems untersucht. Feuerwanzen gehen seit Millionen von Jahren eine mutualistische Beziehung mit einer spezifischen, bakteriellen Darmgemeinschaft ein, die von *Corriobakterium glomerans*, und *Gordonibacter sp.* (Aktinobakterien), *Hungatella sp.* und *Lactococcus lactis* (Firmicutes) sowie *Klebsiella sp.* (Proteobacteria) dominiert ist. Diese Symbionten unterstützen die Ernährung des Wirtes durch die Bereitstellung von B-Vitaminen, die in ihrer Nahrung limitiert sind. Des Weiteren ist der Mitteldarm von Feuerwanzen, der die symbiotischen Bekterien enthält, auch oft von *Leptomonas pyrrhocoris* besiedelt, einem hoch spezialisierten, parasitischen Trypanosomen. Durch ihre hohe Bedeutung können die Symbionten sowohl vertikal (über die Infektion der Eioberflache mit Kot und belecken durch Nymphen) als auch horizontal (via Koprophagie) zwischen Individuen weitergegeben. Ich habe speziell untersucht, ob der Transmissionsweg der Symbionten auch ein Risiko der Weitergabe des Parasiten darstellt und welche Rolle die Symbionten sowie das Immunsystem des Wirtes für den Schutz vor den Parasiten spielen. Ich konnte zeigen, dass die Symbiontenweitergabe in der Tat ein Risiko darstellt, da *L. pyrrocorhis* auf beide Weisen mit weitergegeben werden kann. Des Weiteren konnte ich zeigen, dass die erfolgreiche Co-Infektion des Darmes mit den Symbionten nur in einem niedrigen *L. pyrrhocoris* Titer resultiert, der allerdings die Entwicklung der Tiere verzögert und einen schwachen Einfluss auf die Zusammensetzung der Darmgemeinschaft hat. In Abwesenheit der bakteriellen Symbionten zeichnet sich die Infektion durch einen wesentlich höheren Titer der Trypanosomen aus und sie können sogar vom Darm in die Hämolymphe einwandern, was zu einer ungeregelten Vermehrung und letztendlich dem Tod des Wirts führt. Die Besiedlung der peritrophischen Membran entlang des Darmepithels durch die mutualistischen Bakterien stellt wahrscheinlich eine Barriere dar, die verhindert, dass sich *L. pyrrhocoris* an die Darmwand anheften und durch sie in die Hämolymphe eindringen kann. Ich konnte außerdem zeigen, dass die symbiontischen Darmbakterien selbst nicht von Effektoren des Immunsystems der Wanzen beeinflusst werden, nachdem sie sich in dem zweiten bis dritten Larvenstadium etabliert haben. Die enge Beziehung zwischen den nützlichen Symbionten und ihrem Wirt führte in diesem Fall zur Evolution von Mechanismen, die die Symbionten vor Reaktionen des Immunsystems des Wirtes schützen. Solche Mechanismen können generell eine wichtige Grundlage für die evolutionäre Stabilität von mutualistischen Symbiosen zwischen Tieren und Mikroorganismen darstellen.

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List of publications and author contribution

Chapter two has been published in *Current Opinion in Insect Science*

Onchuru TO, Martinez AJ, Ingham CS, Kaltenpoth M. 2018. Transmission of mutualistic bacteria in social and gregarious insects. Current Opinion in Insect Science 28:50–58.

> *Author contribution*: T.O.O, M.J.A, I.C.S and M.K. did literature review and wrote the manuscript. T.O.O. compiled and edited the manuscript.

Chapter three has been published in *Biology Letters* (*= shared first authorship)

Salem H*, Onchuru TO* Bauer E and Kaltenpoth M. 2015. Symbiont transmission entails the risk of parasite infection. Biology Letters, *11*(12), 20150840.

> *Author contribution*: H.S., T.O.O. and M.K. conceived of the study. T.O.O. and H.S. performed the experiments. H.S. analyzed the data. E.B. performed the transcriptomic analysis. H.S. drafted the manuscript, and all authors reviewed the manuscript.

Chapter four has been published in *Molecular Ecology*

Onchuru TO, Martinez AJ, Kaltenpoth M. 2018. The cotton stainer's gut microbiota suppresses infection of a cotransmitted trypanosomatid parasite. Molecular Ecology 27(16):3408–3419.

> *Author contribution:* T.O.O. and M.K. designed the study. T.O.O. performed the experiments, T.O.O. and A.M. analyzed the data. T.O.O. wrote the manuscript, which M.K. and A.M. reviewed.

Chapter five has been published in *Applied and Environmental Microbiology*

Onchuru TO, and Kaltenpoth M. 2018. Established cotton stainer gut bacterial mutualists evade regulation by host antimicrobial peptides. Applied Environmental Microbiology 85:e00738-19

> *Author contribution:* T.O.O. and M.K. designed the study. T.O.O. performed the experiments, analyzed the data and wrote the manuscript, which M.K. reviewed.

Chapter six has been accepted in *Ecology and Evolution*

Onchuru TO, Kaltenpoth M. 2018. Quantitative PCR primer design affects quantification of dsRNA-mediated gene knockdown. Ecology and Evolution (In print)

> *Author contribution:* T.O.O. and M.K. designed the study. T.O.O. performed the experiments, analyzed the data and wrote the manuscript, which M.K. reviewed.

CHAPTER 1

GENERAL INTRODUCTION

This thesis investigates multipartite interactions between *Dysdercus fasciatus* (Hemiptera: Pyrrhocoridae), commonly referred to as the African cotton stainer bug, its gut bacterial community, and its gut trypanosomatid parasite, *Leptomonas pyrrhocoris* (Kinetoplastida: Trypanosomatidae). More importantly, the consequences of these interactions on the ecology of the insect host are inferred.

1.1. Symbiosis

All organisms live in close and constant interaction with other species. The close and long-term co-existence of two or more species is referred to as symbiosis (Sapp, 1994). The origin of the eukaryotic cell, which makes up multicellular organisms, was as a result of ancient prokaryotic endosymbiotic events (Archibald, 2015; Margulis & Chapman, 1998; Sapp, 1994). Recent studies have demonstrated that the emergence of novel phenotypes and species diversity on earth is partially attributed to symbiotic associations between multicellular organisms and microbes (Douglas, 2015).

Symbiotic associations extend along a dynamic continuum from mutualism, where both partners benefit, to parasitism, where one partner benefits and the other is harmed (Figure 1) (Sapp, 1994). It is common to find mutualistic partners in phylogenetic clades that are strictly parasitic and vice versa, though rare, suggesting recurrent transitions between mutualism and parasitism depending on prevailing conditions (Matsuura et al., 2018; Moran & Wernegreen, 2000). In the past, parasitic interactions amongst organisms have received considerable attention because of the obvious detrimental effects such as disease that these interactions can have on hosts (Levine, 1961). However, recent advances in symbiosis research have led to the appreciation of the positive contribution of symbionts to the ecology, biology, and evolution of eukaryotic organisms (Douglas, 2010, 2015). On the forefront of this research is insect symbiosis, which has immensely contributed to our current understanding of animal-microbe interactions (Douglas, 2014; Engel & Moran, 2013). This success can be attributed to the high diversity of insect-microbe interactions in addition to the experimental tractability and cost-effectiveness of using insects in research.

Figure 1. Outcomes of symbiotic interactions. Symbiosis extends along a continuum from mutualism to parasitism (+ represents benefits, - represents harm)

1.2. Insect symbiosis

Insects are the most diverse group of animals on earth as they represent more than 50% of all described animal species and play a critical role in the functioning of various ecosystems (Grimaldi & Engel, 2005; Schowalter, 2011). This can be attributed to a combination of adaptations that enable insects to successfully adjust to and exploit different ecological niches. Morphological features such as a hard exoskeleton, also referred to as the cuticle, protect insects against predation, desiccation, chemical and mechanical damage, and pathogenic infections, while wings facilitate fast and long distance movement in search of resources or escape from predation and harsh conditions (Schowalter, 2011). Insects also form ecological interactions with other organisms such as flowering plants, insects, and microorganisms that facilitate adaptation and diversification (Buchner, 1965; Douglas, 2015; Grimaldi et al., 2005).

Insects inhabit a wide array of ecosystems ranging from terrestrial to aquatic, tropical to temperate, and rain forests to deserts (Schowalter, 2011). These habitats are known to be rich in microbial composition. For instance, one gram of soil is known to contain up to 5.2×10^4 different 16S rRNA bacteria sequences which corresponds to unique bacteria species based on 100% sequence similarity with sequences in the Ribosomal Database Project (Roesch et al., 2007). Thus, the presence of a diverse array of microbes in ecological niches inhabited by insects implies that these organisms constantly encounter and most likely interact with each other. The recent exponential growth in insect symbiosis research has provided evidence that insect-microbe interactions are ubiquitous. Some of these partnerships are a major source of novelties that influence the ecology and foster evolution of insects (Douglas, 2015; Nalepa, 2015). Primarily, insect-associated microbes play a prominent role in the feeding ecology of insects by enabling them to overcome plant defensive barriers, facilitating digestion of recalcitrant plant polymers, and supplementing nutrient poor diets (Douglas, 2015; Engel et al., 2013; Salem et al., 2017; Schowalter, 2011). Some of the nutrients provided by symbionts are essential for insect development and physiology (Engel et al., 2013). In some Coleopterans, for example, the integrity of the exoskeleton, which protects them against desiccation or predation, depends on the presence or absence of associated bacterial symbionts that produce nutrient precursors necessary for cuticle synthesis (Engl et al., 2018; Vigneron et al., 2014).

Insect-associated symbionts are also known to protect their insect hosts against attacks by natural enemies such as viral and bacterial pathogens, parasites, and parasitoids (Douglas, 2015; Engel et al., 2013). Mechanisms employed by defensive symbionts to protect hosts include mechanical exclusion, competition for host resources (nutrients or colonization niche), chemical warfare, and priming or maturing the host immune systems to effectively defend against invaders (Engel et al., 2013). Additionally, symbionts enable insect hosts to adapt to extreme environmental conditions such as habitats experiencing high temperatures or low humidity (Brumin et al., 2011; Moran & Yun, 2015). Lastly, insect-associated bacterial symbionts act as a source of compounds that influence conspecifics' behaviors such as mate choice and sociality (Douglas, 2015). In chapter two of this thesis, I highlight novel traits conferred by bacterial symbionts in insects with appropriate examples.

1.3. Maintenance of insect-symbiont interactions

Insect symbionts are classified either as obligate or facultative. Obligate (primary) symbionts usually provide hosts with nutrients necessary for survival and are localized in specialized organelles. They are usually evolutionary ancient and are highly specialized to the extent that neither the host nor the symbiont can live without the other. A good example of this symbiosis is that of *Buchnera aphidicola* bacterial endosymbiont, which provisions its aphid host with essential amino acids that it cannot

synthesize nor obtain from its plant sap diets (Douglas, 1998). Facultative (secondary) symbionts on the other hand are not strictly necessary for host survival but enhance host fitness under specific conditions. For example, the secondary bacterial symbionts of the pea aphid confer resistance against parasitoid and fungi attacks (Oliver et al., 2003; Scarborough et al., 2005). These symbionts are not necessarily localized in special symbiont structures like their obligate counterparts thus can often be found colonizing other host tissues such as salivary glands, malpighian tubules, and hemolymph. Although the level of host-symbiont intimacy varies between symbionts, some factors such as symbiont colonization, regulation, and transmission are all important for the maintenance of both obligate and facultative symbiotic associations.

1.3.1. Insects as suitable niches for microbial colonization

Insects possess suitable extracellular and intracellular ecological niches that can be colonized by specific or transitional microbial communities (Douglas, 2015; Engel et al., 2013). These habitats can be categorized as general or specialized depending on the specificity of microbial communities that colonize them. As the name suggests, general habitats refer to those that get colonized by a broad-spectrum of microbes ranging from transient to host-associated microbial communities. These habitats are mostly in direct contact with the external environment and thus more accessible and available to a variety of microbes. They include the cuticle and the gastrointestinal tract (Douglas, 2015; Engel et al., 2013). On the other hand, specialized habitats are frequently colonized by specific symbionts such as those that are tightly associated with the host. These include moderately specialized habitats such as the hemolymph, gut crypts, malpighian tubules, reproductive, and symbiont-housing organs as well as highly specialized habitats such as bacteriocytes (special host cells carrying symbiotic bacteria) which often aggregate to form bacteriomes (Douglas, 2015; Engel et al., 2013). Successful colonization of host-provisioned niches by specific symbionts largely depends on prerequisite host and symbiont adaptations, which influence selection, establishment, maintenance, and regulation of microbial communities that colonize these structures (Lanan et al., 2016; Pontes et al., 2011).

1.3.2. Regulation of insect-associated symbionts

Overproliferation of symbionts or disruption of the normal symbiont community by undesirable microbes can result in the disturbance of symbiotic homeostasis leading to an overall reduction of host and symbiont fitness and eventual breakdown of symbiotic associations. Various mechanisms are known to be responsible for regulating symbionts thus ensuring minimal burden on hosts and symbiont disruption. Processes such as peristalsis, ecdysis, and self or social grooming can result in physical control of microbes in digestive tracts and cuticles (Douglas, 2015). Additionally, insect guts and trachea are lined with matrices, membranes, or filters that select and structure microbial communities that successfully colonize hosts thus ensuring that unwanted symbionts are kept out (Douglas, 2015; Lanan et al., 2016). Only specialized symbionts possessing appropriate virulence factors capable of breaching these mechanical barriers can establish in the matrixes and membranes or advance to specific niches such as the hemolymph, bacteriomes, crypts, or symbiotic organs that require the barriers to be bypassed first (Dale et al., 2001; Pontes et al., 2011).

Physicochemical conditions such as pH, oxygen concentrations, and microbicidal reactive oxygen species (ROS) in insect gut lumens select for and support the growth of specific symbionts rather than transient microbes (Douglas, 2015; Engel et al., 2013). Furthermore, diet and host nutritional status have an impact on the structure and abundance of symbionts (Douglas, 2015; Herren et al., 2014; Wilkinson et al., 2007). In advanced symbiotic relationships, bacteriocytes, bacteriomes, and symbiotic organelles

physically separate and protect symbionts from external disruptions that may be caused by opportunistic microbes and the host immune system, act as a controlled zone for regulating symbiont proliferation, and prevent symbionts from escaping to other host tissues (Login et al., 2011).

In addition to physical separation, insects such as weevils and stink bugs activate localized immune responses in symbiont housing structures to control and regulate symbiont establishment and proliferation (Kim et al., 2014; Login et al., 2011). However, in other mutualistic interactions, host immunity is not actively involved in symbiont control (Kwong et al., 2017). Long-term host-symbiont co-adaptations in these associations have resulted in the evolution of host immune systems to tolerate the presence of symbionts or in the modification of the symbionts' microbe-associated molecular patterns (MAMPs) so as to evade host immune responses or enhance the ability of the symbionts to infect hosts (Douglas et al., 2011; Pontes et al., 2011). The pea aphid, *Acyrthosiphon pisum,* for example, lacks the peptidoglycan recognition proteins (PGRPs) and has an incomplete immune deficiency (IMD) signaling pathway that prevents it from recognizing and mounting an effective immune response against its nutritionally essential *Buchnera aphidicola* endosymbiont (Douglas et al., 2011), thereby allowing successful establishment and maintenance of this endosymbiont.

Microbe-microbe interactions can also influence the composition of the microbial community that ultimately colonizes a host. Synergistic interactions ranging from biofilm formations to metabolic cooperation facilitate successful establishment of the normal gut microbiota of insects such as the honey bee, *Apis mellifera* (Engel et al., 2012; Kešnerová et al., 2017). On the other hand, antagonistic interactions including competition, production of antagonistic metabolites, or host immune system priming can deter the establishment or proliferation of other microbes, thus acting as a mechanism of symbiont regulation and host protection (Kwong et al., 2017).

1.3.3. Transmission of insect symbionts

In order to maintain stability of symbiotic relationships, insect-associated symbionts must be transmitted to and acquired by subsequent generations. Consequently, many hosts rely on vertical and/or horizontal transmission mechanisms to transfer or acquire essential symbionts. In vertical transmission, obligate and in some cases facultative symbionts are transferred directly from parents to the progeny via the germ line, jelly or capsule formations, egg surface smearing, parental care, or specialized secretions containing the symbionts (Onchuru et al., 2018; Salem, Florez, et al., 2015). Transmission bottlenecks can occur when a few individuals of a symbiont that is tightly associated with its host, are passed directly to the next generation. Additionally, living in a nutrient rich and safe environment of the host, which offers limited opportunities for horizontal gene transfer, often result in the loss of redundant genes (Bright & Bulgheresi, 2010). Extreme cases of genome erosion coupled with transmission bottlenecks can lead to the breakdown of symbiotic interactions and/or symbiont replacements (Matsuura et al., 2018)

Horizontal transmission involves acquisition of symbionts anew by subsequent host generations from a free-living symbiont population or from con- or heterospecific organisms (Onchuru et al., 2018; Salem, Florez, et al., 2015). Unlike in vertically transmitted symbionts, horizontally transmitted symbionts commonly maintain stable genomes which allow survival outside their hosts. In addition, they show little or no evidence for co-speciation with their hosts suggesting frequent host switching or multiple origins of symbiotic associations (Bright et al., 2010). Mixed symbiont transmission modes also occur whereby vertical transmission is complemented by horizontal acquisitions from the environment or con/heterospecifics, which increases chances and reliability of symbiont acquisition when one mode of

transmission fails (Bright et al., 2010; Ebert, 2013). Whereas vertical symbiont transmission mechanisms are highly specific and reliable in transmitting desired symbionts only, some horizontal transmission modes often lack elaborate symbiont screening mechanisms making them largely non-specific and thus can be associated with increased risks of transmitting unwanted symbionts (Onchuru et al., 2018).

1.4. Insect trypanosomatid parasites

The gastrointestinal tracts of many species across insect taxa are favorable ecological niches for the development of protozoan parasites, such as those of the Trypanosomatidae family (Maslov et al., 2013). Depending on their life cycle, Trypanosomatidae parasites are either classified as monoxenous or dixenous. Monoxenous trypanosomatids complete their entire lifecycle in invertebrate hosts and they include *Angomonas*, *Blastocrithidia*, *Blechomonas, Crithidia*, *Herpetomonas, Kentomonas, Leptomonas*, *Lotmaria*, *Novymonas, Paratrypanosoma, Sergeia, Strigomonas, Wallaceina,* and *Zelania* genera (Kaufer et al., 2017). Dixenous trypanosomatids on the other hand are pathogens of animals or plants and require invertebrate vectors for transmission and completion of their lifecycle. They include members of the *Endotrypanum, Leishmania*, *Phytomonas*, *Porcisia,* and *Trypanosoma* genera (Kaufer et al., 2017). Dixenous trypanosomatids, especially those of *Trypanosoma* and *Leishmania* genera infect humans and livestock making them medically and economically important. As a result, these parasites have received considerable research attention unlike their monoxenous counterparts, which have been neglected for a long time. However, the description of endosymbiotic associations in some monoxenous genera (*Angomonas, Kentomonas, Novymonas,* and *Strigomonas*) with intracytoplasmic bacteria, some of which are recent associations (Kostygov et al., 2016; Votýpka et al., 2014), has made monoxenous trypanosomatids attractive models for studying the origin of endosymbiosis. Additionally, their close phylogenetic distance to dixenous trypanosomatids makes them ideal candidates for studying evolution of dixeny in trypanosomatids (Flegontov et al., 2016; Kraeva et al., 2015; Lukeš et al., 2014).

1.5. The cotton stainer model system for studying insect-microbe interactions

The African cotton stainer (*Dysdercus fasciatus*) belongs to the heteropteran sub-order of insects, also referred to as true bugs, that is classified under the Hemiptera insect order. It belongs to the Pyrrhocoridae family which has more than 400 described species that mostly inhabit tropical and sub-tropical climates with a few exceptions e.g. *Pyrrhocoris* sp. that are found in temperate climates (Socha, 1993). Bugs of this family are characterized by warning colorations, gregarious behavior, and adaptation to feeding on seeds of Malvales plants (Malvaceae, Tiliaceae, Bombacaceae, and Sterculiaceae) with occasional exploitation of non-Malvales plants (Schaefer & Panizzi, 2000; Socha, 1993). The African cotton stainer feeds on seed bolls of the cotton plant (*Gossypium hirsutum*) resulting in direct damages through mechanical destruction of the developing seeds and indirect damages through the transmission of bacteria and fungi such as *Nematospora gossypii*, the causative agents of internal boll disease (Schaefer et al., 2000). Because of these damages, the African cotton stainer is considered an important economic and agricultural pest (Schaefer et al., 2000).

Most pyrrhocorid bugs are easy to rear and maintain in the laboratory, which has facilitated their extensive use as experimental models for biological research. As early as in the 1960s, *P. apterus* was widely used in the study of insect morphology, physiology, endocrinology, developmental, and reproductive biology among other research areas (Schaefer et al., 2000; Socha, 1993). Its extensive use in experimental research led to the discovery of the 'paper factor' i.e., the juvenile hormone analog in certain papers that inhibits normal development of *P. apterus* nymphs (Slama & Williams, 1965, 1966).

In recent years, *P. apterus* and the closely related species, *D. fasciatus,* (both Pyrrhocoridae) as well as *Riptortus pedestris* (Alydidae) have been widely used to study function, transmission, and regulation of gut-associated microbes (Kaltenpoth et al., 2009; Kikuchi et al., 2012; Kim et al., 2015; Salem et al., 2014, 2013). Regardless of their geographical location and preferred host plant, many members of the Pyrrhocoridae family are characterized by a consistent core gut bacterial community suggesting acquisition from a common ancestor (Figure 2) (Sudakaran et al., 2015). The time of acquisition of these bacterial symbionts by pyrrhocorids coincides with the origin of Malvales plants signifying that symbiont acquisition may have facilitated diversification of bugs into exploiting Malvales plant seed diets (Figure 2b) (Sudakaran et al., 2015). The core gut bacterial community of *Dysdercus* sp. and closely related *Pyrrhocoris* and *Scanthius* genera is highly similar and is mainly comprised of *Hungatella* sp., *Klebsiella* sp., *Coriobacterium glomerans*, and *Gordonibacter* sp., bacterial symbionts (Figure 2b) (Salem et al., 2013; Sudakaran et al., 2012). These gastrointestinal tract bacterial symbionts supplement hosts with B-vitamins that are missing in their Malvales plant seed diets (Salem et al., 2014, 2013).

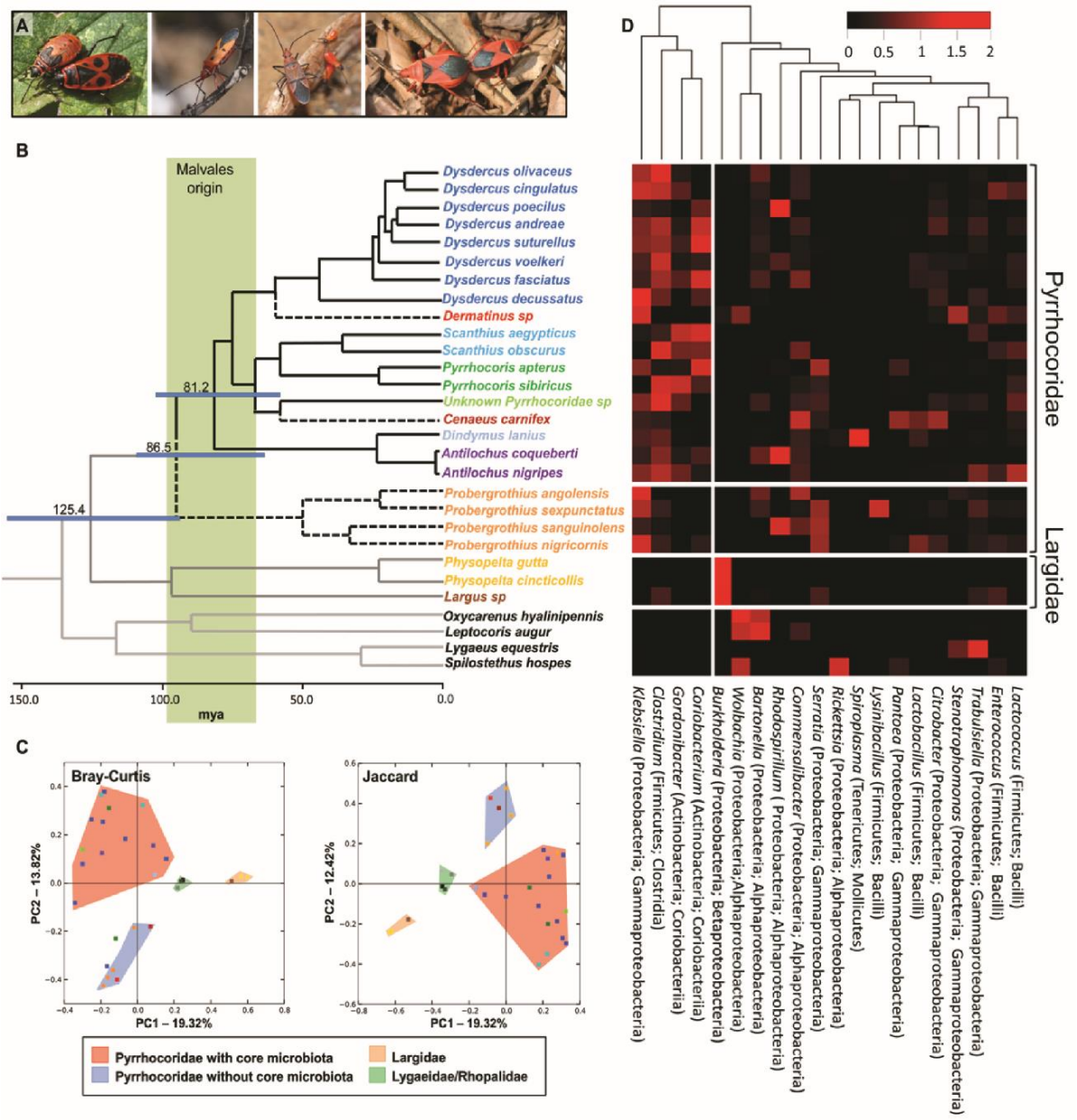

Figure 2. Gut bacterial community composition in Pyrrhocoridae family. Selected Pyrrhocoridae host species i.e. *P. apterus* (adult and nymph)*, Dysdercus singulatus, D. fasciatus* (adult and nymph)*, and Probergrothius sanguinolens,* respectively (A), dated host plant phylogeny showing origin of Malvales plants (B), 2D principal coordinate analysis showing clustering of gut microbial community of different Pyrrhocoridae species (C), relative abundance of microbial taxa as obtained from 454 pyrosequencing of 16S rRNA amplicons (D) (Sudakaran et al., 2015).

The African cotton stainer *D. fasciatus* is frequently parasitized by *Leptomonas pyrrhocoris*, a generalist gut trypanosomatid parasite that co-localizes with the insect's gut bacterial symbionts in the M3 region of the mid-gut where they are mildly virulent. When *L. pyrrhocoris* successfully invades the host hemolymph by breaching the gut epithelial walls, it results in heavily infected individuals that are characterized by sluggish motility, reduced starvation resistance, pale cuticle composition, and increased developmental time (Schaub, 1994). Despite the high prevalence of *L. pyrrhocoris* infections reported in the intestinal tracts of *D. fasciatus* insects sampled from natural populations (Votýpka et al., 2012), many individuals with the parasite in the gut hardly exhibit signs and symptoms of hemolymph invasion, suggesting that *L. pyrrhocoris* is often confined in the insect's intestinal tracts. How hosts and gut bacterial symbionts contribute towards the observed resistance remains unclear. The tight and long-term association of the African cotton stainer insect with a well characterized gut bacterial community and a trypanosomatid parasite coupled with the insect's amenability to experimental manipulations makes it an ideal model for investigating host-symbiont-parasite interactions as I report in this thesis.

1.6. Thesis outline

This thesis aimed to (i) identify the risk involved in the maintenance and transmission of mutualistic symbionts, (ii) investigate the contribution of the gut bacterial community towards resistance of trypanosomatid parasite infections, and (iii) assess the role of host antimicrobial peptides in the regulation of the core gut bacterial symbionts in the African cotton stainer.

In chapter one, I summarize the current understanding of insect symbiosis including novel abilities conferred by insect-associated microbes and how colonization, symbiont regulation, and transmission are essential in the maintenance of symbiotic relationships. I also introduce the African cotton stainer insect as an ideal model system for studying multipartite interactions in insects.

I review in chapter two how social behaviors exhibited by social, sub-social, and gregarious insects facilitate maintenance and transmission of essential bacterial symbionts to offspring and conspecifics. Furthermore, I highlight costs associated with the social transfer of bacterial symbionts and potential implications of this behavior for the evolution of eusociality.

In chapter three, I report on the risk associated with transmission and maintenance of beneficial gut bacterial symbionts in *D. fasciatus*. Firebugs transmit gut bacterial symbionts vertically (via egg surface smearing and probing) and horizontally (via coprophagic behavior) (Kaltenpoth et al., 2009; Salem, Onchuru, et al., 2015). This chapter explores whether these gut bacterial symbiont transmission routes are hijacked by the *L. pyrrhocoris* trypanosomatid for its own transmission.

In chapter four, I investigate the role of the core gut bacterial symbionts in conferring host protection against *L. pyrrhocoris* infections and the mechanism involved in the protection. Additionally, I examine the effect of parasitism on survival and development of the African cotton stainer insect.

The African cotton stainer's gut bacterial symbionts elicit strong host immunological responses characterized by the overexpression of antimicrobials (Bauer et al., 2014). In chapter five, I assess the significance of these immune responses in the regulation of the gut bacterial symbionts by knocking down the expression of key host antimicrobial genes through RNA interference and measuring the effect of this knockdown on host fitness and quantitative composition of the symbiotic gut community.

RNAi technology is an important tool for studying gene functions as used in this thesis to investigate effects of host immune responses on the cotton stainer's gut bacterial composition. RT-qPCR is commonly used to measure the level of gene expression, thus applicable in evaluating gene expression changes following RNAi. In chapter six, I describe the importance of RT-qPCR primer binding position on the target mRNA sequence and the dsRNA used for silencing in the quantification of target gene knockdown.

I conclude in chapter seven by discussing the findings of this thesis in the context of existing knowledge on multipartite interactions in insects. Specifically, I focus on costs and benefits associated with defensive symbionts and how protective symbionts influence host and pathogen evolution. Lastly, I highlight the future research perspectives of multipartite interactions.

1.7. References

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CHAPTER 2

TRANSMISSION OF MUTUALISTIC BACTERIA IN SOCIAL AND GREGARIOUS INSECTS

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Thomas Ogao Onchuru[1], Adam Martinez[1], Chantal Selina Ingham[1], Martin Kaltenpoth[1],*

[1] *Department of Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg-University of Mainz, Johann-Joachim-Becher-Weg 13, 55128 Mainz, Germany*

2.1. Abstract

Symbiotic microbes can confer a range of benefits to social, sub-social, and gregarious insects that include contributions to nutrition, digestion, and defense. Transmission of beneficial symbionts to the next generation in these insects sometimes occurs transovarially as in many solitary insects, but primarily through social contact such as coprophagy in gregarious taxa, and trophallaxis in eusocial insects. While these behaviors benefit reliable transmission of multi-microbial assemblages, they may also come at the cost of inviting the spread of parasites and pathogens. Nonetheless, the overall benefit of social symbiont transmission may be one of several important factors that reinforce the evolution of social behaviors and insect eusociality.

2.2. Highlights

- Mutualistic bacteria confer functional traits to many social and gregarious insects.
- Group living and social behavior shape host microbial communities.
- Social transmission of symbionts entails costs and benefits.
- Social symbiont transfer may reinforce the evolution and maintenance of sociality.

2.3. Symbiont-conferred functional benefits in social and gregarious insects

Many insects engage in mutualistic interactions with bacteria that confer novel traits to their hosts, enabling them to utilize a wide range of previously inaccessible resources or colonize new habitats (Douglas, 2015). In many instances, these partnerships have become so intimate that the partners cannot

survive without each other (Moran et al., 2008). The functional importance of mutualistic bacteria in social and gregarious insects has gained much attention over the past few decades, as they frequently play important roles in host nutritional ecology. In this review, we briefly summarize known functions of mutualistic bacteria in social insects and then focus on their transmission routes from one generation to the next, discussing the possible costs and benefits of social symbiont transfer and its implications for the evolution of social behaviors. As the social transmission of microorganisms between conspecifics occurs from gregarious all the way to eusocial taxa, we will broadly cover bacterial mutualisms within insects across all levels of sociality.

In social and gregarious insects, symbionts have been described or implied to be involved in the biosynthesis of nutrients in bees and bugs (Ben-Yakir, 1987; Kwong & Moran, 2016; Lake & Friend, 1968; Salem et al., 2014), pectin and lignocellulose degradation in bees, termites and wood roaches (Bonilla-Rosso & Engel, 2018; Douglas, 2015; Kwong et al., 2016), and carbohydrate metabolism in ants and bees (Bonilla-Rosso et al., 2018; Zientz et al., 2005). In addition, several bacterial symbionts have putative roles in host nitrogen metabolism (Brune & Dietrich, 2015; Douglas, 2015; Sabree et al., 2009; Zientz et al., 2005). In termites, the hindgut community is essential for nitrogen fixation, recycling and upgrading, mitigating the low nitrogen content of their cellulose-based diet (Brune et al., 2015; Ohkuma, 2008). In ants, putative roles include recycling nitrogenous waste to essential amino acids in *Camponotus* and *Cephalotes* (Douglas, 2015; Hu et al., 2018), nitrogen fixation in certain *Tetraponera* species (Zientz et al., 2005), and providing a tyrosine precursor for cuticle formation in *Cardiocondyla obscurior* (Klein et al., 2016). In fact, symbiotic microbes may be one factor explaining why herbivorous ants can successfully exploit nitrogen-poor arboreal habitats (Russell et al., 2009; Zientz et al., 2005).

Apart from influencing host nutrition, symbionts present an important component of the defensive arsenal in social and gregarious insects, providing protection to host individuals, their food sources and/or nesting environment against pathogens, parasites, and parasitoids (Flórez et al., 2015). In the cockroach *Cryptocercus punctulatus*, the application of feces to the nest plays a putative role in fungal defense. Antifungal compounds in the feces — potentially of microbial origin — may sanitize the nest, preventing growth of antagonistic fungi (Rosengaus et al., 2013). Similarly, in the termite *Zootermopsis angusticollis*, there is evidence that the hindgut microbiota synthesizes multiple functionally active β-1, 3-glucanases with a putative role in fungal pathogen defense (Rosengaus et al., 2014). Recent studies in bees (*Apis mellifera* and *Bombus terrestris*) revealed that individuals with a native, undisturbed gut microbial community were less susceptible to *Lotmaria passim* and *Crithidia bombi* trypanosomatid parasites, respectively, likely due to competitive exclusion of the parasites by bacterial gut symbionts (Koch & Schmid-Hempel, 2011; Schwarz et al., 2016). Other studies observed an effect of the whole gut microbiota and individual bacterial symbionts on the host immune system (Emery et al., 2017; Kwong et al., 2017). Kwong et al., 2017 found that the native, non-pathogenic microbiota of the honey bee *A. mellifera* induces host immune responses, particularly an upregulation of genes coding for the antimicrobial peptides (AMPs) apidaecin and hymenoptaecin in gut tissue and, subsequently, an elevated apidaecin concentration in the gut lumen and hemolymph. Thus, immune priming by the native symbionts may play a role in regulating the microbiota and/or protecting against pathogens. In fungus-farming ants, antimicrobial compounds produced by actinobacterial symbionts protect the fungal cultivars from specialized *Escovopsis* fungal pathogens (Currie, Scott, et al., 1999). Interestingly, recent studies on the burying beetle *Nicrophorus vespilloides* (Coleoptera: Silphidae), which provide parental care for their offspring, suggested a potential food-preserving role of the symbionts in this taxon as well (Shukla et al., 2017; Vogel et al., 2017).

Lastly, growing evidence suggests that symbiotic bacteria can influence host social interactions (Engl & Kaltenpoth, 2018). In the German cockroach, volatiles emanating from feces-associated bacterial symbionts promote gregarious host behavior (Wada-Katsumata et al., 2015). Similarly, in the desert locust *Schistocerca gregaria*, some of the bacterial gut symbionts play a role in host aggregation by producing components of the locust's cohesion pheromone (Dillon et al., 2002). In addition, bacterial symbionts have putative roles in nestmate recognition in eusocial insects (Engl et al., 2018). For example, in the termite *Reticulitermes speratus* and the harvester ant *Pogonomyrmex barbatus*, bacterial associates have an effect on the hosts' chemical profiles and therefore on recognition (Dosmann et al., 2016; Matsuura, 2001).

2.4. Symbiont transmission routes

Insects that depend on symbionts to perform key functions are faced with the challenge of ensuring acquisition or successful transmission of specific symbionts in every generation. Many solitary insects have evolved complex mechanisms that ensure high fidelity of symbiont passage to the next generation, including symbiont invasion of the oocytes prior to embryogenesis, specialized secretions or structures that harbor extracellular symbionts until egg hatching, or organs that allow for the selective colonization of specific environmental bacteria (Bright & Bulgheresi, 2010; Salem, Florez, et al., 2015). While social and gregarious insects can exploit similar mechanisms for symbiont transmission, their frequent contact with conspecifics opens up the possibility for direct symbiont transfer and thereby adds another dimension to the transmission of beneficial microbes. Behaviors such as coprophagy, trophallaxis and shared resources that are predominant in social and gregarious insects facilitate transmission or acquisition of appropriate microbes in addition to the transfer of nutrients or digestive enzymes.

2.4.1. Coprophagy

Coprophagy refers to the consumption of conspecifics' feces after excretion (Figure 1a) and is considered a major force shaping gut microbial communities of gregarious insects such as bugs, beetles, cockroaches and, to some extent, of eusocial bees and termites. In bumble bees and honey bees, for instance, naïve individuals reared in the presence of fresh feces collected from nurse bees acquire bacterial communities similar to those of nurses or wild bees (Koch et al., 2011; Powell et al., 2014). Gregarious Pyrrhocoridae and Reduviidae bugs (Hemiptera) exhibit a high degree of coprophagy, likely as a behavioral adaptation for symbiont acquisition (Baines, 1956; Salem et al., 2013). For instance, when kissing bugs, *Rhodnius prolixus* (Reduviidae), the vectors of Chagas disease, hatch under sterile laboratory conditions and are raised separately from conspecifics, the nymphs lack the important nutritional symbiont *Rhodococcus rhodnii*, resulting in stunted growth (Baines, 1956). However, molting and development can be easily restored when the nymphs are exposed to freshly collected feces or conspecifics harboring the *R. rhodnii* symbiont (Baines, 1956). In *Pyrrhocoris apterus* and *Dysdercus fasciatus* firebugs (both Pyrrhocoridae), initial acquisition of essential microbes is mediated by vertical transmission to offspring via egg surface smearing with feces (Kaltenpoth et al., 2009; Salem, Onchuru, et al., 2015). Additionally, the gregarious nature of these insects facilitates horizontal transmission of microbes when individuals probe conspecifics' feces. Bugs lacking the typical gut microbiota display high symbiont infection frequencies when reared with symbiotic adult conspecifics, contaminated egg shells or feces (Kaltenpoth et al., 2009; Salem, Onchuru, et al., 2015). Coprophagy can also mediate transition between these two modes of transmission that is from horizontal symbiont transfer (uptake of feces from the environment) to vertical transfer, when feces are deposited along with the eggs or offspring (Salem, Florez, et al., 2015).

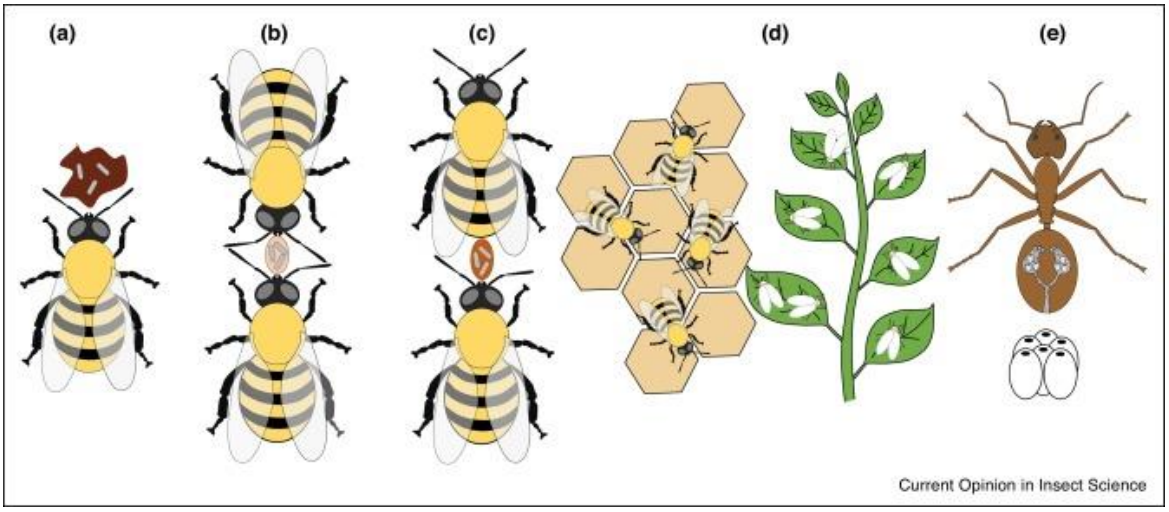

Figure 1. Symbiont transmission routes in social and gregarious insects. (a) Coprophagy, (b) mouth-to-mouth feeding (stomodeal trophallaxis), (c) anus-to-mouth feeding (proctodeal trophallaxis), (d) transmission through shared environment, and (e) transovarial transmission.

Carrion beetles (Coleoptera, Silphidae), which exhibit biparental care, utilize small vertebrate carcasses as an ephemeral nutritional resource for their larvae. When preparing the carcass, they apply anal secretions containing a distinct bacterial community (mostly Xanthomonadaceae, Enterococcaceae, and Enterobacteriaceae) and *Yarrowia* yeasts (Kaltenpoth & Steiger, 2014; Shukla et al., 2017; Vogel et al., 2017). The treated carcass then acts as a medium for vertical symbiont transmission when hatched larvae feed on the secretions, which is reflected in a high similarity of symbiont composition in adult and larval guts as well as prepared carcass surfaces (Shukla et al., 2017; Vogel et al., 2017; Wang & Rozen, 2017). Similarly, transmission of symbionts in *Euoniticellus* dung beetles (Coleoptera, Scarabaeniae) is mediated by maternal fecal secretions deposited in the dung balls together with the eggs. Characterization of the bacterial community composition of larvae, adult females and males, brood balls and maternal secretions revealed that larval gut communities are more similar to female and brood ball communities than to those of males, suggesting that larvae acquire the symbionts vertically upon consuming provisioned maternal secretions (Shukla et al., 2016). In Dictyoptera, coprophagy was likely ancestral (Nalepa et al., 2001) and remains an essential behavior in the transmission of bacterial symbionts in cockroaches and termites. In *Blattella germanica*, whose gregarious nature has been partially attributed to volatile carboxylic acids associated with fecal bacteria (Wada-Katsumata et al., 2015), survivorship and growth of early instar nymphs largely depends on access to nutrients present in conspecifics' feces (Kopanic et al., 2001). Moreover, high similarity of bacterial composition in the feces and gut reveals that this behavior is responsible for inoculation, re-colonization and succession of gut microbiota in the cockroach (Carrasco et al., 2014; Rosas et al., 2018). In termites, coprophagy also mediates the exchange of nutritional fluids, symbiotic protists and bacteria, complementing trophallactic transfer between colony members (see below) (Nalepa, 2015; Ohkuma, 2008).

2.4.2. Trophallaxis

Trophallaxis, the direct transfer of oral fluids via mouth-to-mouth feeding (stomodeal trophallaxis) (Figure 1b) or hindgut content via anus-to-mouth feeding (proctodeal trophallaxis) (Figure 1c), likely evolved from coprophagy (Nalepa et al., 2001). Apart from the transfer of nutrients within a colony (Machida et al., 2001; Nalepa, 2015), many social insect taxa exploit trophallaxis for initial inoculation or re-acquisition of symbionts. In social corbiculate bees (honeybees, bumblebees, and stingless bees),

bioassays demonstrated that both stomodeal and proctodeal trophallaxis are essential for the acquisition and maintenance of the distinctive bee gut microbiota after symbiont-free emergence (Martinson et al., 2012; Powell et al., 2014). When newly emerged bees are exposed to nurse workers or their hindgut homogenates, they mainly acquire core gut symbionts (*Snodgrasella*, *Gilliamella*, *Bifidobacterium*, and *Lactobacillus*), whereas those limited to oral contact with nurse workers are largely colonized by *Lactobacillus* and low amounts of the other taxa (Martinson et al., 2012; Powell et al., 2014). In bumble bees, social contact within the hive has been shown to facilitate symbiont transfer between nestmates, including daughter queens. The latter subsequently transmit the symbionts vertically to their offspring when founding new colonies after hibernation (Koch et al., 2013, 2011).

Similarly, the cellulolytic hindgut communities of endosymbiotic flagellates and bacteria in the eusocial lower termites and in subsocial wood roaches, *C. punctulatus* (Cryptocercidae), are stably maintained by sociality. Group living in termites assures members reliable and continuous access to nutritionally essential gut microbes via trophallaxis (Brune et al., 2015; Lombardo, 2008). Additionally, re-acquisition of microbes lost shortly before ecdysis is achieved by proctodeal trophallaxis between newly molted juveniles and adult conspecifics (Lombardo, 2008; Ohkuma, 2008). In *C. punctulatus*, social contact as well as biparental brood care are key in shaping intestinal flagellate and bacterial communities (Klass et al., 2008). Essential microbes, which take a long time to stably establish in juveniles, and nutrients are repeatedly acquired from adult conspecifics via proctodeal trophallaxis for a period of at least one year until the juveniles gain nutritional and microbial independence (Klass et al., 2008; Lombardo, 2008; Nalepa, 1984, 2015). This behavior also seems to occur in some of their solitary relatives such as *Shelfordella lateralis* (Blattidae), where individuals inoculated with non-native bacteria were shown to re-acquire the native gut microbiota via social contact with conspecific adults (Mikaelyan et al., 2016).

Intimate biparental care of carrion beetles (Silphidae) is key to progeny survival, as it grants larvae access to nutrients and symbionts essential for development. In addition to the transmission of microbes via the carcass surface, acquisition of symbionts by larvae is assured when they engage in stomodeal trophallaxis with adults, whose oral secretions are known to contain symbionts (Shukla et al., 2017). Likewise, eusocial *Polistes* paper wasp males (Vespidae) add oral secretions containing digestive enzymes to pre-chewed pieces of solid food before offering it to their young in regurgitated form (Lombardo, 2008). This process may also include the addition and transmission of relevant gut symbionts.

2.4.3. Environmental transmission

In some insects, direct contact or other forms of exchange between conspecifics or parents and offspring are not necessary for successful symbiont transmission. Rather, symbiotic partners are acquired horizontally from a shared environment or food resource contaminated with symbionts (Figure 1d), as reported in whiteflies, thrips, bees and leaf cutter ants. Caspi-Fluger et al., 2012 and Li et al., 2017 demonstrated that in whiteflies (*Bemisia tabaci*, Hemiptera: Aleyrodidae), which overcome plant defenses when feeding gregariously, plant-mediated horizontal transmission of the secondary endosymbiont *Rickettsia* can occur when infected and non-infected whiteflies of the same or different species share a host plant. These studies observed the transfer of the *Rickettsia* endosymbiont to phloem cells of host plant leaves, as well as its retention and distribution throughout the phloem network before its subsequent re-acquisition by non-infected individuals at high rates (Caspi-Fluger et al., 2012; Li et al., 2017). Similarly, in *Frankliniella occidentalis* (Thysanoptera; Thripidae), which tend to feed gregariously, transmission of symbionts via shared host plants has been demonstrated. Female thrips prefer depositing

eggs on grazed leaves, which are subsequently fed on by hatched larvae that acquire symbionts present in regurgitations or feces of previous feeders (De Vries et al., 2001, 2006).

In bumble bees and honey bees, the bacterial gut symbionts *Snodgrassella*, *Bifidobacterium*, and *Lactobacillus* are taken up during or after natural emergence, when eclosed bees come in direct contact with symbiont-contaminated hive material such as wax, hive frames, pollen, brood cell, honey comb and bee-bread (Koch et al., 2013; Martinson et al., 2012; Powell et al., 2014). Furthermore, intra-colony and inter-colony symbiont transfer frequently happens when bees from the same or different colonies pick up symbionts deposited on shared flowers by previous foragers (Koch et al., 2013), which can then be passed on to nestmates upon return to the hive. Recent examinations of leaf-cutter ant symbioses revealed how nest environments mediate transmission of mutualistic bacteria. In *Acromyrmex* ants, physical contact of newly eclosed symbiont-free worker ants to adult workers and fungus gardens ensures a stable colonization with mutualistic defensive ectosymbiotic *Streptomyces* sp. and *Pseudonocardia* bacteria a few days post-eclosion (Marsh et al., 2014; Poulsen et al., 2003).

2.4.4. Transovarial transmission

In social insects, as in solitary insects, long-term host–symbiont co-evolution can result in strict dependence of symbionts on hosts for survival and transmission. In these circumstances, transovarial transmission is an efficient method of ensuring successful transfer of obligate intracellular symbionts. Transovarial transmission occurs when endosymbiont-filled bacteriocytes or endosymbionts released from bacteriocytes, nurse cells or other organs invade and establish in developing oocytes, eggs or embryos in the mother's ovaries (Figure 1e). In addition to being widespread among solitary insects, this type of transmission has been demonstrated for some social and gregarious taxa such as *Blattabacterium* in *B. germanica* (Sacchi et al., 1988), *Blochmannia* in *Camponotus* ants (Sauer et al., 2002), and *Westeberhardia* in *Cardiocondyla* ants (Klein et al., 2016). In addition, the presence of endosymbionts in reproductive organs, such as those occasionally found in *Camponotus floridanus* male testis follicles (Sauer et al., 2002), suggests that copulation and/or paternal transmission may play a role for symbiont transmission in social or gregarious insects (Watanabe et al., 2014).

2.5. Benefits and costs of social transmission routes

2.5.1. Benefits of social symbiont transmission

Social contact provides more opportunities for effective transmission and maintenance of beneficial microbes than in solitary insects (Figure 2a, Table 1). For example, social bees are known to harbor a more consistent gut microbiota than solitary bees (Martinson et al., 2011), and some sub-social stink bugs exhibit egg-tending behavior, followed by post-hatch symbiont secretions by mothers that ensures offspring uptake of symbionts that may not be able to survive long enough outside the host (Hosokawa et al., 2013). Some microbes may even influence the social behavior of their hosts, like in locusts and cockroaches where their gut bacteria are involved in producing aggregation pheromones, likely enhancing the further transmission of these bacteria when hosts congregate (Dillon et al., 2002; Wada-Katsumata et al., 2015).

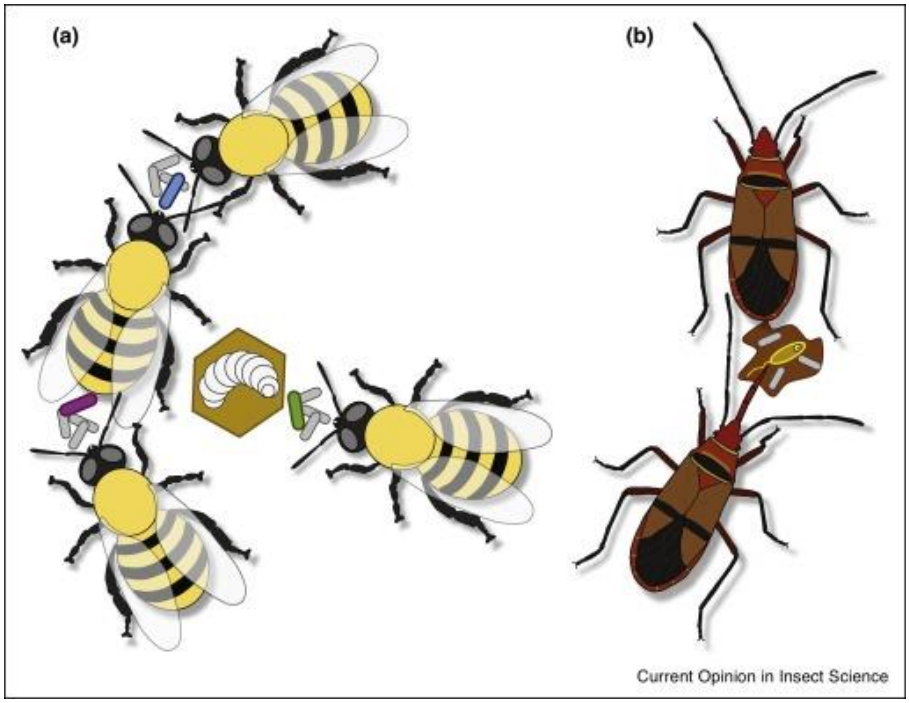

Figure 2. Important benefits and costs of social transmission routes. (a) More opportunities for transmission of multiple beneficial microbes and at different life stages. (b) Parasites, such as trypanosomes, may co-opt the transmission routes of mutualists for their own advantage. For a more detailed account of costs and benefits associated with different transmission routes, please refer to Table 1.

Social behaviors can also promote the establishment of multi-partite symbioses, where multiple microbes together contribute to the overall health of the host (Figure 2a). In a recent study, for example, honeybees were found to gain or lose metabolic potential based on the experimental addition or removal of individual microbes (Kešnerová et al., 2017), suggesting strong selective pressures for behaviors that ensure their maintenance. Other insects may suffer serious fitness defects when transmission routes of one or more of their symbionts are accidentally disrupted, so gregarious behavior as observed in many Heteroptera can be adaptive by increasing the chance of symbiont transmission.

In some cases, mass-provisioning insects transmit their symbiotic microbes in ways that help create an environment that is more suitable for the development of their offspring by inhibiting microbial competitors and pathogens and/or upgrading nutritional content. Carrion beetles, which exhibit cooperative brood care, inoculate animal carcasses with specific bacteria and yeasts that are then transmitted to the larvae and help to preserve the carcass that would otherwise be overgrown with opportunistic microbes (Shukla et al., 2017; Vogel et al., 2017). Beewolves, which provide paralyzed bees for their larvae, have also evolved specialized symbiont deposition behaviors whereby antibiotic-producing *Streptomyces* bacteria left by mothers ensure protection and symbiont transmission to their larvae (Kaltenpoth et al., 2005; Kroiss et al., 2010). And dung beetles, before enclosing their offspring inside dung balls, deposit symbiont-containing 'gifts' that are important for the early nutrition of their offspring inside the dung ball (Shukla et al., 2016).

2.5.2. Cost of co-transmitting parasites and pathogens

In addition to facilitating the transmission of beneficial microbes, social symbiont transmission can also entail significant costs, as social contact may open the door to pathogens that can take advantage of

these transmission routes for their own means (Figure 2b) (Kraus & Page, 1998; Schmid-Hempel, 1998). In social bees such as *A. mellifera* and *B. terrestris*, *Crithidia* trypanosomes were found to infect workers during brood-care of infected larvae (Folly et al., 2017), although other socially acquired bacteria can protect against these infections (Koch et al., 2011; Mockler et al., 2018). Similarly, *Blastocrithidia* and *Leptomonas* trypanosomes in firebugs such as *P. apterus* and *D. fasciatus* are known to co-opt their gregarious hosts' coprophagy and egg-smearing symbiont transmission routes for their own transmission (Figure 2b) (Frolov et al., 2017; Salem, Onchuru, et al., 2015). The foraging behavior of *Acromyrmex* leaf-cutter ant workers appears to be the culprit for acquisition and spread of the fungal garden parasite, *Escovopsis*, to ant nests. It was originally thought that *Escovopsis* is vertically transmitted in fungal pellets carried by ant foundress queens on their nuptial flights, but recent studies instead found that the parasitic fungus sporulates in external ant refuse dumps where spores are spread intraspecifically and interspecifically by sticking to the cuticle of foraging workers from other colonies (Augustin et al., 2017; Currie, Mueller, et al., 1999; Moreira et al., 2015).

Parasitic entomopathogenic fungi, bacteria, and nematodes, too, may spread quickly through social insect societies, as they can be rapidly transmitted, potentially decimating colonies. However, social insects can alleviate these costs and reduce pathogen loads through social allogrooming that removes pathogens and primes the host's immune system (Cremer et al., 2007; Nalepa, 2015; Zhukovskaya et al., 2013). For example, in ants and termites, contact with fungi by either exposure to non-infective doses of spores or trophallaxis with immunized individuals increases their resistance to future pathogen exposures (Konrad et al., 2012; Traniello et al., 2002; Ugelvig & Cremer, 2007). Social immunity is not always effective against all pathogens, however, as proctodeal trophallaxis from *Serratia marcescens*-infected termites was found to increase susceptibility of recipient termites to later encounters with the same pathogen (Mirabito & Rosengaus, 2016).

2.6. Ecological and evolutionary implications

Among insects associated with mutualistic symbionts, selection favors those that successfully endow offspring with their beneficial partners. While the transovarial mode of transmission for intracellular symbionts is shared between some solitary and social insects, they differ in the predominant transmission route for extracellular symbionts (Table 1). Solitary insects mostly rely on depositing fecal droplets, glandular secretions, symbiont capsules or caplets in close contact to the eggs or in the nesting environment (Salem et al., 2017; Salem, Florez, et al., 2015). As these insects usually do not have direct contact with their offspring, depositing the symbionts in locations where they are likely to be encountered by the hatchlings increases the likelihood of successful transmission (Salem, Florez, et al., 2015). By contrast, social insects predominantly exchange symbionts via direct contact, that is, stomodeal or proctodeal trophallaxis (Table 1). Presumably, this mechanism of symbiont transmission has several advantages over the transmission routes that are accessible to solitary insects: Direct transfer reduces the risk of failure to transmit beneficial symbionts and allows for simultaneous provisioning of digestive enzymes that aid digestion in immature individuals (Machida et al., 2001; Nalepa, 2015; Salem, Florez, et al., 2015); the immediate acquisition of microbial partners from conspecifics minimizes the time that the microbes spend outside of their host's body, ensuring the survival of multiple host-adapted symbionts, and although social interactions are known to increase the risk of pathogens spreading across large groups of closely related conspecifics, the direct transfer between individuals in social insect colonies likely reduces the risk of acquiring opportunistic microbes (Meunier, 2015), at least in comparison to an unspecific uptake of feces from the environment or an extended exposure of maternal symbiont-containing secretions to external contamination (Table 1).

Table 1. Demonstrated or hypothesized requirements, benefits and costs associated with different symbiont transmission routes in social and gregarious insects.

	Extracellular transmission route				
	Trophallaxis	**Coprophagy**	**Egg surface smearing**	**Specialized structures**	**Environmental**
A. Requirements for symbiont transmission					
Host social behavior	Eusociality	eusociality/ gregariousness	mostly solitary	mostly solitary	gregariousness
Host adaptations	Behavioral	behavioral	behavioral	metabolic/ structural	Behavioral
Symbiont adaptations	gut passage	gut passage and survival in feces	gut passage and survival on egg surface	survival in specialized structures	Establishment and survival in Environment
B. Benefits of transmission route					
Reliability of symbiont transmission	High	low	medium	high	Low
Additional nutritive uptake	Yes	occasional	occasional	unknown	Yes
Host protection	social immunity	social immunity	egg protection	unknown	Unknown
Maintenance of diverse symbiont community	Yes	Yes	sometimes	no	Unknown
C. Costs of transmission route					
Host metabolic input	Low	low	low	high	Unknown
Risk of parasite co-transmission	High	high	high	low	Unknown
Acquisition of opportunistic microbes	Low	high	high	variable	High

Considering the importance of inter-individual transmission of mutualistic bacteria in social insects, it is tempting to speculate on the role of symbiont transmission in the evolution of sociality. For termites, Cleveland et al., 1934 and Nalepa, 2015 argued that proctodeal trophallaxis was intimately connected to the evolution of sociality, by integrating the social, nutritional, and microbial environment. Arguably, as lower termites need to reacquire their gut protists after each molt, recurrent contact with conspecifics is crucial for maintaining the cellulolytic microbial gut community (Lombardo, 2008; Nalepa, 2015). Even though, as outlined above, many other social insects are associated with microbial symbionts and likewise benefit from their social transfer, most of them only require a single acquisition event for successful and lasting symbiont infection (Marsh et al., 2014; Martinson et al., 2012), rendering frequent contact with conspecifics less important for symbiont transmission. For many eusocial insects, however, beneficial symbionts are as yet uncharacterized (e.g. most eusocial wasps and many ants), so more knowledge is needed to assess whether enhanced symbiont transmission is an important driving force for the evolution of sociality. In addition, quantitative empirical work on costs and benefits associated with different transmission routes (Table 1) remains largely lacking, prohibiting conclusions on the adaptive value of evolutionary transitions between transmission routes. Nevertheless, the opportunity for direct symbiont transmission may have reinforced sociality (Biedermann & Rohlfs, 2017; Lombardo, 2008) and certainly adds another dimension to the costs and benefits of living in groups of conspecifics.

Conflict of interest

Nothing declared.

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CHAPTER 3

SYMBIONT TRANSMISSION ENTAILS THE RISK OF PARASITE INFECTION

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Hassan Salem[1,†], Thomas O. Onchuru[1 †], Eugen Bauer[1,2] and Martin Kaltenpoth[1,3]

[1] *Insect Symbiosis Research Group, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany*
[2] *Luxembourg Centre for Systems Biomedicine, University of Luxembourg, L-4362 Esch-sur-Alzette, Luxembourg*
[3] *Department of Evolutionary Ecology, Johannes Gutenberg University, 55128 Mainz, Germany*

3.1. Abstract

Like many animals, firebugs (Hemiptera, Pyrrhocoridae) rely on behavioural adaptations to successfully endow their offspring with microbial mutualists. To transmit the nutritionally beneficial Coriobacteriaceae symbionts, female firebugs smear egg surfaces with symbiont-containing faecal droplets that are subsequently ingested by newly hatched nymphs through active probing to initiate infection. Alternatively, the symbionts can be acquired horizontally through contact with faeces of infected conspecifics. Here, we report that these adaptations ensuring successful transmission of bacterial symbionts among firebugs are exploited by the specialized trypanosomatid parasite *Leptomonas pyrrhocoris*. Using comparative transcriptomics, fluorescence *in situ* hybridization (FISH) and controlled bioassays, we demonstrate that the transmission cycle of *L. pyrrhocoris* mirrors that of the bacterial mutualists, with high efficiency for both vertical and horizontal transmission. This indicates that the parasite capitalizes on pre-existing behavioural adaptations (egg smearing and probing) to facilitate its own transfer within host populations, adaptations that likely evolved to initiate and maintain an association with beneficial gut symbionts. Thus, the transmission of mutualistic microbes across host generations can entail a significant risk of co-transmitting pathogens or parasites, thereby exerting selective pressures on the host to evolve more specific mechanisms of transfer.

3.2. Introduction

Mutualisms with microorganisms have played an integral role in the evolution of animals (McFall-Ngai et al., 2013). As such, numerous adaptations have evolved, in both host and symbiont, to ensure the successful transfer of beneficial microbes to future host generations (Salem et al., 2015), thereby contributing to the maintenance and evolutionary stability of mutualisms. Similarly, among parasites that obligately depend on their hosts for survival, fitness is largely determined by their success in establishing

infection and transmitting to other hosts (Altizer & Augustine, 1997). Thus, adaptations for successful initiation and maintenance of infection are strongly selected for, and, as a result, represent a fundamental component of the parasites' ecology and evolution (Poulin, 2011).

Firebugs associate with a highly stable gut bacterial community dominated by two actinobacterial symbionts belonging to the Coriobacteriaceae family, as well as members of the Firmicutes and Gammaproteobacteria (Sudakaran et al., 2015, 2012). The two Coriobacteriaceae symbionts *Coriobacterium glomerans* and *Gordonibacter* sp. are vertically transmitted across host generations through the faecal smearing of egg surfaces by females during oviposition, but can also be horizontally acquired through contact with conspecifics (Kaltenpoth et al., 2009). Experimental sterilization of egg surfaces disrupts the transmission cycle of the Coriobacteriaceae symbionts, resulting in aposymbiotic (symbiont-free) firebugs that suffer retarded growth, high mortality and low fecundity (Salem et al., 2013), which is owing to the deficiency in B vitamins that are provided by the symbionts (Salem et al., 2014).

Matching the highly conserved bacterial midgut community associated with firebugs is the specialized epidemiology of trypanosomatids across this insect family (Votýpka et al., 2012). Most striking is the cosmopolitan distribution of a single, mildly virulent flagellate, *Leptomonas pyrrhocoris*, across at least 11 species and four genera of firebugs sampled from eight countries across four continents (Lipa, 2012; Schaub, 1994; Votýpka et al., 2012). Among firebugs, infection by *L. pyrrhocoris* can induce paler coloration, lethargy and diarrhea (Lipa, 2012), as well as increased mortality, reduced starvation resistance and a reduced lifespan in insects that contract the parasite early in development (Schaub, 1994).

Despite the global distribution of *L. pyrrhocoris* among firebugs, little is known about how infection is initiated and maintained. While it is presumed that *L. pyrrhocoris* is horizontally transferred between con- and heterospecifics as mediated by the large aggregations formed by the insects (Votýpka et al., 2012), no study to date has directly reported on the transmission route of *L. pyrrhocoris* within and between host populations.

In this study, we demonstrate that the transmission route of *L. pyrrhocoris* in firebugs mirrors that of the Coriobacteriaceae symbionts. Specifically, we report that while *L. pyrrhocoris* can be acquired horizontally through contact with infected firebugs, the parasite also exploits the symbionts' vertical transmission route via the egg surface for its own transfer among host individuals.

3.3. Results and discussion

Our first insights into the transmission route of trypanosomatids in pyrrhocorid bugs came from comparative transcriptomic analyses of midguts extracted from the firebug *Dysdercus fasciatus* that had either been subjected to egg surface sterilization to rid them of their beneficial Coriobacteriaceae symbionts or left untreated. While the untreated group featured transcripts that could be assigned to the Trypanosomatidae cluster TU61 (associated with the genus *Blastocrithidia*, see (Votýpka et al., 2012), none could be retrieved from firebugs that had been subjected to egg surface sterilization (Figure 1a), suggesting that the method for eliminating the bacterial symbionts also clears infection by the trypanosomatid. This hypothesis is corroborated by fluorescence *in situ* hybridization (FISH) images demonstrating the co-localization of *L. pyrrhocoris* and *C. glomerans* in faecal droplets collected from firebugs that had been artificially inoculated with *L. pyrrhocoris* in the laboratory (Figure 1a).

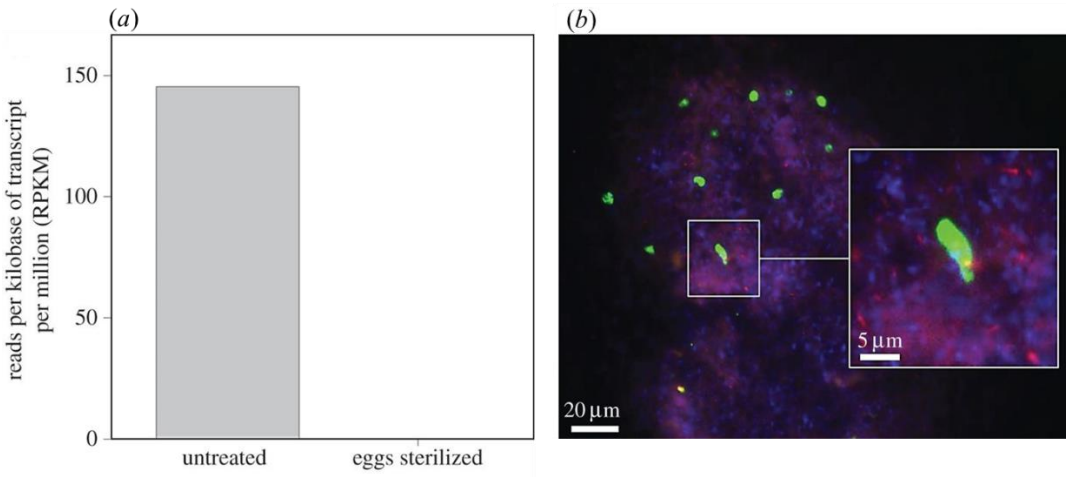

Figure 1. (*a*) Normalized expression values of reads per kilobase of transcript per million reads (RPKM) belonging to the Trypanosomatidae in guts of adult *Dysdercus fasciatus* that had hatched from untreated or surface-sterilized eggs. (*b*) Fluorescence micrograph of *Leptomonas pyrrhocoris* (green) and *Coriobacterium glomerans* (red) in a faecal droplet from *D. fasciatus*. Counterstaining of DNA was done with DAPI (blue).

To directly test for the vertical and horizontal transmission routes of *L. pyrrhocoris* in *D. fasciatus*, we harvested six egg clutches from *L. pyrrhocoris*-infected mating pairs and divided the eggs of each clutch into four groups: (i) untreated, (ii) egg surface-sterilized, (iii) egg surface-sterilized, followed by the inoculation of *L. pyrrhocoris* from pure culture over egg surfaces, and (iv) egg surface-sterilized, then reared upon hatching in the presence of two *L. pyrrhocoris*-infected adult *D. fasciatus*.

Matching the infection dynamics of the Coriobacteriaceae symbionts (Figure 2a, b), *L. pyrrhocoris* (Figure 2c) could be detected with high prevalence in the untreated group (82%). In contrast, the sterilization of egg surfaces resulted in adult firebugs that completely lacked the symbionts as well as *L. pyrrhocoris* (Figure 2a-c; $p < 0.001$, χ^2 test). Spreading cultured *L. pyrrhocoris* over previously sterilized eggs reinstituted the infection at a high frequency (63%; Figure 2c), thereby confirming that the parasite can vertically transmit via the egg surface. Additionally, horizontal transfer of *L. pyrrhocoris* among conspecifics occurred with high efficiency (100%) in firebugs that were subjected to the egg surface sterilization procedure but were subsequently reared in the presence of infected conspecifics (Figure 2c). Collectively, such findings demonstrate that *L. pyrrhocoris* is transmitted across host generations in a manner that is identical to the beneficial Coriobacteriaceae symbionts (Kaltenpoth et al., 2009): vertically via the egg surface and horizontally through contact with infected firebugs, specifically their faeces (Figure 2a,b). This is consistent with recent findings in milkweed bugs and their trypanosomatid parasite, *Leptomonas wallacei* (de Almeida Dias et al., 2014), where transovum propagation of the parasite was demonstrated to mediate vertical transmission. Despite the parallels to the transmission cycle of *L. pyrrhocoris*, it is unclear whether the egg probing behaviour in *Oncopeltus fasciatus* is relevant for symbiont transmission, considering the lack of evidence for vertically transmitted mutualists associated with milkweed bugs (Feir, 1963).

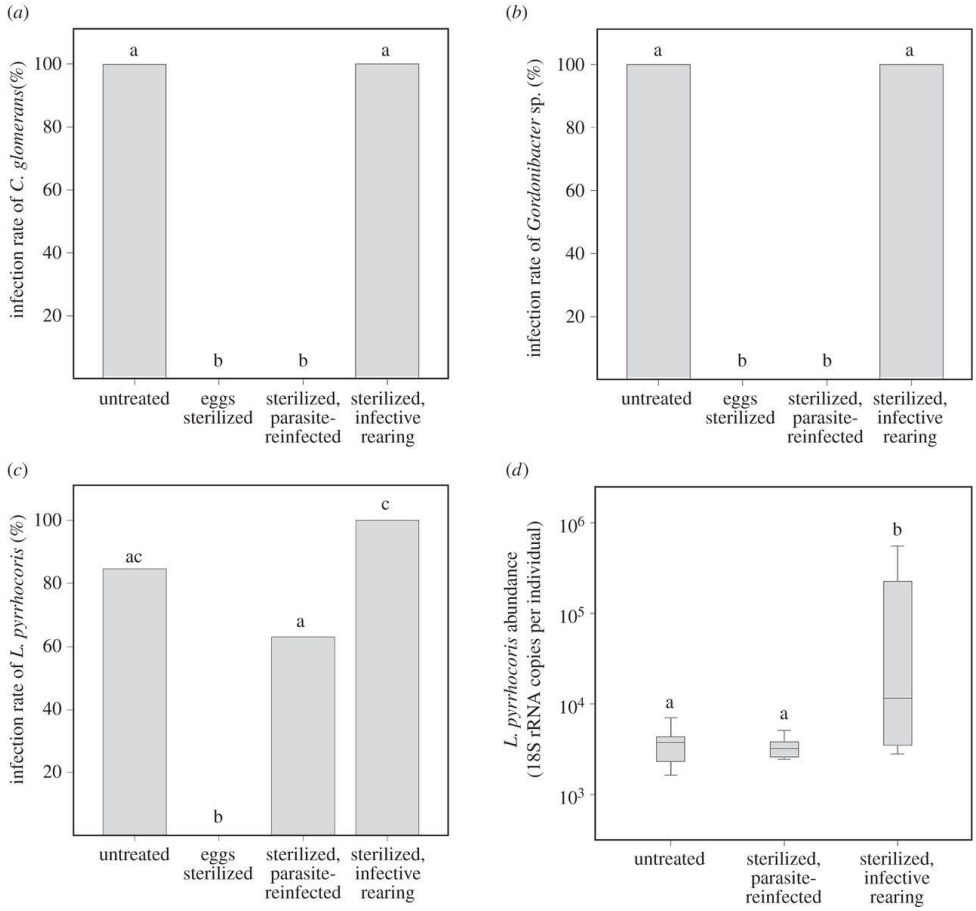

Figure 2. Transmission rates of (*a*) *Coriobacterium glomerans*, (*b*) *Gordonibacter* sp. and (*c*) *Leptomonas pyrrhocoris* in *Dysdercus fasciatus* across four experimental treatments (untreated, eggs surface-sterilized, sterilized then reinfected with *L. pyrrhocoris*, and sterilized then reared in the presence of conspecifics infected with *L. pyrrhocoris* as well as both Coriobacteriaceae symbionts). (*d*) Titre of *L. pyrrhocoris* across infected *D. fasciatus* individuals in the three *L. pyrrhocoris*-harbouring experimental treatments. Parasite abundances represent estimated 18S rRNA gene copy numbers obtained from qPCR assays. Different letters above bars/boxes indicate significant differences ((*a–c*) pairwise χ^2 tests, $p < 0.05$; (*d*) Kruskall–Wallis *H*-test, $p < 0.05$).

When examining parasite titres among infected individuals across the three *L. pyrrhocoris*-harbouring treatments (Figure 2d), we found that parasite load was significantly different across groups ($p = 0.012$, Kruskal–Wallis *H*-test). Specifically, *D. fasciatus* reared in the presence of infected firebugs were found to harbour higher titres of *L. pyrrhocoris* compared with treatments where the trypanosomatids were provisioned over the egg surface, possibly as a consequence of repeated exposure to the parasite via contact with infected conspecifics.

The transmission dynamics reported in this study for *L. pyrrhocoris* are consistent with theoretical predictions implicating bimodal (horizontal and vertical) transfer, alongside low virulence, as hallmarks of globally distributed, specialized parasites (Altizer et al., 1997). Additionally, the high vertical transmission efficiency of the parasite may ultimately select for reduced virulence (Sachs & Wilcox, 2006), owing to the alignment of interest in host and parasite, which may explain the predominance of asymptomatic infections caused by trypanosomatids in insects (Maslov et al., 2013).

Many parasites exploit the ecology of their hosts to initiate infection. For example, *Crithidia bombi*, the trypanosomatid parasite of bumblebees, capitalizes on its host's social organization to spread among

nest-mates following contact with infected individuals (Schmid-Hempel, 1998). *C. bombi* can also propagate within populations of its hosts through the shared use of flowers (Durrer & Schmid-Hempel, 1994), collectively highlighting the adaptive propensity of the parasite to the behavioural as well as feeding ecology of bumblebees. Coprophagy, which often not only contributes towards fulfilling the nutritional requirements of immature insects (Scriber & Slansky, 1981), also facilitates the horizontal transfer of trypanosomatids across a range of bug species (Maslov et al., 2013). In this study, however, we report on how a specialized parasite may have capitalized on pre-existing adaptations for mutualist transmission in an insect to facilitate its own transfer, thereby contributing to a cosmopolitan distribution mirroring that of the host (Votýpka et al., 2012) as well as of the beneficial bacterial associates of this insect family (Sudakaran et al., 2015). Given the widespread occurrence of extracellular symbiont transmission routes in insects (Salem et al., 2015), in particular through the smearing of egg surfaces with faeces or glandular secretions, co-transmission of intestinal parasites is likely a common phenomenon. Hence, the risk of parasite hitchhiking may result in trade-offs that favour the evolution of additional mechanisms ensuring specificity during transfer by the host or new defences against the parasite, particularly if the costs of parasite infection outweigh the benefits of acquiring mutualistic microbes.

3.4. Material and methods

3.4.1. Insect sampling and rearing

Live *D. fasciatus* were originally collected in the Comoé National Park, Côte d'Ivoire, but have since been maintained in the laboratory at the University of Würzburg, Germany, and a subculture 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 28°C and exposed to long light regimes (16 h L : 8 h D cycles).

3.4.2. Illumina-based transcriptome sequencing

RNA was extracted from dissected whole midgut regions (M1–M4) from five symbiotic and aposymbiotic bugs, respectively, resulting in two pooled samples. Transcriptome sequencing of poly-A enriched mRNAs, assembly, annotation and analysis were described previously (Bauer et al., 2014; Salem et al., 2014). The sequence data were deposited in the European Nucleotide Archive, accession number PRJEB6171 (http://www.ebi.ac.uk/ena/data/view/PRJEB6171).

3.4.3. Fluorescence *in situ* hybridization

Faecal droplets were collected from infected adults by pressing the insect's abdomen on a glass slide. Upon drying, the faeces were fixed with 70% and 99% ethanol in succession. Cor653 (Kaltenpoth et al., 2009) and SSUR2 (5'-GAGTCAACACTGCTGGGTGT-3') probes were used to localize *C. glomerans* and *L. pyrrhocoris*, respectively. The SSUR2 probe was designed using the 18S rRNA sequence of *L. pyrrhocoris* (GenBank accession no. JN036653). Hybridization was carried out as described previously (Kaltenpoth et al., 2009).

3.4.4. Experimental set-up

Six egg clutches (approx. 30 eggs each) from different females of *L. pyrrhocoris*-infected *D. fasciatus* were harvested three days after oviposition and kept separately. We then split each of the collected clutches into four experimental treatments: (i) untreated, (ii) surface-sterilized, (iii) surface-sterilized then re-infected with a pure inoculum of *L. pyrrhocoris* (30 µl of approx. 10^5 flagellates μl^{-1}) and (iv) surface-

sterilized and subsequently reared in contact with two *L. pyrrhocoris*-infected adult bugs. The four treatments were provided ad libitum with autoclaved water and a nutrient-rich artificial diet (Salem et al., 2014).

The sterilization of egg surfaces followed the procedure used in (Salem et al., 2013). Briefly, the eggs were submerged in ethanol for 5 min, followed by bleach (12% NaOCl) for 45 s. Residual bleach was removed by washing in autoclaved water.

3.4.5. DNA extraction and PCR screening for *Leptomonas pyrrhocoris* and the Coriobacteriaceae symbionts

All individuals from every experimental treatment were subjected to DNA extraction three days after adult emergence as previously described (Salem et al., 2013). Primers targeting *L. pyrrhocoris*' 18S rRNA gene, SSU Fwd_2 (5'-CTGGTTGATCCTGCCAGTAG-3') and SSU Rev_2 (5'-GAGTCAACACTGCTGGGTGT-3') were then used to screen for the parasite, using the following cycle parameters: 3 min at 94°C, followed by 32 cycles of 94°C for 40 s, 56°C for 1 min and 72°C for 1 min, and a final extension time of 4 min at 72°C. Screening for *C. glomerans* and *Gordonibacter* sp. was performed as previously described (Salem et al., 2013), using the primers Cor_2F/Cor_1R and fD1/Egg_1R, respectively.

3.4.6. Quantitative PCR

To assess parasite infection titres, quantitative PCR for *L. pyrrhocoris* was conducted for samples that were positive for the parasite per diagnostic PCR, using a RotorGene-Q cycler (Qiagen, Hilden, Germany). The final reaction volume of 25 ml included the following components: 1 ml of DNA template, 2.5 ml of SSU Fwd_2 and SSU Rev_2 primers (10 mM), 6.5 ml of autoclaved distilled H_2O and 12.5 ml of SYBR Green Mix (Qiagen, Hilden, Germany).

3.4.7. Statistical analysis

Infection rates of *L. pyrrhocoris, C. glomerans* and *Gordonibacter* sp. across the four experimental treatments were compared using pairwise χ^2 tests (SPSS, Chicago, IL). To compare *L. pyrrhocoris* 18S copy numbers estimated in the qPCRs, Kruskal–Wallis *H*-test with Dunn's *post hoc* comparisons was used as implemented in BiAS v. 7.40 (Epsilon Verlag, Hochheim-Darmstadt, Germany).

Ethics

The work conducted complies with the ethical regulations in Germany.

Data accessibility

Original data on PCR and qPCR results of symbiont and parasite infection are available in the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.187mt.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 4

THE COTTON STAINER'S GUT MICROBIOTA SUPPRESSES INFECTION OF A CO-TRANSMITTED TRYPANOSOMATID PARASITE

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Thomas Ogao Onchuru[1] Adam Martinez[1] and Martin Kaltenpoth[1]

[1]Department for Evolutionary Ecology, Johannes Gutenberg University, Johann-Joachim-Becher-Weg 13, 55128 Mainz, Germany.

4.1. Abstract

The evolutionary and ecological success of many insects is attributed to mutualistic partnerships with bacteria that confer hosts with novel traits including food digestion, nutrient supplementation, detoxification of harmful compounds and defense against natural enemies. *Dysdercus fasciatus* firebugs (Hemiptera: Pyrrhocoridae), commonly known as cotton stainers, possess a simple but distinctive gut bacterial community including B-vitamin supplementing Coriobacteriaceae symbionts. In addition, their guts are often infested with the intestinal trypanosomatid parasite *Leptomonas pyrrhocoris* (Kinetoplastida: Trypanosomatidae). In this study, using experimental bioassays and fluorescence *in situ* hybridization (FISH), we report on the protective role of the *D. fasciatus* gut bacteria against *L. pyrrhocoris*. We artificially infected 2nd instars of dysbiotic and symbiotic insects with a parasite culture and measured parasite titers, developmental time and survival rates. Our results show that *L. pyrrhocoris* infection increases developmental time and slightly modifies the quantitative composition of the gut microbiota. More importantly, we found significantly higher parasite titers and a tendency towards lower survival rates in parasite infected dysbiotic insects compared to symbiotic controls, indicating that the gut bacteria successfully interferes with the establishment or proliferation of *L. pyrrhocoris*. The colonization of symbiotic bacteria on the peritrophic matrix along the gut wall, as revealed by FISH, likely acts as a barrier blocking parasite attachment or entry into the peritrophic matrix. Our findings show that in addition to being nutritionally important, *D. fasciatus'* gut bacteria complement the host's immune system in preventing parasite invasions and that a stable gut microbial community is integral for the host's health.

4.2. Introduction

All animals including insects are in constant exposure to pathogenic infections. As a result, insects have evolved elaborate mechanical, behavioral and chemical defenses as well as a sophisticated innate immune system to protect themselves against antagonists (Buchon et al., 2013; Schowalter, 2011). Physical barriers such as a hard cuticle and the peritrophic matrix in the gut as well as behavioral traits like grooming and the avoidance of infected habitats or individuals form the first line of defense that either prevents infections, inhibits antagonist growth or alleviates infection effects (De Roode & Lefèvre, 2012; Tzou et al., 2002; Zhukovskaya et al., 2013). In some cases, behavioral traits coupled with chemical defenses effectively inhibit pathogen growth (Tragust et al., 2013). When the first line of defense is breached, insects can recognize pathogens and deploy rapid innate immune defenses including cellular (encapsulation, phagocytosis or nodule formation) as well as humoral (antimicrobial peptides, melanization, production of reactive oxygen and nitrogen species) immune responses that target, isolate, and neutralize microbial invaders (Buchon et al., 2013; Marxer et al., 2016).

Apart from the physical mechanisms and classical innate immune system, there is increasing evidence that insects form intimate partnerships with mutualistic bacteria that protect them against viruses, pathogenic bacteria, parasites, fungi, and parasitoids (Bian et al., 2010; Dong et al., 2009; Flórez et al., 2015; Koch & Schmid-Hempel, 2011; Scarborough et al., 2005; Teixeira et al., 2008). Mechanisms deployed by defensive mutualists for host protection include competing with antagonists for limited host resources (Herren et al., 2014; Paredes et al., 2016), production of inhibitory substances such as toxins or antibiotics (Brandt et al., 2017; Flórez et al., 2017; Hamilton, Peng, et al., 2015; Kaltenpoth et al., 2005; Kroiss et al., 2010), priming or maturation of the host immune system (Emery et al., 2017; Kim et al., 2015; Konrad et al., 2012; Weiss et al., 2012, 2011) and improvement of host vigor by modulating nutrient allocation, growth rate or behavior (Gerardo & Parker, 2014). Additionally, host/symbiont-pathogen interactions can facilitate the evolution of reduced pathogen virulence (Ford et al., 2016; Read, 1994), mitigating detrimental fitness effects on the host. On the other hand, symbiont mediated protection can eliminate the selection pressure on host defense genes towards increased resistance (Martinez et al., 2016), which may result in the evolution of low pathogen resistance by the host and dependence on the symbiont for protection (Gerardo et al., 2010).

Trypanosomatid protists (Euglenozoa: Kinetoplastea) are ubiquitous parasites in insects. This is evident by their presence across a wide range of insect taxa within the orders Hymenoptera, Hemiptera, Diptera, and Siphonaptera (Kaufer et al., 2017; Maslov et al., 2013; Schaub, 1994). These eukaryotic parasites are classified into 12 genera within the Trypanosomatidae family and exhibit either a dixenous (complete life cycle in two host species) or monoxenous (complete life cycle in a single host species) lifestyle. Whereas monoxenous trypanosomatids are restricted to invertebrate hosts, dixenous trypanosomatids are parasites of plants and vertebrates but require invertebrate hosts for transmission. Dixenous trypanosomatids such as *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania* species are some of the most studied trypanosomatids due to their economic and/or medical significance, as they cause human and animal African trypanosomiasis, Chagas disease, and different forms of Leishmaniasis, respectively. The study of interactions of these parasites and co-habiting bacteria symbionts within the host vectors has gained much attention in recent times. This is because bacterial symbionts are known to influence the outcome of host-parasite interactions especially in invertebrate hosts. For instance, symbionts enhance maturation of the tsetse fly (*Glossina morsitans*) immune system, which enables the host to resist *Trypanosoma brucei* infections (Weiss et al., 2012, 2011). Such interactions coupled with the experimental and/or genetic tractability of some insect-microbe associations provide a prospective application of

symbionts in disease control strategies (Beard et al., 2001). Despite the huge number of insect taxa associated with monoxenous trypanosomatids and gut microbiota (Engel & Moran, 2013; Kaufer et al., 2017; Maslov et al., 2013; Schaub, 1994), the dynamics and outcomes of host-symbiont-parasite interactions have only been reported in a few taxa such as bumble bees (Koch et al., 2011; Mockler et al., 2018) and honeybees (Schwarz et al., 2016). The study of monoxenous parasites, which has enhanced our understanding of the biology and evolution of their dixenous counterparts (Flegontov et al., 2016), and the ability of these parasites to complete their lifecycle in a single host makes them attractive for use in experimental manipulations. Therefore, research on host-symbiont-monoxenous trypanosomatid interactions can help to expand the knowledge on how bacterial symbionts influence host-parasite interactions and how widespread symbiont-mediated parasite defense is across insects.

African cotton stainers (*Dysdercus fasciatus*) and European firebugs (*Pyrrhocoris apterus*) (both Hemiptera: Pyrrhocoridae) possess a similar, simple and well conserved gut bacterial community composed of *Coriobacterium glomerans, Gordonibacter* sp., *Klebsiella* sp. and *Clostridium* sp. (recently re-classified into the genus *Hungatella*, see Kaur et al., 2014) (Salem et al., 2013; Sudakaran et al., 2012). The Coriobacteriaceae symbionts are known to supplement B-vitamins to the host, which provides an important fitness benefit on the vitamin-limited natural diet of Malvales seeds (Salem et al., 2014). In addition to the gut microbiota, the intestinal tract of these and many other firebug species of the Pyrrhocoridae family can be infested with *Leptomonas pyrrhocoris*, a trypanosomatid parasite that can occur at high frequency in host populations (Votýpka et al., 2012). This eukaryotic parasite maintains stable and consistent infections in *D. fasciatus* populations by hitch-hiking on the bacteria symbionts' vertical and horizontal transmission routes to infect offspring and unrelated conspecifics, respectively (Salem et al., 2015). The transmission of this parasite along with the gut bacteria symbionts in this gregarious insect reveals how non-specific symbiotic bacteria transmission routes, mostly exhibited by social, sub-social, and gregarious insects, increase chances of transmitting and/or acquiring unwanted pathogens (Onchuru et al., 2018). Despite the often high infection frequencies of *L. pyrrhocoris* in host populations (Votýpka et al., 2012), as well as classification of this parasite as pathogenic (Schaub, 1994), natural populations of *D. fasciatus* and *P. apterus* often show minimal or no symptoms of infection. It is not clear however how the hosts and their gut bacterial symbionts, which are co-localized and co-transmitted with the parasites (Salem et al., 2015), contribute towards the management of high infection frequencies. We hypothesize that the interaction of the gut bacterial symbionts and *L. pyrrhocoris* trypanosomatids within the gut or during co-transmission inhibits parasite establishment upon successful transmission.

In this study, we took advantage of the vertical transmission route of the gut bacterial symbionts (Kaltenpoth et al., 2009) to generate dysbiotic (depleted of the core gut bacteria) and symbiotic insects (Salem et al., 2014, 2013), which were artificially infected with *L. pyrrhocoris* to determine the effect of parasites on the host development and survival in the presence and absence of the bacterial symbionts. Furthermore, we aimed to determine if the gut bacterial symbionts play a role in protecting the host from *L. pyrrhocoris* parasite infection by comparing parasite titers of dysbiotic and symbiotic infected individuals. Lastly, we assessed the stability of the core gut bacterial symbionts in symbiotic animals upon parasite infection.

4.3. Materials and methods

4.3.1. Insect sample source and rearing

We used subcultures of *D. fasciatus* insects originally collected in 2001 in Comoé National Park, Côte d'Ivoire that have since been maintained in subcultures at the University of Wurzburg, Germany, at the Max Planck Institute for Chemical Ecology, Jena, Germany and currently at Johannes Gutenberg University in Mainz, Germany. Maintenance of the insects in the lab has no effect on the gut microbiota, which is qualitatively and quantitatively similar to that of field collected insects (Salem et al., 2013; Sudakaran et al., 2015). The experiments were conducted under long light regimes (16-hour day: 8-hour night), 60% humidity and a constant temperature of 26˚C.

4.3.2. Parasite source and culturing

L. pyrrhocoris parasite culture used in this study was originally obtained in 2014 from Jan Votýpka (Department of Parasitology, Charles University in Prague, Czech Republic) and has since been maintained in the laboratory by sub-culturing at the Max Planck Institute for Chemical Ecology, Jena, Germany and currently at Johannes Gutenberg University in Mainz, Germany. The parasites were cultured aerobically at 25˚C in tissue culture flasks (Corning Incorporated, USA) with *Crithidia* medium (ATCC medium: 355) which has been previously used to culture closely related *Leptomonas seymouri* and *Crithidia bombi* trypanosomatid parasites (Jackson, 2010).

4.3.3. Generation of dysbiotic and symbiotic insects

Eleven *D. fasciatus* mating pairs were isolated from the main culture and each maintained separately in sterile cages to lay eggs. Each of the eleven egg clutches was harvested four days post oviposition from the cages and divided into two halves, one of which remained untreated (symbiotic) and while the other one was surface sterilized (dysbiotic). Egg surface sterilization was done as previously described (Salem et al., 2013) by submerging eggs in 95% ethanol for 5 minutes followed by 45 seconds in bleach before thorough washing with distilled water. This procedure removes or significantly reduces core bacterial symbionts, while inflicting no direct negative effects on the developing bug embryos (Salem et al., 2013). Both dysbiotic and symbiotic eggs were incubated at 25˚C and 60% humidity until hatching. To avoid contamination with non-native gut bacteria, hatched nymphs of both groups were fed *ad libitum* with autoclaved linden seeds and autoclaved water. A mixture of B-vitamins (final concentrations of 0.05mg/ml thiamine (B1), 0.1mg/ml riboflavin (B2), 0.2mg/ml nicotinamide (B3), 0.2mg/ml calcium pantothenate (B5), 0.05mg/ml pyridoxine (B6), 0.004mg/ml biotin (B7), 0.05mg/ml folic acid (B9), and 0.2mg/ml cobalamin (B12)) was added to the water of both treatment groups, to compensate for the deleterious consequences of the absence of vitamin-supplementing bacterial symbionts in dysbiotic bugs (Salem et al., 2014).

4.3.4. Experimental set up

Once the insects reached late 2nd instar, both symbiotic and dysbiotic treatments were further sub-divided into two treatments, one of which was infected with the parasite, resulting in a full-factorial design of four treatments per egg clutch, i.e. symbiotic insects with parasites (S+ P+), symbiotic insects without parasites (S+ P-), dysbiotic insects with parasites (S- P+) and dysbiotic insects without parasites (S- P-). Before infection, the insects were starved and deprived of water for 24 hours, after which parasite infection was conducted by feeding insects with a mixture of live parasite culture and crushed linden seeds. An infection load of ~2.4 x 10^3 flagellates was used per insect, as estimated by Neubauer improved

cell counting chamber (Hirschmann techcolor, Germany) under a Zeiss Axio Vert.A1 inverted microscope. This load had been established as the minimal infective dosage that did not result in host mortality. For non-infected groups, the live parasite culture was replaced with sterile *Crithidia* medium used to culture the parasites. The developmental time to adulthood and survivorship of the insects for three weeks after infection was recorded.

4.3.5. DNA extraction and quantitative PCR

Two days after adult emergence, the insects were collected and stored at -60˚C before they were subjected to DNA extraction using the MasterPure™ DNA Purification Kit (Epicentre Technologies) according to the manufacturer's instructions. For the quantification of *L. pyrrhocoris* parasites and gut bacterial symbionts, qPCR targeting the 18S rRNA and 16S rRNA genes, respectively, were set up with specific primers (Table 1). Primers targeting the 16S rRNA gene were designed using sequences of bacteria symbionts isolated from *D. fasciatus* or *P. apterus,* while firebug 18S rRNA primers were designed using consensus 18S rRNA sequences of *D. fasciatus, P. apterus* (red soldier bug) and *Probergrothius angolensis* (*Welwitschia* bug). Gene bank accession numbers of sequences used to design primers are listed in table 1. All primers were designed and specificity determined with Primer-BLAST (Ye et al., 2012). Additionally, specificity was confirmed by blasting sequences of the PCR products amplified by the primers. qPCR was conducted on a Rotor-Gene Q cycler (Qiagen, Hilden, Germany) with a final volume of 10 µl containing 0.5 µl of each primer (10 µM), 5 µl SYBR-mix, 3 µl of qPCR H_2O and 1 µl of either template or standard or negative control (H_2O). The following cycling conditions were used for *L. pyrrhocoris, Gordonibacter* sp.*, Klebsiella* sp.*,* and firebug specific primer sets: 95˚C for 5 mins followed by 95˚C denaturation for 10 secs, 68-60˚C touchdown annealing for the first eight cycles, then 60˚C annealing for the remaining 37 cycles for 15 secs, extension of 72˚C for 10 secs, and a final melting curve analysis from 65˚C to 99˚C with a temperature raise of 1˚C for each step. As for *C. glomerans* and *Hungatella* sp. primers assays, the same conditions were used, but annealing was adjusted to 70-64˚C touchdown for the first six cycles, then 64˚C for the remaining 39 cycles. Quantification of each target gene was performed with the Rotor-Gene Q software using their respective external standard curves amplified with similar conditions from serial dilutions (10^{10} to 10^2 copies/µl) of purified PCR product of that gene. Concentration of each gene for every sample was normalized with the respective concentration of the host 18S rRNA gene.

Table 1: Primers and probes used for the quantification (qPCR), high-throughput profiling (Illumina sequencing), and localization (FISH) of *D. fasciatus* gut bacteria and *L. pyrrhocoris* parasite.

qPCR Primers			
Name	**Sequence (5' to 3')**	**Reference**	**Target**
SSU_Fwd_4	CGCGAAAGCTTTGAGGTTAC	(44)	*L. pyrrhocoris* 18S rRNA
Lepto-R	TTGGATCTCGTCCGTTGAC		
Clost_243_Fwd	CGTCTGATTAGCCGGTTGG	This study (JX406495.1)	*Hungatella* sp. 16S rRNA
Clost_359_Rev	TGCCTCCCGTAGGAGTTTG		
Dfas_Kleb1308_F	GGATCAGAATGCCACGGTGA	This study (JX406498.1)	*Klebsiella* sp. 16S rRNA
Dfas_Kleb1414_R	CGCCCTCCCGAAGGTTAAG		
Gord-Uni-For	GCATCGGGATAACGCAAGGA	This study (KP142900.1)	*Gordonibacter* sp. 16S rRNA
Gord_278_Rev	AGTCTGGGCCGTATCTCAGT		
Corio 16S-2F	GGTACAGCGGGATGCGATG	This study (FJ554837.1)	*C. glomerans*. 16S rRNA
Cor_Dfas_1R	CCCCGTGAGGGTTGGGCC		
Firebug18S-1F	CGGTGCTCTTTACCGAGTGT		Firebug 18S rRNA

Firebug18S-1R	AACGTCGCAATACGAATGCC	This study (KP142855.1, KP142869.1 & KP142870.1 consensus)	
Sequencing primers (annealing region)			
U515F	GTGYCAGCMGCCGCGGTA	(50)	Universal bacteria 16S rRNA
U927R	CCCGYCAATTCMTTTRAGT		
Probes			
Lepto-R-Cy5	CY5-TTGGATCTCGTCCGTTGAC	(44)	*L. pyrrhocoris* 18S rRNA
Cor653-Cy3	CY3-CCCTCCCMTACCGGACCC	(46)	*C. glomerans*. 16S rRNA

4.3.6. Fluorescence *in situ* hybridization (FISH)

Nymphs with normal gut bacterial symbionts were collected from our main cultures, infected with *L. pyrrhocoris* parasites as described above and then allowed to mature. Adult insects were anaesthetized with CO_2 gas for three minutes and then the M3 section was dissected out on a cold block under aseptic conditions and fixated in formaldehyde (4% in PBS) at 4˚C for 24 hours and subsequently washed in running water for 20 mins to remove fixative. The samples were then dehydrated with increasing concentrations of butanol (30%, 50%, 70%, 80%, 90%, 96%, and absolute butanol) at room temperature and transferred into infiltration solution (50 ml Technovit 8100 basic solution + 0.3 g hardener I) for 24 hours at 4˚C and embedded in 967 µl of infiltration solution containing 33 µl of hardener II for 24 hours. After polymerization, the guts were sectioned with a Microm HM355S microtome (Thermo Fisher Scientific, Germany) and mounted on glass slides coated with poly-L-lysine (Kindler, Germany). FISH was conducted on the gut sections to localize the *C. glomerans* symbiont and the *L. pyrrhocoris* parasite within the *D. fasciatus* M3 mid-gut region according to a previously described protocol (Kaltenpoth et al., 2009). As we were interested in the localization of *L. pyrrhocoris* in relation to the bacterial symbionts present in the lumen and the peritrophic matrix along the gut walls, we selected *C. glomerans* which has been reported in both sites (Sudakaran et al., 2012). Cor-653R-Cy3 labelled probe (Sudakaran et al., 2012) and Lepto-R-Cy5 labelled probe (Salem et al., 2015) were used to stain *C. glomerans* and *L. pyrrhocoris,* respectively, while DAPI (4', 6-diamidino-2-phenylindole) was used for general DNA staining.

4.3.7. High-throughput microbiota profiling

We pooled DNA samples, one from each replicate, for the two symbiotic treatments (S+ P+ and S+ P-) and performed Illumina sequencing of bacterial 16S rRNA amplicons to establish overall effects of parasite infection on the gut microbial community composition. The variable V4-V5 region of the 16S rRNA gene was amplified with universal primers, 515F and 909R (Ellis et al., 2013) and sequenced on Illumina Miseq V3 platform (StarSEQ, Mainz, Germany). The resulting sequences were analyzed with QIIME2 v.2018.2 (Caporaso et al., 2010). DADA2 (Divisive Amplicon Denoising Algorithm 2) method was used for dataset denoising, filtering sequences, removing chimeras, read-pairing, 100% OTU clustering and picking representative sequence from each cluster (Callahan et al., 2016). The resulting sequences were assigned taxonomy with the Silva 128 release database using 99% consensus taxonomy (Quast et al., 2012; Yilmaz et al., 2013). Mitochondria and chloroplast OTUs were discarded and the resulting feature tables were then used to visualize the data.

4.3.8. Data analysis

With the dependent design of four treatments (symbiotic and dysbiotic with or without parasites) for each egg clutch, Friedman and Wilcoxon-matched pairs tests would have been the suitable tests to assess

differences across treatments. However, we did not use these statistical tests because of low or no survivorship in some replicates of dysbiotic groups, which resulted in missing data for some replicates that impairs the use of tests relying on dependent data. Instead, differences in developmental time, firebug 18S rRNA copies and gut microbe abundances between treatments were tested using Kruskal-Wallis test with Dunn's *post hoc* tests with Holms correction for multiple comparisons, while differences in parasite titers between symbiotic and apo-symbiotic parasite-infected treatments were tested with Wilcoxon-Mann-Whitney-test using BiAS v. 7.40 software. Survival probabilities between the four treatments were plotted based on Kaplan-Meier models using the rms package (Harrell, 2013) as implemented in R 3.4.1, while pair-wise differences in survivorship and effect of symbiont and/or parasite interactions on survivorship were checked by cox mixed-effects models using the coxme package (Therneau, 2015) as implemented in R 3.4.1.

4.4. Results

A total of 149 insects emerged from the four treatments (S+ P- = 57, S+ P+ = 53, S- P- = 19, and S- P+ = 20). Time to adulthood data for all 149 insects was used to compare developmental time across treatments, while survival probabilities were calculated using insect survivorship data until week three after the commencement of the experiment. DNA was successfully extracted from 133 insects that emerged (S+ P- = 48, S+ P+ = 51, S- P- = 15, and S- P+ = 19) and used to quantify parasite and symbiont titers.

4.4.1. Symbiont and parasite localization

Fluorescence *in situ* hybridization localized both the *C. glomerans* symbiont and the *L. pyrrhocoris* parasites in the M3 region of the mid-gut. *C. glomerans* was abundantly present on the peritrophic matrix lining the gut epithelial cells, while *L. pyrrhocoris* was predominantly localized within the M3 gut lumen, away from the gut walls (Figure 1).

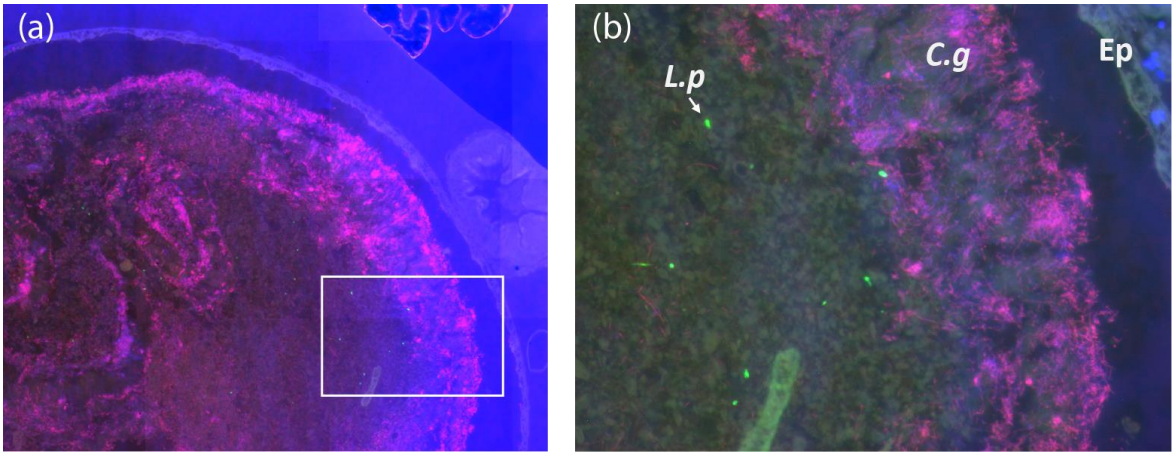

FIGURE 1: Parasite and symbiont localization in the M3 region of *D. fasciatus'* mid-gut. M3 gut cross-section (a) with the rectangle highlighting magnified region (b). *C. glomerans* cells (*C.g.,* magenta) are abundantly present on the peritrophic matrix lining the gut epithelial cells (Ep) of the gut wall, while *L. pyrrhocoris* (*L.p.,* green) flagellates are located in the M3 lumen (see arrow for an example).

4.4.2. Parasite titers of dysbiotic and symbiotic insects infected with *L. pyrrhocoris*

To determine whether gut bacterial symbionts interfere with *L. pyrrhocoris'* establishment or development within *D. fasciatus* hosts, we compared normalized parasite titers of emerged individuals from the two

parasite infected treatments (Figure 2). The absolute abundance of the gene used for normalization, the firebug 18S rRNA gene, was not significantly different across treatments (p = 0.07, Kruskal-Wallis H-test) (Supporting Information Figure 1). However, parasite titers of dysbiotic insects (S- P+) were significantly higher by more than one order of magnitude compared to those of symbiotic counterparts with intact gut bacterial community (S+ P+) (p = 0.008, Wilcoxon-Mann-Whitney-test).

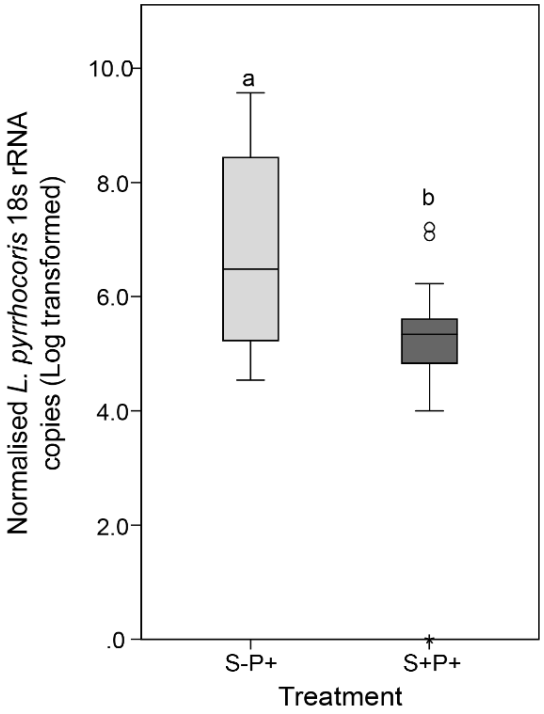

FIGURE 2: *L. pyrrhocoris* titers of parasite infected *D. fasciatus* with perturbed (S- P+) or native (S+ P+) gut bacterial community. Significant differences are represented by different letters above boxes (Wilcoxon-Mann-Whitney-test, p = 0.008). S+ P+ = symbiotic with parasite, S-P+ = dysbiotic with parasite. The circles are outlier values and the star is an extreme value.

4.4.3. Parasite and symbiont effect on host development and survival

To test the effect of symbiont removal and subsequent *L. pyrrhocoris* parasite infection on insect development, we compared developmental time to adulthood for the 149 insects that emerged from all four treatments (Figure 3). A Kruskal Wallis H-test revealed that developmental times differed significantly between treatments ($p < 0.05$). Dunn's *post hoc* tests with Holm correction for multiple comparisons showed that symbiotic insects without parasites (S+ P-) developed faster than symbiotic insects with parasites (S+ P+), dysbiotic insects with parasites (S- P+) and dysbiotic insects without parasites (S- P-) ($p < 0.05$). However, developmental times did not differ significantly between symbiotic insects with parasites (S+ P+) and both dysbiotic treatment insects (S- P+ and S- P-) ($p > 0.05$).

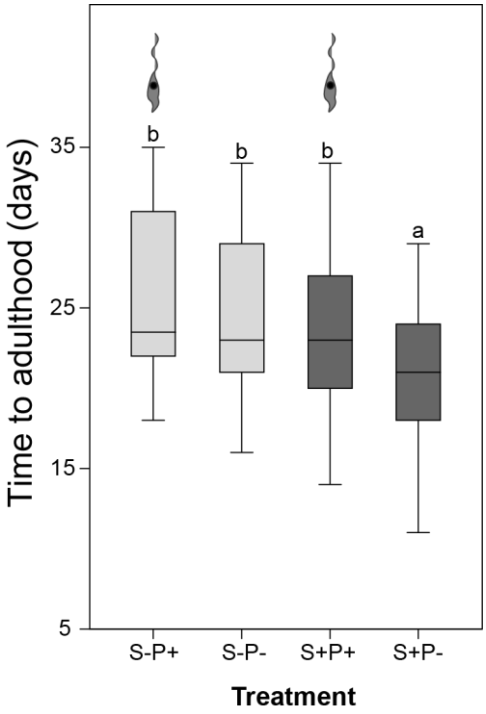

FIGURE 3: Effect of *L. pyrrhocoris* parasite infection on *D.fasciatus* developmental time. Symbiotic insects without parasites (S+ P-) developed significantly faster than symbiotic insects with parasites (S+ P+) and both dysbiotic insects with and without parasites (S- P+, S- P-). Significant differences are represented by different letters above boxes (Kruskal-Wallis H-test with Dunn *post hoc* tests with Holm correction for multiple comparisons, $p < 0.05$).

To elucidate the effect of symbiont presence or absence and parasite infection status on host mortality, we compared host survivorship across the four treatments for three weeks post-parasite exposure (Figure 4). A Cox mixed-effects model revealed that host survival was only significantly affected by symbiont status ($p = 0.004$), but not significantly affected by either parasite status (p=0.073) or symbiont-parasite interactions ($p = 0.83$). However, parasite-infected individuals generally showed lower survivorship in both symbiotic and dysbiotic treatments (Figure 4).

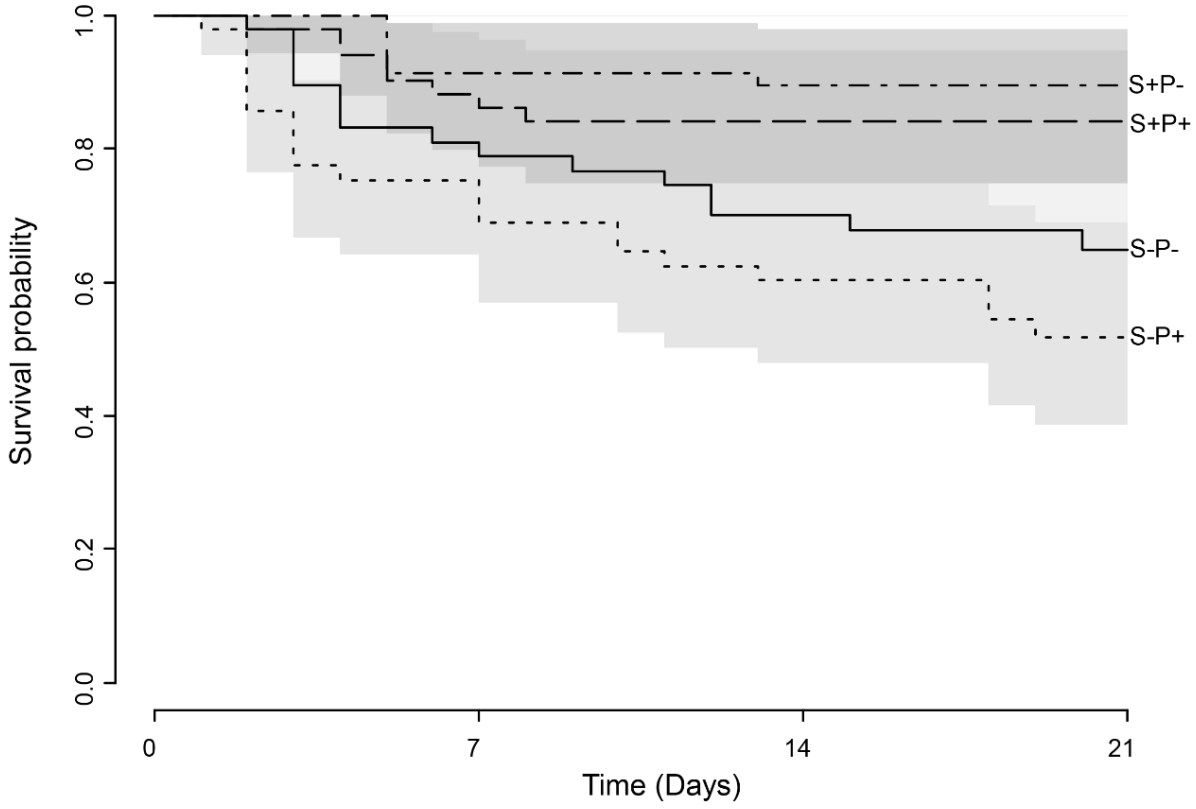

FIGURE 4: *D. fasciatus* survival after egg surface sterilization and/or *L. pyrrhocoris* parasite exposure. Symbiont presence significantly influenced host survival (p = 0.004), but parasite infection (p = 0.073) or symbiont and parasite interaction (p=0.83) did not (Cox mixed effects model). S+ P+ = symbiotic with parasite, S+ P- = symbiotic without parasite, S- P+ = dysbiotic with parasite, and S- P- = dysbiotic without parasite.

4.4.4. Effect of egg surface sterilization and parasite infection on gut microbiota

The egg surface sterilization protocol successfully decreased the abundance of all four core members of the *D. fasciatus* gut bacterial community, as expected (Figure 5). Normalized abundance of *Hungatella* sp. (Figure 5a), *Klebsiella* sp. (Figure 5b), and *C. glomerans* (Figure 5d) 16S rRNA copies were significantly different between treatments ($p<0.01$, Kruskal Wallis H test). However, Dunn's *post hoc* tests revealed that only *Hungatella* sp. and *Klebsiella* sp. symbionts were significantly reduced in both dysbiotic treatments when compared to the two symbiotic treatments ($p<0.01$) (Figure 5a,b). Although the abundance of *Gordonibacter* sp. and *C. glomerans* was also decreased in dysbiotic treatments, this difference was not significant for both S+ P- and S+ P+ in the case of *Gordonibacter*, and for S+ P+ in *Coriobacterium,* because some untreated individuals showed low bacterial titers (Figure 5c-d) ($p > 0.05$).

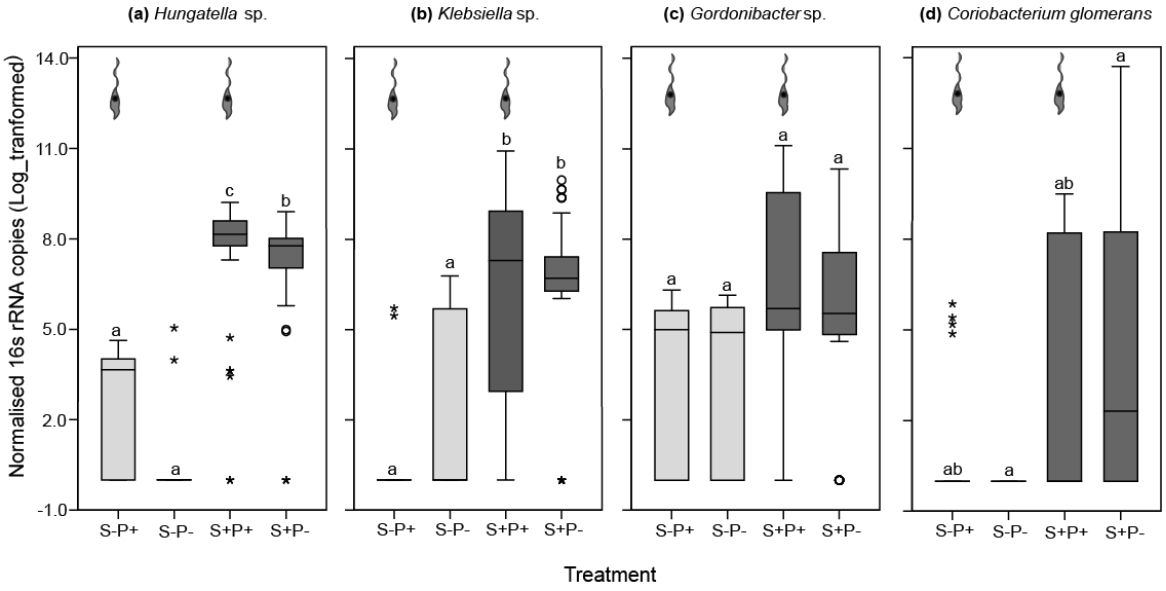

FIGURE 5: Impact of egg surface sterilization and/or *L. pyrrhocoris* parasite exposure on the abundance of *D. fasciatus* core gut symbionts. Egg surface sterilization reduced the abundance of (a) *Hungatella* sp. (b) *Klebsiella* sp. (c) *Gordonibacter* sp. and (d) *C. glomerans* gut symbionts, but this reduction was not significant for *Gordonibacter* sp. as well as the parasite-containing *C. glomerans* treatment. Parasite infection in symbiotic insects increased the abundance of *Hungatella sp.* (a), but had no significant effect on other symbionts (b, c, d). Significant differences are represented by different letters above boxes (Kruskal-Wallis H-test with Dunn's *post hoc* tests with Holm correction for multiple comparisons, $p < 0.05$). S+ P+ = symbiotic with parasite, S+ P- = symbiotic without parasite, S- P+ = dysbiotic with parasite, and S- P- = dysbiotic without parasite. The circles are outlier values and the stars are extreme values.

To assess the effect of parasite infection on *D. fasciatus'* native gut microbiota, we compared the abundance of the four core gut bacterial symbionts between the two symbiotic treatments (with and without parasites). Dunn's *post hoc* tests with Holm correction for multiple comparisons after Kruskal Wallis H-tests revealed that *L. pyrrhocoris* parasite infection significantly increased the abundance of *Hungatella* sp. ($p = 0.023$) but did not significantly affect *Klebsiella* sp., *Gordonibacter* sp., or *C. glomerans* titers ($p > 0.05$). Illumina sequencing results confirmed the increased abundance of *Hungatella* after parasite infection, while the other core microbes experienced smaller differences in abundance (Supporting Information Figure 2). Additionally, parasite infection resulted in a decrease of gut microbiota diversity as demonstrated by a lower number of bacteria genera recorded in parasite infected animals (Supporting Information Figure 2).

4.5. Discussion

In this study we investigated complex multipartite interactions between *D. fasciatus* and its closely associated gut bacteria and intestinal parasites. Specifically, we sought to; (a) determine parasite localization in relation to gut bacteria symbionts, (b) investigate whether gut bacterial communities play a role in inhibiting *L. pyrrhocoris* parasite infections, and (c) understand effects of this trypanosomatid infection on the host and its gut microbes.

With FISH experiments, we demonstrated that *C. glomerans* symbionts colonize the M3 mid-gut region as previously described (Sudakaran et al., 2012), together with *L. pyrrhocoris* parasites. *C. glomerans* symbionts were mainly localized on the peritrophic matrix along the gut epithelial wall, while *L. pyrrhocoris* parasites were mostly present in the gut lumen (Figure 1). By contrast, studies on the localization of a

closely related parasite, *Leptomonas wallacei*, in milkweed bugs (*Oncopeltus fasciatus*, Hemiptera: Lygaeidae) revealed that the parasite is attached directly to the perimicrovillar membranes of the midgut (Romeiro et al., 2003). However, no study has reported on the presence of bacterial symbionts in this insect yet, so it is conceivable that the localization of bacterial symbionts on the peritrophic matrix along the gut epithelium displaces *L. pyrrhocoris* and prevents them from colonizing this niche in *D. fasciatus*. Co-localization of parasites with bacterial symbionts in pyrrhocorid bugs is relevant for their vertical and horizontal co-transmission, since the parasites can be easily transferred along with the bacteria to the feces, which are applied to the egg surface by female bugs and exchanged via coprophagy (Frolov et al., 2017; Salem et al., 2015).

The gut bacterial community of *D. fasciatus* is well characterized with *Hungatella* sp. *Gordonibacter* sp., *Klebsiella* sp. and *C. glomerans* symbionts being the consistently present bacteria taxa (Salem et al., 2013; Sudakaran et al., 2015, 2012). The Coriobacteriaceae symbionts, *C. glomerans* and *Gordonibacter* sp., are essential in supplementing hosts with B-vitamins (Salem et al., 2014, 2013). However, our new findings reveal an additional role of the gut bacterial community of acting as an extended "immune phenotype", which hinders successful *L. pyrrhocoris* parasite establishment or proliferation within the host. We demonstrate experimentally that dysbiotic insects possess higher parasite titers compared to insects with intact gut microbiota upon artificial infection with *L. pyrrhocoris* parasites, suggesting a direct or indirect effect of the bacterial symbionts on the parasite. Antagonistic interactions of insect bacterial symbionts and host pathogens occurs through the production of bioactive metabolites, competition for host resources, host immune priming or through improving host vigor (Flórez et al., 2015; Gerardo et al., 2014). In *D. fasciatus*, the occurrence of bacterial symbionts on the peritrophic matrix lining the gut wall is the most likely explanation for variability in the observed parasite titers. Trypanosomatids are known to colonize the guts of insects by adhering to the midgut peritrophic matrix (Frolov et al., 2017; Hamilton, Votypka, et al., 2015; Maslov et al., 2013; Romeiro et al., 2003). In fact, mutations in the major component of this matrix, drosocrystallin, increase the severity of entomopathogenic bacterium, *P. entomophilla,* and *Jaenimonas drosophilae* trypanosomatid infections in *Drosophila* (Hamilton, Votypka, et al., 2015; Kuraishi et al., 2011), and knockdown of PpPer1, a molecular component of the peritrophic matrix in the sand-fly *Phlebotomus papatasi,* results in higher *Leishmania* infection loads (Coutinho-Abreu et al., 2013). These findings demonstrate the importance of the peritrophic matrix in mediating trypanosomatid infections. In *D. fasciatus* firebugs, colonization of this matrix by *C. glomerans* (Figure 1) and other members of the core microbiota (Sudakaran et al., 2012) may result in a barrier that can interfere with *L. pyrrhocoris* establishment or proliferation. Limiting the parasites' access to the peritrophic matrix and gut epithelium may result in oxygen deprivation, since the M3 region shows a steep oxygen gradient with an anoxic center (Sudakaran et al., 2012). In the absence of oxygen, metabolism, motility, and proliferation of *Leishmania* promastigotes is strongly inhibited demonstrating that these trypanosomatids depend on respiration for energy generation (Van Hellemond & Tielens, 1997; Van Hellemond & Van Der Meer, et al., 1997), a process that may be shared amongst members of the trypanosomatidae family, including *L. pyrrhocoris* parasites.

Protection of insect hosts by the gut microbiota is not unique to firebugs. Honey bees with disturbed gut microbial communities have a higher susceptibility to *Lotmaria passim* trypanosomatid infections (Schwarz et al., 2016). In the bumble bee, *Bombus terrestris,* inoculation of symbiont-free insects with a complete gut bacterial community through fecal feeding resulted in lower parasite titers compared to insects inoculated with an isolated gammaproteobacterial symbiont or deprived of their core gut bacterial symbionts upon infection with *Crithidia bombi* parasites (Koch et al., 2011). Further studies with field

caught bees showed a negative correlation between *Crithidia bombi* infection and the presence of a betaproteobacterial symbiont *Snodgrassella* (Koch et al., 2011). Additionally, recent results from *Bombus impatiens* studies indicate that a higher microbiome diversity, high bacteria abundance and the presence of *Gilliamella*, *Lactobacillus* Firm-5 and *Apibacter* core gut bacteria is associated with low *Crithidia bombi* infection titers (Mockler et al., 2018). In other *Bombus* species, infection with *Crithidia* is negatively associated with the abundance of *Gilliamella* symbiont and positively associated with the abundance of non-core bacterial symbionts (Cariveau et al., 2014). Honey bee core gut bacterial symbionts (*Gilliamella* and *Snodgrassella*), which are also bumble bee core symbionts, have the potential to form biofilm barriers that may prevent parasite invasions into the epithelial gut wall, thereby protecting the host (Engel et al., 2012). Gut bacteria mediated protection has also been reported in the locust *Schistocerca gregaria.* Infection density with *Serratia marcescens* pathogenic bacteria is negatively correlated with the diversity of the locust's gut bacteria (Dillon et al., 2005).

Successful *L. pyrrhocoris* infection significantly increased host developmental time (Figure 3). These results agree with earlier findings that showed slight increase in developmental time of *Pyrrhocoris apterus* and *Rhodnius prolixus* bugs infected with *L. pyrrhocoris* and specific strains of *Trypanosoma cruzi,* respectively (Peterson et al., 2015; Schaub, 1994). Prolonged development may reduce the reproductive output of individuals by delaying sexual maturity and increasing mortality before reproduction due to a high predation risk (Benrey & Denno, 1997; Prokopová et al., 2010). Additionally, we observed significant differences in developmental times between dysbiotic and symbiotic treatments without parasites (S- P- and S+ P-) (Figure 3), despite supplementing their diet with additional B-vitamins. This suggests that the gut bacteria symbionts may have additional nutritional roles beyond the B-vitamin supplementation that has been previously reported (Salem et al., 2014). There was no significant difference between the dysbiotic (S- P+ and S- P-) treatments suggesting that the trypanosomes have a negative developmental effect on the host only in the presence of gut bacterial symbionts.

Under our laboratory conditions, parasite-infected bugs showed lower survivorship than control individuals, but this difference was not significant (Figure. 4). This finding concurs with high survival rates of *Rhodnius prolixus* bugs infected with certain strains of *Trypanosoma cruzi* (Peterson et al., 2015), suggesting the low virulence of some monoxenous trypanosomatid parasites. However, severity of trypanosomatid infection can be context-dependent (Brown et al., 2000; Schwarz et al., 2016). In bumble bees, virulence of its associated gut trypanosomatid, *Crithidia bombi,* is greatly influenced by host nutritional status. When fed *ad libitum*, infected bumble bees showed no mortality, while under starvation conditions, mortality increased up to 50% (Brown et al., 2000). As for honey bees, susceptibility to *L. passim* infections is determined by stress conditions (Schwarz et al., 2016). Since our experiments were performed in controlled conditions and insects fed *ad libitum*, such condition-dependent fitness consequences of *L. pyrrhocoris* parasite infection would have gone undetected. In addition, the close association of *D. fasciatus* with its gut bacterial symbionts and parasites that are co-localized and co-transmitted together (Salem et al., 2015; Sudakaran et al., 2015) presents opportunities for host/symbiont-parasite co-evolution, which may result in reduced parasite virulence as was shown previously for pathogenic *Staphylococcus aureus* and the *Enterococcus faecalis* protective symbiont that were allowed to evolve together in their *Caenorhabditis elegans* host (Ford et al., 2016). Lastly, the long term culturing of the parasite in a media, outside the host, may have declined its virulence over time and therefore, the observed fitness effects on the host may be underestimated.

Stability of any symbiotic system depends on the homeostatic state of its partners. In addition to habitat, diet, developmental stage and host phylogeny (Yun et al., 2014), also pathogen infections can influence

gut microbial composition in insects. This has been described in *Trypanosoma cruzi* infected triatomine bugs (Díaz et al., 2016), West Nile Virus infected *Culex pipiens* mosquitoes (Zink et al., 2015), *C. bombi* infected bumble bees (Koch et al., 2011) and *L. passim* parasite infected honey bees (Schwarz et al., 2016). In *D. fasciatus,* our results from qPCR and Illumina sequencing of 16S rRNA amplicons revealed that parasite infection has a minor effect on the gut microbiota composition (Figure 5), increasing *Hungatella* abundance and reducing overall diversity (Figure 5 & Supporting Information Figure 2). The increase in *Hungatella* may be as a result of a decrease in competition for resources due to the decrease in bacterial diversity. The non-significant effect we see on most core microbes (Figure 5) may be explained by the time of parasite infection: We infected the insects 10 days (late 2nd instar nymphs) post-hatching, a time when the core bacteria have already been established (Sudakaran et al., 2012). Further studies are necessary to elucidate the effect of simultaneous symbiont and parasite co-establishment or symbiont establishment after parasite infection on the final composition of the gut bacterial community. Parasite infection both during and after establishment of the core symbiotic microbiota are ecologically relevant scenarios, considering the occurrence of vertical parasite co-transmission with the bacterial symbionts via the egg surface as well as horizontal transmission in nymphal stages through the host's feces (Salem et al., 2015).

In conclusion, these results expand our current understanding of the essential role gut bacterial symbionts play in the ecology of their *D. fasciatus* host. In addition to the already established nutritional role (Salem et al., 2014), our results show that the gut bacterial community is integral to *D. fasciatus'* health, as it interferes with parasite proliferation, most likely through competitive exclusion. Antagonistic interactions between symbionts and pathogens are medically relevant, especially in the context of insect-vectored diseases. The study of *L. pyrrhocoris* monoxenous trypanosomatids has enhanced the understanding of *Leishmania* (Flegontov et al., 2016), a dixenous trypanosomatid, which is transmitted by sandflies and is responsible for causing human Leishmaniasis. Therefore, *D. fasciatus* provides a good model system for studying insect-symbiont-pathogen interactions, which can be inferred for controlling insect vectored human and animal pathogens through the generation of paratransgenic bacterial symbionts that lower or alter vector competence. This has been demonstrated in *Rhodnius prolixus,* where its essential symbiont, *Nocardia rhodnii*, has been engineered to interfere with the development of *Trypanosoma cruzi,* the causative agent of Chagas disease in humans (Beard et al., 2001).

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Data accessibility

The following data are available from the Dryad repository (https://doi.org/10.5061/dryad.pp39377): gut microbiota and parasite abundances measured by qPCR, insect developmental time and survivorship, and high-throughput gut microbiota profiling data and analysis.

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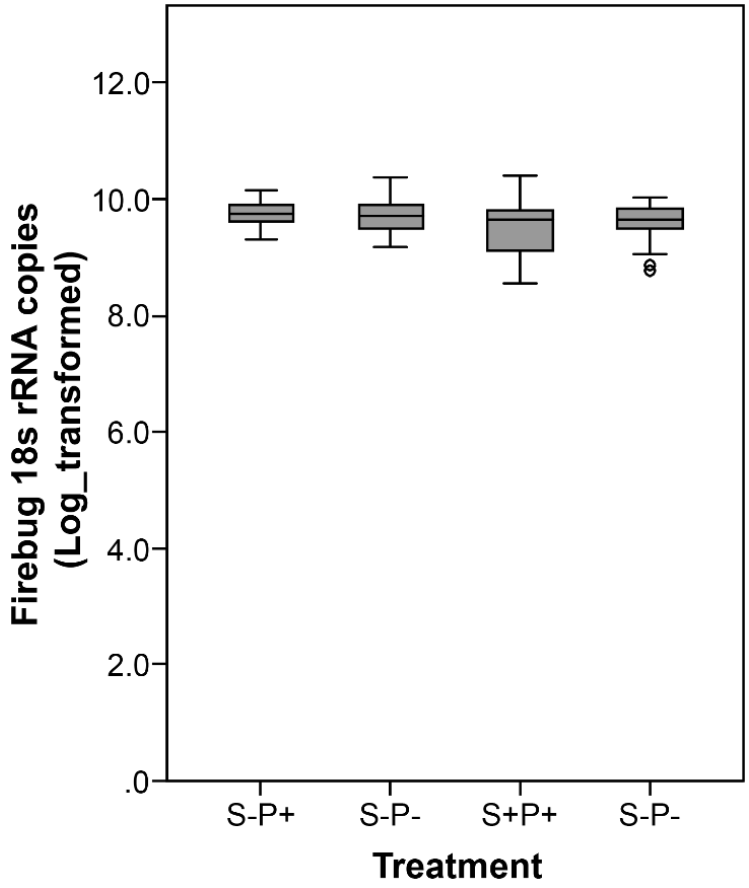
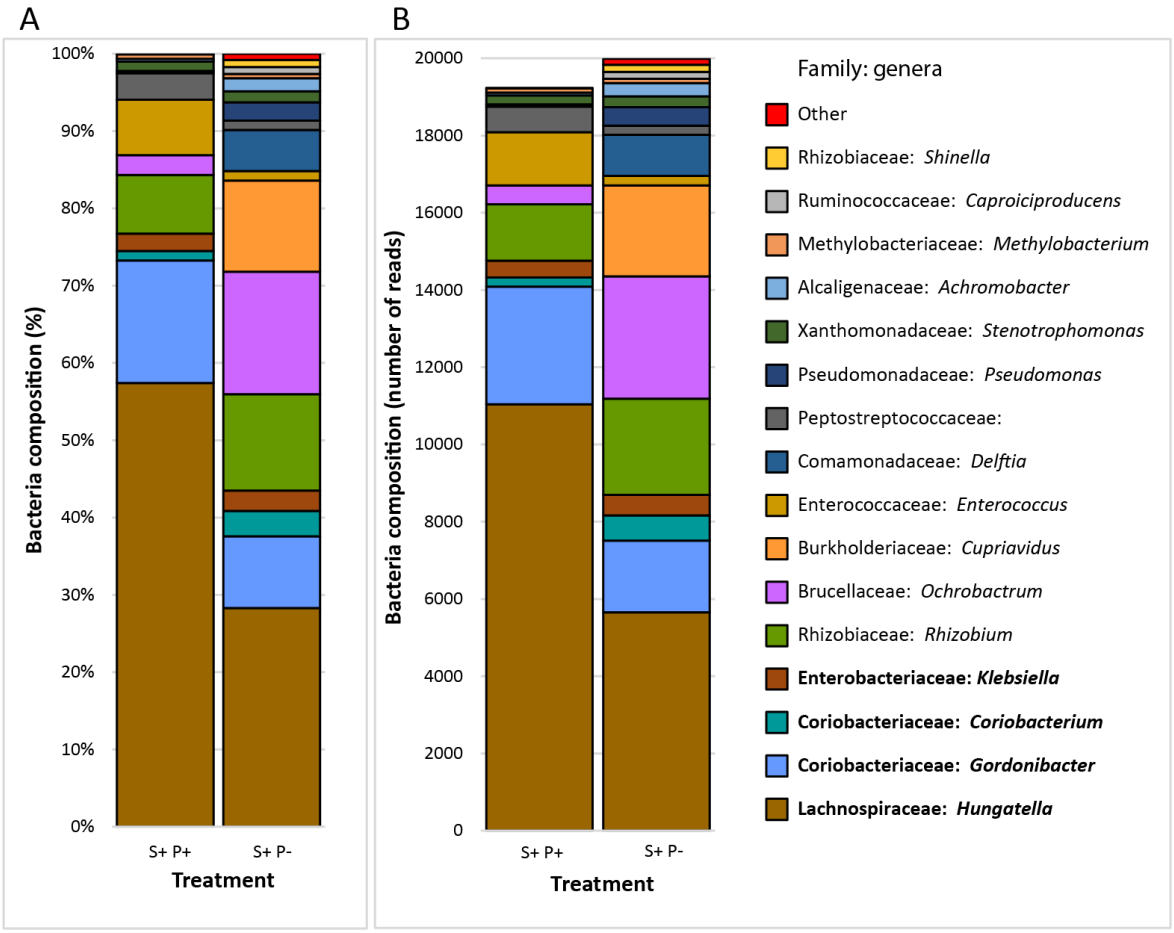
4.7. Supporting Information



SUPPLEMENTARY FIGURE 1: Abundance of the firebug 18S rRNA normalization gene was similar across treatments. (Kruskal-Wallis H-test with Dunn's *post hoc* tests with Holm correction for multiple comparisons, $p > 0.07$).



SUPPLEMENTARY FIGURE 2: Effect of *L. pyrrhocoris* infection on the gut bacterial community composition of *D. fasciatus*. Bacteria composition in percentage (A) and composition in number of reads (B). Infected firebugs show a decreased number of bacterial genera and a higher abundance of *Hungatella*, *Gordonibacter*, and *Enterococcus,* but decreased amounts of *Ochrobactrum, Cupriavidus, Deftia, Coriobacterium,* and *Pseudomonas*.

CHAPTER 5

ESTABLISHED COTTON STAINER GUT BACTERIAL MUTUALISTS EVADE REGULATION BY HOST ANTIMICROBIAL PEPTIDES

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Thomas Ogao Onchuru[1] and Martin Kaltenpoth[1]

[1]Department for Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg University Mainz, Hanns-Dieter-Hüsch-Weg 15, 55128 Mainz, Germany.

5.1. Abstract

Symbioses with microorganisms are ubiquitous in nature and confer important ecological traits on animal hosts, but also require control mechanisms to ensure homeostasis of the symbiotic interaction. In addition to protecting hosts against pathogens, animal immune systems recognize, respond to and regulate mutualists. The gut bacterial symbionts of the cotton stainer bug, *Dysdercus fasciatus,* elicit an immune response characterized by the upregulation of C-type lysozyme and the antimicrobial peptide pyrrhocoricin in bugs with their native gut microbiota compared to dysbiotic insects. In this study, we investigated the impact of the elicited antimicrobial immune response on the established cotton stainer gut bacterial symbiont populations. To this end, we used RNAi to knock down immune genes hypothesized to regulate the symbionts and subsequently measured the effect of this silencing on host fitness and on the abundance of the major gut bacterial symbionts. Despite successful downregulation of target genes by both ingestion and injection of dsRNA, silencing of immune genes neither had an effect on host fitness nor on the qualitative and quantitative composition of established gut bacterial symbionts, indicating that the host immune responses are not actively involved in the regulation of the nutritional and defensive gut bacterial mutualists. These results suggest that close associations of bacterial symbionts with their hosts can result in the evolution of mechanisms ensuring that symbionts remain insensitive to host immunological responses, which may be important for the evolutionary stability of animal-microbe symbiotic associations.

5.2. Importance

Animal immune systems are central for the protection of hosts against enemies by preventing or eliminating successful infections. However, in the presence of beneficial bacterial mutualists, the immune system must strike a balance of not killing the beneficial symbionts while at the same time preventing enemy attacks. Here, using the cotton stainer bug, we reveal that its long-term associated bacterial symbionts are insensitive to the host's immune effectors, suggesting adaptation to the host's defenses, thereby strengthening the stability of the symbiotic relationship. The ability of the symbionts to elicit host immune responses but remain insensitive themselves may be a mechanism by which the symbionts prime hosts to fight future pathogenic infections.

5.3. Introduction

Insects are the most diverse and successful group of animals on earth (Grimaldi & Engel, 2005; Schowalter, 2011). This can be attributed not only to their morphological and behavioral adaptations but also to ecological interactions with other organisms, including microorganisms, which confer insect hosts with novel traits, allowing them to expand into diverse ecological niches (Douglas, 2015; Schowalter, 2011). Insect-associated mutualistic bacteria can be essential for host nutrition and digestion, defense against natural enemies, detoxification of harmful compounds, adaptation to challenging environments, and host behavior manipulation (Berasategui et al., 2017; Bosch & Welte, 2017; Douglas, 2015; Engl, Eberl, et al., 2018; Engl & Kaltenpoth, 2018; Feldhaar, 2011; Flórez et al., 2015; Moran & Yun, 2015). Thus, mutualistic bacteria are integral to the functional ecology of their insect hosts, and concordantly, hosts have evolved mechanisms ensuring reliable and efficient acquisition, maintenance, and transmission of the beneficial bacterial partners (Onchuru, Martinez, Ingham, et al., 2018; Salem, Florez, et al., 2015).

While mutualistic bacteria confer their hosts with novel capabilities, their regulation is essential to avoid uncontrolled proliferation, which can be costly to the host (Oliver et al., 2006). Accordingly, insect hosts have evolved mechanical, nutritional, chemical, and immunological mechanisms to ensure maintenance of mutualistic bacterial populations necessary for their needs. In some symbiotic relationships, insect hosts such as aphids and *Sitophilus oryzae* beetles have evolved specialized cells called bacteriocytes that physically confine and restrict the growth of their bacterial symbionts (Login et al., 2011; Nakabachi et al., 2005). In other mutualistic relationships, hosts are known to restrict their extracellular symbionts in specialized structures such as mid-gut crypts, antennal reservoirs, or larval symbiont bearing organs, as reported e.g. for stinkbugs, beewolves, and *Lagria* beetles, respectively (Flórez et al., 2017; Kaltenpoth et al., 2005; Kikuchi et al., 2011).

In addition to confinement and restriction in specialized cells or structures, availability and amount of essential nutrients required by the microbial partner play an important role in the establishment or proliferation of symbionts (Herren et al., 2014; Wilkinson et al., 2007). For instance, the population densities of *Spiroplasma poulsonii,* the *Drosophila melanogaster* endosymbiont, and *Buchnera aphidicola,* the pea aphid endosymbiont, correlate positively with the host lipid and nitrogen levels, respectively (Herren et al., 2014; Paredes et al., 2016; Wilkinson et al., 2007). This suggests that diet and host nutritional status have an impact on symbiont proliferation. This is supported by simulation experiments by Mitri et al., 2016, who demonstrated that nutrient limitation on microbial colonization surfaces can drive the structure and functioning of microbial assemblages. In addition, intra- and interspecific antagonistic or cooperative interactions amongst co-colonizing symbionts can directly influence their composition and abundance in the host (Scherlach & Hertweck, 2017). The dominant gut bacterial symbionts of the

honey bee, *Snodgrassella alvi* and *Gilliamella apicola,* for example, have complementary metabolic capabilities essential for joint resource utilization and cross-feeding interactions, which subsequently affects their abundance and ability to jointly colonize the host (Kešnerová et al., 2017; W. K. Kwong et al., 2014; Scherlach et al., 2017).

Insects are also known to possess an elaborate innate immune system that not only defends them against pathogens, but also has the ability to recognize and regulate bacterial mutualists (Kim et al., 2015; Login et al., 2011; Nakabachi et al., 2005; Park et al., 2018; Uvell & Engström, 2007). For instance, the *Burkholderia* symbiont of the bean bug *Riptortus pedestris,* which is confined to specialized mid-gut crypts, is highly susceptible to the insect's humoral immune responses (Kim et al., 2016; Park et al., 2018). A strong immunological response characterized by the upregulation of c-type lysozyme and pyrrhocoricin-like and rip-thanatin antimicrobial peptides in the midgut efficiently controls *Burkholderia* symbiont populations in the crypts (Kim et al., 2014; Park et al., 2018). Likewise, *Sitophilus* sp. weevils' *ColA* antimicrobial peptide is not only important for containing the *Sitophilus* primary endosymbiont within the bacteriocyte but also for regulating symbiont growth by inhibiting cell division (Anselme et al., 2008; Login et al., 2011; Maire et al., 2018). While our knowledge of the interactions between the insects' immune system and beneficial microbes has increased considerably in the past decades, a general understanding of the molecular mechanisms underlying the maintenance of a mutualistic microbiota while at the same time ensuring an efficient defense against antagonists remains lacking.

The African cotton stainer bug, *Dysdercus fasciatus* (Hemiptera: Pyrrhocoridae), possesses a simple and stable core bacterial community in the mid-gut, which is composed of *Hungatella* sp., *Klebsiella* sp., *Coriobacterium glomerans*, *Gordonibacter* sp., and unknown Rickettsiales bacteria (Salem et al., 2013; Sudakaran et al., 2012). These gut symbionts supplement the host with B-vitamins that are limiting in their seed-based diet, and they were recently shown to provide protection against a trypanosomatid parasite, *Leptomonas pyrrhocoris* (Onchuru, Martinez, & Kaltenpoth, 2018; Salem et al., 2014, 2013). Due to their functional importance, the symbionts are maintained in host populations through both vertical and horizontal transmission routes (Kaltenpoth et al., 2009; Salem, Onchuru, et al., 2015), which are also exploited by the *L. pyrrhocoris* parasite for its own transmission within *D. fasciatus* populations (Salem, Onchuru, et al., 2015). Dysbiotic insects (deprived of core gut bacteria and parasites) can be generated by interrupting the symbiont and parasite transmission routes (Kaltenpoth et al., 2009; Salem et al., 2013; Salem, Onchuru, et al., 2015), allowing investigation of the gut bacterial symbionts' contribution to host fitness and physiology as well as host-symbiont-parasite interactions. Comparative transcriptomics of cotton stainer insects with native gut bacterial community and dysbiotic insects revealed a differential expression of genes of the insect's innate immune pathways, i.e. Imd, Toll, JAK/STAT, and Phenoloxidase pathways (Bauer et al., 2014). In particular, c-type lysozyme and the antimicrobial peptide (AMP) pyrrhocoricin showed significantly higher expression levels in insects with native bacteria, while the expression levels of the AMPs hemiptericin and defensin were upregulated in dysbiotic insects (Bauer et al., 2014).

Here, we hypothesized that the antimicrobial effectors overexpressed in *D. fasciatus* in the presence of native gut microbial symbionts may be involved in the regulation of the cotton stainer's gut bacterial community. To test this hypothesis, we established an efficient RNAi (RNAi)-mediated gene knockdown procedure, which we used to silence the expression of key immune genes of the Toll and Imd pathways. We subsequently measured the effect of silencing on insect fitness correlates (developmental time, weight and survival rates) and quantified the abundance of core bacterial

community to determine the interaction between the host immune genes and the essential nutritional and defensive gut bacterial symbionts.

5.4. Results

5.4.1. Optimal dsRNA delivery method in cotton stainers

To determine the optimal method for delivering dsRNA to achieve significant knockdown of *D. fasciatus'* genes, we exposed bugs to dsRNA for the c-type lysozyme gene by either feeding or injection and subsequently compared knockdown efficiency and the durations of silencing for both methods for three weeks. Our results show that both feeding and injection efficiently delivered c-type lysozyme dsRNA molecules, resulting in a significant knockdown of up to two orders of magnitude in the first and second week after dsRNA exposure (Figure 1a and b) (Mann-Whitney-U tests, $p < 0.05$). Even though the expression levels for both methods remained lower in the knockdown treatments compared to the controls throughout the third week, the differences were no longer significant (Figure 1a and b) (Mann-Whitney-U tests, $p > 0.05$).

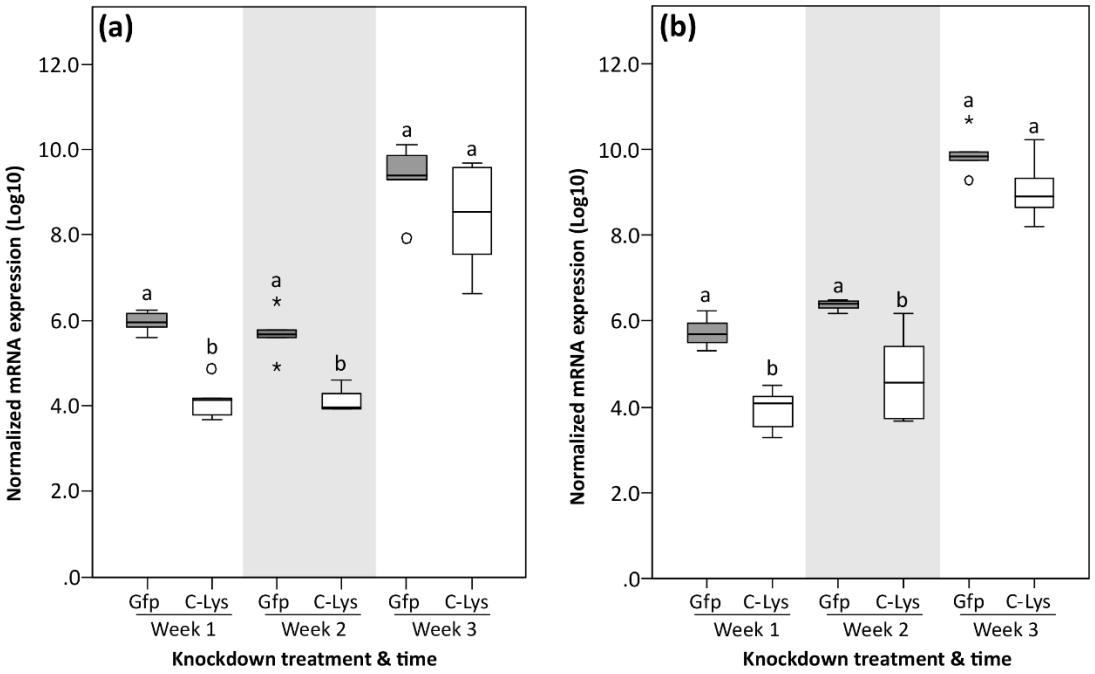

FIGURE 1. Efficiency of RNAi mediated knockdown of D.fasciatus' c-type lysozyme after dsRNA feeding (a) and dsRNA injection (b). Dark boxes represent control knockdown individuals exposed to dsRNA for the green fluorescent protein (Gfp), white boxes show expression levels in bugs treated with dsRNA targeting c-type lysozyme (C-Lys). Significant differences between treatments and controls are represented by different lowercase letters above boxes (Mann-Whitney U test). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the range, circles represent outliers, and stars represent extreme values.

5.4.2. RNAi mediated knockdown of immune genes in *D. fasciatus*

To study the role of the immune system in the regulation of established gut bacterial symbionts, we silenced the expression of candidate immune genes by RNA interference (RNAi) in late 2nd instar *D. fasciatus* nymphs, a stage where the core bacterial community is already mostly established (Sudakaran et al., 2012). By feeding the respective dsRNA to the bugs, we silenced genes encoding the immune effectors c-type lysozyme, pyrrhocoricin, two forms of defensin (defensin 1 and defensin 2), and

hemiptericin (Figure 2, black in grey boxes). We also targeted genes upstream in the Toll and Imd pathways, respectively, encoding Dorsal and Tab (Figure 2, green) that enhance the expression of effector genes, as well as Cactus and NF-kB inhibitor (Figure 2, red) that inhibit the expression of effector genes (Tzou et al., 2002).

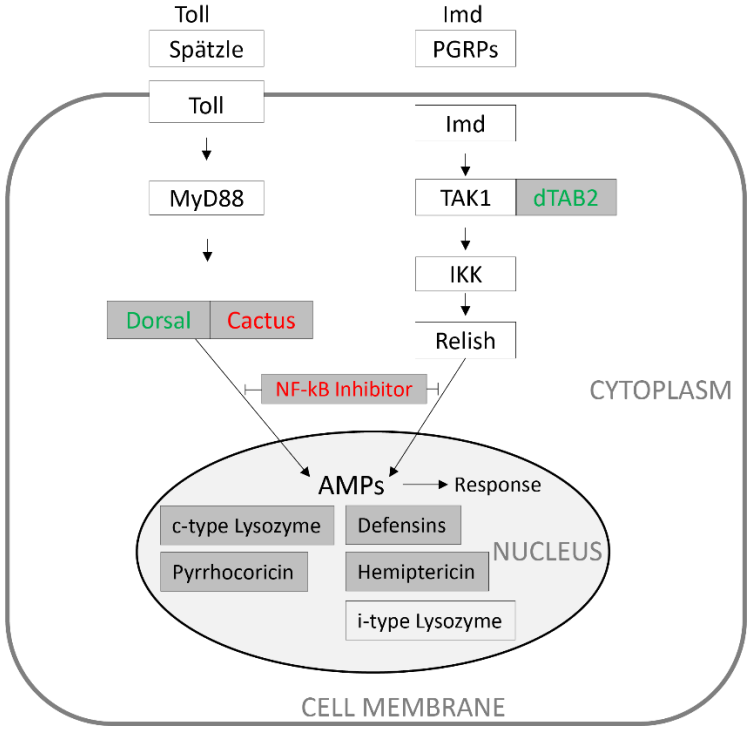

FIGURE 2. Schematic diagram of the insect Toll and Imd pathways, and silenced genes in this study. Grey boxes represent all genes targeted for knockdown, genes in red inhibit transcription of effector genes, while genes in green enhance transcription of effector genes. Figure modified from reference (Bauer et al., 2014).

Quantitative PCRs one week after RNAi treatment revealed that the expression levels of the target genes in the knockdown treatments were lower than that of control individuals fed dsRNA of the green fluorescent protein (GFP) gene by at least one order of magnitude, except for NF-kB inhibitor and hemiptericin. The transcript levels of c-type lysozyme and pyrrhocoricin, which were previously found to be significantly overexpressed in the presence of *D. fasciatus'* native gut bacterial symbionts (Bauer et al., 2014), were significantly reduced by two and one orders of magnitude, respectively, after knockdown (Figure 3e and f). Wilcoxon-signed rank tests revealed that our knockdown strategy significantly decreased the expression levels of all targeted genes except for hemiptericin (Figure 3, Wilcoxon-signed rank test, $p < 0.05$). Although there were lower transcript levels of hemiptericin in the knockdown treatments compared to the controls, this difference was not significant (Figure 3g, Wilcoxon-signed rank test, $p = 0.496$).

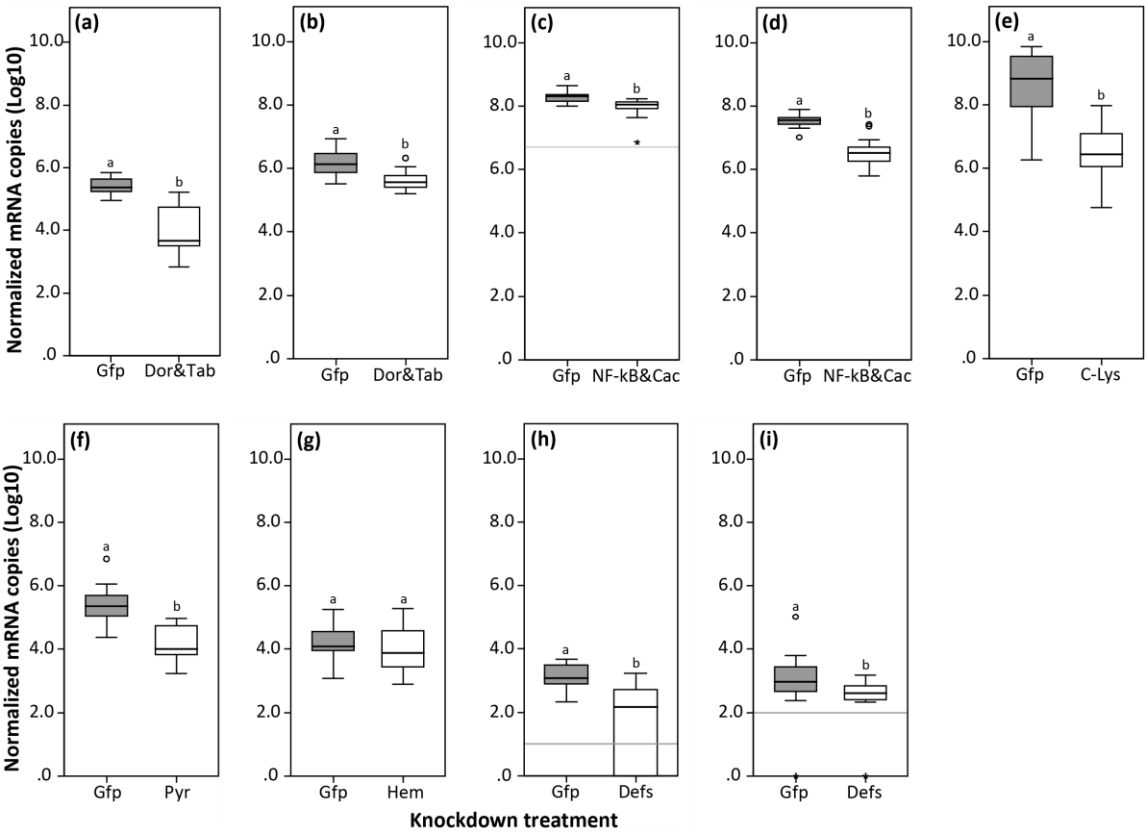

FIGURE 3. Expression of target immune genes in *D. fasciatus* nymphs one week after knockdown. RNAi mediated knockdown resulted in significant decreases in the expression of Dorsal (Dor) (a), Tak1 binding protein (Tab) (b), NF-kappa B inhibitor (NF-kB) (c), Cactus (Cac) (d), c-type Lysozyme (C-Lys) (e), Pyrrhocoricin (Pyr) (f), defensin 1 (h) and defensin 2 (i) (Defs) in comparison to control individuals fed with dsRNA targeting the GFP gene, while Hemiptericin (Hem) (g) showed an insignificant decrease in transcript levels after knockdown. Significant differences are represented by different lowercase letters above boxes (Wilcoxon Signed-Rank Test). Detection threshold is 0 if not indicated by the grey horizontal line (negative control in the qPCR). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the range, circles represent outliers, and stars represent extreme values.

5.4.3. Impact of immune gene knockdown on insect fitness

Once we established that the target genes had been successfully knocked down, we sought to evaluate the effect of knockdown on insect fitness correlates, i.e., developmental time (time between knockdown and adult emergence), weight upon adult emergence, and survival rate until adulthood (survivorship from the time of RNAi treatment until emergence). Across the seven treatments, developmental time and insect weight upon adult emergence were not significantly different (Figure 4a and b) (Friedman test, developmental time: χ^2 (6) = 5.282, p = 0.508; weight at emergence: χ^2 (6) = 8.816, p = 0.184). Similarly, there were no differences in survival rates between each of the six knockdown treatments and the control treatment (Figure 5) (Cox mixed effects model, $p > 0.05$).

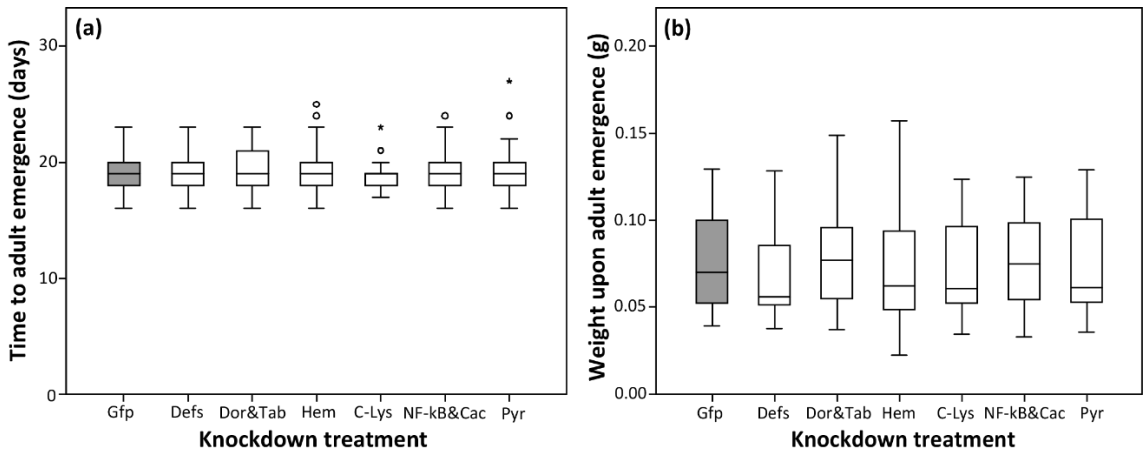

FIGURE 4. *D. fasciatus* developmental times (a) and weights upon adult emergence (b) after RNAi-mediated knockdown of immune genes. Times between gene knockdown and adult emergence (a) and weights after adult emergence (b) were not significantly different across treatments (Friedman test with Dunn-Bonferroni post hoc tests). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the ranges, circles represent outliers, and stars represent extreme values. Gfp, Green fluorescent protein; Defs, Defensins; Dor, Dorsal; Tab, Tak1 binding protein; Hem, Hemipterricin; C-Lys, c-type Lysozyme; NF-kB, NF-kappa B inhibitor; Cac, Cactus; Pyr, Pyrrhocoricin.

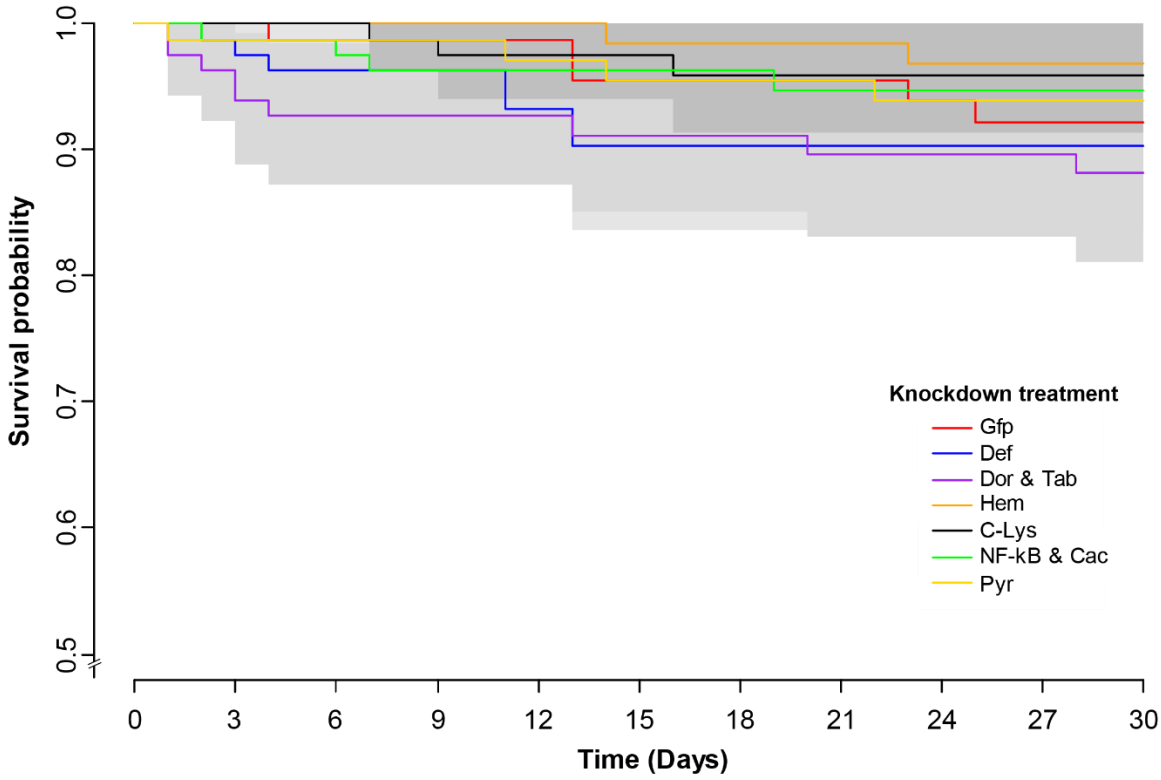

FIGURE 5. *D.fasciatus* survival rates after RNAi mediated knockdown of target immune genes. Survival rates of insects in each immune gene knockdown treatment were not significantly different from those of the control treatment ($p > 0.05$, Cox-mixed effects model). Grey blocks represent 95% confidence levels. Gfp, Green fluorescent protein; Defs, Defensins; Dor, Dorsal; Tab, Tak1 binding protein; Hem, Hemipterricin; C-Lys, c-type Lysozyme; NF-kB, NF-kappa B inhibitor; Cac, Cactus; Pyr, Pyrrhocoricin.

5.4.4. Impact of immune gene knockdown on the composition of the *D. fasciatus* gut bacterial community

Establishment of the core members of the gut bacterial community of firebugs (*Hungatella* sp., *C. glomerans*, *Gordonibacter* sp. and *Klebsiella* sp.) occurs in the 2nd instar stage (Sudakaran et al., 2012). To determine if *D. fasciatus'* immune system is actively involved in the regulation of the already established gut bacterial symbionts, we knocked down the expression of target genes in the late 2nd instar nymphs and quantified by quantitative PCR (qPCR) the 16S rRNA copy numbers of the core gut bacterial symbionts one week after knockdown as well as after emergence as adults. After one week of RNAi knockdown, normalized 16S rRNA copy numbers of *C. glomerans* and *Hungatella* sp. in nymphs were statistically significantly different across treatments (Figure 6a and c) (Friedman test: *C. glomerans*, $\chi 2\,(6) = 16.286$, $p = 0.012$; *Hungatella*, $\chi 2\,(6) = 14.971$, $p = 0.02$). However, Dunn-Bonferroni post hoc tests did not reveal any significant differences between the control and any of the six knockdown treatments. Instead, significant differences were observed between c-type Lysozyme (C-lys) and Dorsal and Tak1 binding protein (Dor&Tab) treatment ($p < 0.05$) as well as c-type Lysozyme (C-Lys) and defensins (Defs) treatment ($p < 0.05$) for *C. glomerans* (Figure 6a) and between Pyrrhocoricin (Pyr) and Dorsal and Tak1 binding protein (Dor&Tab) knockdown treatment for *Hungatella* sp. ($p = 0.05$) (Figure 6c). Although significant, the symbiont abundance in the treatments was decreased by less than one order of magnitude. Normalized 16S rRNA copy numbers of *Gordonibacter* sp. and *Klebsiella* sp. were not significantly different across the seven treatments (Figure 6b and d) (Friedman test: *Gordonibacter*, $\chi 2\,(6) = 4.041$, $p = 0.671$; *Klebsiella*, $\chi 2\,(6) = 12.143$, $p = 0.059$).

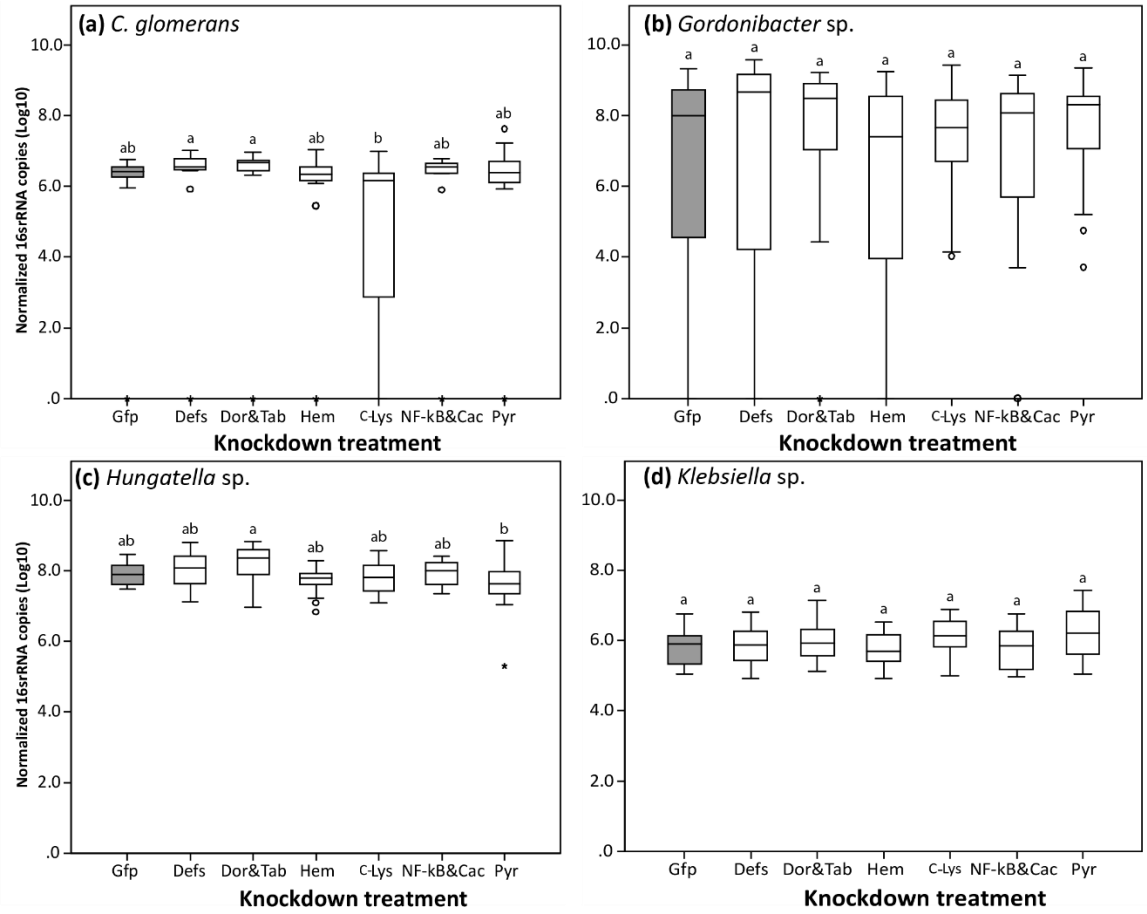

FIGURE 6. Abundances of the core bacterial taxa in *D. fasciatus* nymphs one week after knockdown of key immune genes. Significant differences of 16S rRNA copies of *C. glomerans* (a),

Gordonibacter sp. (b), *Hungatella* sp. (c), and *Klebsiella* sp. (d) as revealed by qPCR are indicated by different lowercase letters above the boxes (Friedman test with Dunn-Bonferroni post hoc tests). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the range, circles represent outliers, and stars represent extreme values. Gfp, Green fluorescent protein; Defs, Defensins; Dor, Dorsal; Tab, Tak1 binding protein; Hem, Hemiptericin; C-Lys, c-type Lysozyme; NF-kB, NF-kappa B inhibitor; Cac, Cactus; Pyr, Pyrrhocoricin.

Similarly, adults that emerged from the dsRNA-treated nymphs showed minor changes in the bacterial community, with only *Hungatella* sp. showing statistically significant differences across treatments (Figure 7c) (Friedman test: $\chi2\,(6) = 16.071$, $p = 0.013$). Dunn-Bonferroni post hoc tests revealed statistically significant differences between the Gfp control treatment and both the Pyr and Defs knockdown treatments ($p < 0.05$). *Hungatella* abundance in these treatments was decreased by about one order of magnitude compared to the Gfp controls. The abundances of *C. glomerans, Gordonibacter,* and *Klebsiella* did not differ significantly across treatments (Figure 7a, b, and d) (Friedman test: *C. glomerans*, $\chi2\,(6) = 12.429$, $p = 0.053$; *Gordonibacter*, $\chi2\,(6) = 7.745$, $p = 0.257$; *Klebsiella*, $\chi2\,(6) = 1.469$, $p = 0.962$).

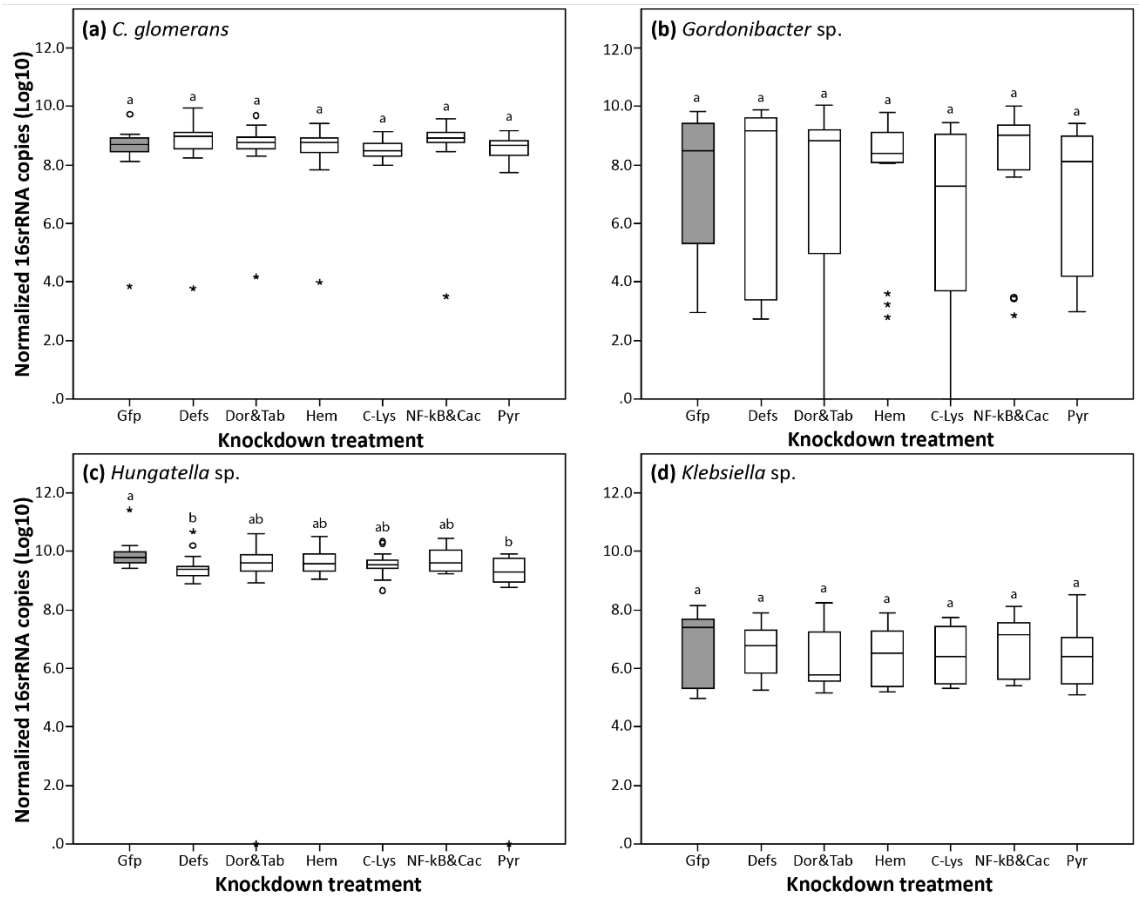

FIGURE 7. Abundances of the core bacterial taxa in adult *D. fasciatus* that emerged from nymphs treated with dsRNA silencing target immune genes. Significant differences of 16S rRNA copies of *C. glomerans* (a), *Gordonibacter* sp. (b), *Hungatella* sp. (c), and *Klebsiella* sp. (d) as revealed by qPCR are indicated by the different lowercase letters above the boxes (Friedman test with Dunn-Bonferroni post hoc tests). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the range, circles represent outliers, and stars represent extreme values. Gfp, Green fluorescent protein; Defs, Defensins; Dor, Dorsal; Tab, Tak1 binding protein; Hem, Hemiptericin; C-Lys, c-type Lysozyme; NF-kB, NF-kappa B inhibitor; Cac, Cactus; Pyr, Pyrrhocoricin.

5.5. Discussion

In addition to defending hosts against pathogenic infections, animal immune systems play an important role in the molecular cross-talk of hosts and their beneficial microbes in many animal-bacterial symbioses (Kim et al., 2015; Login et al., 2011). Here, we studied the interaction of the pyrrhocorid bug *D. fasciatus* with its nutritional and defensive gut bacterial symbionts (*Coriobacterium glomerans, Gordonibacter* sp., *Hungatella* sp., and *Klebsiella* sp.) via the host immune system. Using a target gene knockdown approach, we report that after establishment, *D. fasciatus'* gut bacterial symbionts were not affected by the insect's antimicrobial peptides, although some of them are overexpressed in the presence of the bacterial symbionts. Concordantly, the insect's developmental time, weight gain, and survival rate were not significantly affected by the knockdown of immune genes.

The success of RNAi-mediated gene knockdown in insects is highly variable across species as well as genes or even life stages of the same insect (Scott et al., 2013). This is because of the difficulty associated with the delivery, uptake, processing, and trafficking of dsRNA molecules required to trigger RNAi and the variability in the transcript suppression period (Scott et al., 2013; Shukla et al., 2016). In our experiments, we tested the efficiency of the two traditional dsRNA delivery methods that are commonly used in insect gene function studies to knockdown the expression of important cotton stainer immune genes. Our results show that dsRNA molecules delivered by both injection and feeding were readily taken up by the cotton stainer and processed into siRNAs to initiate posttranscriptional gene silencing as witnessed by the significant knockdown of the target genes (Figures 1 and 3). In other hemipteran insect species such as *Pyrrhocoris apterus* (Pyrrhocoridae), *Oncopeltus fasciatus* (Lygaeidae), and *Rhodnius prolixus* (Reduviidae), RNAi via injection has been used successfully to study genes associated with their growth and development (Hughes & Kaufman, 2000; Konopova et al., 2011). Our results provide more evidence on the applicability of the injection method in the delivery of dsRNA for RNAi studies in heteropteran insects. Additionally, we show that feeding is an equally reliable technique for conducting successful RNAi-mediated silencing experiments, both in terms of the degree and the duration of knockdown (Figures 1). Being a non-invasive and simpler procedure compared to injection, feeding is more applicable in large scale RNAi experiments. For example, in the honey bee (*Apis mellifera)*, where both injection and feeding methods efficiently deliver dsRNA for gene knockdown (Amdam et al., 2003; Hunter et al., 2010), feeding has been used successfully in the ecological application of RNAi in improving honeybee health and resistance against ecologically important viral infections (Hunter et al., 2010).

Removal of the essential cotton stainer gut bacterial symbionts not only affects host fitness but also changes the expression pattern of the host immune genes (Bauer et al., 2014). Transcriptome and qPCR analyses showed a higher expression of c-type lysozyme and pyrrhocoricin in bugs harboring native gut bacteria compared to dysbiotic bugs (Bauer et al., 2014). This suggested that these immune effectors might be involved in the regulation of the beneficial gut bacterial symbionts. Contrary to this expectation, however, knockdown of these immune genes did not have an effect on host insect fitness or on the abundance of the established core gut bacterial symbionts throughout the insect's developmental time (Figures 4-6), indicating that the symbionts are insensitive to the host immune effectors. The cotton stainer's gut bacterial symbionts are important in B-vitamin supplementation and protection against *L. pyrrhocoris* infections (Onchuru, Martinez, & Kaltenpoth, 2018; Salem et al., 2014, 2013). Similarity in insect fitness correlates (developmental time, survivorship, and weight at emergence) between the different knockdown treatments and the control treatment affirms that all seven treatments had access to the essential symbionts supplying B-vitamins required for development. This is corroborated by the

qualitative and quantitative consistency of the core gut bacterial symbionts across the seven treatments (Figure 6 and 7). Thus, the symbionts may have adapted and become insensitive to the host immune responses that they trigger, stabilizing this nutritional and protective mutualism. The ability of the gut bacterial symbionts to elicit the cotton stainer's immune responses may be a mechanism by which they stimulate or prime the firebug immune system to fight pathogenic infections, thereby protecting the host (Emery et al., 2017; Flórez et al., 2015; Weiss et al., 2012). A similar effect has been observed in honey bees where individuals with high levels of hymenoptaecin and apidaecin antimicrobial peptides as a result of harboring native gut bacterial symbionts are better protected upon infection with *Escherichia coli* (Waldan K Kwong et al., 2017).

The insensitivity of gut bacterial symbionts to AMPs as suggested by our results is contrary to results reported for other insects such as bean bugs *(Riptortus pedestris)* and *Sitophilus* grain weevils, where host antimicrobial peptides are actively involved in the regulation of established symbionts (Kim et al., 2016; Login et al., 2011; Maire et al., 2018; Park et al., 2018). Nevertheless, our findings agree with other studies demonstrating resistance of symbionts to host AMPs. Similar to the cotton stainers, honey bees mount an innate immune response against their core gut bacterial symbionts (Emery et al., 2017; Waldan K Kwong et al., 2017). In particular, honey bees harboring natural gut bacterial communities show a higher expression of hymenoptaecin and apidaecin antimicrobial peptides in the gut and hemolymph as compared to bees with perturbed gut bacterial communities (Waldan K Kwong et al., 2017). Investigations into the function of these two antimicrobial peptides through *in vitro* experiments revealed that the major honey bee gut bacterial symbionts (*Snodgrasella alvi*, *Lactobacillus* Firm-5, *Bifidobacterium* sp., and *Gilliamella apicola*) are resistant to one or both AMPs (Waldan K Kwong et al., 2017) suggesting that the elicited AMPs are not directly involved in regulating the symbionts. Similarly, in the tsetse fly *Glossina morsitans morsitans*, the innate immune system does not seem to be involved in the regulation of its *Wigglesworthia* and *Sodalis* endosymbionts, although these symbionts activate the tsetse fly's humoral and cellular immunity (Weiss et al., 2012, 2011). Furthermore, a strong immune response characterized by a higher expression of antimicrobial peptides as a result of *E. coli* and trypanosomatids challenge does not to affect the titers of the two endosymbionts (Rio et al., 2006). Therefore, our findings corroborate these studies demonstrating that bacterial symbionts can be recognized by the host resulting in the activation of the host immune responses to which the bacterial symbionts remain insensitive.

Pathogens successfully infect hosts by evading immune detection due to the lack of immune elicitors or by avoiding killing through the suppression of host defense mechanisms (52). Likewise, long-term co-evolution of beneficial bacterial symbionts with hosts may result in the adaptation of the symbionts to the hosts' immune system in a way that they are not recognized as foreign or remain insensitive to the host immune responses (Pontes et al., 2011; Vallet-Gely et al., 2008). The association of *D. fasciatus* and other pyrrhocorids with their characteristic symbiotic bacteria for approximately 80 million years (Sudakaran et al., 2015) may have resulted in the evolution of resistance mechanisms by the symbionts aimed at evading the host antimicrobial immune responses, as suggested for other symbiotic bacteria (Cullen et al., 2015; Pontes et al., 2011; Vallet-Gely et al., 2008). For instance, *Sodalis glossinidius,* a secondary endosymbiont of the tsetse fly encodes a gene cluster whose expression products are responsible for the modification of the negative charge of lipid A, a component of the cell membrane lipopolysaccharides (Pontes et al., 2011). This modification interferes with the ability of the tsetse fly's cationic AMPs to bind to the endosymbiont's cell membrane resulting in resistance to these AMPs (Pontes et al., 2011). Mutation of the regulatory systems for this gene cluster results in the susceptibility of *S. glossinidius* to the host's cationic AMPs and inability to colonize the host (Pontes et al., 2011).

Similarly, *Bacteroides thetaiotaomicron,* a human gut bacterium, encodes an enzyme responsible for the alteration of the negative charge on the lipopolysaccharides leading to decreased binding ability of AMPs responsible for membrane disruption (Cullen et al., 2015). In *Riptortus pedestris*, the composition of the cell membrane lipopolysaccharides of its *Burkholderia* symbiont is not only essential for successful host colonization but also for symbiont titer regulation (Kim et al., 2015). As some of the cotton stainer gut bacteria are not easily cultivable, it is difficult at the moment to investigate whether any changes in their cell membrane composition are responsible for the ability of the gut bacterial symbionts to evade regulation by host AMPs.

Our findings do not, however, rule out other possible host mechanisms regulating the bacterial symbionts. Other than activating the expression of AMPs, the gut bacterial symbionts may also elicit other immune responses such as the production of reactive oxygen species and cellular immune mechanisms, which could be important in symbiont regulation (Tzou et al., 2002). In addition to providing a surface area for the adherence of gut bacterial symbionts, the peritrophic matrix of *D. fasciatus* provides the physico-chemical conditions and nutrients that can influence symbiont growth dynamics (Mitri et al., 2016; Sudakaran et al., 2012). For instance, the oxygen gradient present in the M3 mid-gut region of firebugs can act as a selection and regulation mechanism that influences microbial community growth dynamics within the mid-gut (Onchuru, Martinez, & Kaltenpoth, 2018; Sudakaran et al., 2012). Additionally, co-colonization of the cotton stainers' peritrophic matrix by the different members of the gut bacterial community presents an opportunity for either antagonistic or synergistic interactions between symbionts that can greatly influence composition and stability of the gut microbial community (Kešnerová et al., 2017; Speare et al., 2018).

In conclusion, our results indicate that the cotton stainer AMPs do not regulate already established gut bacterial symbionts, as knockdown of the AMPs that are overexpressed in the presence of the symbionts (or any other AMPs) did not change the qualitative and quantitative composition of the gut bacterial community. We speculate that the ability of the gut bacterial symbionts to elicit host immune responses may be a mechanism of immune priming to enhance the host's protection against future pathogenic infections. The insensitivity of the symbionts to the host immune responses may be due to the evolution of resistance against the host AMPs during the long term association of the symbionts with the host. However, colonization succession studies of firebug gut bacterial symbionts show that the core gut bacterial symbionts are qualitatively and quantitatively established in the 2nd instar (Sudakaran et al., 2012). Since we performed our knockdown experiments in the late 2nd instar stage when the core symbionts had already established, we cannot rule out that the investigated AMPs are important in shaping the gut microbial community during host colonization and early development. We therefore propose further investigation into the role of AMPs before and during symbiont establishment.

5.6. Materials and methods

5.6.1. Insect culture source

We used cotton stainer insect cultures that are currently maintained in the laboratory at the Johannes Gutenberg University Mainz. The insects were originally collected in Comoé National Park, Côte d'Ivoire in 2001 and were previously maintained at the University of Würzburg, Germany, and at the Max Planck Institute for Chemical Ecology, Jena, Germany. The composition and the abundance of the cotton stainer's gut microbial community remained unaffected despite the long-term maintenance in the laboratory (Sudakaran et al., 2015). All experiments in this study were conducted in Fitotron® standard

growth chambers (Weiss Technik, Leicestershire, UK) under controlled environmental conditions: temperature of 26˚C, 60% humidity, and long light regimes of 16-hour day and 8-hour night.

5.6.2. Double stranded RNA (dsRNA) preparation

To generate dsRNA for the target genes, we used *D. fasciatus* transcript sequences from a previous transcriptomic study (Bauer et al., 2014). Candidate sequences for dsRNA generation were first checked for the possibility of off-target knockdown by blasting them against a local BLAST database generated using all *D. fasciatus* transcript sequences. Only the unique regions of these sequences that had no matching sequences in the database other than the intended target were selected for further processing. Using Primer-BLAST, we designed specific primers for these unique regions of the target sequences and used them for a diagnostic PCR. A 12.5µl PCR reaction was set up containing 6.4µl H_2O, 1.5µl reaction buffer S, 1.5µl dNTPs mix, 1µl of each primer (10µM), 0.1µl of Peqlab DNA polymerase (Peqlab, Erlangen, Germany) and 1µl of the cDNA template synthesized from *D. fasciatus* total RNA. The following PCR conditions were used: denaturation at 94°C for 30s, annealing at 58°C for 40s, extension at 72°C for 45s, and a final extension at 72°C for 4 minutes. The PCR product was run on an agarose gel, purified with the innuPREP PCRpure kit (Analytik Jena, Jena, Germany), and sequenced with an ABI 3130 capillary sequencer (Applied Biosystems, California, USA). Specificity of the primers was further confirmed by blasting the primer sequences and their respective PCR products against the local database. Once the primers' specificity was confirmed, T7 RNA polymerase promoter sequence was added to the primers' 5' end and then used to perform a second PCR. The PCR reaction conditions stated above were used, but the annealing temperature was increased by 5°C after the first 5 cycles because of the additional T7 RNA polymerase promoter sequence. The PCR product with the T7 RNA polymerase promoter sequence was purified as stated above and used as a template for dsRNA synthesis using MEGAscript® RNAi kit (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's protocol. Briefly, a 20µl reaction was set up containing ~0.1 - 0.2 µg of the template, 2µl of each of the dNTPs (75mM each), 2µl of the T7 reaction buffer, 2µl of the T7 enzyme mix, and the rest nuclease free water. The reaction components were mixed by gentle pipetting and then incubated at 37°C for 12 hours for dsRNA synthesis. The transcribed dsRNA was mixed with 1µl TURBO DNase and incubated at 37°C for 30 minutes to digest the DNA template. The DNA-free dsRNA was checked on a low percentage agarose gel and once it was confirmed to be of the expected size, it was purified using the ethanol precipitation protocol. Briefly, 30µl of nuclease free water and 5µl of 3M sodium acetate were added followed by the addition of 150µl absolute ethanol. The solution was mixed thoroughly by vortexing and incubated at -20°C for one hour, after which it was centrifuged at 4°C and 15,000 rpm for 15 minutes. The pellet was washed once with 200µl of 70% ethanol and air dried for 15 minutes before it was dissolved in 50µl of nuclease free water. RNA concentration was determined using the Varioskan™ LUX multifunction microplate reader (Thermo Scientific, Massachusetts, USA) before storage at -20°C until use.

5.6.3. Establishing the most efficient method for dsRNA delivery

To establish the optimal method for delivering dsRNA to knock down cotton stainer target genes, two *D. fasciatus* egg clutches (~35 eggs each) were collected from the main cultures, one for each of the two methods investigated, i.e., feeding and injection. The eggs were maintained in the conditions specified above until hatching. The newly hatched nymphs were fed *ad libitum* with autoclaved water and linden seeds diet until they reached the late second instar, when they were each divided into two groups i.e., knockdown and control treatments. RNAi was performed by depriving the insects of water for 24 hours and then feeding or injecting them with dsRNA of c-type lysozyme to test for the efficiency of feeding

and injection methods of dsRNA delivery, respectively. One microliter of dsRNA with a concentration of ~2μg*μl^{-1} was used per insect for both methods. For the feeding method, a droplet of the dsRNA was offered to the group of insects in a cage, while for the injection method, the insects were first anaesthetized with CO_2 for one minute and then attached to a suction pump before dsRNA was injected into the first abdominal segment using the ES-blastocyst injection pipette straight with spike needle (Biomedical instruments, Zöllnitz, Germany) mounted to the CellTram vario manual microinjector (Eppendorf, Hamburg, Germany). For the controls, mock knockdown was done using dsRNA targeting the transcript for green fluorescent protein (GFP). After dsRNA delivery, the insects were reared in their respective cages and fed *ad libitum* with autoclaved water and linden seeds diet. To evaluate the efficiency and duration of knockdown for each of the two dsRNA delivery methods, 3-6 bugs, depending on the number surviving after silencing, were sampled on day 7, day 14 and day 21 after the RNAi knockdown procedure for RNA extraction and subsequent qPCR.

5.6.4. Experimental set-up to investigate the role of immune genes in symbiont regulation

Fifteen adult *D. fasciatus* mating pairs were collected from the main cultures and kept in small sterile box cages (14cm x 8cm x 6cm) until they laid eggs. Egg clutches with >35 eggs were collected for this experiment and incubated in sterile petri dishes lined with moist filter papers at 26°C and 60% humidity until hatching. The newly hatched nymphs were fed *ad libitum* with autoclaved water and linden tree seeds diet until they reached the late 2nd instar (~7 days after hatching), when they were randomly divided into seven groups of equal size. RNAi targeting single genes or a combination of two genes/two isoforms of the defensin gene was performed as per the feeding protocol described above for all the seven groups: Dor & Tab (Dorsal and Tab), NF-kB & Cac (NF-kB inhibitor and Cactus), Defs (Defensin 1 and defensin 2), Hem (Hemiptericin), Pyr (Pyrrhocoricin), C-Lys (C-type Lysozyme), and Gfp (Control). All knockdown treatments were maintained on autoclaved water and linden seed diets in sterile cages until the end of the experiment. Survival rates, developmental time to adulthood, measured as the duration from silencing until 50% of the bugs in a replicate treatment had molted into adults, and weight at emergence, computed as the average weight of individuals within each replicate treatment, were recorded to determine the effect of knockdown on *D. fasciatus* fitness. One week post-RNAi, one insect was sampled from each of the 7 treatments for all 15 replicates and subjected to RNA extraction and qPCR to check for knockdown success and the stability of the core gut microbial community. Additionally, upon reaching adulthood, the M3 mid-gut region was dissected from one insect per replicate treatment to check for the effect of knockdown on the core gut bacteria upon completion of the developmental period of the insects.

5.6.5. RNA extraction, cDNA synthesis, and qPCR

Total RNA was extracted from the whole insect for the nymphs or M3-gut region for the adults using the innuPREP RNA Mini Isolation Kit (Analytik Jena, Jena, Germany) following the manufacturer's protocol. Concentration and quality (optical density (OD) 260/280 and OD 260/230, respectively) of extracted RNA samples was determined using a Varioskan™ LUX multifunction microplate reader (Thermo Scientific, Massachusetts, USA). QRT-PCR was done using a Quantitect® Reverse Transcription kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines. Briefly, genomic DNA was removed by setting up a 14μl reaction mixture comprising of ~0.2μg of RNA template, 2μl of gDNA wipeout buffer, and the rest RNase free water. DNA was digested by incubating the mixture at 42°C for 2 minutes, followed by the addition of 1μl of the RT primer mix, 4μl of the Quantiscript RT buffer and 1μl of the Quantiscript reverse transcriptase enzyme. This mixture was then incubated at 42°C for 30 minutes for cDNA synthesis, after which the enzyme was inactivated at 95°C for 3 minutes

and the generated cDNA stored at -20°C until required for qPCR to measure gene expression or bacterial abundance.

To determine if RNAi mediated knockdown of target genes was successful, qPCRs targeting immune genes were set up with gene-specific primers (Table 1), which were designed and specificity determined as described above. For the quantification of core gut bacterial symbionts, qPCR targeting the 16S rRNA genes were set up with specific primers for each of the symbionts as described previously (Onchuru, Martinez, & Kaltenpoth, 2018). A qPCR was set-up with a final reaction volume of 10µl containing 0.5µl of each primer (10 µM), 5µl SYBR-mix, 3µl of qPCR H_2O, and 1µl of either template or standard or negative control (H_2O). An additional qPCR targeting *D. fasciatus'* 18S rRNA was performed for normalizing the expression of knockdown genes and abundance of the core gut bacterial symbionts. qPCR was conducted on the Rotor-Gene Q cycler (Qiagen, Hilden, Germany) with cycling conditions as described previously (Onchuru, Martinez, & Kaltenpoth, 2018). Quantification of the copy number of the expressed immune genes and bacterial 16S rRNA was determined using Rotor-Gene Q software as previously described (Onchuru, Martinez, & Kaltenpoth, 2018). Gene expression levels and abundance of the core bacterial symbionts were normalized with host 18S rRNA copy numbers prior to data analyses.

Table 1: Primers used to quantify (qPCR) the expression of target gene transcripts. Sequences of the genes can be retrieved from European Nucleotide Archive in the Sequence Read Archive database under accession number PRJEB6171 (Bauer et al., 2014)

Primer name	Primer sequence	Target gene	Sequence ID
Cact-1F	5'-GGCCTGATCTCTTCGCCTAC-3'	*D.fasciatus* cactus	Dfas-16185
Cact-1R	5'-AACAAAAAGGCAGTCGTCGC-3'		
Nf_kappa_qPCR_1F	5'-ACTCTTCCGGTCCTCTCGAA-3'	*D.fasciatus* Nf-Kappa inhibitor	Dfas-48512 & 53732
Nf_kappa_qPCR_1R	5'-AGCTTAACACGCTCGACCAA-3'		
Dorsal_1F	5'-CCGGCTCTTTAGCCAACATC-3'	*D.fasciatus* dorsal	Dfas-36948
Dorsal_2R	5'-ACAGTTGCCAAGGTTGAAACA-3'		
Lyso_For_1	5'-CTTTCCAACCCTGAATGCTC-3'	*D.fasciatus* c-type lysozyme	Dfas-30397
Lyso_Reverse	5'-AGCACGGACTACGGACTGTT-3'		
Hemi_1_qpc_for	5'-TGAAGGCTCAGGGTAAC-3'	*D.fasciatus* hemiptericin	Dfas-00011 & 46208
Hemip_2_ rev	5'-GTTTTCCTGTGCATCGCTGT-3'		
Pyrrho qpc for	5'-GCCAGAGCTTGAACAGGAA-3'	*D.fasciatus* pyrrhocoricin	Dfas-00911 & 33105
Pyrrho qpc rev	5'-TGTTGTATATCGGCCTTGGA-3'		
Tab_qPCR_1F	5'-AAAGGCCACCAGTTGTCAGG-3'	*D.fasciatus* tak1 binding protein	Dfas-09234 &30553
Tab_qPCR_1R	5'-TGCAGCTAAACGGGCACTAA-3'		
Def_for	5'-CAACTTTCCAAACAAATCCACA-3'	*D.fasciatus* defensin 1	Dfas-33854
Dfas_Def_1R	5'-ACTGTCTTCTTGCAGCTCCC-3'		
Defensin-1F	5'-GGGTGTGAACCACTGGGATT-3'	*D.fasciatus* defensin 2	Dfas-51099
Defensin-1R_Modified	5'-TATGCGCCGCTATGGTC-3'		

5.6.6. Data analysis

All our experiments were performed by splitting up the same egg clutches and distributing the individuals equally across treatments, resulting in each replicate consisting of siblings. Therefore, our data met the requirements to be analyzed with paired test statistics, since individuals in each treatment were related. Accordingly, we used Wilcoxon-signed rank test to assess differences in relative gene expression between the knockdown vs the control treatments. However, we used Mann-Whitney U tests to compare knockdown success when determining the efficient method to deliver dsRNA for knocking down genes in *D. fasciatus* because of unequal sample size distribution for the injected treatments where the invasive procedure resulted in the death of some individuals. Friedman test with Dunn-Bonferroni post hoc tests was used to compare differences in the times of development, weights of emerged individuals and symbiont abundances across the different treatments. These tests were done using SPSS statistics 23.0 (IBM, NY, USA). Survival probabilities of insects were compared across the different treatments as described previously, by using Cox mixed effects models as implemented in R 3.4.1 (Onchuru, Martinez, & Kaltenpoth, 2018).

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CHAPTER 6

QUANTITATIVE PCR PRIMER DESIGN AFFECTS QUANTIFICATION OF dsRNA-MEDIATED GENE KNOCKDOWN

Accepted in Ecology and Evolution

Thomas Ogao Onchuru[1] and Martin Kaltenpoth[1]

[1]Department for Evolutionary Ecology, Institute of Organismic and Molecular Evolution, Johannes Gutenberg University - Mainz, Hanns-Dieter-Hüsch-Weg 15, 55128 Mainz, Germany.

6.1. Abstract

RNA interference (RNAi) is a powerful tool for studying functions of candidate genes in both model and non-model organisms and a promising technique for therapeutic applications. Successful application of this technique relies on the accuracy and reliability of methods used to quantify gene knockdown. With the limitation in the availability of antibodies for detecting proteins, quantitative PCR (qPCR) remains the preferred method for quantifying target gene knockdown after dsRNA treatment. We evaluated how qPCR primer binding site and target gene expression levels affect quantification of intact mRNA transcripts following dsRNA-mediated RNAi. The use of primer pairs targeting the mRNA sequence within the dsRNA target region failed to reveal a significant decrease in target mRNA transcripts for genes with low expression levels, but not for a highly expressed gene. By contrast, significant knockdown was detected in all cases with primer pairs targeting the mRNA sequence extending beyond the dsRNA target region, regardless of the expression levels of the target gene. Our results suggest that at least for genes with low expression levels, quantifying the efficiency of dsRNA-mediated RNAi with primers amplifying sequences completely contained in the dsRNA target region should be avoided due to the risk of false negative results. Instead, primer pairs extending beyond the dsRNA target region of the mRNA transcript sequences should be used for accurate and reliable quantification of silencing efficiency.

6.2. Introduction

The discovery of RNA interference (RNAi) revolutionized the study of gene functions in eukaryotes. This gene regulatory mechanism utilizes double-stranded RNA (dsRNA) or short interfering RNA (siRNA) molecules to direct homologous-dependent interference of gene activity (Novina & Sharp,

2004; Scott et al., 2013). The presence of dsRNA in eukaryotic cells triggers the RNAi machinery to initiate reactions leading to the methylation of histone proteins or destruction of mRNA transcripts resulting in transcription or translation inhibition, respectively (Novina et al., 2004). This process begins with the cleavage of free dsRNA in the cytoplasm by RNaseIII endonuclease dicer into small interfering RNAs (siRNAs) that are picked up by the RNA induced silencing complex (RISC), a multi-protein complex, which degrades the sense strands of the siRNAs and uses the antisense strands as guides for the destruction of target complementary mRNA transcripts before they are translated into proteins (Novina et al., 2004; Scott et al., 2013). Alternatively, the antisense strands can recruit enzymes that methylate the histone proteins leading to the formation of a silenced chromatin, thereby inhibiting transcription (Novina et al., 2004).

Naturally, RNAi regulates development and physiology, suppresses transposon activity, and provides defense against RNA virus infections in many organisms using endogenously expressed microRNAs or exogenously introduced viral dsRNA (Ambros, 2004; Obbard et al., 2009). However, this mechanism can also be exploited artificially to study functions of endogenous eukaryotic genes of interest through the introduction of synthetic dsRNA or siRNA molecules that trigger the host's natural RNAi machinery to silence the respective genes, which allows investigation into their specific functions. The artificial injection of dsRNA for a gene encoding the myofilament protein into the nematode *Caenorhabditis elegans* led to the discovery of dsRNA-mediated RNAi silencing (Fire et al., 1998). Following its discovery, dsRNA-mediated RNAi became a powerful research tool for understanding gene functions as well as a promising therapeutic candidate for the management of genetic disorders (Agrawal et al., 2003; Seyhan, 2011).

The success and extent of RNAi silencing differs between hosts, life stages, and genes of the same organism. This may be due to variability in the stability of dsRNA molecules *in vivo,* their uptake by target cells, and *in vivo* amplification and transmission of the silencing signal between cells to facilitate systemic or transgenerational silencing (Scott et al., 2013; Wang et al., 2016). In addition to these endogenous challenges, successful use or application of RNAi can be influenced by external technical factors, including the methods used for administering the dsRNA and measuring its success (Herbert et al., 2011; Holmes et al., 2010; Scott et al., 2013). It is therefore important that reliable and precise methods are used to evaluate efficacy and specificity of gene silencing following RNAi to avoid false positive or false negative results and consequently wrong conclusions (Herbert et al., 2011; Holmes et al., 2010). In addition to phenotypic observations, there are two standard methods for assessing the success of RNAi-mediated gene silencing: real-time quantitative PCR (RT-qPCR) for quantifying depletion of relevant mRNA transcripts relative to controls, and western blotting or immunofluorescence for measuring the reduction in the amount of target proteins (Agrawal et al., 2003; Scott et al., 2013).

While it is known that accurate quantification of target mRNA transcript levels by RT-qPCR after gene knockdown with exogenous siRNAs depends on the selection of primer binding site relative to the siRNA cleavage site (Herbert et al., 2011; Holmes et al., 2010), it is unclear how RT-qPCR primer selection affects quantification of dsRNA-mediated RNAi gene knock down, especially in relation to the expression levels of target genes. Our first insights into the importance of primer design in the accurate measurement of dsRNA gene silencing came about when we were studying the role of *Dysdercus fasciatus*' (Hemiptera: Pyrrhocoridae) immune genes in the regulation of its gut bacterial symbionts (Onchuru & Kaltenpoth, 2019). Following dsRNA-mediated RNAi, transcript levels of genes under low expression remained unchanged or were even higher in the treatment groups compared to the controls, while transcript levels of the highly expressed target genes decreased significantly as expected. To identify if

this unexpected result was due to primer design and/or target gene expression levels, we designed two primer pairs for three genes with different levels of expression, respectively; one primer pair targeted the part of the mRNA transcript sequence that was entirely complementary to the dsRNA used for silencing and the other amplified a sequence extending beyond the region complementary to the dsRNA construct within the target mRNA. We report that the use of qPCR primers targeting a sequence that is completely contained within the dsRNA construct can lead to false negatives or an underestimation of gene knockdown in genes with low expression levels.

6.3. Materials and Methods

Total RNA extracted from *D. fasciatus* using the innuPREP RNA Mini Isolation Kit (Analytik Jena, Jena, Germany) was used for cDNA synthesis with Quantitect® Reverse Transcription kit (Qiagen, Hilden, Germany) as per the manufacturer's guidelines. This cDNA was used as a template for the synthesis of dsRNA for *D. fasciatus'* antimicrobial peptide genes defensin 1 and defensin 2, which have low expression levels, and c-type lysozyme gene with a higher expression level using MEGAscript® RNAi kit (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's protocol. The expression levels of the three genes are based on a previously published transcriptomic data which reported the normalized gene transcripts of the c-type lysozyme to be at least 200 times higher than those of the different isoforms of the defensin genes (Bauer et al., 2014). The synthesized dsRNA was used for *in vivo* RNAi gene knockdown that was performed by feeding 15 replicate individuals of second instar *D. fasciatus* nymphs that had been deprived of water for 24 hours with dsRNA for defensins (i.e. defensin 1 and defensin 2 in combination) or c-type lysozyme, or nonsense dsRNA for GFP for the control group, respectively. In pilot experiments, we found that there was no difference in the degree of silencing when the two defensin genes are silenced independently or together.

One week after dsRNA feeding, total RNA was extracted from one nymph per replicate treatment and used for cDNA synthesis as described above to measure gene knockdown success. Two qPCR primer sets (Table 1) were designed for each gene with primer BLAST using their respective sequences obtained from a previously described *D. fasciatus* transcriptome (Bauer et al., 2014). One primer set amplified the section of the target mRNA sequence that was entirely complementary to the dsRNA sequence used for silencing, while the second primer set amplified the mRNA sequence extending beyond the region that is complementary to the dsRNA construct (Figure 1). Specificity of the primers was determined by blasting their sequences and those of their respective PCR products against a local BLAST database that was created using the *D. fasciatus* transcriptome. Blasting of the PCR product sequences also allowed us to exclude the possibility of off-target gene silencing.

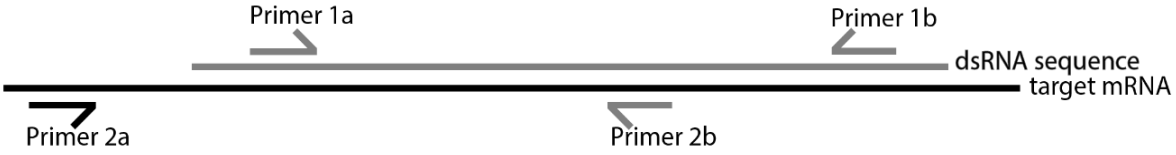

Figure 1. Schematic illustration of selected qPCR primer binding sites relative to the location of the dsRNA construct within the target mRNA. Primers were designed to amplify the mRNA sequence section that is complementary to the dsRNA used for silencing (Primer set 1) or amplify a sequence extending beyond the boundaries of the dsRNA construct within the target mRNA transcript (Primer set 2).

Quantitative PCR was set-up using these two primer sets to quantify gene knock down success. The 10 µl qPCR reaction mixture contained 0.5 µl of each primer (10 µM), 5 µl SYBR-mix, 3 µl of qPCR H_2O and 1 µl of either the cDNA template or a standard or a negative control (H_2O). The reaction mixture was run on the Rotor-Gene Q cycler (Qiagen, Hilden, Germany) with the following cycling conditions: 95˚C initial denaturation for 5 mins followed by 95˚C denaturation for 10 secs, touchdown annealing for 15 secs at 68-60˚C for the first eight cycles, then 60˚C annealing for the remaining 37 cycles for 15 secs, extension of 72˚C for 10 secs, and a final melting curve analysis from 65˚C to 99˚C with a temperature raise of 1˚C for each step. Quantification of each target gene was performed with the Rotor-Gene Q software as described by Onchuru et al., (Onchuru et al., 2018) using external standard curves amplified with similar conditions from serial dilutions (10^{10} to 10^2 copies/µl) of purified PCR product of the respective gene. The transcript copy numbers of each gene were normalized with the number of host 18S rRNA transcripts that were quantified by qPCR using previously described primers (Table 1) (Onchuru et al., 2018) and conditions described above. Differences in the normalized transcript copy numbers between treatments and controls were evaluated using Wilcoxon-signed rank tests and plotted using boxplots as implemented in SPSS Version 23 (IBM, NY, USA). Part of the data analyzed here, i.e., gene expression using primers targeting mRNA sequence extending beyond the dsRNA target site, is published in (Onchuru et al., 2019).

Table 1. qPCR primers used in measuring dsRNA-mediated gene knockdown

Target gene	Primer Name	Product size (bp)	Primer sequence	Target site of RT-qPCR primers	Pairing
Defensin1	Dfas_Def_1F	239	GTCCTTCTCCTGGTCTTCGC	inside dsRNA	Set 1
	Dfas_Def_1R		ACTGTCTTCTTGCAGCTCCC	inside dsRNA	
Defensin1	Def_for	273	CAACTTTCCAAACAAATCCACA	outside dsRNA	Set 2
	Dfas_Def_1R		ACTGTCTTCTTGCAGCTCCC	inside dsRNA	
Defensin2	Dfas_Def_2F	224	CTCGCACCTTCCTCCTTTGT	inside dsRNA	Set 1
	Dfas_Def_2R		CTATGGTCGCTGTCTCGGC	inside dsRNA	
Defensin2	Defensin-1F	173	GGGTGTGAACCACTGGGATT	inside dsRNA	Set 2
	Defensin-1R_Modified		TATGCGCCGCTATGGTC	outside dsRNA	
c-type Lysozyme	Lyso_For_2	168	CCTCTGGCACTTGGTCTTCC	inside dsRNA	Set 1
	Lyso_Rev_2		AACAGCCACTACTGGTGCAA	inside dsRNA	
c-type Lysozyme	Lyso_For_1	163	CTTTCCAACCCTGAATGCTC	outside dsRNA	Set 2
	Lyso_Reverse		AGCACGGACTACGGACTGTT	inside dsRNA	
18S rRNA	Firebug18S-1F	198	CGGTGCTCTTTACCGAGTGT	Firebug 18S rRNA (Onchuru et al., 2018)	
	Firebug18S-1R		AACGTCGCAATACGAATGCC		

6.4. Results

We compared the effect of qPCR primer binding site within the mRNA transcript sequence on the accurate quantification of gene knockdown following dsRNA-mediated RNAi for three genes with different expression levels. Quantifying transcript levels of target genes with primers targeting the mRNA sequence that is complementary to the dsRNA used for gene silencing (primer set 1, Figure 1) indicated a significant increase or an insignificant decrease of transcript levels in treated animals compared to the controls for genes with low expression levels (Figure 2), but not for a highly expressed gene (Figure 3). With this primer pair, expression of defensin 1 gene increased significantly by 119% in the knockdown group compared to the controls (Figure 2A, Wilcoxon-signed rank test, Z=-2.953, p = 0.003) while the expression of defensin 2 gene in the knockdown group decreased insignificantly by 25% (Figure 2B, Wilcoxon-signed rank test, Z=-0.966, p = 0.334). However, for the highly expressed c-type lysozyme gene, a significant knockdown of up to 99% was observed with this primer set (Figure 3, Wilcoxon-signed rank test, Z=-3.237, p = 0.001).

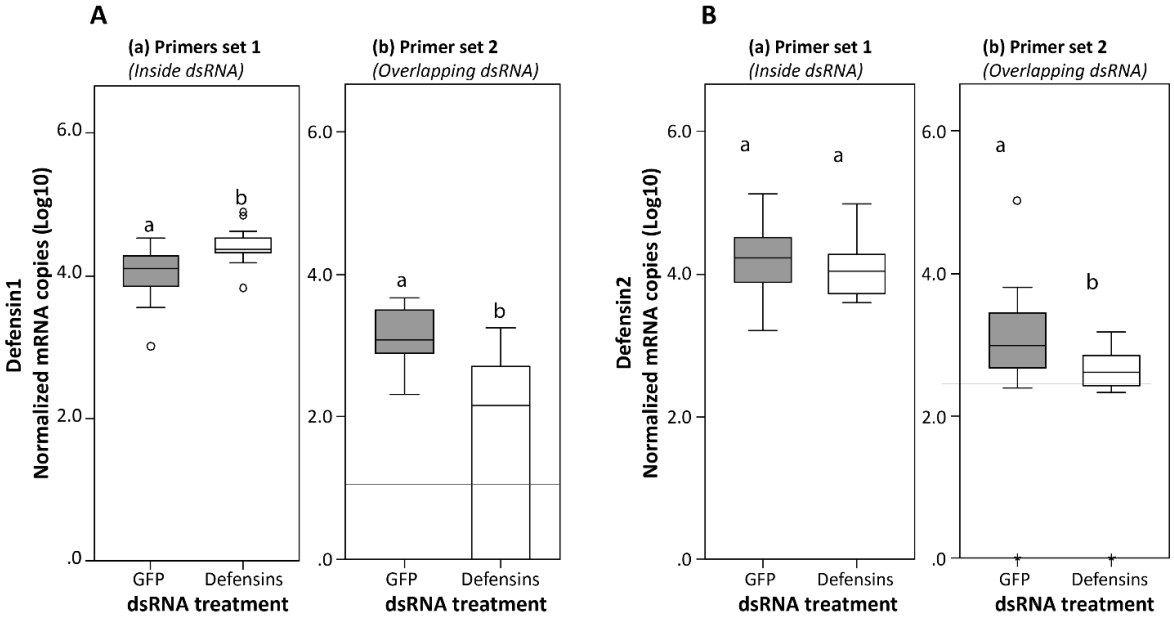

Figure 2. Quantification of transcript levels of (A) defensin 1 and (B) defensin 2 genes with low expression levels using two different primer sets. (a) Quantification with primers binding and amplifying the mRNA transcript sequence that is complementary to the dsRNA sequence used for silencing, and (b) quantification with primers binding and amplifying a sequence of the target mRNA transcript extending beyond the dsRNA construct. Significant differences are indicated by different letters above the boxes. Boxes comprise 25–75 percentiles, lines in boxes represent medians, whiskers denote the range, and circles represent outliers. Detection threshold is 0 if not indicated by the grey horizontal line (negative control in the qPCR).

On the other hand, measuring gene knockdown with primers targeting the sequence extending beyond the dsRNA construct within the target mRNA transcript (primer set 2, Figure 1) revealed a significant gene knockdown for all three genes, regardless of the gene's expression levels. With this primer set, a decrease of 93%, 84%, and 94% was recorded for c-type lysozyme, defensin 1, and defensin 2 genes, respectively, in the knockdown group compared to the controls (Figure 2 and 3, Wilcoxon-signed rank test, Z=-3.067, Z=-2.953, and Z=-2.329, respectively, $p < 0.05$).

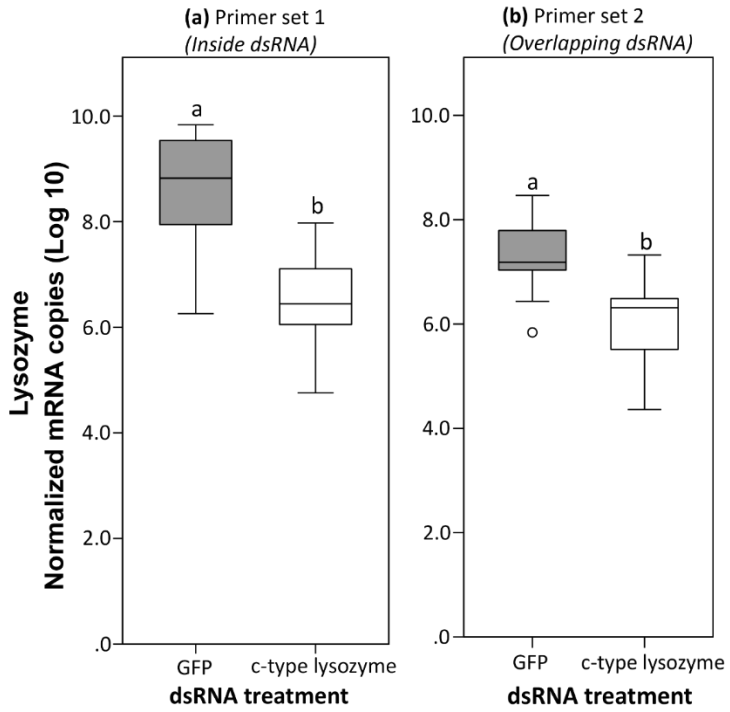

Figure 3. Quantification of transcript levels of the highly expressed c-type lysozyme gene using two different primer sets. (a) Quantification with primers binding and amplifying the mRNA transcript sequence that is complementary to the dsRNA sequence used for silencing, and (b) quantification with primers binding and amplifying a sequence of the target mRNA transcript extending beyond the dsRNA construct. Significant differences are indicated by different letters above the boxes. Boxes comprise 25–75 percentiles, lines in boxes represent medians, whiskers denote the range, and circles represent outliers.

6.5. Discussion

Interfering with the activity of genes through RNAi-mediated gene silencing and evaluating the host phenotypic changes is a powerful approach to determine specific gene functions. To confirm that the observed phenotypic changes are due to the knockdown of target genes and not off-target effects, the concentrations of residual target gene transcripts and proteins are measured with RT-qPCR and western blotting, respectively. In this study, we sought to understand the importance of qPCR primer design and host gene expression levels on the quantification of residual mRNA transcripts following dsRNA-mediated RNAi. Our findings suggest that the target gene expression levels and the choice of primer binding site relative to the mRNA sequence targeted for silencing are important factors to consider when designing qPCR primers for evaluating RNAi success. Specifically, for genes with low expression patterns, quantification of mRNA transcripts with primers targeting an amplicon that is contained within the dsRNA target region resulted in an underestimation of the degree of silencing or in false negative results. However, this problem could be circumvented by using primers targeting an amplicon extending beyond the dsRNA target region within the mRNA transcript.

Efficacy and duration of RNAi-mediated gene silencing varies not only between organisms but also between genes of the same organism (Scott et al., 2013; Wang et al., 2016). This disparity may be explained by variation in the RNAi machinery or extracellular enzymatic capacity of different organisms or tissues to degrade exogenous dsRNA molecules, which affects their stability *in vivo* and consequently silencing efficiency (Scott et al., 2013; Spit et al., 2017; Wang et al., 2016). Additionally, in some organisms, the silencing signal can be amplified by different mechanisms e.g. the RNA-dependent RNA polymerase (RdRp), which uses siRNAs generated from the diced primary dsRNA molecule as primers

to copy the target mRNA resulting in the formation of secondary dsRNA (Sijen et al., 2001). Our findings suggest that variation of RNAi between genes may be as a result of quantification errors influenced by improperly designed RT-qPCR primers and differences in gene expression. In *D. fasciatus* insect, a significant reduction of the target mRNA transcripts is achieved for up to two weeks following dsRNA-mediated RNAi (Onchuru et al., 2019), a clear indication that either the dsRNA remains stable *in vivo* or the silencing signal is amplified resulting in sustained knockdown for this duration. The presence of residual primary or *in vivo* amplified dsRNA molecules can influence quantification of gene knockdown during qPCR. Stable dsRNA molecules may be extracted during total RNA extraction, reverse transcribed and quantified during RT-qPCR. This results in an overestimation of the gene expression levels in the target knockdown treatments as compared to controls leading to an underestimation of gene knockdown or complete false negatives.

Our results corroborate the findings of other studies showing the importance of primer design in the quantification of intact mRNA transcripts after RNAi. In siRNA-mediated RNAi experiments, the choice of RT-qPCR primer binding position relative to the siRNA-mediated cleavage site has an effect on the quantification of target mRNA transcripts (Herbert et al., 2011; Holmes et al., 2010). For example, after siRNA-mediated cleavage, degradation of the generated 3' mRNA fragment may be blocked; hence the use of primers targeting this fragment leads to an underestimation of the degree of RNAi-mediated gene silencing (Holmes et al., 2010; Mainland et al., 2017). On the other hand, using primers too close to the siRNA cleavage site results in false positive results when contaminating siRNA molecules extracted with total RNA inhibit RT-qPCR (Herbert et al., 2011).

RNAi is a useful tool for research and therapeutics (Agrawal et al., 2003; Seyhan, 2011). To successfully exploit this technique, however, a number of considerations must be taken into account when designing dsRNA-mediated RNAi experiments. Besides optimizing dsRNA dosage, delivery, and cellular uptake with cell membrane penetrating peptides and increasing stability of dsRNA in the extracellular environment by knocking down dsRNases to improve RNAi efficiency (Spit et al., 2017), accurate quantification of successful RNAi gene silencing is key to avoiding incorrect conclusions. With RT-qPCR being the most common and widely used method for the quantification of intact target mRNA transcripts after silencing, its reliability is essential regardless of the gene or organism being studied. Based on our findings, we recommend the use of primers amplifying the mRNA transcript sequence extending beyond the dsRNA target region of the mRNA transcript to ensure accurate quantification of RNAi gene knock down, especially in genes with low expression patterns.

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Data accessibility statement

The gene expression raw data measured by qPCR are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.28n8d6t.

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CHAPTER 7

GENERAL DISCUSSION

In the preceding chapters, I reported on host-symbiont-parasite multipartite interactions using the experimentally tractable African cotton stainer insect (*Dysdercus fasciatus*) that is associated with both mutualistic and parasitic microbes. In this chapter, I discuss these findings in the context of fundamental symbiosis questions, i.e., (i) what is the ecological relevance of symbiotic associations, (ii) how are symbiotic associations established and maintained, and (iii) what are the costs and evolutionary consequences associated with being in symbiotic partnerships?

7.1. Pyrrhocorid-associated bacterial symbionts as drivers of ecological diversification

Many species of the Pyrrhocoridae family whose gut bacterial communities have been profiled are characterized by consistent gut bacterial symbionts that are distinct from those of individuals of the closely related Largidae family (Gordon et al., 2016; Sudakaran et al., 2015). The mid-gut bacterial community of Pyrrhocoridae, especially in the M3 section, is relatively simple (< 20 bacterial species) and is largely dominated by *C. glomerans* and *Gordonibacter* sp. (Actinobacteria), *Hungatella* sp. and *Lactococcus lactis* (Firmicutes), and *Klebsiella* sp. (Proteobacteria) symbionts (Gordon et al., 2016; Sudakaran et al., 2015). Regardless of the geographical location or the preferred host plant, these bacteria are consistently present across different genera of the Pyrrhocoridae family with a few exceptions such as the genus *Probergrothius,* which conspicuously lacks most of the characteristic core bacterial symbionts (Sudakaran et al., 2015, 2012). Thus, it is likely that the split of the *Probergrothius* clade from the rest of the Pyrrhocoridae happened before the core gut bacterial community was acquired by the common ancestor of the rest of the Pyrrhocoridae genera (Sudakaran et al., 2015). The time of acquisition of the characteristic core gut bacterial symbionts coincides with the origin of Malvales plants, suggesting that the acquisition of the core microbiota may have facilitated diversification of these phytophagous bugs into exploiting Malvales plant seed diets (*Gossypium*, *Tilia*, *Hibiscus*, *Adansonia*, *Ceiba*, and *Sterculia*) (Imtiaz, 1987; Sudakaran et al., 2015). This is corroborated by the preference of *Probergrothius angolensis,* which has distinct abundances of the core gut bacterial symbionts from those of species feeding on Malvales plants, on seeds of a distantly related *Welwitschia mirabilis* host plant (Panizzi & Grazia, 2015; Schaefer & Panizzi, 2000; Wetschnig & Depisch, 1999).

Maintenance of a specific gut microbial community across Pyrrhocoridae generations and populations is attributed to the reliability of vertical and horizontal mixed-mode of symbiont transmission. Vertical transmission happens when females smear egg surfaces with fecal excretions containing the gut

symbionts which are subsequently probed by the newly hatched nymphs (Kaltenpoth et al., 2009; Salem et al., 2015), while horizontal transmission occurs when individuals within a population engage in cannibalistic and coprophagic behaviors that are enhanced by the gregarious nature of pyrrhocorids (Kaltenpoth et al., 2009; Schaefer et al., 2000). Taking advantage of these transmission modes, Salem et al., 2013 generated dysbiotic *Pyrrhocoris apterus* and *D. fasciatus* insects (both Pyrrhocoridae) through egg surface sterilization and rearing of the insects under sterile conditions with *ad libitum* feeding. By comparing fitness correlates of bugs with and without the core gut bacterial symbionts, they demonstrated the ecological relevance of the bacterial symbionts for the two firebug species. Experimental perturbation of the core microbiota resulted in increased nymphal developmental times and a significant decrease in survivorship, mating frequencies, and lifetime reproduction success (Salem et al., 2013). When dysbiotic insects were allowed to re-acquire conspecific and heterospecific symbionts, normal fitness was restored with conspecific symbionts but not heterospecific symbionts which further confirmed the importance of the symbionts and the high degree of host-symbiont specificity in these insects (Salem et al., 2013). Alternatively, fitness was restored by supplementing the firebugs' artificial diet with B-vitamins, indicating that the core gut bacterial community plays an important role in nutrient provisioning (Salem et al., 2014).

In addition to the nutritional role, I report in chapter four that the gut microbial community protects *D. fasciatus* against infections caused by a co-localized and a co-transmitted trypanosomatid parasite, *Leptomonas pyrrhocoris,* putatively via competitive exclusion (Onchuru, Martinez, & Kaltenpoth, 2018). When *L. pyrrhocoris* is confined in the intestinal tracts of hosts, it causes asymptomatic infections characterized by low parasite titres, diarrhea, and slightly increased nymphal developmental times (Schaub, 1994; Tanada & Kaya, 2012). Occasionally, serious infections associated with lethargy, pale cuticle coloration, reduced starvation resistance, and even death occur when the parasite invades the host hemolymph (Schaub, 1994; Tanada et al., 2012). The asymptomatic nature of *L. pyrrhocoris* infections I have observed in natural and laboratory firebug populations may be, to some extent, a result of the presence of defensive gut bacterial symbionts that confine and interfere with parasite establishment in the guts of hosts, highlighting their additional ecological relevance. Since *L. pyrrhocoris* is a generalized parasite of pyrrhocorids (Votýpka et al., 2012), symbiont-conferred protection is likely wide-spread across many species with the characteristic gut bacterial community. This finding indicates that the firebug gut bacterial symbionts (*Coriobacterium glomerans*, *Gordonibacter* sp., *Hungatella* sp., and *Klebsiella* sp.) are engaged in multi-partite and multi-functional mutualistic interactions important for host protection and nutrition. Multi-functional symbionts are common in other insect taxa associated with gut bacterial symbionts. In the honeybee (*Apis mellifera*), for example, a normal gut microbiota protects against *Lotmaria passim, Serratia marcescens,* and *Nosema* infections, facilitates the digestion of pollen through pectin degradation, and promotes host development via metabolism and hormonal signaling (Bonilla-Rosso & Engel, 2018; Kwong et al., 2017; Schwarz et al., 2016; Zheng et al., 2017). Likewise, the *Burkholderia* endosymbiont of *Riptortus* stinkbugs that is localized in mid-gut-associated crypts enhances host innate immunity (Kim, Lee, et al., 2015), helps in the degradation of fenitrothion (organophosphorus pesticide), which has neurotoxic effects to insects (Kikuchi et al., 2012), and significantly contributes to host growth, survival, and reproduction, suggesting a nutritional role (Kikuchi et al., 2007). Gut microbial communities might be generally multi-functional as this has also been reported in vertebrates. In humans and mice, for instance, gut bacterial symbionts breakdown indigestible dietary components, stimulate host immunity, and protect against pathogenic infections as well as chronic conditions (Lozupone et al., 2012; Tremaroli & Bäckhed, 2012)

7.2. Sociality, insect immunity, and symbiosis

Insects have evolved complex immune systems and intricate behaviors to counter threats from natural enemy attacks, i.e., viral, bacterial, and fungal pathogens as well as parasites, parasitoids, and predators. Some insect taxa live in groups where intrinsic collective and individual behaviors complement innate immune defenses in protecting against invaders. Social behaviors such as communal immunization, allogrooming, nest cleaning, and removal of diseased or dead nest mates as well as the active transfer of body fluids with immune components are integral aspects of social insects' immunity and nutrition (De Roode & Lefèvre, 2012; Machida et al., 2001; Nalepa, 2015; Zhukovskaya et al., 2013).

In addition to social immunity, both solitary and social insects possess elaborate cellular and humoral innate immune systems that protect against invaders (Lemaitre & Hoffmann, 2007; Strand, 2008). Central to insect immune responses are pattern-recognition receptors (PRRs) that are responsible for recognizing microbe-associated molecular patterns (MAMPs) and activating various cellular and humoral immune defenses. Immediately upon recognition, invading organisms are attacked by constitutive hemocyte-modulated cellular immune defenses (phagocytosis, encapsulation, melanization, and nodulation) and microbicidal reactive oxygen species (ROS) present along epithelial cells which are the primary points of pathogen invasion (Lemaitre et al., 2007). These defense mechanisms ensure rapid separation, entrapment, and killing of invading pathogens at the point of entry before they cause systemic infections. At advanced stages of infection, humoral immune responses are triggered through the activation of Toll, IMD, JAK/STAT, and JNK pathways leading to the production of peptides and enzymes with activity against bacteria, fungi, and parasites that may have survived constitutive defenses (Yi et al., 2014). Upon production, these bioactive substances kill or block pathogens by suppressing cell division and growth through the inhibition of bacterial protein synthesis or induction of cell lysis by increasing permeability of the cell membranes (Yi et al., 2014).

Recent studies show that in addition to protecting insects against invaders, the innate immune system of insects also plays a central role in the recognition, qualitative and quantitative regulation, and localization of mutualistic symbionts as reported for bean bugs, weevils, and bees (Douglas, 2014). In the bean-bug *R. pedestris,* a strong immunological response characterized by an upregulation of a pyrrhocoricin-like and thanatin antimicrobial peptides and c-type lysozyme in the midgut before molting effectively decreases the abundance of its *Burkholderia* symbionts (Kim et al., 2014). Knockdown of these antimicrobial effectors results in an increase of the *Burkholderia* symbiont titers suggesting their importance in regulating the symbiont populations within the mid-gut associated crypts (Park et al., 2018). Similarly, the antimicrobial peptide *ColA* of *Sitophilus* weevils is highly expressed in the bacteriocytes housing the primary endosymbionts to not only prevent their escape to other host tissues but also to regulate their proliferation by inhibiting cell division (Anselme et al., 2008; Login et al., 2011; Maire et al., 2018).

One challenge arising from mutualistic interactions is how hosts maintain mutualistic symbionts while concurrently ensuring a competent defense against pathogens, considering that both mutualists and pathogens share similar MAMPs responsible for activating host immune responses. In addition to the seclusion of mutualistic symbionts in specialized host cells to prevent them from interacting with host immune systems, recent research shows that long-term coevolution of symbionts with their hosts can lead to host-mediated quenching of the mutualists' but not a pathogens' MAMPs resulting in the activation of host immune responses towards pathogens but not the mutualistic symbionts (Maire et al., 2019). In *Sitophilus zeamais* weevils, for instance, some of the peptidoglycan recognition proteins in the

bacteriomes have evolved to enzymatically degrade mutualist's peptidoglycans thereby preventing their escape from the bacteriomes to the rest of the body where they cause a systemic activation of the host immune system (Maire et al., 2019). Conversely, in some symbiotic interactions, beneficial symbionts successfully activate host immune responses which do not necessarily regulate or directly control the activating symbionts. For instance, in *D. fasciatus* and *Apis mellifera*, the core gut bacterial symbionts elicit antimicrobial immune responses that have no effect on their composition and function (Bauer et al., 2014; Kwong et al., 2017; Onchuru & Kaltenpoth, 2019). Experimental investigations into the influence of the honeybee antimicrobial responses on their gut bacterial symbionts revealed that the symbionts are resistant to the honey bee AMPs (Kwong et al., 2017). Similarly, my findings in *D. fasciatus* reveal that the core gut bacterial symbionts, i.e., *C. glomerans, Gordonibacter* sp., *Hungatella* sp., *and Klebsiella* sp. are insensitive to the AMPs that are highly expressed in insects containing these symbionts. Successful knockdown of the genes encoding these antimicrobials neither had an effect on the qualitative nor quantitative composition of the gut bacterial symbionts, suggesting that the symbionts are not controlled by the host AMPs. How the *D. fasciatus'* nutritional and defensive gut bacterial symbionts manage to remain insensitive to AMPs that are expressed in their presence remains a key question for future studies.

In other host-symbiont associations where mutualists are not affected by host immune defenses, modification of the symbionts' surface membranes interferes with AMPs' activity. For example, the secondary endosymbiont of the tsetse fly, *Sodalis glossinidius,* and the human gut bacterium, *Bacteroides thetaiotaomicron,* are known to encode enzymes responsible for the alteration of the negative charge of lipid A, the biologically active component of lipopolysaccharides, which subsequently interferes with the binding and activity of the host cationic antimicrobial peptides (Cullen et al., 2015; Pontes et al., 2011). The importance of a symbiont's membrane composition on its interaction with the host immune system has also been demonstrated in *R. pedestris*. The *Burkholderia* symbiont, which is acquired from the environment by *R. pedestris* after every generation, must lose the O-antigen on its cell surface in order to establish a mutualistic association with its host (Kim, Son, et al., 2015). Successful isolation and *in vitro* culturing of the dominant *D. fasciatus* gut bacterial symbionts will facilitate investigations into whether alteration of the symbionts' cell membranes is involved in the evasion of host antimicrobial regulation.

7.3. Costs and benefits of defensive symbiosis

7.3.1. Host and symbiont fitness trade-offs

Defensive symbionts complement host immune defenses in protecting against natural enemies. Occasionally, hosts entirely depend on these symbionts for defense against invaders which can result in the formation of intimate mutualistic interactions. Generally, tight evolutionary associations of symbionts with their hosts lead to dependence of symbionts on hosts for nutrition, proliferation, and transmission, especially when the symbionts are restricted to host environments. This can bear a cost on growth, development, and reproduction of hosts resulting in trade-offs between utilizing limited resources for essential processes and redirecting them towards symbiont maintenance (Vorburger & Perlman, 2018). Here I discuss how mechanism of protection, symbiont density and diversity, enemy occurrence or infection status, and specificity of symbiont-conferred protection can be associated to fitness trade-offs in hosts or their defensive symbionts.

The strength of protection provided by some defensive symbionts is dependent on symbiont density whereby individuals with lower symbiont titres have low protection compared to those with higher symbiont titres (Lu et al., 2012). Additionally, the strength of protection can also depend on the

defending symbiont strain (Vorburger et al., 2018). In *D. fasciatus*, I have shown that the gut bacterial symbionts protect against *L. pyrrhocoris* infections by adhering to the peritrophic matrix and likely block entry of this parasite into the hemolymph (Onchuru, Martinez, & Kaltenpoth, 2018). Therefore, maintaining a high diversity and abundance of the gut bacterial symbionts may enhance resistance against *L. pyrrhocoris* infections. However, maintaining a high symbiont density and diversity or a specific symbiont strain to achieve higher protection can have negative side-effects on host fitness as seen in *Drosophila*, *Aedes*-derived cell lines, and aphids. In *Drosophila* flies, egg hatch rates, male fertility, and fecundity of individuals infected with antiviral protecting *Wolbachia* symbionts are all negatively correlated with symbiont density in the host somatic tissues (Martinez et al., 2015). Furthermore, *Wolbachia* strains conferring greatest antiviral protection are associated with significantly lower host fitness levels in the absence of the virus (Chrostek et al., 2013; Martinez et al., 2015). Similarly, the extent of dengue virus inhibition by *Wolbachia* symbiont in *Aedes*-derived cell lines is directly proportional to the symbiont densities per cell, but possessing the symbiont significantly decreases host cell proliferation rates (Frentiu et al., 2010). Since *Wolbachia* symbionts rely on host fitness for maintenance and vertical transmission, their negative effects on host survival, fecundity, lifespan, and hatch rate not only affect host population dynamics but also the symbiont's fitness levels and frequencies within host populations (Martinez et al., 2015).

Fitness costs of maintaining diverse defense symbionts for maximum protection have been demonstrated in aphids. In addition to harboring the primary endosymbiont *Buchnera aphidicola*, the pea aphid also maintains several secondary endosymbionts including *Serratia symbiotica* and *Hamiltonella defensa*, which defend against *Aphidius ervi* parasitoid attacks (Oliver et al., 2010). Although each symbiont provides resistance to parasitoid attacks in a single infection, resistance is enhanced during a superinfection, i.e., double infection with both *S. symbiotica* and *H. defensa* (Oliver et al., 2006). However, superinfected aphids experience low fecundity, higher mortality rates, longer generation times, and significantly lower fresh weights at maturity, indicating that maintaining diverse symbionts compromises other host physiological processes (Oliver et al., 2006). In the presence of parasitoids, defensive phenotypes conferred by symbionts lead to high densities of the protected aphid species (Sanders et al., 2016). This can subsequently affect the composition and the abundances of other aphid species and their specialist parasitoids within the community leading to a community collapse, especially when the different aphid species share a common resource (Sanders et al., 2016). Unlike in *Drosophila, Aedes* cell lines, and aphids, I did not see any fitness costs in *D. fasciatus* due to the presence of the protective gut bacterial symbionts. Any costs may have been masked by the nutritional function of the symbionts as a result of a favorable benefit to cost ratio.

The mode of symbiont-conferred protection can induce additional costs to mutualistic partners besides the standard costs associated with symbiont maintenance. Generally, production of bioactive compounds, which are common in interference mechanisms, is associated with slow growth rates of producers (Riley & Wertz, 2002). Additionally, toxins produced against invaders may cause collateral damage to hosts which may subsequently force hosts to invest additional resources in mechanisms for repairing or avoiding undesired collateral damages (Vorburger et al., 2018). As for defensive symbionts that confer host protection via immune priming (Flórez et al., 2015), symbiont presence may results in a continuous activation of the host immune system regardless of the pathogen infection status. In many insects, overstimulation of host immunity is associated with low reproduction rates due to resource re-allocation (Maire et al., 2019; Schwenke et al., 2015). The core gut bacterial symbionts of *D. fasciatus*

activate the production of antimicrobial peptides whose function and cost has not been described yet (Bauer et al., 2014).

In addition to posing direct fitness costs to hosts, defensive symbionts can also cause indirect ecological costs by interfering with ecologically relevant host traits. Defensive behaviors (aggressiveness and escape reactions) of pea aphids, for example, are greatly reduced in individuals protected against parasitoid attacks by the *H. defensa* symbiont compared to individuals lacking this protective symbiont (Dion, Polin, et al., 2011). Reduction in the defensive behaviors is associated with increased susceptibility of the aphids to predatory ladybird attacks that are normally avoided by the behavioral responses (Polin et al., 2014).

Possessing defensive symbionts does not always guarantee protection, especially when invaders actively evolve counter-adaptations to symbiont-conferred defenses. For example, *Lysiphlebus fabarum* parasitoids of aphids show increased abilities to parasitize aphids possessing the *H. defensa* strain they evolve with, but not aphids possessing other *H. defensa* symbiont strains (Dennis et al., 2017). As a result, aphid hosts may be faced with the challenge of replacing symbiont strains from time to time to keep up with the evolving parasitoid or maintaining a diverse array of symbionts and strains to limit parasitoid counter-adaptation measures. Metagenomic analysis of the gut bacterial community of *D. fasciatus* has shown that the main bacterial symbiont, *Coriobacterium glomerans,* which mostly colonizes the peritrophic matrix of the insect in dense populations and likely block invasion of the hemolymph by the parasite (Onchuru, Martinez, & Kaltenpoth, 2018), is maintained in two strains although the function of each of the strains is not yet clear (Bauer, personal communication). As I have already discussed above, maintenance of multiple strains or symbionts might require additional host resources which could consequently impose costs on host fitness (McLean et al., 2018; Oliver et al., 2006).

When symbionts rely on their hosts for maintenance and transmission, their fitness is directly tied to the host's interests. Symbionts residing in hosts are under tight host regulation which limits their proliferation and restricts interactions with other microbes, especially for bacteriome-associated symbionts, thereby limiting opportunities such as horizontal gene acquisition that are common amongst their free-living counterparts. Although *D. fasciatus'* gut bacterial symbionts remain insensitive to the host antimicrobials expressed in their presence, other regulation mechanisms such as gut physicochemical conditions (pH and oxygen concentrations) and microbicidal reactive oxygen species (ROS) might be important in restricting their proliferation within the gut. Non-specific transmission of these symbionts via egg surface smearing and coprophagic behavior ensures maintenance of stable genomes that allows them to survive outside the host.

The presence and variation in the abundance of pathogens that defensive symbionts protect against can indirectly affect defensive symbiont fitness by influencing the symbiont's frequencies within the population. In the absence or low abundance of pathogens, selection pressures drive hosts towards the symbiont-free condition because of fitness costs associated with possessing symbionts while at higher abundances of pathogens, selection favors hosts with defensive symbionts as the benefits of possessing the symbionts outweigh costs associated with their maintenance (Hopkins et al., 2017). In the absence of *L. pyrrhocoris* infections, *D. fasciatus* maintain a stable gut bacterial community, as it is essential in supplementing the insect's nutrient-poor seed diet with B-vitamins. However, presence of the parasite slightly interferes with the abundance and diversity of the gut bacterial symbionts (Onchuru, Martinez, & Kaltenpoth, 2018).

7.3.2. Transmission of opportunistic microbes

To maintain beneficial symbionts across host generations and populations, hosts evolve vertical and/or horizontal transmission mechanisms that transfer or facilitate acquisition of essential mutualistic symbionts. Depending on the specificity of a transmission mechanism, symbiont transfer can open windows for the transfer and acquisition of opportunistic microbes. Non-specific symbiont transmission mechanisms such as trophallaxis, coprophagy, and environmental acquisition that are prevalent in social (bees, ants, and termites) and gregarious (Reduviidae and Pyrrhocoridae bugs) insects play an important role in the establishment of multi-partite symbioses, as reviewed in chapter two, but are characterized by high chances of transmitting or acquiring unintended microorganisms (Konrad et al., 2012; Onchuru, Martinez, Ingham, et al., 2018; Tragust et al., 2013; Traniello et al., 2002). In some instances, primary pathogen exposures during social and gregarious interactions increase susceptibility of individuals to subsequent exposures (Mirabito & Rosengaus, 2016).

In *D. fasciatus* and its close relative *P. apterus*, both of which exhibit gregarious behaviors, transmission patterns of their trypanosomatid parasites, *L. pyrrhocoris* and *Blastocrithidia papi,* respectively*,* mirror those of their mutualistic gut bacterial symbionts, suggesting that the parasites hitch-hike on symbiont transmission routes (Frolov et al., 2017; Salem et al., 2015). The adoption of the symbionts' vertical (egg surface smearing and probing) and horizontal (coprophagic behavior) transmission mechanisms may have reinforced the parasites' ability to establish high infection frequencies in different geographical host populations (Votýpka et al., 2012). Thus, although group living increases reliability of obtaining a stable microbial community, this behavior presents risks of facilitating rapid transmission and acquisition of pathogens amongst highly genetically similar nest mates which may lead to colony collapse when pathogens are strongly virulent.

7.4. Defensive symbionts as drivers of host and parasite evolution

Defensive symbionts complement host immune defenses in protecting against natural enemies through the production of bioactive metabolites, competition for limiting resources, priming host immune defenses, and improving host vigor (Flórez et al., 2015; Ford & King, 2016; Kroiss et al., 2010; Pan et al., 2012; Paredes et al., 2016). Thereby, symbiont-mediated protection can have short-term and long-term repercussions on the evolution of defensive symbionts, host immunity, and virulence of invaders. Natural enemies can nonetheless counter-adapt symbiont conferred defenses, resulting in the reduction or loss of the symbiont protective trait after a few host generations (Dennis et al., 2017; Dion, Zélé, et al., 2011). For example, experimental coevolution of *Enterococcus faecalis* protective symbiont with pathogenic *Staphylococcus aureus* bacteria in *Caenorhabditis elegans* host resulted in a significant reduction of pathogen virulence after ten generations as a result of adaptation to the defensive microbe (Ford, Kao, et al., 2016). Equally, *Aphidius ervi* and *Lysiphlebus fabarum* parasitoids of aphids rapidly counter-adapt to the protection provided by *H. defensa* symbionts and after a few generations, parasitism rate is similar or even higher in protected than in unprotected aphid lines (Dennis et al., 2017; Dion, Zélé, et al., 2011).

The mechanism of symbiont-mediated defense can have an impact on pathogen evolution dynamics. When symbionts defend the host via interference mechanisms, which is the most commonly reported mode of protection in described defensive systems (Ford & King, 2016), parasites may evolve counter-adaptive mechanisms such as altering target cell membrane receptors or producing counteractive compounds to resist or neutralize symbiont toxins (Vorburger et al., 2018). Such investments are costly as they require re-allocation of resources by the parasite which may ultimately lead to the evolution of low parasite virulence. Conversely, symbiont-mediated protection through competition for shared host

resources might drive pathogens towards higher virulence as increased exploitation of common resources is essential for higher parasite fitness (Alizon et al., 2013; Vorburger et al., 2018).

In hosts, protection conferred by defensive symbionts can interfere with the evolution of innate host resistance mechanisms due to decreased selection pressures on host defensive genes to evolve better protection. In *Drosophila melanogaster*, the presence of *Wolbachia* endosymbiont, which confers strong protection against *Drosophila* C virus (DCV), interferes with the evolution of host immunity against DCV (Teixeira et al., 2008). In an evolution experiment, *Wolbachia*-mediated resistance against DCV infections increased when the protective symbiont and the virus evolved together. However, the copy number of the *D. melanogaster* allele that is responsible for resistance against DCV decreased concurrently due to redundancy and relaxed selection (Martinez et al., 2016). A severe reduction of the frequency of this virus resistance allele from the host can lead to the evolution of *D. melanogaster* dependence on the symbiont for protection as it may have occurred in aphids. Despite lacking the Imd pathway genes responsible for recognition, signaling, and killing of microbes (Gerardo et al., 2010), aphids overcome fungi infections and parasitic wasp attacks by harboring defensive facultative endosymbionts (Oliver et al., 2010). Arguably, relaxed selection for aphid immune defenses as a result of the aphid lifestyle, i.e., teaming up with defensive and nutritional bacterial symbionts, may have facilitated the loss of the essential innate immune genes.

The mode of acquisition and transmission of a pathogen can influence evolution of virulence. Adaptation to vertical transmission mechanisms mostly facilitates evolution of low symbiont virulence compared to horizontal transmissions (Sachs & Wilcox, 2006). As with many other monoxenous trypanosomatids, *L. pyrrhocoris* parasites confined in guts of pyrrhocorids cause asymptomatic infections that are characterized by mild virulence (low mortality rates) (Koch & Schmid-Hempel, 2011; Onchuru, Martinez, & Kaltenpoth, 2018). Although *L. pyrrhocoris* parasite still relies on frequent horizontal transmission, it is likely that its adaptation to the symbionts' vertical transmission mechanism might have facilitated evolution of low virulence since its fitness levels are tied to host survival and reproduction.

7.5. The future of host-symbiont-parasite interactions

Many of the described host-parasite coevolution theories assume simple interactions where evolutionary counter-adaptations occur between two species, i.e., the host and its parasite. However, despite the increasing evidence of symbiont-mediated protection, there is little knowledge on how defensive symbionts influence host-parasite coevolutionary dynamics in multipartite associations. In symbiont-conferred protection, it is possible that a symbiont-parasite arms race takes prevalence over host-parasite coevolution, especially when protection is based on direct interactions between symbionts and natural enemies. For instance, production of toxins by protective symbionts against pathogens can cause reciprocal adaptive responses from the pathogens (Dion, Zélé, et al., 2011; Vorburger et al., 2018). How symbionts and pathogens evolve this counter-adaptive measures within the host environment remains a key research area for future studies.

Some symbionts are known to improve host vigor and fitness through nutrient supplementation which enhances its potency to ward off natural enemies (Flórez et al., 2015; Gerardo & Parker, 2014). Therefore, any disturbance on the homeostasis of the essential symbionts or symbiont provisioned nutrients by pathogens can alter host fitness and subsequently interfere with its defense abilities. *D. fasciatus,* for example, relies on its gut bacterial symbionts for the supplementation of B-vitamins that are limiting in the insect's natural Malvales seed diets (Salem et al., 2014). Arguably, its gut trypanosomatid

parasite, *L. pyrrhocoris,* likewise depends on the gut bacterial symbionts for B-vitamin supply for growth and development, as the parasite lacks B-vitamin synthesis pathways (Flegontov et al., 2016). This implies that symbiont produced B-vitamins may be enhancing *L. pyrrhocoris* infections and that *D. fasciatus* directly competes with the parasite for the symbiont supplied essential B-vitamins. Recent studies in the tsetse fly show that its essential nutritional and defensive endosymbiont, *Wigglesworthia,* enhances parasitism by the folate auxotrophic African trypanosomes through the supply of this essential B-vitamin that the parasites require for growth and development within the insect host (Rio et al., 2019). Since this is a relatively new field in symbiosis research, future studies should characterize how commonly symbiont-mediated advancement of antagonism occurs across insect taxa. Additionally, its influence on host-mutualist relationships remains largely unknown, hence the need to assess the impact of parasite-mediated within-host competition, i.e., symbiont and parasite competition or host and parasite competition for public goods, on the stability of mutualistic interactions.

Defensive symbionts significantly improve host fitness in the presence of natural enemies, and there are recommendations for their exploitation as biological control agents to combat pests and diseases of medical and agricultural importance. For example, *Wolbachia* and *Sodalis glossinidius* endosymbionts of *Aedes aegypti* mosquitoes and tsetse flies, respectively, have significant potential for preventing incidences of dengue viral and human African trypanosomiasis (HAT) infections, respectively (Aksoy et al., 2008; Hoffmann et al., 2015). Using defensive symbionts that co-evolve with the target pathogen may be a particularly sustainable strategy when combating pathogens that develop resistance to drugs. This is because the symbionts can reciprocate parasite adaptations compared to drugs which have few options for countering parasite adaptations. However, for defensive symbionts to be fully exploited as biological control agents, future studies must address reproducibility of laboratory experimental results in natural environments with additional and ecologically relevant confounding factors. Furthermore, symbiont-parasite coevolutionary dynamics must be addressed to determine short and long-term stability and effectiveness of the defensive symbionts, e.g., to understand how quickly pathogens adapt to symbiont protection (Vorburger et al., 2018). Suggestions have also been put forward to genetically engineer defensive symbionts to improve their protective ability or confer them with additional traits that can favor their selection and distribution in target populations. Before these recommendations are implemented, however, extensive ecological risk assessments must be conducted to determine the impact of such technologies on natural ecosystems.

7.6. References

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ORIGINALITY STATEMENT

I certify that the intellectual content of this thesis is the product of my own work. Further, I confirm that I have clearly referenced, in text and in references, all sources used in this work. This work has not been previously, or concurrently, submitted to any other faculty or University for the award of any degree. Published sections of this thesis and contributions of each collaborator have been adequately acknowledged at the beginning of this thesis.

Mainz, 12th June 2019

Onchuru, Thomas Ogao

"A capacity, and taste, for reading gives access to whatever has already been discovered by others." — Abraham Lincoln