Defensive symbiosis in *Drosophila*: from multiple infections to mechanism of defense

by

Phineas T. Hamilton
B.Sc., University of Victoria, 2006
M.Sc., University of Victoria, 2010

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biology

Defensive symbiosis in *Drosophila*: from multiple infections to mechanism of defense

by

Phineas T. Hamilton
B.Sc., University of Victoria, 2006
M.Sc., University of Victoria, 2010

Supervisory Committee

Dr. Steve Perlman, Supervisor
(Department of Biology)

Dr. Brad Anholt, Departmental Member
(Department of Biology)

Dr. Ben Koop, Departmental Member
(Department of Biology)

Dr. Brian Starzomski, Outside Member
(Department of Environmental Studies)

Supervisory Committee

Dr. Steve Perlman, Supervisor
(Department of Biology)

Dr. Brad Anholt, Departmental Member
(Department of Biology)

Dr. Ben Koop, Departmental Member
(Department of Biology)

Dr. Brian Starzomski, Outside Member
(Department of Environmental Studies)

ABSTRACT

Multiple infections within the same host are now understood to be common and important determinants of the outcomes of disease processes. Multiple infections are particularly important in insects, which are often infected by vertically transmitted symbionts that are passed from the mother to her offspring. In many cases, these symbionts have evolved to confer high levels of protection against co-infecting parasites, pathogens, or other natural enemies. Despite widespread examples of symbiont-mediated defense, there are key outstanding questions in the ecology and evolution of defensive symbiosis. These include the mechanisms through which protection is conferred, the specificity of defensive effects against different parasites and pathogens, and the overall roles of defensive and other symbioses in host communities and ecosystems.

To address these questions, I used a model of defensive symbiosis in which the bacterium *Spiroplasma* protects the woodland fly *Drosophila neotestacea* from the nematode parasite *Howardula aoronymphium*. First, I conducted a series of experiments

that included transcriptome sequencing of *D. neotestacea* infected by *Howardula* and *Spiroplasma* to uncover the mechanistic basis of defense in this symbiosis. Through these experiments, I found evidence of a putative protein toxin encoded by *Spiroplasma* that might contribute to defense. Following this, we characterized the protein as a novel member of a class of toxins known as ribosome-inactivating proteins (RIPs). RIPs are important virulence factors in bacteria such as enterohemorrhagic *E. coli*; I exploited recent approaches for quantifying RIP activity to design sensitive assays that demonstrate that *Howardula* suffers a high degree of ribosome cleavage specific to RIP attack during *Spiroplasma*-mediated defense. This is among the first demonstrations of a mechanism of defense against a specific enemy in an insect defensive symbiosis.

I next worked with collaborators to culture and characterize a novel trypanosomatid parasite of *Drosophila* that I uncovered during the above transcriptome sequencing. Trypanosomatids are protist parasites that are common in insects, and the causes of important human diseases that include Chagas disease and African sleeping sickness. Despite *Drosophila*'s history as an important model of infection and immunity, little is known of its trypanosomatid parasites, and we describe this parasite as a new genus and species: *Jaenimonas drosophilae*, the first trypanosomatid formally described from a *Drosophila* host. We conduct a series of experiments to understand infection dynamics, immune responses and interactions with other parasites and symbionts within the host, beginning to establish *Drosophila-Jaenimonas* infections as a tractable model of trypanosomatid infection in insects.

Finally, though examples of ecologically important defensive symbioses accumulate, an understanding of their overall roles in ecosystems is lacking. I close with a synthesis of the ways in which symbioses - defensive or otherwise - can affect ecosystem structure and function through their effects on food webs. This work will help to develop a conceptual framework to link reductionist findings on specific symbioses to larger scale processes.

Contents

List of Tables

List of Figures

ACKNOWLEDGEMENTS

Research is a collaborative process, and this work wouldn't have been possible without the help of many people. I've done my best to acknowledge the contributions of everyone involved throughout this dissertation.

I owe thanks to many others, including Brad Anholt and Jean Richardson, whose mentoring during my Masters research led me to pursue a PhD. Thanks to Brian Starzomski and Ben Koop, whose guidance on my supervisory committee was indispensable. Other students were also a source of insight and inspiration: David Stuss, Ben Sutherland, Steve McGehee, Pavel Kratina, Jong Leong, and the incomparable Cuong Hieu Le, along with the many members of the Perlman Lab, provided me with friendship and perspective along the way. Steve Perlman, my PhD supervisor, was instrumental to all the work presented here, making the whole thing possible on so many levels.

I'd also like to thank my family for their support and encouragement on this path. Most, I'd like to thank Sarah, without whom I'd never have known about *Spiroplasma*, *Drosophila*, or any of the other creatures I study here.

My funding during this work was provided by NSERC, University of Victoria Scholarships, and a Sinergia grant to Steve Perlman from the Swiss National Science Foundation. Much of the work was made possible by access to Compute Canada's WestGrid network.

Chapter 1

An introduction: from multiple infections to defensive symbiosis

Historically, infectious disease has garnered little attention in ecology. Certainly, isolated and striking examples of diseases such as myxomatosis in feral rabbits or chestnut blight spreading across North America drew substantial attention from ecologists and ecosystem scientists [4, 5]. By and large, though, infection has not been a primary consideration when trying to understand the species interactions that structure natural systems, being subordinate to other well-studied processes such as competition and predation [6, 7]. This has recently begun to change: dramatic and recent declines in many species from infectious disease, often exacerbated or precipitated by human activity, have led to a surge of recent interest. There is now rapidly growing literature exploring the effects of disease on ecosystems and vice versa (e.g. [8–13]), in some ways following a similar growth in evolutionary theories of disease and infection from decades ago [14].

Alongside these developments, it is increasingly evident that most infections do not occur in isolation. Multicellular organisms are typically infected by one or more parasites or pathogens much of the time, and these infections may have strong effects on each other that can dictate the dynamics of disease transmission and the ultimate effects on the host [13, 15–19]. From a human perspective, this is probably best exemplified by HIV, which interacts strongly with other infections through suppression of host immunity, and appears responsible for the resurgence of other diseases, such as tuberculosis in parts of Africa [20]. Other examples such as antagonism between hookworms and *Giardia* in the human gut [21] underscore the likely importance of

multiple infections across disease systems. Strong interactions between infectious agents appear common in many natural systems as well [18, 19, 22, 23], although work on elucidating these interactions has only recently really begun [13, 24, 25].

From an evolutionary perspective, interactions between co-infecting parasite species or genotypes within the same host have provided some of the strongest rationale for why parasites evolve towards virulence, most simplistically where parasites aggressively exploit the host in a push to gain a transmission advantage over competing parasites within the same host [25, 26]. Experimental findings support this - for example more virulent rodent malaria strains appear to be superior competitors [27]. In fact, in cases where parasites have strong competing interests for transmission opportunity, the evolution of direct interference between parasites may even be predicted to evolve [28]. One recent study even found the evolution of 'soldier' castes in snail-infecting trematodes that protect the host against exploitation from additional trematode infections [29]. In this respect, diverse parasite strategies to protect the host can evolve when other natural enemies - parasite or otherwise - seek to exploit the host in a manner the conflicts with parasite interests. Parasite infections can even become beneficial if they interfere sufficiently with other more virulent infections or lethal predators. Such defensive symbioses were initially characterized as isolated examples in diverse systems, from defensive fungal endophytes of grasses [30] to bacteria that protect crustaceans from fungal infection [31] to light-emitting *Vibrio* bacteria that aid in the crypsis of bobtail squid [32], but defensive symbioses increasingly appear to be common [33], if not ubiquitous.

Insects are remarkable among terrestrial animals in that they typically have intimate associations with microbes that are vertically transmitted, or inherited from the mother to her offspring [34, 35]. Many insect symbioses have even evolved to be obligate for the host, providing otherwise limiting nutrients necessary for host success, such as is classically observed with *Buchnera aphidicola* bacterial symbioses of pea aphids [36]. In contrast, other symbioses are facultative in that they are not essential for host survival. These non-essential associations have been intensively studied due to the ways in which symbionts can selfishly distort host reproduction to further their own transmission [37–39], for example by killing males (the non-transmitting sex) in infected lineages to increase female fitness (the transmitting sex) [37]. Conversely, the evolution of symbiont-mediated defense can be also predicted based on the interests of vertically transmitted infections that rely on successful host reproduction for transmission [28], and many striking examples are accruing: insect symbioses

that protect diverse hosts from enemies that include parasitoid wasps, RNA viruses, parasitic nematodes, fungal pathogens and predatory spiders have now been documented [19, 22, 40–44]. The factors that enable the initiation of mutualistic symbioses are not always clear [45], but some bacterial lineages that associate with insects comprise both pathogens and defensive mutualists [46], suggesting that such defensive properties can be early initiators of symbiotic mutualisms, in insects and beyond.

In some cases insect symbionts can suppress the replication of other parasites to such an extent that they are now being exploited to control the spread of insect-vectored diseases. This is currently being attempted through the mass releases of *Aedes* mosquitos in Australia that have been artificially infected with a strain of *Wolbachia*, an inherited symbiont known to interfere with viral replication in multiple hosts [22, 47], in order to suppress Dengue virus transmission to humans [48, 49], and similar approaches are also being explored for *Anopheles* vectors of malaria [50, 51]. *Wolbachia* can also selfishly distort host reproduction using a strategy known as cytoplasmic incompatibility (CI) in which *Wolbachia*-uninfected females in a population are at a reproductive disadvantage, providing a ready drive mechanism for the spread of symbiont-encoded traits into host populations and increasing its prospects as an agent to transform vector populations [39, 49, 52]. Despite this surge in applied interest from a biocontrol perspective, it remains unknown precisely how insect symbionts effect defense in most cases, particularly against specific enemies in naturally occurring defensive associations [53, 54], and a better understanding of these factors is needed.

The bulk of the work presented in this dissertation seeks to better understand defensive symbiosis through studying an association in which a common and widespread woodland fly, *Drosophila neotestacea*, is protected by a bacterium in the genus *Spiroplasma*. [19]. It is my hope that through studying the ecology, evolution, and mechanisms of symbiont-mediated defense in this system we may gain a deeper understanding of processes both affected by and governing disease outcomes.

Drosophila neotestacea suffers high rates of infection by the parasitic nematode *Howardula aoronymphium* in the wild. Across North America ∼25% of individuals are infected, and this infection sterilizes flies [55, 56]. Recently, however, flies were uncovered that were no longer sterilized by infection, and the reproductive-stage nematode 'motherworms' infecting these flies were much smaller, demonstrating that these flies have substantially greater resistance and tolerance to the parasite [19]. Remarkably, this resistance is caused by an inherited bacterial symbiont - *Spiroplasma*,

a Gram-positive bacterium in the class *Mollicutes*. Moreover, the selective advantage conferred by this protection has led to the recent spread of *Spiroplasma* across the range of *D. neotestacea* [1, 19]. Other studies have further shown striking effects of *Spiroplasma* on *Howardula* population dynamics: *Howardula* goes rapidly extinct in experimental microcosms in the presence of *Spiroplasma* [3], underscoring the importance of this association to the ecology of both *D. neotestacea* and *Howardula*. While this system provides one of the clearest examples of a dynamic and ecologically relevant defensive symbiosis, many aspects of the association remain unresolved, such as what are the community-level consequences of this defense, and what is the mechanism through which *Spiroplasma* confers defense.

In Chapter 2, I sought to uncover the mechanistic basis of *Spiroplasma*-mediated defense against *Howardula*. As mentioned, one of the major unresolved questions in the study of insect defensive symbioses has been how protection is actually achieved, and various hypotheses have been put forward to account for the defensive properties of diverse defensive associations [33]. These include, roughly, symbiont-mediated priming, or up-regulation, of host immunity [57–59], competition for limiting resources between parasite and defensive symbiont [60], or production of toxic factors by the symbiont to directly attack invading parasites [61, 62]. Using a series of experiments and a conceptual framework derived from ecological theory on interspecific competition [63,64], I systematically investigated the importance of each of the above mechanisms in this defensive symbiosis. Through these experiments, it became apparent that the best-supported mechanism of protection was through the production of toxins by *Spiroplasma*, with the finding that *Spiroplasma* encodes a potential protein toxin that might specifically target *Howardula*. I also uncovered evidence of an undiagnosed trypanosomatid parasite infecting fly lines in our lab. The bioinformatic analyses for this project were done in collaboration with Ben Koop and Jong Leong, whose assistance with transcriptome assembly and interpretation was indispensable; this work was published in Molecular Ecology [65] with their contributions reflected in their shared authorship. Deeper investigations of this protein toxin and trypanosomatid parasite form the basis of Chapters 3 and 4, respectively.

Following the identification of a potential toxin encoded by *Spiroplasma*, I sought to link this toxin to *Spiroplasma*'s defensive properties. In insect symbioses, there are now multiple examples of known toxins that are produced by symbionts [61, 66], and also putative toxins identified that appear to function in defense [62]. Rarely, if ever, though, have studies successfully characterized and linked specific toxins to eco-

logically meaningful defense against specific enemies. From an applied perspective, understanding the mechanisms that underpin ecologically relevant defensive symbioses, such as we see in *Spiroplasma*'s protection of *D. neotestacea*, will be essential to the goal of exploiting insect symbioses to interfere with the transmission of human disease. To this end, I collaborated with biochemists Martin Boulanger and Fangni Peng, who were able to express and purify this putative toxin for use in a number of experiments that I performed in Chapter 3.

Following expression, I was able to characterize the protein and confirm its function, and that it is a member of a class of toxins known as ribosome-inactivating proteins (RIPs). RIPs include important toxins from a human standpoint, such as the Shiga-like toxins of enterohemorrhagic strains of *E. coli*, and ricin, a plant toxin notable for its extreme cytotoxicity and potential as a bioterrorism agent [67, 68]. Building on recently developed molecular assays for RIP activity [69, 70], I was able to design and apply novel assays for RIP activity against *Howardula* and *Drosophila* during *Spiroplasma*-mediated defense, confirming that *Howardula* is specifically attacked by a RIP during defense. This provides some of the strongest support for the role of a specific effector in an insect defensive symbiosis to date, as well as intriguing opportunities to further investigate the coevolution of toxins and a natural enemy in defensive symbioses. This work is currently in review, with Fangni and Martin as coauthors.

In Chapter 4, I systematically investigate the trypanosomatid parasite uncovered in Chapter 2. RNA sequence data from Chapter 2 showed this parasite to be a trypanosomatid, one of a lineage of protist parasites that are widespread in insects but also well known as the causes of severe diseases in humans that include American Chagas disease, African sleeping sickness, and diverse leishmaniases. Intriguingly, *Drosophila* species have been extensively used as animal models of infection and immunity [71], but their relationships with trypanosomatids are mostly uncharacterized, despite high prevalence of infection in the wild [72]. This is largely due to the fact that to date, there appear to be no cultured and described trypanosomatids that naturally infect *Drosophila*. In collaboration with Jan Votýpka, Vyacheslav Yurchenko, and Julius Lukeš - parasitologists with deep expertise in trypanosomatids - I was able to culture this parasite, which we subsequently described as a new genus and species: *Jaenimonas drosophilae*, named for John Jaenike, who has contributed greatly to an understanding of the ecology and evolution of woodland *Drosophila* and their parasites and symbionts.

Using *Jaenimonas*, I performed a number of infection experiments to test the susceptibility of diverse *Drosophila* host species to *Jaenimonas*, to quantify its fitness effects, and to measure the genetic responses of the host to infection, aided by Nathan Bird, an Honours student at the time. Through further collaboration with Anna Dostálová and Bruno Lemaitre, we were also able to experimentally test the contribution of multiple host defense pathways to resistance to *Jaenimonas* using *D. melanogaster* mutants for key immune genes. This work was recently published in mBio [73], and begins to establish *Drosophila* as a powerful and flexible model to investigate trypanosomatids in their insect hosts. Following this characterization, I conducted additional experiments to examine the interactions between trypanosomatids, *Howardula*, and *Spiroplasma* in *D. neotestacea*, and these findings are included as a so-far unpublished supplement to Chapter 4.

In Chapter 5, I return to a more ecological consideration of insect symbiosis, and symbiosis in general. Despite a rapidly accumulating literature on symbioses in natural systems, a major hurdle remains in understanding their relative contributions to ecosystem structure and function [53]. This is partly because many studies on symbiosis are necessarily reductive, and partly because the prevailing framework for studying ecosystem-level processes as networks of species deals poorly with mixed interaction types that include symbiotic mutualisms [74, 75]. Closing on a synthesis and exploration of these issues, I hope to provide insight into ways by which localized and small-scale findings on diverse symbioses, defensive or otherwise, might be better understood on ecosystem scales to provide direction for tackling this important and unresolved aspect of symbiosis research.

Collectively, this work begins to resolve multiple important aspects of the continuum encompassing multiple infections and symbiotic mutualisms. Using *Spiroplasma* in *D. neotestacea* I present strong evidence that a novel ribosome-inactivating protein underlies host defense, with implications that extend well-beyond this particular interaction. The characterization of *Jaenimonas drosophilae* in *Drosophila* provides a model for investigating trypanosomatid interplay with diverse other infectious agents, and also opportunities to tractably explore aspects of a medically important lineage of parasites in their insect hosts. Finally, by considering the effect of defensive and other symbioses on food web structure and function, I hope to clarify the important yet understudied effects that I believe symbioses to exert in ecosystems.

Chapter 2

Transcriptional responses in a *Drosophila* defensive symbiosis

Abstract

Inherited symbionts are ubiquitous in insects and can have important consequences for the fitness of their hosts. Many inherited symbionts defend their hosts against parasites or other natural enemies; however, the means by which most symbionts confer protection is virtually unknown. We examine mechanisms of defense in a recently discovered case of symbiont-mediated protection, where the bacterial symbiont *Spiroplasma* defends the fruit fly *Drosophila neotestacea* from a virulent nematode parasite, *Howardula aoronymphium*. Using quantitative PCR of *Spiroplasma* infection intensities and whole transcriptome sequencing, we attempt to distinguish between the following modes of defense: symbiont-parasite competition, host immune priming, and the production of toxic factors by *Spiroplasma*. Our findings do not support a model of exploitative competition between *Howardula* and *Spiroplasma* to mediate defense, nor do we find strong support for host immune priming during *Spiroplasma* infection. Interestingly, we recovered sequence for putative toxins encoded by *Spiroplasma*, including a novel putative ribosome inactivating protein, transcripts of which are up-regulated in response to nematode exposure. Protection via the production of toxins may be a widely used and important mechanism in heritable defensive symbioses in insects.

Introduction

It has become increasingly evident that many animals harbor microorganisms that protect against natural enemies [31, 33, 53]. These defensive symbioses have long resisted study for a number of reasons, including that most host microbiomes are incredibly complex and most symbionts cannot be reared without their hosts. Demonstrating the role of defensive microbes in the wild is also challenging, as it requires intimate knowledge of the host's natural enemies.

Perhaps the best-studied animal defensive symbioses are found in insects, which are commonly infected with facultative bacterial symbionts that are transmitted primarily from mothers to their offspring, often in the egg cytoplasm [39, 76, 77]. While they are not strictly required for the insect host to survive, facultative maternally-transmitted symbionts can act as dynamic sources of heritable variation in host populations, and can increase their own frequency by providing benefits to their hosts, such as by protecting them against natural enemies [41, 78, 79]. Defensive heritable symbioses have now been demonstrated in diverse insects, including aphids, fruit flies, and beetles, protecting them against a wide range of organisms, including parasitic wasps and nematodes, pathogenic fungi and RNA viruses, and predatory spiders [19, 22, 41, 43, 61, 80]. That defense against diverse parasites can result from infection by taxonomically disparate symbionts suggests that it is a common if not predominant aspect of insect symbioses.

The mechanisms by which symbionts actually defend their hosts remain virtually unknown. Interest in this subject has surged in recent years due to their potential use in controlling human diseases that are vectored by insects (eg. [48, 50]). For example, *Aedes* and *Anopheles* mosquitoes have been artificially infected with strains of the inherited symbiont *Wolbachia* that block replication of Dengue virus and malaria [50, 57]. In addition to determining whether a defensive symbiont will provide protection in novel hosts, the specific mechanism of defense can potentially affect the long-term persistence of the symbiont and the ability of the natural enemy to evolve counter-resistance.

Three contrasting mechanisms have been proposed to explain how a symbiont could be expected to block parasite growth and lead to defense of the host [33]; all have parallels within a more classical ecological framework, being analogous to exploitative, interference and apparent competition between species [63,64,81,82]. First, exploitative competition between symbionts and parasites within a host for limiting

factors could limit parasite growth, decreasing parasite-induced pathology and leading to defense. This would likely be mediated through competition for a shared and limiting resource, and provides an indirect mechanism though which a symbiont and parasite could interact. While exploitative competition can be a common feature of coinfecting parasites [83] with important implications for the evolution of parasite virulence [26], it has yet to be convincingly demonstrated as a mechanism for host protection in heritable symbionts - although recent work suggests that competition for cholesterol may play a role in *Wolbachia*'s defense against RNA viruses in *Drosophila* [60].

Second, symbionts have been proposed to defend their hosts by priming the host immune system [33, 58, 84], increasing the ability of the host to respond effectively to attack by a parasite. This is analogous to apparent competition, in which an increase in one species leads to an increase in a predator that negatively affects a competitor [82]. In the case of apparent competition between interacting parasites, the host immune system is typically considered to take the place of the predator [64, 85], and as such should be evident in symbiont up-regulation of host immunity. The best evidence for immune priming in defensive symbiosis has come from studies of mosquitoes artificially infected with *Wolbachia* that have shown that many host immune genes are constitutively up-regulated during symbiont infection [47, 84].

Last, symbionts may directly attack parasites or other enemies, analogous to interference competition. Interference competition arises when one species directly antagonizes another [63, 81], possibly through preemption of space or the production of toxic factors. Interference competition is common in bacteria, which produce a ubiquity of bacteriocin toxins, among others, that target closely-related competitors [86], and is also probably the best-substantiated mode of defense by heritable symbionts. In *Paederus* beetles, a heritable *Pseudomonas* symbiont produces a toxin that can deter predation by spiders [61]. In aphids harboring the defensive symbiont *Hamiltonella defensa*, defense against parasitoid wasps is linked to toxins produced by a bacteriophage associated with *Hamiltonella* [62].

Here, we performed experiments aiming to test which of the above-described mechanisms explains a defensive symbiosis in *Drosophila*. *Drosophila neotestacea* is a widespread North American woodland fly that is commonly infected by a virulent parasitic nematode, *Howardula aoronymphium* [55]. Parasitism by *Howardula* normally sterilizes flies. Remarkably, flies harboring the inherited defensive bacterial symbiont *Spiroplasma* are no longer sterile when infected with nematodes, and

Howardula infection intensity is dramatically decreased [19]. The selective benefit conferred by *Spiroplasma* has led to its rapid spread throughout *D. neotestacea* [1], but the means by which *Spiroplasma* defends its host remains completely unknown.

First, in order to test for exploitative competition, we measured the intensity of *Spiroplasma* infection in *Howardula*-infected and uninfected flies over time, using quantitative PCR, with the hypothesis that *Spiroplasma* and *Howardula* would exhibit compensatory dynamics if in exploitative competition for common resources (i.e. *Spiroplasma* infection intensity would decrease in the presence of *Howardula*). Second, to test for apparent competition in the form of immune-priming, we performed an RNA-sequencing experiment to look for evidence that *Spiroplasma* up-regulates host immunity in a manner that could explain defense. Last, we screened *Spiroplasma* transcripts assembled during RNA-sequencing for evidence of *Spiroplasma*-encoded genes that could account for defense, such as toxins that could underlie interference competition between *Spiroplasma* and *Howardula*. Our results show little support for exploitative competition or immune priming; rather, we uncovered putative *Spiroplasma* toxins that may be involved in defense.

Materials and Methods

Drosophila and *Howardula* stocks

Spiroplasma infected [S+] and uninfected [S-] lines of *D. neotestacea* originally collected in 2006 in Hartford, Connecticut were maintained as described elsewhere [1]. These lines initially also harbored *Wolbachia*, which we selectively eliminated by treatment with rifampicin [19]. We introgressed S+ and S- fly lines for six generations prior the start of the experiment [1]. *Howardula aoronymphium* used in this experiment were also originally collected in Hartford in 2006, and were maintained in the lab in *Drosophila falleni*.

Spiroplasma-Howardula competition: experimental design

To test for effects of *Howardula* on *Spiroplasma* infection intensity we exposed the S+ fly line to infective *Howardula* nematodes: we collected eggs by allowing flies to oviposit on mushroom-agar (100g blended *Agaricus bisporus*, 100 mL water, 5 g sucrose, 2.5 g agar and 0.1 g methyl paraben as a mold inhibitor) plugs in petri

dishes overnight. Small mushroom *(A. bisporus)* pieces were infected with 400 infective juvenile nematodes produced by grinding *Howardula*-infected *D. falleni* in Ringer's solution. We added the same volume of ground *D. falleni* in Ringer's from a *Howardula* uninfected line to mushroom pieces for *Howardula*-unexposed (control) treatments. 20 eggs were transferred to infested (*Howardula* exposed treatment) or uninfested (*Howardula* control) mushroom pieces. Mushrooms were transferred to plastic vials containing moistened cheesecloth and a cotton dental plug maintained at 22°C, and monitored fly development.

Individual flies were collected at 5 day intervals (larvae at 5 days, pupae at 10 days, adults at 15, 20, and 25 days), and stored at -20°C. DNA was extracted from individual flies using 75 μL PrepMan Ultra (Applied BioSystems, Foster City, CA). Relative *Spiroplasma* infection intensity was measured using quantitative PCR, standardizing dnaA copy number primer with the host gene triose phosphate isomerase that we developed for *D. neotestacea* using Primer3 [87, 88]. For quantification, we used 20 μL reactions with iTaq Universal SYBR Green Supermix (BioRad) according to the manufacturer's instructions, on a BioRad CFX-96 thermal cycler, with duplicate technical replicates and 1/100 dilutions of template DNA. Primer efficiencies were validated using 5 $\times$ 10-fold serial dilutions of S+ *D. neotestacea* DNA (efficiencies and cycling conditions in Table A.1). Relative infection intensities were calculated using the efficiency-controlled method of [89]. Flies exposed to nematodes were diagnosed as *Howardula*-infected or uninfected based on triplicate PCR screens using the aor primer set [3] (see Table A.1 for reaction conditions).

We tested for *Howardula* effects on *Spiroplasma* infection using an analysis of covariance (ANCOVA) with crossed treatment (*Howardula*-exposed uninfected; *Howardula*-exposed infected; *Howardula* unexposed) and sampling day as predictor variables and relative *Spiroplasma* infection intensity as the response variable, omitting flies collected at the first time point when no *Howardula* were detected from this analysis. Throughout experiments, $\log_{10}$ or $\log_2$ transformations of response variables were used for statistical analyses as appropriate.

Transcriptome Sequencing: Experimental Design

We used a 2 $\times$ 2 factorial experimental design, with *Spiroplasma* infection and *Howardula* exposure as crossed factors to assess the transcriptional response of the *D. neotestacea* host to *Spiroplasma* and *Howardula*, resulting in 4 libraries for sequencing.

Experimental *Howardula* (HA) infections were generated as detailed above, but included S- lines, to allow for assessment of a total of 4 experimental treatments (HA-S-; HA+S-; HA-S+; HA+S+), with 10 vials (containing 20 eggs per vial) set up for each treatment.

One day after eggs had been placed on mushrooms, we collected five *D. neotestacea* eggs from each treatment, and made additional fly collections every five days as flies developed, until six collections had been made (26 days from egg oviposition), giving an aggregate sample of 30 egg, larval, pupal and adult *D. neotestacea* from each treatment. When adult flies emerged (< 24hrs), they were transferred to solitary individual vials to ensure they remained unmated for the course of the experiment. At each collection time point, we initiated collections at 9 AM and randomized the order of treatments collected to minimize confounding effects of collection time on host gene expression, and also collected flies at random from vials of each treatment. Flies were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

We extracted total RNA from aggregate fly samples for each treatment using a Qiagen RNEasy Kit (Qiagen, USA) after homogenizing flies in Qiagen Buffer RLT in a Mini Beadbeater 8 for 30 sec with 1 mm silica-zirconium beads (BioSpec Products). Total RNA was provided to the Michael Smith Genome Sciences Centre for subsequent polyA enrichment for mRNA transcripts, library construction and sequencing of 75 bp paired-end transcripts using an Illumina HiSeq 2000 according to established protocols (www.bcgsc.ca). Because this mRNA enrichment selects for eukaryotic transcripts, the majority of *Spiroplasma* transcripts were not recovered. One library was constructed and lane sequenced per experimental condition.

Bioinformatics Analysis

We pooled raw reads from each experimental condition and assembled a de novo reference transcriptome using Trinity [90], with a minimum K-mer count of 3. Trinity has the ability to distinguish putative spliced isoforms of each assembled gene. We found substantial similarity between numerous assembled isoforms and even genes in our transcriptome assembly. Thus, to improve the quality of our de novo assembly, we simplified the assembly through an elimination of redundancy by taking a single representative of transcripts that were > 98% similar over a minimum length of 300 bp, as determined by BLASTn [91]. This simplified transcript set was further screened by only retaining putative transcripts that were characterized as full-length [92], those

that had a significant match to the SwissProt or Gene Ontology protein databases (E-value $< 10^{-}5$), and those that did not show any sequence homology to a known protein but had a predicted open reading frame ≥ 300 bp.

Expression levels for each contig in this reduced assembly were determined by independently mapping raw reads in each experimental condition back to the reduced assembly using the 'RNA-Seq Analysis' tool in the CLC Genomics Workbench software package.

Because the assembled transcriptome was expected to contain transcripts from both the host (*Drosophila*) and parasite (*Howardula*), as well as other potential infectious organisms, including *Spiroplasma*, we used a BLASTx search against the nr (non-redundant) database using standalone BLAST+ 2.2.26 to assign putative taxonomy to each contig. Contigs with BLAST e-values of $< 10^{-4}$ and the top hit as *Drosophila* were characterized as host transcripts, whereas contigs with the top hit as a nematode genus were characterized as *Howardula*. We expected to find *Drosophila* transcripts in all treatments, whereas *Howardula* transcripts should only be present in *Howardula*-exposed treatments. Indeed, this was the case, with the vast majority of putative *Drosophila* transcripts (99.7 %) found in all four treatments, and 97.7 % of *Howardula* transcripts present in only two. To ensure that all possible *Spiroplasma* transcripts were recovered, we also subjected the initial (unreduced) Trinity assembly to this BLASTx search. Once putative taxonomy was assigned to contigs, additional searches were performed against the FlyBase database for all *Drosophila melanogaster* predicted proteins in order to assign (E-value $< 10^{-4}$) gene annotations to contigs predicted to be from *Drosophila*. We searched against the SwissProt database to assign annotations to transcripts from other organisms. Transcripts predicted to belong to organisms (potential gut commensals, etc) not the focus of this study were not further analyzed.

The *DESeq* package [93] in R/BioConductor v. 2.14 [94] was used to test for differential expression (DE) of transcripts in response to treatments. P-values were calculated for each *Drosophila* transcript for main effects of *Howardula* exposure and *Spiroplasma* infection, and the statistical interaction between the two. We assessed the significance of effects using an adjusted P-value of 0.10 (Benjamini-Hochberg correction). This slightly less stringent corrected P-value was used due to the relatively low power of the limited technical replication of our experiment and our concern with type II error (i.e. failing to find *Spiroplasma* effects on immune gene expression when present). While this experiment also allowed us to assess the effect of *Spiroplasma*

on *Howardula* gene expression, we found that the substantially lower (nearly ten-fold) *Howardula* read counts in S+ treatments precluded meaningful analysis without further replication.

Finally, we used the *GOSeq* package [95] in BioConductor to test for Gene Ontology enrichment in host transcripts with common responses to infection. DE transcripts were divided into five categories: those having a significant positive (up-regulated) or negative (down-regulated) response to *Howardula* or *Spiroplasma*, or having a significant interaction between infection types (i.e., non-additive effects of *Howardula* and *Spiroplasma* infection) and tested for GO term enrichment in each of these categories based on GO associations for *Drosophila melanogaster* genes in FlyBase.

Quantitative PCR of Gene Expression Levels

To verify our transcriptomic analysis in an independent experiment, we selected a subset of transcripts responding significantly to treatments and designed RT-qPCR primers to evaluate their expression levels (Table A.1). To do this, we repeated experimental infections to generate additional replicate samples appropriate for qPCR-based gene expression analysis. We performed experiments and reared flies as described above, but collected single adult flies from each treatment only once adult flies were 7-days old (∼20 days post oviposition). We also diagnosed nematode exposed flies as infected or uninfected, giving a total of six treatments: nematode infected, nematode uninfected and nematode unexposed (control), each with and without *Spiroplasma*. Altogether, 30 flies were included (5 from each treatments; one S+ line individual was diagnosed as S- using PCR and was analyzed as such) in this analysis.

RNA and DNA from individual flies was extracted using Trizol-LS (Invitrogen), eluting RNA into 10 μL of RNase-free water. We confirmed successful DNA extraction by amplifying a portion of the *D. neotestacea* tpi gene (Table A.1) and diagnosed *Howardula* infection status using PCR. Flies were considered to be negative for *Howardula* if we successfully amplified host DNA but failed to amplify *Howardula* in triplicate PCR reactions. Because we uncovered a cryptic trypanosomatid infection in our experiment during analysis (see results below) we additionally screened these flies for trypanosomatid infection using primers targeting the trypanosomatid spliced leader rRNA sequence [96] (Table A.1); despite successful amplification from other lab and wild collected flies, we were unable to amplify trypanosomatid DNA in these flies,

strongly suggesting they were uninfected by these parasites at the time of collection. Individual flies from S+ treatments were confirmed to be *Spiroplasma*-infected using *Spiroplasma*-specific primers prior to data analysis (Table A.1). All PCR reactions contained positive and negative (no-template) controls.

RNA fractions from each fly were treated with DNAse I (Ambion) to digest contaminating DNA, and RNA quality was verified by electrophoresis on a 1 % agarose gel. Remaining RNA (5 μL) was used as template for random-primed cDNA synthesis using the Invitrogen Superscript III cDNA Synthesis Kit (Invitrogen).

We measured relative mRNA transcript abundance for genes of interest normalizing expression against the reference gene rpl28 from *Drosophila* and using 1/20 dilutions of cDNA, but in otherwise the same manner as detailed for the qPCR of *Spiroplasma* infection above. Primers were developed for genes of interest using Primer3, and primer efficiency was verified using a 5 $\times$ 5-fold dilution series for more highly expressed transcripts, or a 5 $\times$ 2-fold dilution series for less abundant transcripts. Primer specificity was verified by melt-curve analysis and gel electrophoresis of products; efficiencies and cycling conditions for each primer set are presented in Table A.1.

Data were analyzed using a 2 $\times$ 3 ANOVA in R, with *Spiroplasma* and *Howardula* status (unexposed, exposed uninfected, infected) crossed, and the $\log_2$ of the relative expression value of the gene of interest as the response. For one gene of interest (lysozyme X), some (4/30) flies had no detectable transcript abundance; relative expression values for these flies were included in statistical analyses at the expression level of the lowest reliably detected sample.

We also designed primers to amplify the putative RIP toxin of *Spiroplasma* (Table A.1), normalizing expression level against *Spiroplasma* dnaA transcript abundance. Initial qPCR analysis of expression level in week-old flies was suggestive of an effect of *Howardula*, but not conclusive; to further examine expression patterns we generated an additional 32 S+, *Howardula*-exposed and control flies for analysis as outlined above, and tested *Spiroplasma* RIP expression in them when they were day-old adults ($\sim$13 days post oviposition; 31/32 flies were confirmed as *Spiroplasma* positive with PCR and were used in analysis); we also quantified *Spiroplasma* infection intensity in the DNA fraction from these flies (absolute quantification of DNA standardized to 40 ng/μL) to examine infection intensity effects on RIP expression. We analyzed *Howardula* and *Spiroplasma*-density effects on RIP expression using ANCOVA.

Results

Spiroplasma Density

We did not detect any evidence of an effect of *Howardula* exposure or infection on the intensity of *Spiroplasma* infection in *D. neotestacea* (Figure 2.1: ANCOVA of $\log_2$ transformed infection intensity: infection main effect, $F_{2,47} = 1.06$, $P = 0.355$; infection × time interaction, $F_{2,47} = 1.64$, $P = 0.21$). Based on this, *Spiroplasma* growth appears independent of the presence of *Howardula*; this is counter to the expectation of decreased *Spiroplasma* growth in the presence of *Howardula* if they are in exploitative competition.

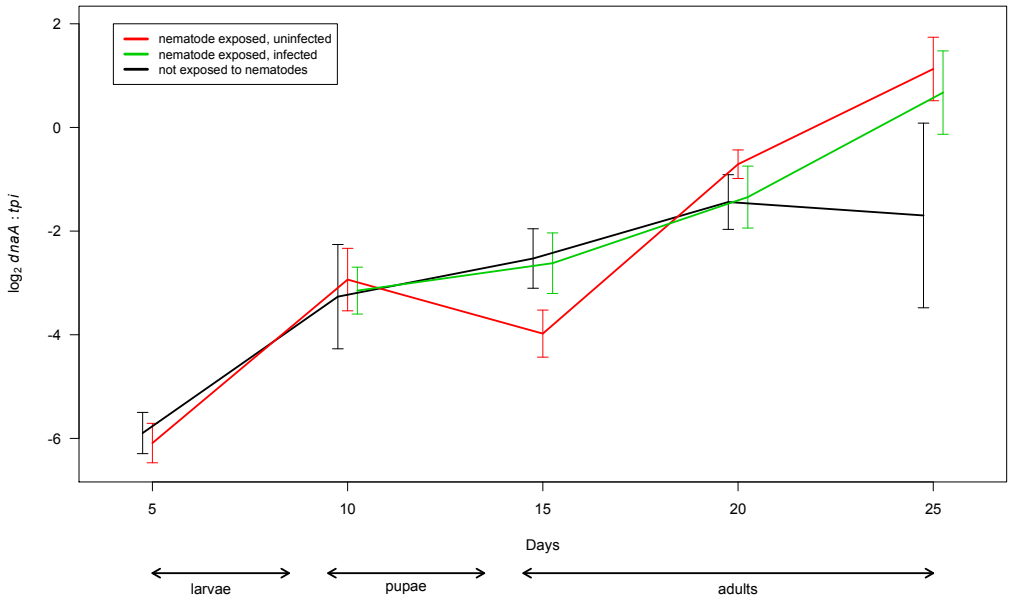

Figure 2.1: *Spiroplasma* infection intensity (mean ± SE of $\log_2$ of *Spiroplasma* dnaA normalized against host tpi) over the development of *Drosophila neotestacea*. Error bars are staggered for clarity. There were no discernible effects of *Howardula* exposure or infection status on *Spiroplasma* infection (N = 69).

RNA Sequencing Gene IDs and Numbers

Patterns of transcript expression across treatments indicated that taxon classification was accurate, in that the vast majority of putative *Drosophila* transcripts (99.7 %) occurred in all four treatments, while those of *Howardula* were limited to HA+

treatments (97.7 %). Illumina sequencing provided an average of over 3×10^8 raw 75 bp paired-end reads per treatment that successfully mapped back to the assembled transcriptome. Initial Trinity assembly produced a transcriptome of 129,379 putative isoforms of 65,144 genes, for an average of 1.99 isoforms per gene. Subsequent reduction of this assembly yielded a total of 30,575 transcripts with predicted ORFs longer than 300 bp, 28,520 (93 %) of which we were able to provide with a putative taxonomy from BLASTx against the non-redundant database (Figure 2.2). Of these, 13,650 (50 %) were tentatively identified as *Drosophila*, and 12,651 (93 %) had annotations in FlyBase (E-value $< 10^{-}4$). We also recovered 6,648 putative nematode transcripts (Figure 2.2).

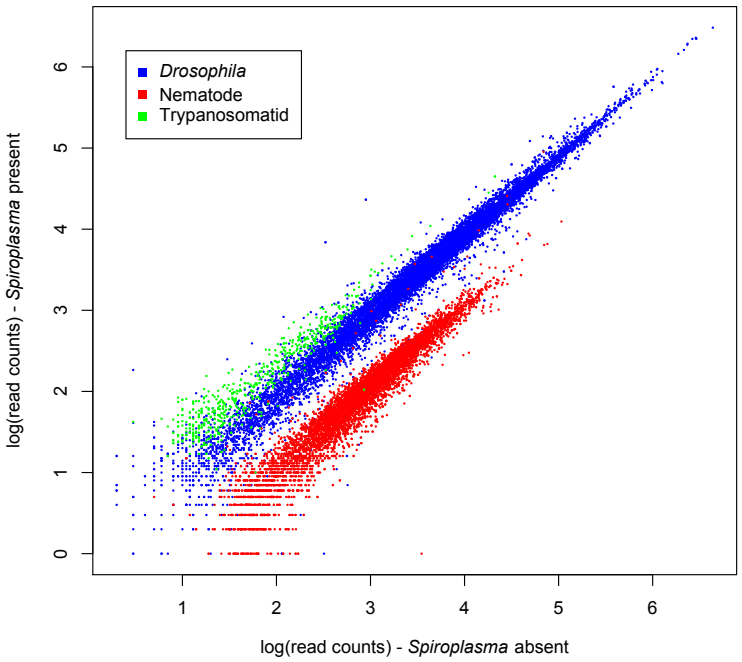

Figure 2.2: Raw expression levels of assembled transcripts in the reduced transcriptome, with taxonomy assigned by BLASTx against the non-redundant database. Treatments containing both host and parasites are shown (S-HA+ and S+HA+), with total numbers of contigs for the host and parasites presented in legend. Nematode transcript abundance is ∼10 fold lower in the S+ line.

We also recovered 568 transcripts that appeared to originate from a cryptic trypanosomatid infection in our samples (Figure 2.2). Most trypanosomatid reads occurred only in *Howardula*-exposed treatments (505/568 = 89 %, most likely reflecting

a low number of read-mapping errors). Dissection and microscopic examination subsequently confirmed that *Howardula* infected lab fly lines also harbored trypanosomatids; thus many of the *D. neotestacea* exposed to *Howardula* to generate the transcriptome were likely coinfected with trypanosomatids.

Screening the unreduced Trinity assembly for putative *Spiroplasma* genes based on expression pattern and annotation as *Spiroplasma* or related *Mycoplasma* yielded 49 probable *Spiroplasma* transcripts. Few of these (21) had high quality SwissProt annotations, and their low number and relative sequencing depth precluded rigorous tests of differential expression. However, we did find some transcripts of interest encoded by *Spiroplasma*, including two putative toxins: a homolog of *Clostridium* epsilon toxin and a putative ribosome-inactivating protein (RIP). Sequence overlap of these two contigs suggests that they are immediately adjacent in the *Spiroplasma* genome and may be co-transcribed. Based on Illumina read counts the putative RIP was ∼3 fold up-regulated in *Howardula*-exposed flies and is likely among the more highly-expressed *Spiroplasma* genes, given the low overall number of *Spiroplasma* transcripts recovered. This RIP is also clearly associated with *Spiroplasma*; portions of the contig encoding this gene have high sequence homology to other *Spiroplasma* (BLASTn to *Spiroplasma citri*; E-value 2×10^{-47}).

Transcriptional Responses of *Drosophila*

Tests for differential expression in *DESeq* found evidence ($P_{adj} < 0.1$) of *Spiroplasma* or *Howardula* effects on the expression of 693 transcripts. Most of these (324) were up-regulated in response to *Howardula* exposure, whereas fewer (144) were down-regulated. Only 139 genes responded significantly to *Spiroplasma* infection, with 57 up-regulated and 82 down-regulated. An additional 150 genes showed a statistical interaction between *Howardula* and *Spiroplasma*.

Gene Ontology enrichment analysis of transcripts with common expression patterns using *GOSeq* recovered multiple statistically significant functional categories (Table A.2). Additionally, to visualize patterns of host immune-gene expression, we selected all transcripts having GO terms for immune response (GO:0006955), innate immunity (GO:0045087), defense (GO:0006952), or response to stress (GO:0006950) and heat-mapped their expression across treatments (Figure 23; n=167 transcripts). Figure 2.3 clearly demonstrates that these transcripts are responding predominately to *Howardula* exposure rather than *Spiroplasma* infection, although the majority of

their responses were not statistically significant.

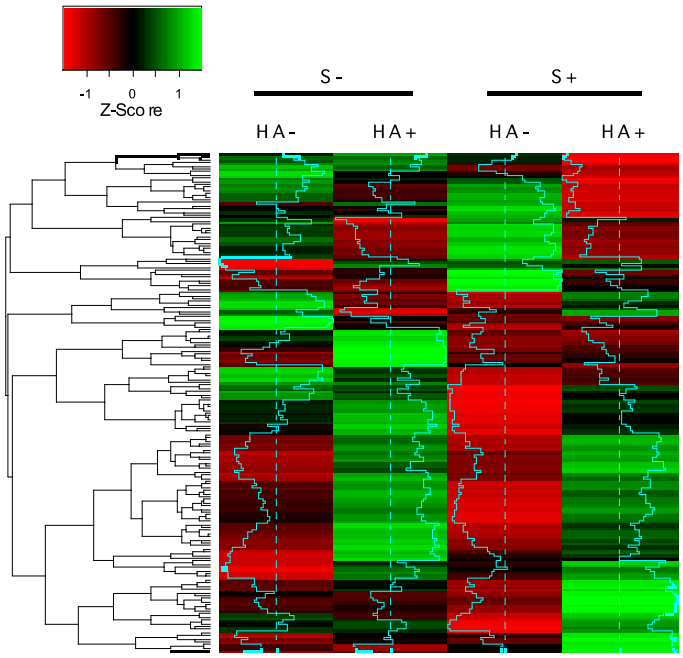

Figure 2.3: Heatmap of expression levels of transcripts with immune, stress or defense function, based on Gene Ontology. Transcripts are clustered on the y-axis based on similarity in expression levels across treatments (Euclidean distance of scaled responses), with the blue trace reflecting the relative magnitude of expression across transcripts (as Z-scores). Expression patterns demonstrate a large immune gene response to *Howardula* (HA) exposure but little response to *Spiroplasma* (S-); note that most of these transcripts are not statistically significant in their responses to treatments.

More specifically, among transcripts significantly down-regulated in *Howardula*-exposed treatments, there was substantial enrichment of GO terms relating to egg development, mitosis and protein translation (Table A.2). This is consistent with an infection that sterilizes the host, and serves as a useful phenotypic anchor that corroborates our ability to detect biologically significant effects of infection with this experimental design.

Numerous transcripts with potential immune activity were up-regulated in response to *Howardula* exposure. These predominately included lectins with carbohydrate binding activity, numerous fibrinogen-like domain containing proteins potentially involved in defense, and proteases. Interestingly, GO enrichment analysis

did not find significant enrichment of immune-related genes in *Howardula*-exposed treatments, although many such genes were elevated at a non-significant level (Figure 2.3). There were many genes involved in clotting substantially up-regulated in response to nematode exposure, including fondue. Also, genes (23) involved in chitin metabolism were up-regulated in response to *Howardula*, possibly in a defensive capacity, although this is not well established in insects [97, 98]. It is also possible that many of these genes may be responding to trypanosomatid infection. In contrast to a recent study of *Drosophila melanogaster* infection by axenic nematodes [99], we did not find convincing evidence of up-regulation of anti-microbial peptides (AMPs) in response to *Howardula* exposure, although numerous differences in methods preclude direct comparison with this study. We did find moderate up-regulation of one isoform of defensin, although another isoform in the dataset did not respond in this way.

We found no clear evidence of host immune priming by *Spiroplasma*: GO enrichment analysis failed to find any statistically significant GO categories enriched among transcripts up-regulated during *Spiroplasma* infection, although a few potential immune genes were affected. These were predominately proteases, although pro-phenol oxidase A1 that may be involved in a defensive melanization response was also moderately up-regulated [71]. There were no AMPs strongly up-regulated in response to *Spiroplasma*.

Host immune modulation by *Spiroplasma* could also be evident in the potentiation of host defense genes during infection, which would be apparent in a *Spiroplasma* $\times$ *Howardula* statistical interaction on expression level. Of the 150 transcripts showing such an interaction, only 14 had a peak of expression in S+HA+ treatments. Among these were two proteases, a fibrinogen-like domain containing protein, a nimrod protein - involved in phagocytosis - and a putative allergen that could potentially be involved in host defense. While it is not possible to rule out that one or more of these few genes underlies *Spiroplasma*'s defensive properties, comparison with studies that more strongly support immune-priming makes this unlikely [58, 59], as the up-regulation seen here is idiosyncratic and of low magnitude.

RT-qPCR Validation

Our independent validation of differential expression from the assembled transcriptome was generally in agreement with transcriptomic results. This analysis was more complex in that we diagnosed individual *Howardula*-exposed flies as infected or unin-

fected: we took this approach to allow an additional level of resolution in interpreting our transcriptomic results. Also, the generation of new biological samples, the use of alternative methods to isolate RNA and construct cDNA libraries, and sampling flies collected at a single time point were different from the transcriptomic analysis, but we are encouraged that we successfully recovered the main trends in the data under such different conditions. Furthermore, we were unable to amplify trypanosomatid DNA in these samples, suggesting they were likely free of this confounding infection. With the five individuals we analyzed from each of the six experimental conditions, we found that four target genes gave results consistent with those of RNA-sequencing, in terms of direction of fold change and a significant response to *Howardula* exposure. The two AMPs we measured gave more variable results, which we discuss below.

The immune-related genes (lysozyme x, a c-type lectin, and cysteine-proteinase 1) that we measured were all consistent with the transcriptome in their significant up-regulation during *Howardula* exposure. Interestingly, they also shared a response in that they responded strongly and positively to nematode exposure but were relatively suppressed during nematode infection (Figure A.1). This apparent immune activation and suppression is intriguing, and could reflect successful defense against nematode infection.

More subtle patterns of expression in response to *Spiroplasma* were also recovered in some cases, although not always statistically significantly; an additional validation gene, spermidine synthase, showing down-regulation in S+ lines in the transcriptome was also down-regulated in response to *Spiroplasma* in week-old flies using RT-qPCR (Figure A.1; $P = 0.08$). In contrast, while lysx was down-regulated in response to *Spiroplasma* in the transcriptome, we did not detect an effect during qPCR (Figure A.1).

Two AMPs that responded in the transcriptome - attacin-C and diptericin-B - followed a different pattern of expression during RT-qPCR. However, these genes were characterized by idiosyncratic and extreme up-regulation in a few flies (2 out of 30). We suspect that a few such flies possibly responding to a cryptic bacterial infection rather than experimental treatments drove the low-level differential expression of these genes seen in our RNA-sequencing data. We did find that the expression of the two genes was highly correlated across treatment conditions in both the RNA-sequencing and qPCR results ($r = 0.92$ and log-log $r = 0.71$, respectively), suggesting the observed discrepancy represents biological variation rather than technical error.

Spiroplasma Putative RIP response

RT-qPCR of the putative *Spiroplasma* RIP in one-day old flies corroborated its strong response to treatments (Figure 2.4: ANCOVA; $F_{2,27} = 7.2$, $P = 0.003$), and also revealed that its relative expression increased with higher *Spiroplasma* infection intensity ($F_{1,27} = 37.0$, $P < 0.001$ respectively). Interestingly, up-regulation occurred only in *Howardula*-exposed but uninfected flies, and a strong statistical interaction between *Howardula*-status and *Spiroplasma* intensity was driven by increased per-capita RIP expression in *Howardula*-exposed but uninfected flies (Figure 2.5: $F_{2,27} = 4.2$, $P = 0.03$). *Spiroplasma* infection intensity itself, however, did not respond significantly to treatments (ANOVA; $P = 0.95$), corroborating our experiments on exploitative competition (i.e. no effect of *Howardula* on *Spiroplasma* density).

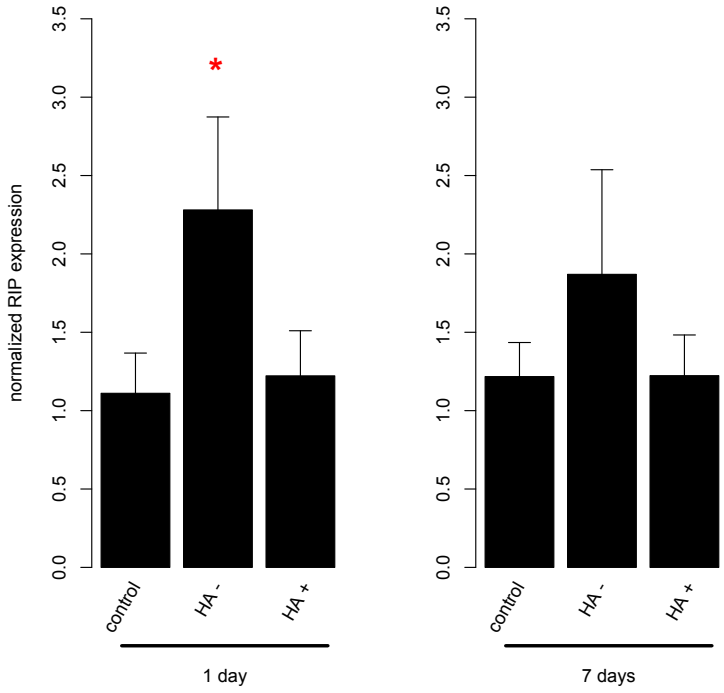

Figure 2.4: Expression of the putative *Spiroplasma* ribosome-inactivating protein in one-day (n=31) and seven-day (n=14) old flies. Experimental treatments are un-exposed (control), nematode-exposed and uninfected (HA -), and nematode exposed and infected (HA +). Expression is significantly higher in day-old nematode - flies. Flies in the seven-day experiment were also screened for trypanosomatid infection; all flies tested negative

Discussion

There has been a recent surge in research on defensive symbionts [41, 100], but little is known about the mechanisms by which they provide protection. Transcriptome sequencing is a promising approach that begins to solve this problem. In this study we tested the predominant hypotheses for how symbiont-mediated protection occurs using a recently discovered *Drosophila* defensive symbiosis. We applied a framework of competition between species developed for free-living competitors but also applied to coinfecting parasites [63, 64], and while we found no evidence of either apparent or exploitative competition in our transcriptomic or *Spiroplasma* density experiments, we did find multiple putative toxins and a pattern of *Spiroplasma* infection suggestive of interference competition. As such, applying this conceptual framework to symbiont-parasite interactions provides an opportunity to link mechanistic bases of defense to a larger body of ecological and evolutionary theory [63, 81, 82].

The bulk of this work examined the transcriptional response of the *Drosophila* host to *Spiroplasma* and *Howardula*. While there was not strong evidence of immune priming, we also found *Spiroplasma* to have little effect on host gene expression at all, with relatively few genes responding significantly to infection. This is consistent with a recent microarray study of *Spiroplasma* infection in *Drosophila melanogaster* showing relatively little host response to infection [101], as well as studies that examined the expression of specific immunity genes [102, 103]. It may be surprising that there is little effect on host gene expression despite the fact that unlike *Wolbachia*, *Spiroplasma* typically occurs extracellularly and at high densities in the hemolymph, where numerous immune effectors are active [71]. However, *Spiroplasma* has no cell wall and it has been proposed that it evades, and perhaps even suppresses host immunity [102, 104].

Our results contrast with recent studies that have shown constitutive up-regulation of host immune genes when *Wolbachia* infections are established in novel mosquito hosts [58, 84] that may account for reduced vector competence. Similar to our results, though, immune-priming by *Wolbachia* appears reduced or absent in native infections [59, 105]. Some symbionts may also interact with host-immunity by triggering the production of reactive oxygen species that are toxic to parasites and pathogens [58, 106]. To examine this, we looked at the expression of the genes dual oxidase (duox) and NADPH oxidase (nox) in our transcriptome and found no significant up-regulation under any experimental conditions. Furthermore, we found no

significant enrichment of function for oxidoreductase activity among *Spiroplasma* up-regulated genes during our Gene Ontology analysis (GO:0016491 enrichment: $P_{adj} = 1$), making it unlikely that these pathways contribute to defense in this system.

There was little effect of *Spiroplasma* on host gene expression, but we did detect a clear response to *Howardula* exposure, in terms of systematic down-regulation of transcripts related to egg production and reproduction, as would be expected from a parasite that sterilizes its host. Surprisingly little is known about the insect immune response to parasitic nematodes [98], and our dataset provides some interesting clues in this regard. For example, we found up-regulation of potential clotting factors, including fondue, which has recently been implicated in *D. melanogaster*'s defense against entomopathogenic nematodes [107], as well as many genes containing fibrinogen-like domains and numerous lectins with carbohydrate binding activity that are potentially important in insect defense. Intriguingly, quantitative-PCR consistently suggested that some probable defense genes were up-regulated in flies that were exposed to nematodes but did not become infected (i.e. flies that had successfully defended against nematode attack). However, the presence of the trypanosomatid infection in the transcriptome makes it unclear what is necessarily driving this response. The presence of these interacting parasites and symbionts makes this a promising system to evaluate the dynamic interplay between infectious agents in *Drosophila*, but it unfortunately precludes a clear analysis of *Howardula*'s effects on host immunity at this time.

In general, little is known about the effect and distribution of trypanosomatids in *Drosophila* though they appear to be diverse and ubiquitous parasites of *Drosophila* [108–110]. Trypanosomatids and *Howardula* occupy different tissues in *D. neotestacea*, with the former predominately infecting the host midgut (personal observation), while *Howardula* infect larval flies by piercing the cuticle and reside in the hemolymph of adults. These parasites likely cause divergent responses in the host, but *Drosophila* immune responses to both are poorly defined [98, 111]. Importantly, trypanosomatid coinfection has no effect on the defensive properties of *Spiroplasma* (unpublished data). Thus, although trypanosomatid infection complicates our ability to examine host immune response to *Howardula* in this study, the defensive effects of *Spiroplasma* are robust to it, and should still be observable.

Our primary intent in our RNA sequencing experiment was to sequence host mRNA, but our *de novo* transcriptome assembly also produced transcripts for putative *Spiroplasma* genes. Our very high depth of sequencing that allowed us to

capture low abundance transcripts helps to explain our sequencing of bacterial genes even after mRNA enrichment. Among these *Spiroplasma* sequences are two putative toxins, including an apparently *Spiroplasma* encoded ribosome inactivating protein (RIP), with no sequenced homolog yet found in other *Spiroplasma*. This putative RIP shows up-regulation in response to nematode exposure in both transcriptomic and qPCR analyses, and is also increased at higher *Spiroplasma* densities in day-old flies. Interestingly, similar to some assayed immune genes, up-regulation occurs in nematode exposed but uninfected flies (Figure 2.4). As yet, we are uncertain of the reason for this; though we have not found evidence that *Spiroplasma* prevents nematode infection (unpublished data), it is probable that the flies diagnosed as uninfected here were attacked by nematodes during exposure (i.e. juvenile nematodes pierced the cuticle to invade the fly) but managed to shed the infection, potentially helping to explain the pattern of up-regulation. It is also interesting that expression of this putative toxin was detected in all experimental conditions, even control flies.

RIPs are important virulence factors in bacteria; for example, phage-mobilized RIP Shiga or Shiga-like toxins are responsible for the virulence of human toxigenic *E. coli* strains [112]. RIPs are also capable of being highly specific against certain tissue types and/or organisms due to differential rates of endocytosis into the cell where they exhibit their toxic activity through the N-glycosidic cleavage of ribosomes [112], providing a plausible mechanism for toxin specificity in defensive symbiosis. Intriguingly, phage-encoded RIPs have been implicated in one of the best-studied insect defensive symbioses, the γ-proteobacterium *Hamiltonella defensa* that protects its aphid host against parasitoid wasps [62,113]. We cannot rule out that the putative RIP in *Spiroplasma* is mobilized by a phage, although we found no evidence for such a phage in the transcriptome. However, while the *Spiroplasma* RIP has high homology to the A subunit of type I and II RIPs, the phage-encoded *Hamiltonella* RIP bears low homology to the B_5 subunit of type II RIPs [114], suggesting that these putative toxins may not be directly comparable.

We propose that toxins encoded by *Spiroplasma* could underlie host defense through interference competition in this system, although further work characterizing the function of putative *Spiroplasma* toxins will be necessary to demonstrate this conclusively. Nonetheless, *Spiroplasma* and *Howardula* co-inhabit the fly hemocoel in close proximity, providing ample opportunity for *Spiroplasma*-derived factors to interact with *Howardula*. Further, interference competition can be expected to result in an asymmetrical effect of one parasite on the other [63], concordant with *Spiroplasma*'s effects

on *Howardula*.

Together, these results provide the strongest support for a model of interference competition involving *Spiroplasma*-encoded toxins mediating defense. But is there reason to believe that interference may be more generally true of inherited defensive symbioses in insects? While there is evidence for all forms of competition [58, 60], we suggest that interference competition will predominate in heritable defensive symbioses, largely due to the vertical transmission of symbionts. Theory has long recognized that facultative heritable symbionts must walk an evolutionary tightrope: they are transmitted through the successful reproduction of their hosts, but are also expected to impose metabolic costs that lead to decreased relative fitness of infected hosts [76, 77]. While on the one hand this trade-off can help to explain the evolution of conditional mutualisms such as defense, it also implies strong selection against heritable symbionts that have high metabolic costs in their hosts. Such costs may be exacerbated by strong exploitative competition between parasites and symbionts, or constitutive up-regulation of immunity. This may also explain why immune priming can be pronounced in artificial lab-established symbiont infections relative to naturally occurring ones. For example, *Wolbachia* infections established in *Anopheles* mosquitoes produce an enormous amount of reactive oxygen species that appear to block malaria transmission; an added consequence is an extreme reduction in host fecundity [51]. This balance may be particularly important for symbionts that do not manipulate host reproduction and appear to rely on the selective advantage of defense for their maintenance and spread such as *Hamiltonella* in pea aphids and *Spiroplasma* in *D. neotestacea* [1, 78] Producing parasite-specific toxins is a very effective evolutionary strategy for heritable symbionts, as it may allow targeted effects on natural enemies in the absence of collateral damage to the host that would result in decreased host fitness and thus symbiont transmission. Given the known specificity of toxicity by RIPs, they make attractive candidates for defensive factors in this respect. Indeed, toxins are increasingly observed in the genetic repertoire of insect symbionts [61, 66, 113] and may be important to diverse symbioses.

Chapter 3

A novel ribosome-inactivating protein in a *Drosophila* defensive symbiont

Abstract

Vertically transmitted symbionts that protect their hosts against parasites and pathogens are well known from insects, yet the underlying mechanisms of symbiont-mediated defense are largely unclear. A striking example of an ecologically important defensive symbiosis involves the woodland fly *Drosophila neotestacea*, which is protected by the bacterial endosymbiont *Spiroplasma* when parasitized by the virulent nematode, *Howardula aoronymphium*. The benefit of this defense strategy has led to the rapid spread of *Spiroplasma* throughout the range of *D. neotestacea*, though the molecular basis for this protection has been unresolved. Here, we show that a novel *Spiroplasma*-encoded ribosome-inactivating protein (RIP) related to Shiga-like toxins from enterohemorrhagic *E. coli* plays a central role in conferring protection to *D. neotestacea*. We first show that recombinant *Spiroplasma* RIP catalyzes depurination of 28S ribosomal RNAs in a cell-free assay, and of *Howardula* ribosomes in vitro at the canonical RIP target site within the α-sarcin/ricin loop of 28S rRNA. Next, we show that *Howardula* parasites in *Spiroplasma*-infected flies have the majority of their rRNA degraded at the α-sarcin/ricin loop and show a signal of depurination consistent with RIP-dependent modification. Notably, host rRNA is largely unaffected, highlighting the targeted specificity of the protection mechanism. Collectively, our

study identifies one of the first toxins discovered from an insect defensive symbiosis and suggests an underlying RIP-dependent mechanism in defense.

Introduction

Symbiosis is now recognized to be a key driver of evolutionary novelty and complexity [76, 115], and symbioses between microbes and multicellular hosts are understood as essential to the health and success of diverse lineages, from plants to humans [116]. Insects, in particular, have widespread associations with symbiotic bacteria, with most insect species infected by maternally transmitted endosymbionts [34]. Though many insect symbionts perform roles essential for host survival, such as supplementing nutrition, others are facultative and not strictly required by their hosts. These facultative symbionts have evolved diverse and intriguing strategies to maintain themselves in host populations despite loss from imperfect maternal transmission and metabolic costs to the host. These range from manipulating host reproduction to increase their own transmission [38, 39], such as by killing male hosts, to providing context-dependent fitness benefits [77]. Recently, it has become clear that different insect endosymbionts have independently evolved to protect their hosts against diverse natural enemies that so far include pathogenic fungi [42], RNA viruses [22,43], parasitoid wasps [41], parasitic nematodes [19], and predaceous spiders [40, 61]. This suggests that defense might be a common aspect of many insect symbioses and demonstrates that symbionts can serve as dynamic and heritable sources of protection against natural enemies [77].

Despite a growing appreciation of the importance of symbiont-mediated defense in insects, key questions remain. Most demonstrations of defense have been under laboratory conditions, and the importance of symbiont-mediated protection in natural systems is unclear in most cases [53]. At the same time, the proximate causes of defense are largely unknown, although recent studies have provided some intriguing early insights: a *Pseudomonas* symbiont of rove beetles produces a polyketide toxin thought to deter predation by spiders [61]; *Streptomyces* symbionts of beewolves produce antibiotics to protect the host from fungal infection [117]; and bacteriophages encoding putative toxins are required for *Hamiltonella defensa* to protect its aphid host from parasitic wasps [62], while the causes of other naturally occurring defensive symbioses are unresolved. From an applied perspective, the ongoing goal of exploiting insect symbioses to arrest disease transmission to humans from insect vectors [49]

makes a deeper understanding of the factors contributing to ecologically relevant and evolutionarily durable defensive symbioses urgently needed.

Here, we investigate the mechanism underlying one of the most striking examples of an ecologically important defensive symbiosis. *Drosophila neotestacea* is a woodland fly that is widespread across North America and is commonly parasitized by the nematode *Howardula aoronymphium*. Infection normally sterilizes flies [118]; however, when flies harbour a strain of the inherited symbiont *Spiroplasma* - a Gram positive bacterium in the class *Mollicutes* - they remarkably tolerate *Howardula* infection without loss of fecundity, and infection intensity is substantially reduced [19]. The benefit conferred by this protection lends a substantial selective advantage to *Spiroplasma*-infected flies, and has led to *Spiroplasma*'s recent spread across North America, with symbiont-infected flies rapidly replacing uninfected ones [1]. *Spiroplasma* is a diverse and widespread lineage of arthropod-associated bacteria that can be commensal, pathogenic, or mutualistic [46]. Maternal transmission has arisen numerous times in *Spiroplasma*, including strains that are well known as male-killers [46]. In addition to defense against nematodes in *D. neotestacea*, other strains of *Spiroplasma* have recently been shown to protect flies and aphids against parasitic wasps and pathogenic fungi, respectively [44,119], but in no case is the mechanism of defense understood.

We previously found that the defensive *Spiroplasma* from *D. neotestacea* encodes a putative ribosome-inactivating protein (RIP) toxin, and speculated that it might play a role in defense [65]. RIPs are widespread across plants and some bacteria and include well-known plant toxins of particular human concern such as ricin, as well as important virulence factors in human toxigenic strains of *E. coli* and *Shigella* (26, 27). RIPs characteristically exert their cytotoxic effects through depurination of eukaryotic 28S ribosomal RNAs (rRNA) at a highly conserved adenine in the α-sarcin/ricin loop (SRL) of the rRNA by cleaving the N-glycosidic bond between the rRNA backbone and adenine (28, 29). While the proliferation of RIPs across different lineages implies functional significance, their ecological roles are unclear, although they appear to have antiviral or other defensive roles in some cases (27, 30). Here, we show that *Spiroplasma* expresses a functional RIP distinct from previously characterized toxins that appears to contribute to *Spiroplasma*'s defense against *Howardula*. This work sheds light on the mechanisms employed in defensive associations that protect hosts from disease, and confirms ecological roles for RIPs in a tripartite defensive symbiosis.

Results

Spiroplasma strains encode diverse RIP-like sequences

Earlier RNA sequencing assemblies from *Spiroplasma*-infected *D. neotestacea* recovered sequence of a putative RIP encoded in a 403 amino acid ORF (hereafter *Sp*RIP) [65]. Characterized RIPs are typically of two types: type I toxins consist of a conserved catalytically active A chain (typically ∼30 kDa) that in type II toxins may be linked to a B or B_5 subunit (e.g., for ricin and Shiga toxins, respectively) that serves as a lectin and facilitates toxin entry into target cells [67, 120]. The ORF of *Sp*RIP was predicted to encode an N-terminal signal peptide, followed by a disordered region of 70 AAs, and a ∼300 AA C-terminal region homologous to characterized RIP A chains. While this is substantially longer than typical for monomeric type I toxins, we found no convincing bioinformatic evidence for the presence of a B chain homologous to those characterized from Shiga-like toxins or type II plant RIPs.

BLASTp searches recovered putative RIPs encoded by other *Spiroplasma* strains, including the recently sequenced defensive and male-killing MSRO strain of *Spiroplasma poulsonii* from *D. melanogaster* [121]. Phylogenetic analysis of these protein sequences with selected seed sequences from the PFAM database (PF00161) placed RIP-like sequences from *Spiroplasma* strains with bacterial RIPs such as the Shiga-like toxins (Figure 3.1), and alignments confirmed the presence of the known conserved catalytic residues of RIPs in *Sp*RIP.

Spiroplasma expresses a functional RIP

To characterize *Sp*RIP, we expressed and purified the protein following codon optimization for *E. coli* (Figure 3.2; signal peptide removed; Ala31 through His403). This yielded a 44 kDa protein that degraded to a stable protein of 34 kDa after 2 weeks in HBS at 4°C (Figure 3.2). Consistent with our expectation, mass spectrometric analysis confirmed this to be a result of proteolysis of the ∼70 residues of the N-terminal region predicted to be disordered. Subsequent assays were performed using this stable protein.

We used a modified, highly sensitive RT-qPCR based assay to assess depurination activity [69, 70]. In brief, depurination at the SRL leaves an abasic site following attack. These assays exploit the property of reverse transcriptases to incorporate dAMP opposite this abasic lesion during reverse transcription, resulting in a quan-

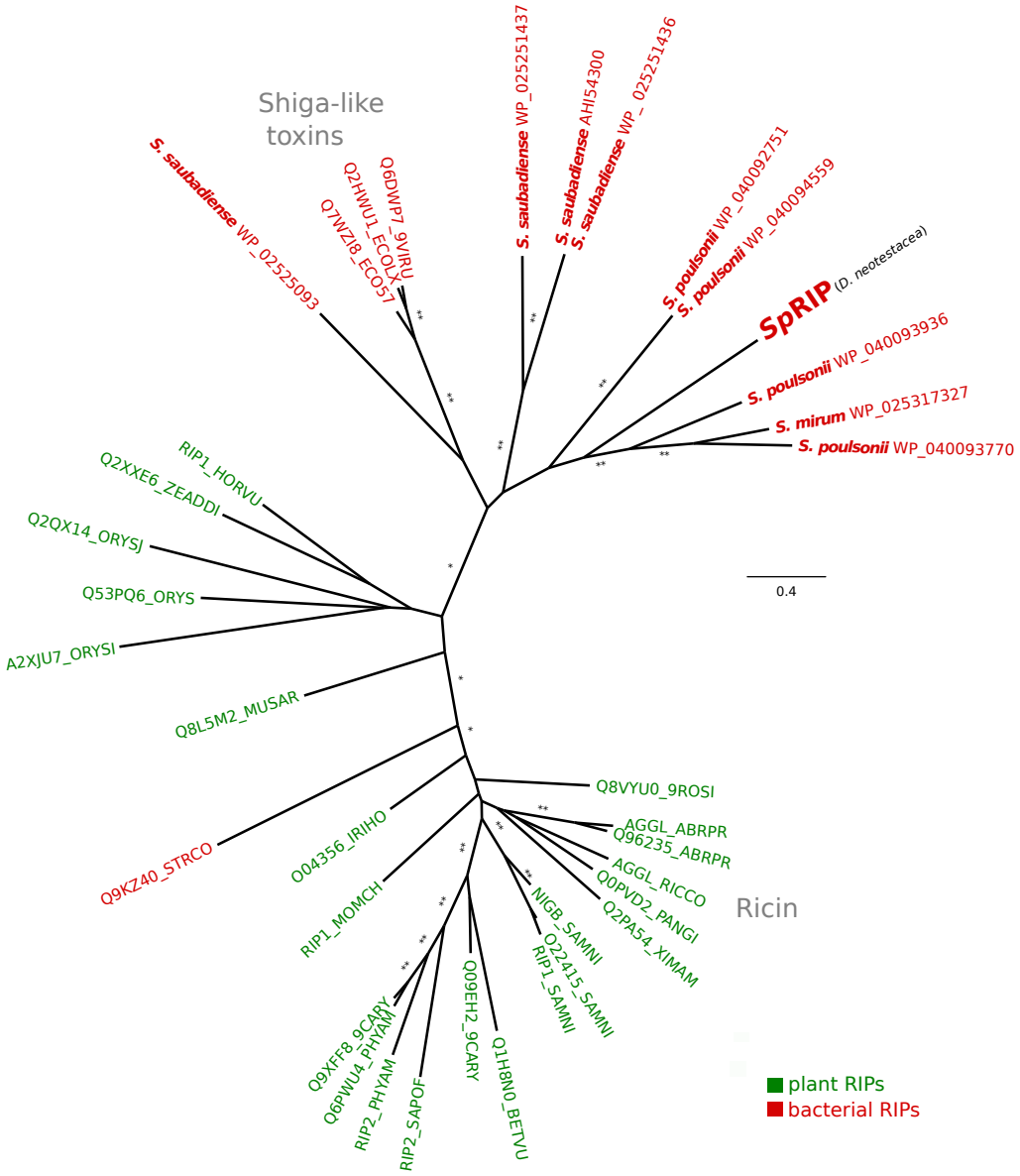

Figure 3.1: *Spiroplasma* strains encode divergent RIP-like sequences. Maximum-likelihood phylogeny of *Sp*RIP from the defensive *D. neotestacea* strain of *Spiroplasma*, aligned with other putative *Spiroplasma* RIPs and selected RIP seed sequences from the PFAM database (PF00161). Support values are calculated based on 1000 bootstraps using FastTree; the scale bar denotes substitutions per site. Single and double asterisks represent >70% and 90% bootstrap support, respectively.

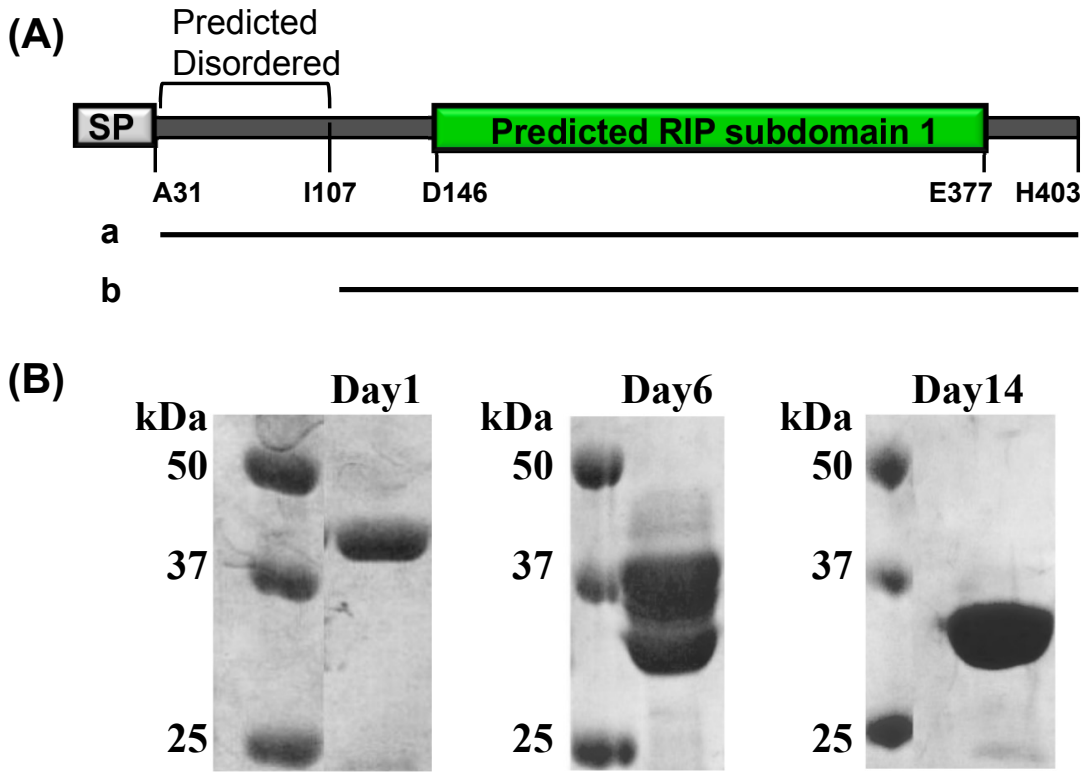

Figure 3.2: Purified recombinant *Sp*RIP degrades into a stable product over two weeks time at 4°C. (A) Schematic of *Sp*RIP domain prediction. SP, signal peptide. The black horizontal line a represents the recombinant protein (Ala31 to His403) produced in this study. b represents the stable degradation product from purified recombinant *Sp*RIP. (B) SDS-PAGE analysis of *Sp*RIP (44 kDa) incubated at 4°C. Figure courtesy of Fangni Peng.

tifiable signature shift from T (the complement of A) to A at the site of depurination in resultant cDNA. To exploit this, we developed qPCR primers to rabbit 28S rRNA for use in cell-free rabbit reticulocyte lysate-based assays.

Incubating reticulocyte lysate with *Sp*RIP led to a ∼50% decrease in the abundance of the cDNA representing intact 28S rRNA relative to negative controls (Figure 3.3A - hereafter intact template; $t_{3.94} = 11.68$, $P < 0.001$), and correspondingly, more than a 1000-fold increase in cDNA representing depurinated rRNA (Figure 3.3B - hereafter depurinated template; $t_{2.18} = 42.22$, $P < 0.001$). A 4 × 5-fold serial dilution of *Sp*RIP also confirmed depurination across a range of concentrations, with clear dose dependence to < 0.1 μM (Fig 3.4; $\log_2$(depurination) vs. $\log_5$([*Sp*RIP]) $R^2 = 0.92$, $P < 0.001$). This supports a depurination function for *Sp*RIP, with enzyme-dependent depurination likely proceeding through cleavage of the N-glycosidic bond, as observed in other RIPs.

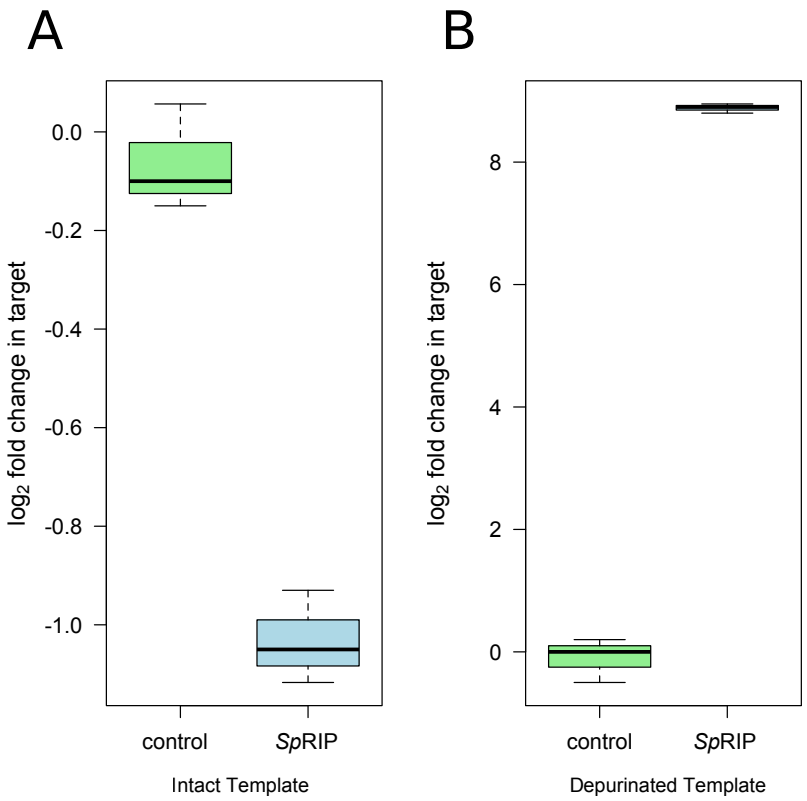

Figure 3.3: *Sp*RIP depurinates rabbit 28S rRNA at the site of RIP attack in cell-free assays. Abundance of cDNA representing intact (A) and depurinated (B) rRNA template after incubation with 5.25 μM of recombinant *Sp*RIP for 30 min at 30°C (N = 6). *Sp*RIP significantly decreases the abundance of intact template and increases the abundance of depurinated template ($P < 0.001$ for both).

*Sp*RIP depurinates *Howardula* ribosomes in vitro

To confirm *Sp*RIP activity against *Howardula* nematode ribosomes, we designed a RT-qPCR assay to measure depurination of *Howardula* rRNA. This assay was able to specifically differentiate *Howardula* rRNA from that of the fly host, with cDNA reverse transcribed from nematode-uninfected fly negative controls yielding no amplification.

We harvested live *Howardula* by grinding infected *Drosophila falleni* (*Spiroplasma*-negative) in insect Ringer's solution, and incubated this homogenate with recombinant *Sp*RIP at 21°C for 4 hours, using a lower temperature to avoid directly killing nematodes during incubation. Incubation with the toxin again dramatically increased the abundance of depurinated template by more than 2000-fold (Figure 3A and B; $t_{2.38} = 18.34$, $P < 0.001$). In contrast to the reticulocyte lysate assay, there was

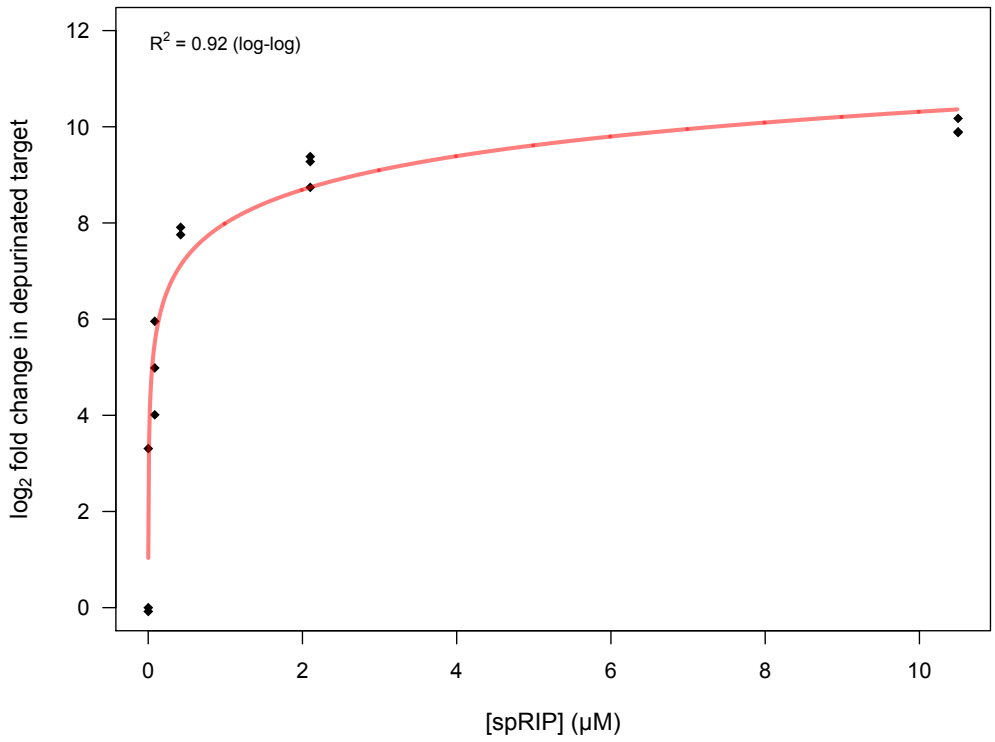

Figure 3.4: *Sp*RIP causes dose-dependent depurination of rabbit 28S rRNA in cell free assays (N = 14).

no substantial decrease in the abundance of intact template under these conditions ($t_{2.94} = 0.51$, $P = 0.65$). We further incubated single nematode motherworms to limit substrate availability, again not observing appreciable depletion of intact 28S rRNA under these conditions despite large increases in depurinated ribosomes (data not shown; $t_{3.89} = 0.31$; $P = 0.77$ and $t_{2.03} = 8.68$, $P = 0.01$, respectively), suggesting that a proportion of *Howardula* ribosomes might not be accessible to the stable *Sp*RIP lacking the predicted disordered region used in this in vitro system.

RNA-sequencing shows depurination of *Howardula* 28S rRNA at the α-sarcin/ricin loop in the presence of *Spiroplasma*

To test for evidence of *Howardula* attack by a RIP in vivo we revisited RNA-seq reads generated during a previous experiment, in which we sequenced RNA of *D. neotestacea* and *Howardula* in the presence and absence of *Spiroplasma* infection

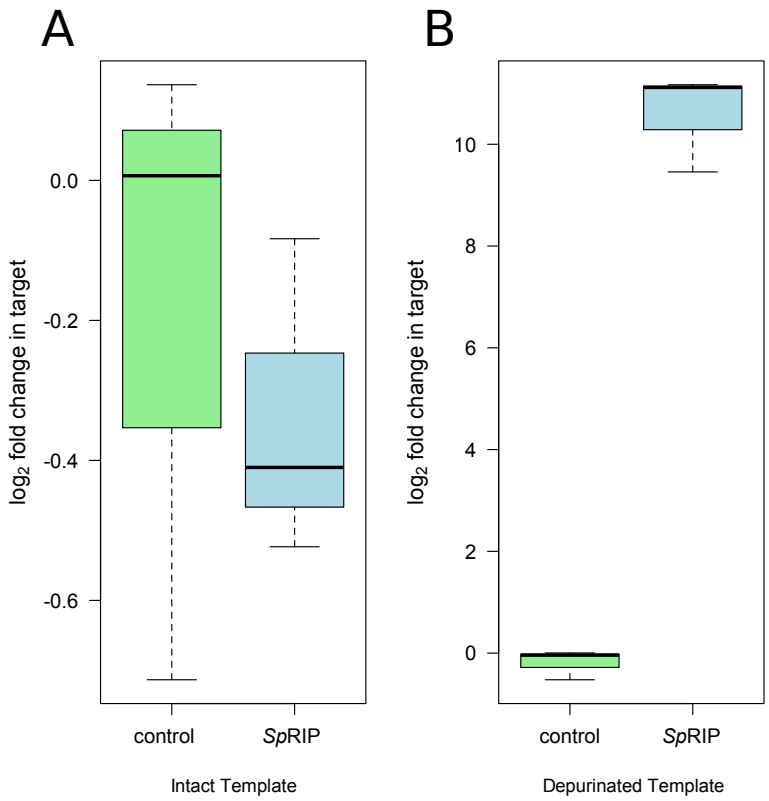

Figure 3.5: *Sp*RIP depurinates *Howardula* 28S rRNA at the site of RIP attack in assays with live *Howardula*. Abundance of cDNA representing intact (A) and depurinated (B) rRNA template after incubation with 5.25 μM of recombinant *Sp*RIP for 4 hours at 21°C (N = 6). While abundance of depurinated template is significantly increased, intact template is not clearly decreased ($P < 0.001$ and $P = 0.65$, respectively).

[65]. We reasoned that signal of depurination should be observed in reads mapping to the SRL of *Howardula* 28S rRNA given the reliance of the RNA-seq on reverse transcription during library construction, causing a shift in the read at the site of depurination in cleaved rRNA.

Mapping raw reads to near full-length 28S rRNA for *Howardula* revealed a highly significant signal of depurination with a shift from A to T (or the complement) in 3.8% of reads mapping to the adenine target of RIPs in *Spiroplasma*-infected flies ($P < 10^{-180}$; coverage = 2,807 reads). In contrast, this signal was not present in *Howardula* reads from *Spiroplasma*-uninfected flies ($P > 0.1$; coverage = 15,389). The same analysis mapping a subset of raw reads to *D. neotestacea* 28S rRNA also

revealed significant evidence of depurination, but to a much lesser extent, with a shift detectable in only 0.4% of reads ($P < 10^{-17}$; coverage = 4,822). Again, there was no evidence of depurination in the absence of *Spiroplasma* ($P > 0.1$; coverage = 3,485). This near 10-fold greater depurination of *Howardula* vs. *D. neotestacea* rRNA suggests substantial differences in exposure and/or susceptibility of host vs. parasite ribosomes to a RIP in the presence of *Spiroplasma*, as we would expect.

qPCR confirms that *Spiroplasma* depurinates *Howardula* 28S rRNA in vivo and dramatically decreases intact rRNA

We applied the RT-qPCR assay for depurination of *Howardula* 28S rRNA to *Howardula*-infected adult flies, infected and uninfected with *Spiroplasma*, collected 1-day post-eclosion. *Howardula* 28S rRNA with an intact SRL was dramatically decreased in the presence of *Spiroplasma* (Figure 3.6A; $t_{8.88} = 3.37$ $P = 0.008$). Because this assay is normalized to an upstream region of rRNA not predicted to be affected by RIPs, this may be interpreted as an ∼6-fold surplus of *Howardula* rRNA degraded at the SRL vs. intact *Howardula* rRNA in the presence of *Spiroplasma*. Correspondingly, the depurinated template was ∼20-fold more detectable (Figure 3.6B $t_{10.53} = 3.36$ $P = 0.007$). Together, these assays show that the majority of *Howardula* 28S rRNA is degraded - specifically at the RIP target in the SRL - when *Spiroplasma* is present, and that a significant proportion is further depurinated in a manner specific to attack from a RIP (Figure 3.6B). Depurination at the SRL is known to be a potent inducer of apoptosis [120, 122], and renders the rRNA backbone susceptible to cleavage, likely accounting for the relatively modest accumulation of depurinated rRNA coincident with dramatic SRL-specific degradation.

To corroborate RNA-sequencing results and ensure depurination is specific to *Howardula*, we designed an RT-qPCR assay for *D. neotestacea* rRNA (Table 1). We assayed fly ovaries - host tissue known to be high in *Spiroplasma* density - in gravid females with and without *Spiroplasma*, as well as in the whole-fly samples that showed depurination of the *Howardula* SRL (above). Though there appeared to be slightly elevated RIP-specific depurination in host rRNA in the presence of *Spiroplasma* (Figures 3.6C and A.2), this was not significant above controls in either assay at these sample sizes ($P = 0.30$ and $P = 0.14$ for whole flies and ovaries, respectively), and was substantially lower than that observed in *Howardula* in the same samples. This confirms the greater level of depurination in the parasite vs. host, demonstrating that

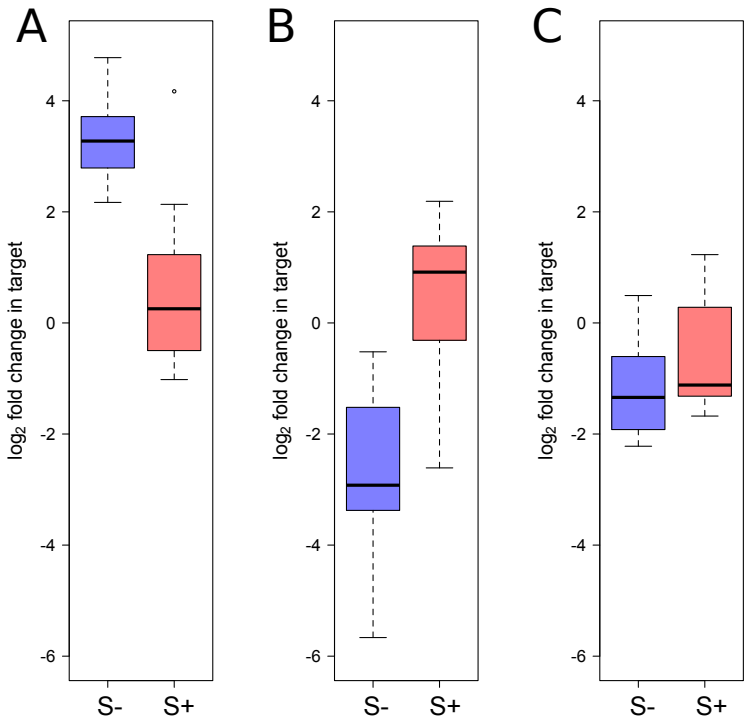

Figure 3.6: *Spiroplasma* depurinates *Howardula* 28S rRNA in vivo, and dramatically reduces the relative abundance of intact 28S rRNA. Intact *Howardula* rRNA is reduced ∼6 fold in the presence *Spiroplasma* (A) (P = 0.008), while the abundance of depurinated template representing RIP-induced depurination increases ∼20-fold (B) (P = 0.007). *Drosophila* host rRNA is not significantly depurinated in the same samples (C) (P = 0.16). cDNA used in analysis was reverse transcribed from whole one-day-old *Howardula*-infected flies with (S+) and without (S-) *Spiroplasma* infection (N = 13)

.

*Sp*RIP selectively targets *Howardula*.

Discussion

Recent years have seen an increasing awareness that symbiotic associations can be critical in protecting multicellular hosts against parasites and pathogens. Many host-associated microbes produce metabolites that are known or suspected to function in defensive capacities [61,66,123], but the effectors that defend against specific enemies in maternally transmitted insect endosymbionts, whose success is typically intimately linked to that of the host, are poorly understood. This is largely due to the difficulty

of working with uncultivable symbiotic lineages: here neither *Howardula* nor *Spiroplasma* can currently be grown outside of the host, precluding many approaches to establishing function in these systems. There is great interest in exploiting insect symbioses to interrupt disease transmission from insects to humans, and a lack of understanding of the mechanisms underlying evolutionarily durable defensive symbioses impedes a full evaluation of the efficacy of these strategies.

Here, we show that a defensive symbiosis currently sweeping North American populations of a common woodland *Drosophila* employs a divergent RIP that affects a virulent and common nematode parasite. While some other facultative symbionts of insects are known to produce potent toxins, such as the pederin produced by *Pseudomonas* symbionts of *Paederus* rove beetles [61], *Spiroplasma* in *D. neotestacea* is remarkable for the extent to which the association has been selected upon due to its defensive properties, resulting in its rapid spread across North America. It is also remarkable due to the extent to which a prevalent parasite, *Howardula*, is affected. This association thus allows exploration not only of *Spiroplasma*'s defensive effectors, but also of ways in which *Howardula* might counter-evolve to mitigate them. Understanding the mechanisms responsible for this defense can thereby clarify the proximate causes of ecologically relevant defensive symbioses, as well as the ways in which they might be mitigated by their targets.

In vivo, *Spiroplasma* infection leads to depurination of *Howardula* ribosomes to a much greater extent than in the *Drosophila* host, demonstrating substantially greater targeting of a *Spiroplasma*-associated RIP to the parasite. Indeed, effective targeting of invading parasites would be expected of a toxin functioning in defense. It is unclear how this specificity is achieved in the case of *Sp*RIP; there is substantial precedent for specificity of type I plant RIPs, which can have highly varying toxicities against different cell lines in vitro, though the molecular basis is mostly unknown [68]. Similarly, the B_5 subunit of Shiga-like toxins - the closest characterized relatives of *Sp*RIP - binds specifically to the glycosphingolipid Gb3 of mammalian cells, triggering toxin endocytosis into Gb3-bearing cells and leading to heightened toxicity against specific tissues and cell types [120]. Whether the predicted disordered region of *Sp*RIP might function similarly as a ligand, specifically binding to receptors of *Howardula* and other parasites is unclear, but is suggested by the lack of a strong decrease in intact rRNA in in vitro assays against *Howardula* with our recombinant *Sp*RIP lacking this region. In addition, a potential pore-forming toxin is encoded directly upstream of *Sp*RIP [65], and it might be that such factors provide entry for *Sp*RIP into *Howardula*

cells, potentiating toxicity.

Intriguingly, *Sp*RIP is the first characterized of what appears to be a relatively diverse array of RIPs encoded by different *Spiroplasma* strains (Figure 3.1), some of which are primarily known as either insect pathogens or male-killers, and one of which - the MSRO strain of *S. poulsonii* - is also defensive against parasitoid wasps [119]. Many, but not all of these putative toxins have retained the essential residues of RIPs, while some also possess extensive modifications that include uncharacterized C-terminal domains of hundreds of amino acids. This conservation and proliferation of RIP-like sequences across *Spiroplasma* strains suggests functional importance in some capacity, and it is tempting to speculate that they might play roles in other defensive symbioses or in male-killing. Indeed, the apoptotic hallmarks of MSRO-induced male-killing bear similarity to those induced by RIPs in other systems [124]. Putative Shiga-like toxins are also encoded in the genomes of phages that are essential to the protection against parasitoid wasps that is conferred to aphids by *Hamiltonella* [113], and it will be interesting to test whether these also target ribosomes.

Recent studies that transfer *Spiroplasma* strains to new host species have revealed interesting variation in the fitness consequences and defensive properties of novel host-*Spiroplasma* associations. When established in new host species, the *D. neotestacea* strain of *Spiroplasma* successfully protects against nematode infection (42). On the other hand, although other strains - including at least one predicted to encode RIPs - were able to stably persist in *D. neotestacea*, only the native strain protected against *Howardula* [125], suggesting that particular *Spiroplasma*-encoded RIPs might be specific to different parasites or pathogens. This is consistent with the high degree of divergence observed between *Spiroplasma* RIPs (Figure 3.1), as well as our finding of *Howardula*-specific RIP targeting. With respect to effects on the host, others have suggested that some degree of mis-targeting of toxins contributes to the virulence that is sometimes observed in novel (laboratory-initiated) *Spiroplasma* infections [126], and our findings are also consistent with this possibility.

In sum, we present evidence of a novel toxin involved in a *Drosophila* defensive symbiosis and find that *Howardula* suffers a much greater degree of RIP-induced rRNA depurination than the *Drosophila* host due to *Spiroplasma*; whether RIPs are acting in concert with other factors in this association remains to be determined. The continued goal of understanding the complex interactions that underpin ecologically important symbioses require a deeper understanding of these factors, as does the aim of exploiting defensive symbioses to limit disease transmission to humans. Our

findings shed light on these factors, while also revealing a novel function for RIPs in nature as players in this tripartite defensive symbiosis.

Materials and methods

Phylogenetic Analysis

Putative RIPs from *Spiroplasma* were accessed using BLASTp searches against the NCBI nr database with *Sp*RIP as a query, and were included based on a low E-value and high degree of coverage. We aligned these and selected RIP sequences from the PFAM seed database using kalign [127] and constructed maximum likelihood trees (1000 bootstraps) with FastTree [128] following model selection in MEGA [129].

Expression and Purification of *Sp*RIP

The gene encoding full length *Sp*RIP was codon optimized for expression in E. coli and synthesized by GenScript, with the region coding for the mature protein (Ala31 through His403 - signal sequence removed) subcloned into a modified pET28a expression vector containing an N-terminal TEV protease-cleavable hexahistidine tag.

Recombinant *Sp*RIP was produced using the *E. coli* BL21 codon plus strain. Chemically competent cells were transformed and grown in 2XYT media containing 50 μg/ml ampicillin and 35 μg/ml chloramphenicol at 37°C with shaking. Overnight culture was diluted 20 fold into 1 L ZYP-5052 autoinduction media at the same antibiotic concentration and grown for 4 hours at 37°C before the temperature was reduced to 30°C for overnight cell growth.

Bacterial cells were harvested by centrifugation and lysed by French press. We purified protein in the cell lysate by Ni-NTA batch bind. Briefly, the cell lysate was diluted in a Ni-NTA binding buffer (20 mM HEPES, pH 8.0, 1 M NaCl, 30 mM imidazole), and incubated with 2 ml Ni-NTA slurry at 4°C for 1 hour with stirring. Following the incubation, the recombinant protein was eluted from Ni-NTA resin in 5 ml fractions with 250 mM imidazole in elution buffer (20 mM HEPES, pH 8.0, 1 M NaCl). Elutions were pooled and buffer exchanged into HEPES-buffered saline (HBS: 20 mM HEPES, pH 7.5, 150 mM NaCl), with 2% glycerol and 0.5 mM EDTA. The N-terminal hexahistidine tag was cleaved with TEV protease using the established protocol from Sigma-Aldrich. The TEV treated RIP was further purified by cation

exchange chromatography using 0 - 1.0 M gradient of NaCl in 20 mM HEPES buffer, pH 6.8 and finally by size exclusion chromatography in HBS (20 mM HEPES, pH 7.5, 300 mM NaCl, with 2% glycerol). Fractions were analyzed by SDS-PAGE and the monomeric fractions as defined by SEC elution profile were pooled and concentrated.

Incubation of the purified recombinant protein (44 kDa) at 4°C for two weeks in HBS resulted in a stable degradation product of 34 kDa as shown by SDS-PAGE (Figure 2). To identify the sequence of the proteolyzed fragment, the 34 kDa band was excised from gel, reduced, alkylated, and in silico digested with trypsin. Mass spectrometric analysis of the digested peptides was done with a Voyager DE-STR mass spectrometer (Applied Biosystems) using mass range 800-3500. For comparison with the MS captured peptide masses, the full-length recombinant protein sequence was submitted to Protein Prospector - MS digest server (UCSF), which reports the predicted trypsin-digested peptide masses. The MS data showed that ∼70 residues from the N-terminus were proteolyzed.

The final yield was of *Sp*RIP was 0.72 mg purified protein L^{-1} cell culture. BBE31, a surface protein of *Borrelia burgdorferi*, was purified by the same method and was used alternatively to BSA as a negative control in incubations, showing that *Sp*RIP's activity did not a result from contamination from the expression system.

Bioinformatics for RNA-sequencing

RNA-sequencing reads originating from [65] were used to test for evidence of depurination of the 28S rRNA SRL at the level of cDNA. In brief, a factorial experiment was conducted in which we sequenced the metatranscriptome of *D. neotestacea* in the presence and absence of *Spiroplasma* and *Howardula* infection. Targeted re-assemblies of *Howardula* and *Drosophila* 28S rRNA were conducted in Geneious 7 (Biomatters, Ltd) to obtain near full-length 28S rRNAs spanning the conserved SRL for both species and raw reads (or a random subset thereof) from *Howardula*-infected libraries with and without *Spiroplasma* were mapped to these assemblies (default low sensitivity setting), and P-values for variants called in Geneious. Raw sequence reads have been deposited under the NBCI SRA PRJNA295093.

Design and validation of RT-qPCR for depurination

For rabbit, *Howardula*, and *Drosophila* ribosomes we designed RT-qPCR assays following the methods of [69, 70] (Table A.2). In summary, primers were designed with

the 3' terminal base of either the forward or reverse primer complementary to the site of depurination, with separate primers designed to detect intact (A) vs. depurinated (T) template, and a secondary mismatch to increase specificity [69, 70]. The reverse primer for each assay was designed in Primer3 [87], and chosen to bind to a region of divergence between *Howardula* and *Drosophila* for those assays. A second normalizing primer set for each assay was designed for upstream rRNA regions not predicted to be affected by RIPs.

All assays were tested for target specificity using synthetic DNA (IDT gBlocks) with and without a transversion to T at the predicted site of depurination. In all cases, no cross-amplification of the non-target template occurred until ∼12 C_t later than target amplification, indicating primer pairs are ∼4000 × more specific to their target templates, making this the saturation limit of the assay. Because samples with no depurination will cross amplify at this point, fold-changes should be interpreted in a relative manner - changes to reaction conditions that affect specificity will affect baseline measures of depurination. Fold change in targets was calculated using the $\Delta\Delta C_t$ method, normalized to amplification of rRNA upstream of the site of depurination, and mean C_t values for each target in each separate experiment or a reference sample from the control treatment when standardizing control samples to 0 was desired [70]. Efficiencies and R^2 values (Table A.2) for primers for detection of intact and depurinated template were calculated using 5 × 10-fold serial dilutions of synthetic DNA or random-primed cDNA (for the *Howardula* normalizing primer set only).

Total RNA was extracted from samples (reticulocyte lysate, whole flies, or nematode motherworms) using Trizol (Invitrogen). For each experiment either 500 or 1000 ng of RNA was reverse transcribed using SuperScript II (Invitrogen) and random primers following quantification with a NanoDrop spectrophotometer. Consistent with others, we found that delays in reverse transcription or freeze-thaw cycles dramatically decreased detectability of depurinated rRNA, so RNA was reverse transcribed immediately following RNA extraction. qPCR reactions were run at 1/10 dilutions in duplicate 10 or 20 μL reactions on a BioRad CFX96 thermal cycler with BioRad SsoFast EvaGreen Supermix. Two cDNA samples for tests of in vivo depurination which could not be reliably amplified with normalizing primer sets were excluded from analysis (2/21). Control samples with no expected depurination in which the primer set for depurinated template failed to amplify were conservatively assigned the highest reliably amplified Ct value for the primer set for the experiment

during analysis. All statistical analyses were conducted in R v.3.1.2 [94], primarily using linear models (Welch's t-test) with $\log_2$ transformations of response variables to meet test assumptions. For in vivo tests of depurination, *Spiroplasma*-infected and uninfected *D. neotestacea* were reared and infected with *Howardula* as detailed in [65].

Chapter 4

Infection dynamics and immune response in a newly described *Drosophila*-trypanosomatid association

Abstract

Trypanosomatid parasites are significant causes of human disease, and are ubiquitous in insects. Despite the importance of *D. melanogaster* as a model of infection and immunity, and a long awareness that trypanosomatid infection is common in the genus, no trypanosomatid parasites naturally infecting *Drosophila* have been characterized. Here, we establish a new model of trypanosomatid infection in *Drosophila* - *Jaenimonas drosophilae*, gen. et sp. nov. As far as we are aware, this is the first *Drosophila*-parasitic trypanosomatid to be cultured and characterized. Through experimental infections, we find that *D. falleni*, the natural host, is highly susceptible to infection, leading to a substantial decrease in host fecundity. *Jaenimonas* has a broad host range, readily infecting a number of *Drosophila* species, including *D. melanogaster*, and oral infections of *D. melanogaster* larvae resulted in the induction of numerous immune genes, and when injected into adult hemolymph, *Jaenimonas* kills *D. melanogaster*, although interestingly, neither the Imd nor Toll pathways are induced, and Imd mutants do not show increased susceptibility to infection. In contrast, mutants deficient for Drosocrystallin, a major component of the peritrophic

matrix, are more severely infected during oral infection, suggesting that the peritrophic matrix plays an important role in mediating trypanosomatid infection in *Drosophila*. This work demonstrates that the *Jaenimonas-Drosophila* system can be a powerful model to uncover the effects of trypanosomatids in their insect hosts.

Introduction

Trypanosomatids are kinetoplastid parasites that include the causal agents of major human diseases, such as African sleeping sickness, American Chagas disease, and diverse leishmaniases. The trypanosomatids causing these diseases have complex dixenous lifecycles that alternate between vertebrates and blood-feeding insects, and understanding trypanosomatid interactions with their insect vectors has been a major focus of research [130].

The vast majority of trypanosomatids have simple monoxenous lifecycles [110,131], however, infecting only invertebrate hosts, and monoxenous trypanosomatids are evolutionarily ancient, while the dixenous lifestyle is derived [132]. Insects are the most common hosts of monoxenous trypanosomatids, with dipterans (flies) and heteropterans (true bugs) showing particularly high rates of infection. Intriguingly, flies of the genus *Drosophila* are commonly infected by trypanosomatids in the wild [72, 108, 109, 133] with infections first documented over a century ago. However, there has been very little work on *Drosophila*-trypanosomatid interactions, and no trypanosomatids from *Drosophila* hosts have been grown in culture and formally described. Only a handful of studies have directly examined the effects of trypanosomatid infection on *Drosophila* fitness, and these have used uncharacterized and/or mixed parasites from wild flies [134], or parasites derived from other insect hosts. For example, the only study to examine the immune response of *Drosophila* to trypanosomatids used infections with *Crithidia* parasites of bumblebees and mosquitoes [111]. This lack of attention is especially surprising given that *Drosophila* has become one of the most important animal models of infection and immunity [71].

Here, we report the successful cultivation and first formal molecular and morphological description of a trypanosomatid parasite naturally infecting a *Drosophila* species. Molecular characterization shows this isolate to be highly divergent from previously described flagellates, representing a new genus and species, which we name *Jaenimonas drosophilae* gen. et sp. nov.

We conduct a series of experiments to investigate the mode of transmission, tem-

poral dynamics of infection, and fitness consequences of *J. drosophilae* (hereafter *Jaenimonas*) in its native host, *Drosophila falleni*. We find that *Jaenimonas* rapidly establishes infections in larvae that persist through the development of the fly, leading to a reduction in host fecundity, and that adult flies readily transmit infections to each other. We also show that *Jaenimonas* has a wide host range, establishing high-intensity infections in diverse *Drosophila* species.

Finally, having established that *Jaenimonas* readily infects *D. melanogaster*, we take advantage of the wealth of resources and tools associated with this model organism to begin to examine the immune response of *D. melanogaster* to native trypanosomatids. Transcriptomic (RNA-seq) analysis reveals an active immune response in infected larvae. In contrast to work on tsetse-trypanosome interactions, we find that mutants deficient in the Imd immune pathway do not show increased susceptibility, although mutants for Drosocrystallin, a key component of the *Drosophila* peritrophic matrix (PM), show greater susceptibility to infection. *Jaenimonas* shows substantial promise as a highly tractable model to investigate the interactions of an understudied lineage of parasites with the most established insect model of disease.

Results

Isolation, primary characterization and subcloning of a new trypanosomatid species

We initially uncovered evidence of an infection in a laboratory line of *D. falleni*; dissections of flies from this line revealed trypanosomatid-like cells localized to the midgut of many specimens. This fly line has presumably been stably infected since its collection in West Hartford, CT in 2006. This line had been used to maintain a *Drosophila*-parasitic nematode, *Howardula aoronymphium*. We subsequently established a trypanosomatid-infected *D. falleni* line that was free of nematodes. This infection has persisted in lab culture (i.e. vials containing *D. falleni*, where adults transmit the infection to larvae and other adults) for over two years. We established axenic cultures of the parasite in BHI media [96]. Laboratory (infected *D. falleni*) and cultured isolates were named dfal-01-lab and dfal-01, respectively. Using the primary culture of dfal-01, we performed successive rounds of clonal isolation to generate a single clonal trypanosomatid line (dfal-01.02).

Morphological and ultrastructural characterization

Light microscopy of dfal-01.02 revealed uniform cells with typical promastigote morphology (Figure 4.1A) [110, 135]. Importantly, the dfal-01.02 cultured cells were morphologically indistinguishable from those observed in situ in *D. falleni* (detailed size ranges in taxonomic description below).

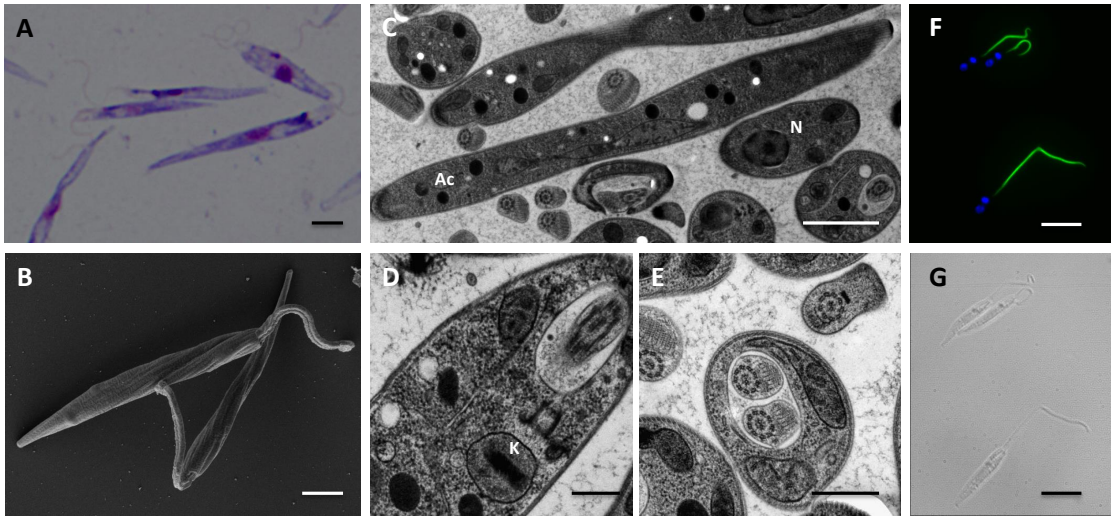

Figure 4.1: Morphology of *Jaenimonas drosophilae* sp. n. in axenic culture. A) Light microscopy (Giemsa-stained), B) SEM, and C-E) HPF-TEM of *Jaenimonas*. C), D) Longitudinal sections reveal typical features of trypanosomatids such as nucleus (N) and kinetoplast (K) as well as presence of acidocalcisomes (Ac). E) Cross-section of the cell through the flagellar pocket displays extremely well developed paraflagellar rod supporting flagella even within the pocket. F), G) *Jaenimonas* stained with L8C4 mouse monoclonal antibody against *T. brucei* paraflagellar rod 2 (PFR2). Scale bars are 1 μm (A-C, F-G) and 500 nm (D-E). Figure courtesy of Jan Votypka and Vyacheslav Yurchenko

We next analyzed the dfal-01.02 cells by scanning electron microscopy (Figure 4.1B) and high-pressure freezing electron microscopy [136, 137]. SEM confirmed that the dfal-01.02 cells were typical elongated promastigotes with well-developed pellicu-

lar ridges (Figure 4.1B), though the flagellum was considerably thicker than other trypanosomatids [135, 138]. HPF-TEM revealed all the typical trypanosomatid features such as an oval nucleus, basal bodies, glycosomes, and an electron-dense kinetoplast disc within a reticulated mitochondrion rich with tubular cristae (Figure 4.1C-D). One peculiarity is an extremely well-developed paraflagellar rod. Notably, it was detectable even within the flagellar pocket (Figure 4.1E).

Phylogenetic analysis

We sequenced the SSU (18S) rRNA, glyceraldehyde-GAPDH, and spliced-leader (SL) RNA genes of the laboratory and cultured isolates. The SSU rRNA sequence obtained was 99% similar to one originating from an uncultured trypanosomatid (KC183713) from *D. ananassae* collected in Hawaii [139]. For both SSU rRNA and gGAPDH sequences, the next closest homologs in GenBank belonged to *Herpetomonas* and *Strigomonas* species ($> 90\%$). For phylogenetic reconstruction SSU rRNA and gGAPDH sequences of dfal-01.02 were concatenated and aligned with a set representing major trypanosomatid clades (Figure 4.2). The cultured species is distinct from all previously described clades of monoxenous trypanosomatids [110]. The SL RNA gene is the most suitable marker for determining relationships between closely related species, sub-species, or populations of insect trypanosomatids [96, 140]. Analysis of this gene also confirmed a unique sequence, with its position on the phylogenetic tree broadly correlated with the novel clade inferred from the SSU rRNA marker, with the most similar SL sequences in GenBank also from uncultured trypanosomatids from *D. ananassae* [139]. Sequences shared 95% identity, clearly indicating that they represent the same typing unit (TU173 [110]), and based on the 90% threshold rule belong to the same species [141].

Taxonomic summary

Class Kinetoplastea Honigberg, 1963 emend. Vickerman, 1976

Subclass Metakinetoplastina Vickerman, 2004

Order Trypanosomatida Kent, 1880

Family Trypanosomatidae Doflein, 1901

Genus *Jaenimonas* gen. n., Votpka and Hamilton, 2015

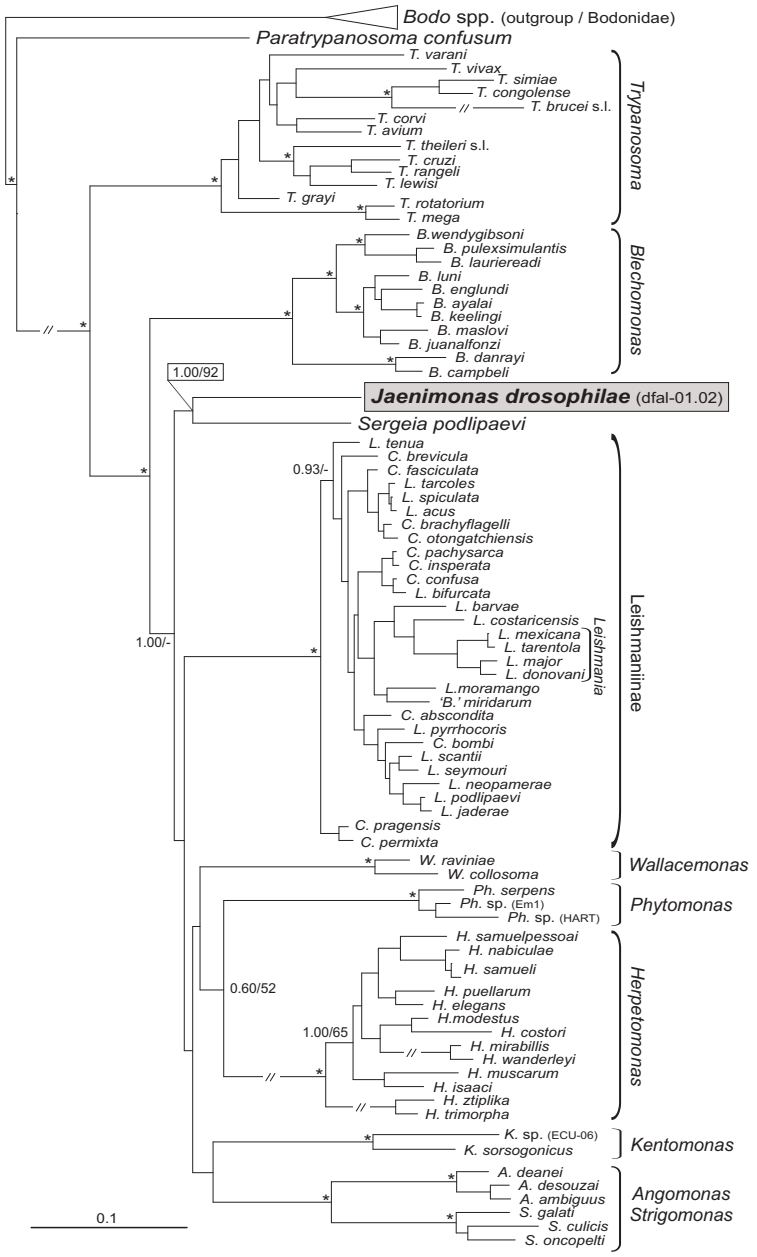

Figure 4.2: *Jaenimonas* is a novel and deep-branching trypanosomatid lineage. SSU rRNA-gGAPDH-based Bayesian phylogenetic tree. Bootstrap values from Bayesian posterior probabilities (5 million generations) and bootstrap support for ML analysis (1,000 replicates) shown. Dashes indicate bootstrap support below 50% or different topology. Asterisks represent >90% bootstrap support and Bayesian posterior probability of >0.95. Double-crossed branches are at 50% of original length. The tree was rooted with *Bodo saltans* sequences, the closest free-living relative of trypanosomatids. The scale bar denotes the number of substitutions per site. Figure courtesy of Jan Votypka

Generic diagnosis: A well-supported monophyletic group of monoxenous trypanosomatids of invertebrate hosts (Diptera: Drosophilidae) without bacterial endosymbionts. It is defined by a set of unique sequences of the SSU rRNA, gGAPDH and SL RNA genes. Molecular phylogenetic analyses confirm this genus as a new member of the family Trypanosomatidae that cannot be associated with any valid genus.

Etymology: The generic name honours Dr. John Jaenike from the University of Rochester, USA, who has made important contributions to the field of host-parasite ecology and evolution, with much of his research focused on natural populations of *Drosophila*. "monas" (Greek) - monad; third declension (monas) feminine; the word monas is included in many generic names of flagellates.

Jaenimonas drosophilae sp. n. Votpka and Hamilton, 2015 (Figure 4.1). Species diagnosis and description: Cultured *J. drosophilae* cells are of the typical promastigote morphology. Cells range from 11.4 and 20.3 m in length and between 0.9 and 3.1 m in width, with flagellum measuring from 6.2 to 16.6 μ m. The kinetoplast disk varies between 114 and 210 nm in thickness and 310 and 670 nm in diameter. The species is identified by the unique sequences KP260534 (SSU rRNA), KP260535 (gGAPDH), and KP260536 (SL RNA), and belongs to typing unit TU173.

Type host: *Drosophila falleni* Wheeler, 1960 (Diptera: Drosophilidae). The xenotype (2006/dfal-01) is deposited at the Royal British Columbia Museum.

Site: Intestine (midgut).

Type locality: Vicinity of West Hartford, CT, USA (4146'04"N; 7245'14"W).

Type material: Hapantotype (Giemsa-stained slide 2006/Dfal-01/S), axenic culture of the primary isolate (Dfal-01) and clonal line (Dfal-01.02) are deposited in the research collections of respective institutions in Prague, Ostrava, and Ceske Budejovice, Czech Republic.

Etymology: The species name is derived from the name of the typical host, *Drosophila* spp.

Remarks: Based on the sequences of SSU rRNA (KC183713), and SL RNA (KC183707, KC183708, and KC183709), the environmental samples from *D. ananassae* captured in the vicinity of Captain Cook, Hawaii, also belong to the same species, *J. drosophilae*.

Jaenimonas infects *Drosophila* larvae, persisting through the development of the fly

We developed a per os larval infection model to assay the effects of *Jaenimonas* in *Drosophila* by exposing newly-hatched larvae to a homogenate of *Jaenimonas*-infected

D. falleni adults in insect Ringer's solution. This homogenate was added to fly food (mushroom or banana, depending on host species) at 1 day post-oviposition (dpo). To establish the number of parasites required to initiate infections, we exposed *D. falleni* larvae to a serial dilution of *Jaenimonas* (from ∼62,500 to ∼2,300 cells), finding that the majority of larvae become infected even at the lowest dose, confirming substantial infectivity (Figure A.3).

Adding high densities ($> 10^5$ trypanosomatids per exposure) of cultured (i.e. axenic) *Jaenimonas* to larval food produced some gut infections, but at low intensities compared to fly homogenate. We therefore used fly homogenate in subsequent experiments. Changes in infectivity are not uncommon in cultured trypanosomatids [142], so we were not surprised to find similar effects in *Jaenimonas*, though the reason for this effect here is not known.

Quantifying infections over time revealed rapid acquisition and persistence of infection in *D. falleni*. Flies exposed to *Jaenimonas* at 1 dpo showed high intensity infections when sampled as larvae at 5 dpo. This was evident qualitatively as masses of parasites visible in the larval midgut and PM upon dissection, and also quantitatively from qPCR of whole larvae (Figure A.4). qPCR following the same fly cohort over time showed that infection persisted and increased modestly as flies developed (Figure A.4; linear model (LM) of log-transformed genome equivalents (GEs); $t_{45} = 3.64$, $P < 0.001$). High infection rates in all life stages following larval exposure demonstrated persistent infection through fly development and metamorphosis: mean infection prevalence overall was 82.5 ± 5.9 % (mean ± se) with no appreciable decrease in infection frequency as flies developed (binomial GLM LRT; $\chi^2_2 = 0.75$, $P = 0.39$).

To confirm *Jaenimonas* persistence through metamorphosis we dissected pupae that were exposed as larvae and found that, over pupation, infection consistently became sequestered in a structure we identified as the yellow body, based on its morphology and location within pupae [143].

We also observed *Jaenimonas* transmission among adult flies by co-housing uninfected flies with those from the stably-infected line. After 5 and 7 days, 44 ± 6% of recipient (uninfected line) flies were found to be infected (71 ± 9% of flies from the donor line were infected). Only 24 ± 7% of uninfected-line flies had obviously replicating infections though, with the remaining new infections evident as only a few free-swimming trypanosomatids in the adult gut, suggesting that adults may be more resistant to acquiring infection than larvae.

Jaenimonas exposure decreases host fecundity

We measured a number of fitness parameters of *D. falleni*, chosen a priori, following larval exposure to *Jaenimonas* or a mock control. These were egg-to-adult survival, egg-to-adult development time, adult wing area (as a proxy for fly size), and adult fecundity (mature eggs in week-old females). Of these, the most affected was female fecundity, with exposed flies having on average 34% fewer eggs (Figure 4.3B: GLMM LRT; $\chi^2_1 = 5.95$, P = 0.015). The number of flies surviving to adulthood was also somewhat lower in trypanosomatid-exposed treatments, though not significantly so (Figure 4.3A: GLMM; $\chi^2_1 = 3.34$ P = 0.068). Neither fly development time nor wing size of flies appeared to be affected by exposure (Figure 4.3A & 4.3C: LMMs; P > 0.5).

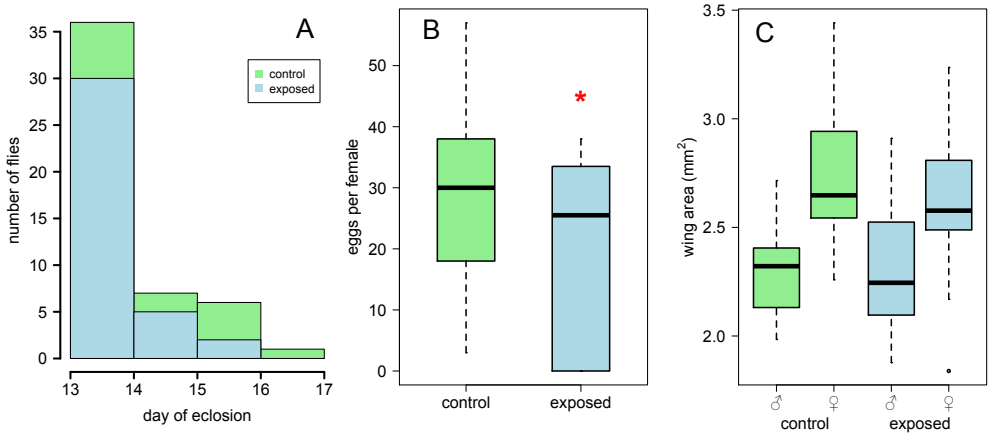

Figure 4.3: *Jaenimonas* decreases female fecundity; results from oral exposure of *D. falleni* larvae at 1 dpo. A) development time and number of emerging flies (bars overlaid, not stacked), B) eggs per 7-day old female, and C) wing area of 7-day old males and females. Exposed females have significantly fewer eggs than unexposed controls (P = 0.014).

Jaenimonas has a wide host range

We exposed larvae from three *Drosophila* species to *Jaenimonas*: *D. falleni* and *D. neotestacea* (both subgenus *Drosophila*), which share a mushroom-feeding niche and are sympatric in parts of their range [56], and *D. melanogaster* (OregonR), which is a distant relative (subgenus Sophophora) not known to breed on mushroom [144]. Infection rates and intensities were quantified in exposed 5-day old larvae and 5-day

old adults from the same experimental cohort using qPCR.

Larvae of all species became infected at high rates (Figure 4.4A). Species differed in their susceptibility, with *D. falleni* and *D. melanogaster* infected at the highest and lowest rates, respectively (species main effect; $\chi^2_2 = 30.38$, $P < 0.001$).

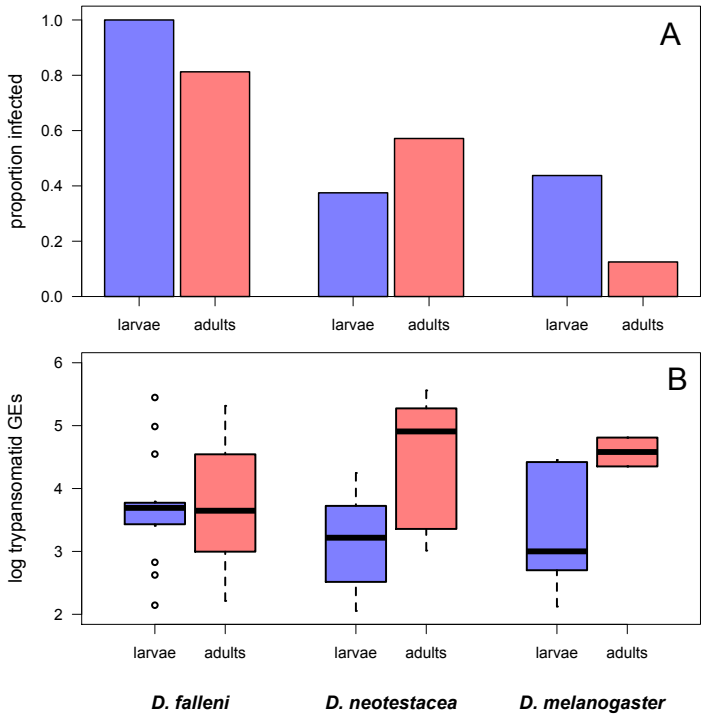

Figure 4.4: *Jaenimonas* has a broad host range. A) Infection rates of larvae and flies following exposure to *Jaenimonas* orally at 1 dpo (62,500 cells per replicate of 20 fly eggs; 16 larvae and flies screened per species, N= 96) and B) infection intensities expressed as genome equivalents (GEs) measured via qPCR. Infection rates are significantly higher in larvae ($P < 0.001$) and lowest in *D. melanogaster* adults (species × stage interaction: $P = 0.017$)

Infections persisted through metamorphosis in all species and were present in 5-day old adults. In *D. melanogaster*, however, there was a pronounced bottleneck in infection over metamorphosis, with substantially lower infection rates in adults than larvae (Figure 5.6A; binomial GLM LRT; species × stage interaction; $\chi^2_2 = 8.11$, $P = 0.017$). Intensity of infection, as measured by qPCR, did not detectably differ between species (Figure 4.4B; $F_{2,46} = 0.26$, $P = 0.79$).

Oral infection with *Jaenimonas* induces an immune response in *D. melanogaster* larvae

To test for immune gene and other transcriptional responses to infection we conducted RNA-sequencing of infected *D. melanogaster* larvae matched to mock-infected controls. Tests for differential expression on multiple biological replicates using Tophat and Cufflinks [145] identified 122 genes that responded significantly to infection after multiple test correction (Cuffdiff Q value < 0.05). Of these, 54 were up-regulated in response to exposure, and 68 were down-regulated (Figure 4.5).

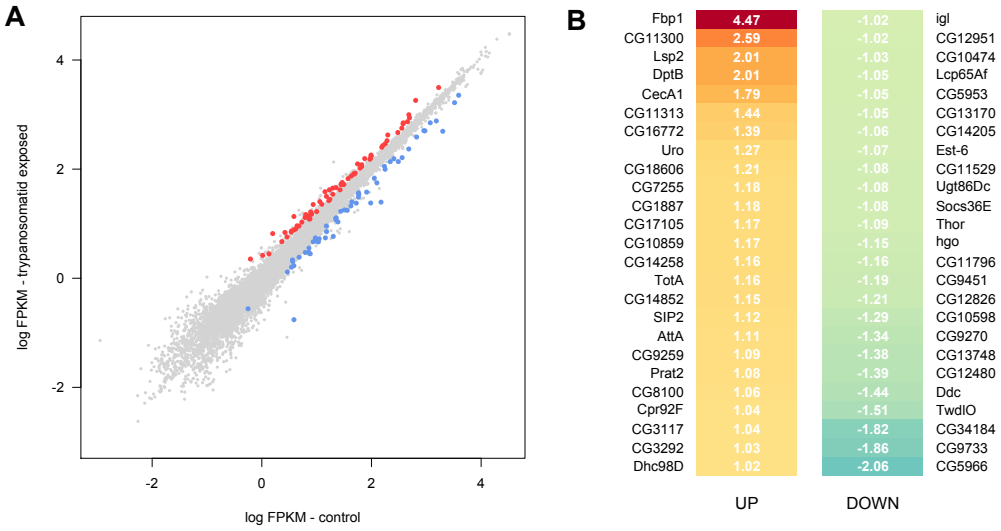

Figure 4.5: Transcriptional response of *D. melanogaster* larvae to Jaenimonas exposure. Oral exposures were conducted using ~62,500 *Jaenimonas* at 1 dpo, and larvae were sampled and pooled for analysis at 5 dpo. A) mean FPKM (fragments per kilobase per million reads) of *D. melanogaster* genes in exposed and control (mock exposure) larvae. B) The top 25 up- and down-regulated genes with $\log_2$ fold changes (N = 5 libraries)

Among up-regulated genes, Gene Ontology enrichment analysis using DAVID [146] predominately identified enrichment of GO terms representing defense response, with 8 of the 54 up-regulated genes having immune-related functional annotation (Table 5.1). Among these were 3 antimicrobial peptide genes (AMPs): Diptericin B, Cecropin A1, and Attacin A, that were as much as 4-fold up-regulated (for DptB) in response to infection. Interestingly, one of the most up-regulated genes was CG11313, a serine protease specific to the *melanogaster* subgroup, evolving under positive selection, present in larval hemolymph clots, and also up-regulated in response to par-

Table 4.1: Gene Ontology Enrichment analysis for genes up- and down-regulated in response to *Jaenimonas* exposure in *D. melanogaster* larvae. Selected gene ontology terms are shown to limit redundancy. Analysis was based on genes identified as differentially expressed by Cufflinks (Q < 0.05) and conducted using the online tool DAVID against a *D. melanogaster* background.

GO term	Accession	Ontology	No. of Genes	P_{adj}
	up-regulated (n=54)			
Defense Response	GO:0006952	BP	8	<0.001
Pigment Metabolic Process	GO:0042440	BP	4	<0.001
Extracellular Region	GO:0005576	CC	11	<0.001
Larval Serum Protein Complex	GO:0005616	CC	3	0.002
Oxygen Transporter Activity	GO:0005344	MF	7	<0.001
	down-regulated (n=68)			
Aromatic AA Metabolic Process	GO:0009072	BP	4	<0.001

asitoid attack [147–149]. PPO1 and PPO2, key enzymes in the melanization defense response [150], were also both up-regulated - unexpectedly, given a lack of obvious melanization response observed during infection, but possibly in response to wounding of the gut epithelium.

Among genes down-regulated in response to infection, there was only one enriched GO term, corresponding to metabolism of aromatic amino acids (Table A.1, Figure 4.5). Many genes relating to host nutritional status were also strongly affected by *Jaenimonas*, either up- or down-regulated (e.g. Fbp1, Lsp2, CG5966), which is not surprising since we might expect heavy gut infections to affect host nutrition and metabolism.

Imd deficient *D. melanogaster* do not show increased susceptibility *Jaenimonas* infection

The Imd pathway is the major regulator of intestinal immune response to oral bacterial infection in *Drosophila* [151]. The up-regulation of larval AMPs associated with the Imd pathway (above) prompted us to assay whether Imd deficient flies were more susceptible to the parasite. We challenged larvae deficient in the transcription factor Relish - which mediates the Imd pathway response [152] - with parasites and screened adult flies for infection. The proportion of infected Relish flies did not differ from wild type control lines (Figure 4.6A GLM; χ^2_2=2.66, P=0.45). Thus, while *Jaenimonas* induced expression of several AMPs in larvae, the Imd pathway does not appear to constitute a major component of resistance to the parasite.

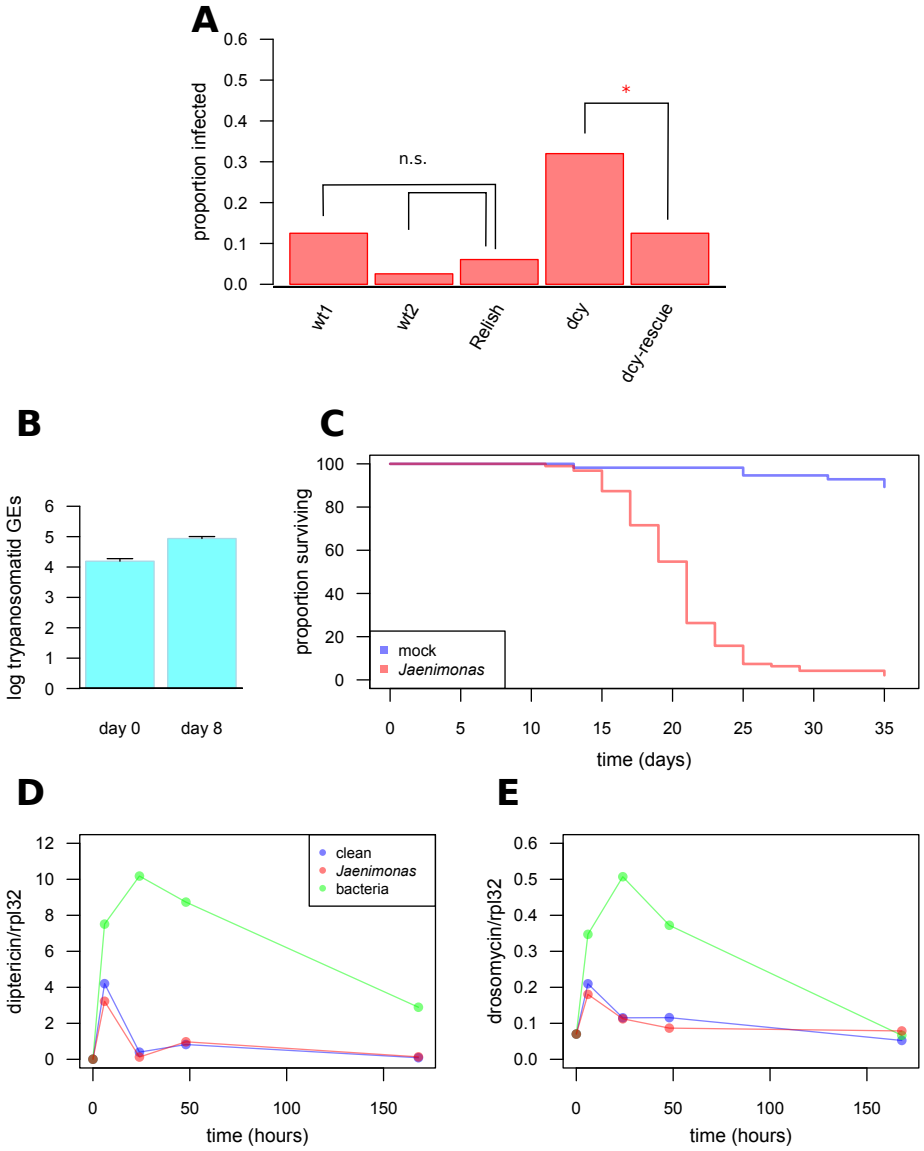

Figure 4.6: *D. melanogaster* immune response to *Jaenimonas*. A) Infection rates of adults following oral larval exposure to *Jaenimonas*. Drosocrystallin knock-out (dcy) flies are significantly more infected than the control line (P=0.029), but the proportion of infected Relish E20 flies is not different control lines (wt1 = white 1118, wt2 = OregonR; $P > 0.05$) (N = 146). B) Intensity of *Jaenimonas* infection in *D. melanogaster* after injection of parasites in the hemolymph of adult flies. Intensity is significantly higher on day 8 ($P = 0.038$). C) Parasites cause strong mortality when injected ($P < 0.001$). D), E) Normalized induction of Diptericin (D) and Drosomycin (E) expression after *Jaenimonas* or bacteria injected into the hemolymph of adult *D. melanogaster*. Data courtesy of Anna Dostalova.

The peritrophic matrix serves as a barrier to *Jaenimonas* infection in the intestine

The PM has been implicated as an important barrier to trypanosomatid establishment in dipterans [153]. To assess its importance in *Drosophila* resistance to *Jaenimonas*, we infected a line deficient in the Drosocrystallin (dcy) protein. Dcy is a component of the PM in adult flies, and its deletion results in a reduction of PM width, an increase in its permeability, and increased susceptibility to bacterial infection [154]. Dcy-deficient flies had substantially higher rates of infection than control dcy-rescue flies, in terms of adult infection levels following larval exposure (Figure 4.6A; χ^2_1=4.74, P=0.029), suggesting a role of an intact PM as a barrier to the establishment and/or persistence of *Jaenimonas* in the midgut.

Systemic infection with *Jaenimonas* kills *D. melanogaster* but does not induce a pronounced immune response

We explored the impact of *Jaenimonas* on the host immune response by directly injecting the parasite into the *D. melanogaster* body cavity. qPCR confirmed that *Jaenimonas* survives and proliferates in the host hemolymph, multiplying ∼5× by 8 days after injection (Figure 4.6B; P = 0.037). *Jaenimonas* injection caused very high mortality, albeit with a late onset consistent with slow replication within the host (Figure 4.6C; log rank test: P < 0.001). This effect, however, varied with host genotype, with some strains being more or less susceptible (representative Oregon R strain shown). We measured immune induction in systemic infections through expression of Diptericin and Drosomycin, two antimicrobial peptides commonly used as read-outs for activation of the Imd and Toll pathways. Interestingly, there was no observable up-regulation of either AMP above background levels, even upon injection of high doses of parasites (∼14 000 parasites/fly; Figure 4.6D-E), suggesting that though variation in resistance appears to exist, it is not clearly linked to Imd and Toll pathway competence.

Discussion

Although it has been known for over a century that trypanosomatids are pervasive parasites of *Drosophila*, they have received scant attention. To date, no *Drosophila*-

specific trypanosomatids have been formally described, save some early morphological descriptions that appear to be apocryphal due to the notorious difficulty of identifying trypanosomatids without molecular markers (e.g. *Trypanosoma drosophilae* in [133]), and most aspects of trypanosomatid biology within *Drosophila* hosts remain unknown. These include the diversity, systematics, and effects of these parasites in their hosts, which is remarkable given the intense study that the genus receives as a model of infection and immunity.

Here, we begin to address this deficiency by describing a new trypanosomatid genus and species from *Drosophila*. Though isolated from *D. falleni*, we find that *Jaenimonas* has a broad host range, easily infecting the distantly related *D. melanogaster*. Previous molecular screens in a wide range of wild *Drosophila* have found sequences of *J. drosophilae* in *D. ananassae* collected in Hawaii [139], thus confirming *Jaenimonas* to be geographically widespread and with a broad host range, infecting both *Sophophora* and *Drosophila* subgenera.

Oral infection of larval *Drosophila* demonstrated that infection persists through the life of the fly, including through metamorphosis, when the fly gut undergoes a near-complete rearrangement that can also purge it of dominant microbes [155]. Early work on *Crithidia* infection in mosquitoes demonstrated that some infections persist from larval mosquitoes to adults [156], and early accounts of *Drosophila* trypanosomatids suggest this to be common [72, 133, 157]. Interestingly, the main observed difference between the *Drosophila* species we challenged was persistence of the infection after pupation, as *D. melanogaster* adults, but not *D. falleni* or *D. neotestacea*, were less infected than larvae. Whether this is due to differential immune response, or specific structural rearrangements during pupation is unclear, although it is interesting that there was a clear effect of the integrity of the PM on adult infection rates in *D. melanogaster*.

We observed *Jaenimonas* transmission to both larvae and adults, as well as between adult flies, but suspect that transmission to larvae is an especially important component of the epidemiology of these parasites. Characteristics of the *Drosophila* life history suggest that larval infections would be important: many *Drosophila* breed and reach high larval densities on ephemeral or patchy food resources [144], with substantial opportunity for density-dependent pathogen transmission from adults to larvae, or among larvae. Indeed, many *Drosophila*-specific nematode parasites - such as *Howardula* - and parasitoid wasps have life cycles that rely entirely on transmission to larvae [158], suggesting that many parasites of *Drosophila* commonly exploit larval

aggregation.

Though trypanosomatids have mostly been considered relatively benign in their insect hosts [110], there have been few direct experiments performed so far that test this. In fact, we found a pronounced negative effect of *Jaenimonas* exposure in the laboratory, where it lead to ∼1/3 reduction in female fecundity. Ebbert et al. [134] also found costs of trypanosomatid infection in larval *D. melanogaster* - larvae that were fed infected adult carcasses took longer to pupate. In many cases, trypanosomatid virulence in insects is also context-dependent: the virulence of *Crithidia bombi* in bumblebees, for instance, increases under times of nutritional or environmental stress [159]. It is likely then that the effect we see under laboratory conditions is conservative.

Jaenimonas exposure in *D. melanogaster* larvae induced a modest but clear change in host gene expression, with a number of immune-related transcripts up-regulated (Figure S3), including 3 AMPs. Induction of AMPs has also been demonstrated in tsetse and bumblebees exposed to trypanosomatids [130, 160], and AMPs have been shown to have trypanocidal effects in these systems [160]. AMP levels in our *Jaenimonas* exposures were low (i.e. ∼4-fold for DptB) however, compared to those induced by pathogenic gut bacteria [151], and this may be due to localization of the response to the gut (we measured gene expression in whole larvae). It is also possible that the induced AMPs could be an indirect byproduct of other gut microbes affected by and/or interacting with *Jaenimonas*: a number of recent studies have found that gut bacteria have important effects on immune response to trypanosomatids [100,161]. Relatively low but significant increases in AMP expression are also consistent with an early study that examined *D. melanogaster* immune response following oral exposure to mosquito and bumblebee trypanosomatid parasites [111].

Interestingly, mutant Imd deficient *D. melanogaster* did not show higher levels of *Jaenimonas* infection, further suggesting that the AMP induction observed in our larval transcriptome may not be a direct consequence of *Jaenimonas* infection. This also appears very different from tsetse flies, in which the Imd pathway appears to play an important role in limiting trypanosome infections [160]. Of course, there are many crucial differences between tsetse-*Trypanosoma* and *Drosophila*-*Jaenimonas* interactions, including the importance of a bloodmeal in trypanosome transmission and the presence of Wigglesworthia, obligate nutritional symbionts of tsetse that also play a critical role in immunity to parasites [162].

In contrast to Imd mutants, flies deficient in Drosocrystallin were more permis-

sive to *Jaenimonas*. An intact PM thus appears to be an important component of *Drosophila* defense against trypanosomatids, similar to what has been shown for bacterial enteric infections [154]. The PM has also been implicated in resistance to trypanosomatids in other dipterans, where it can act as a physical barrier, as has been suggested for *Leishmania* development in sand flies, or it could modulate host immune responses, as proposed for tsetse [162]. Our results favor the former scenario, but further study will be necessary to identify the precise role of the PM in resistance to *Jaenimonas*.

When injected into host hemolymph, *Jaenimonas* proliferated causing high mortality, but we did not observe an up-regulation of either Diptericin or Drosomycin, readouts for the Imd and Toll pathways, respectively. This is in contrast to the up-regulation of Diptericin, Drosomycin and Drosocin observed when *Crithidia* from mosquitoes and bumblebees was injected in *D. melanogaster* [111]. Still, induction of AMPs in that study was low when compared to that provoked by injection of bacteria [71], and the authors did not observe a clear negative relationship between AMP induction and parasite-induced mortality. Thus, our results suggest that *Jaenimonas* is not recognized by the fly immune system when injected - at least the Toll and Imd pathways. This might be because as a eukaryote it lacks easy determinants of recognition.

In sum, this is the first to be characterized of an apparently diverse and ubiquitous lineage of parasites from *Drosophila*, and allows the exploitation of a rich body of knowledge and experimental tools to study insect-trypanosomatid interactions, particularly monoxenous infections. In addition to beginning to unravel the ecology and dynamics of *Drosophila*-trypanosomatid associations, the infection of *D. melanogaster* makes *Jaenimonas* an excellent model for understanding insect immunity to trypanosomatids more generally.

Materials and Methods

Rearing flies

Drosophila falleni and *D. neotestacea* used in this study were maintained in plastic vials containing ∼5 ml Carolina *Drosophila* medium (Fisher Scientific), a small piece of *Agaricus bisporus* mushroom, and a cotton wick. Flies were kept in an incubator at 21°C on a 12L:12D cycle. *D. melanogaster* were either maintained on *Drosophila*

medium with the addition of yeast, or on grape juice agar (6.2 g agar, 58.8 g Farigel wheat, 58.8g yeast, 100 ml grape juice, 4.9 ml propionic acid, and 26.5 ml methyl-4-hydroxybenzoate solution (400g/L in 95% ethanol) per L of water). *D. melanogaster* were also kept at 25C after systemic infection, and for oral infection of mutant lines.

Parasite isolation and establishing of cultures and clonal lines

Drosophila falleni were originally collected in West Hartford, USA in 2006. For parasite isolation, insects were dissected and examined under a microscope as described previously [163, 164]. The primary isolate was cultivated in Brain Heart Infusion (BHI) medium (Sigma-Aldrich) supplemented with 10 μg/ml of hemin (Jena Biosciences) and antibiotics as reported previously [96]. All subsequent passages were done in the same media with pH adjusted to 6.5. Clonal isolates were obtained using the limiting dilution methods as described earlier with modification [165]. In brief, parasites were counted using a hemocytometer, serially diluted to a density of 16 cells per 22 ml (10 ml conditioned, 12 ml unconditioned) of media, and plated in a 96 well plate (200 μl/well). The cloning procedure was repeated twice.

We established a *Jaenimonas*-infected fly line by passaging infection to a parasite-free *D. falleni* line (also collected in West Hartford). This recipient line remained stably infected for the course of experiments, and a trypanosomatid-uninfected version of the same line was homogenized for negative controls during experimental infections.

Light and electron microscopy

Light microscopy of Giemsa stained smears was done as described elsewhere [138]. We performed standard measurements for 50 cells. Fluorescence microscopy was done by staining with L8C4 mouse monoclonal antibody against *T. brucei* paraflagellar rod 2 [166], counterstained with DAPI, with Alexa Fluor 488 dye goat anti mouse as a secondary antibody. Scanning electron microscopy (SEM) and high pressure freezing transmission electron microscopy (HPF-TEM) were performed as described elsewhere [136].

PCR amplification, cloning and sequencing

Total genomic DNA was isolated from 10 ml of axenically grown cultures using High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the man-

ufacturer's protocol. 18S ribosomal RNA (SSU) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) genes were amplified from 10 to 100 ng of total genomic DNA using primers S762-S763 [164] and M200-M201 [96], respectively and sequenced directly. The gene encoding SL RNA was amplified using the primer pair M167-M168 [136], cloned using InsTA PCR Cloning Kit (Thermo Fisher) and sequenced.

Phylogenetic analyses

We aligned 18S (SSU) rRNA and gGAPDH gene sequences of selected trypanosomatids using Kalign (53), and the resulting alignments were edited manually using BioEdit to remove fast-evolving regions (mainly for SSU rRNA) that prevented unambiguous alignment [167]. The final concatenated dataset contained 84 taxa and included 3,168 characters. Phylogenetic analyses of the SSU and gGAPDH datasets were performed with MrBayes 3.2.2 (Bayesian criteria: rates for six different types of substitution; proportion of invariant sites and shape parameter of the -correction for the rate heterogeneity with four discrete categories were allowed to vary; the covarion model was used to allow the rate heterogeneity along the tree; the Markov chain Monte Carlo was run 5 million generations) and PhyML for maximum likelihood (ML: the best-fitting model [GTR + I + Γ of the sequence evolution was determined using Modeltest 3.7 and bootstrapped with 1000 replicates) [127, 168–170].

Quantitative PCR of *Jaenimonas*

We developed a qPCR assay for *Jaenimonas* based on the SSU rRNA gene (dtrypF: 5'-CTTACGGGAATATCCTCAGCAC-3', dtrypR: 5'-CTGTCCGGATCTGGTAAAGTTC-3'), designing primers in Primer3 [87]. Quantitative PCR was conducted using a BioRad CFX96 thermal cycler and SsoFast EvaGreen MasterMix (BioRad) according to the manufacturer's recommended reaction and thermal cycling conditions (20 μL reactions, 60°C annealing temperature). We generated standard curves for trypanosomatid quantification using duplicate reactions of a 6 $\times$ 10-fold serial dilution of DNA extracted from *Jaenimonas* pure culture, counted using a hemocytometer. This spanned a dynamic range from 10 to 10^6 trypanosomatid genome equivalents (GEs), and provided highly reproducible results across this range ($R^2 > 0.9875$ on all quantitation standard curves; efficiency $> 83\%$).

DNA was extracted using PrepMan Ultra (Life Technology). Flies (larvae, pupae

or adults) were added to 2 mL screw cap cryotubes with 50 μL PrepMan Ultra and $\sim 10 \times 1$ mm silica/zirconia beads (BioSpec Products). Samples were disrupted for 15 secs on a miniBeadBeater 16 (BioSpec Products), and extraction was completed according to the manufacturer?s protocols. Quantitative PCR reactions were run in duplicate using a 1/10 dilution of template DNA in water; any samples with replicates > 0.5 C_t apart were rerun.

When collecting larvae for screening, we rinsed larvae in distilled water prior to DNA extraction to reduce any external trypanosomatid contamination. Still, because we also often observed free-swimming trypanosomatids in larval guts where no heavily replicating infection was apparent, we also set a cutoff of 100 *Jaenimonas* GEs to indicate an infection.

Experimental infections

To challenge larvae with trypanosomatids we first collected eggs from *Drosophila* left to oviposit overnight on slices of mushroom agar in petri dishes [65]. Eggs were transferred in groups of 10 or 20 to either a small piece of mushroom (for mushroom feeding species) or organic banana, then transferred to plastic vials containing moistened cheesecloth. For initial infection trials using *Jaenimonas* from pure culture, we attempted applying different doses to mushroom or pieces, ranging from $\sim 5.0 \times 10^4$ to 1.5×10^5 cells in brain-heart infusion (BHI) during their exponential growth phase (to achieve the higher intensity doses we supplemented growth media with 5% FBS).

Ultimately, we observed the highest experimental infection rates by adding *Jaenimonas* derived by grinding infected *D. falleni* in insect Ringer's solution, applying 6.25×10^4 *Jaenimonas* from this homogenate to mushroom, banana, or semi-defined media, one day after oviposition. An equal volume of *D. falleni* slurry from the matched uninfected line, with an equal ratio of ground flies in insect Ringer's, was added to negative controls. This infection protocol was repeated for a number of assays outlined below. We additionally tested for infectivity of lower doses by serially diluting this homogenate in a 3-fold dilution (4 dilutions from $\sim$62500 to $\sim$2300 cells), and exposing *D. falleni* larvae as above. Larvae were screened for infection by dissection, and only considered infected if substantial masses of clearly replicating trypanosomatid cells could be discerned in the midgut and PM.

We tested for adult-adult transmission by co-housing 5 infected-line and 5 uninfected-line *D. falleni* in vials (8 vials total = 80 flies). To differentiate flies from these lines,

we combined infected-line males with uninfected-line females and vice-versa. At two time points (5 and 7 days after cohabitation), flies were dissected and screened for trypanosomatids by light microscopy, and the mean infection rates per vial calculated.

Dynamics of infection

We quantified infection dynamics by exposing *D. falleni* larvae to *Jaenimonas* as above and monitoring infection with qPCR of whole flies. Flies were collected at 5, 10, 15 and 25 dpo and subjected to DNA extraction and qPCR (N = 56). Upon eclosion (∼14 dpo), flies were transferred to solitary vials containing a small amount of *Drosophila* medium and mushroom to prevent adult-adult transmission. Data were analyzed using a linear model (LM) to test for time effects on log-transformed trypanosomatid GEs, and a binomial GLM to test for time effects on infection prevalence.

Fitness effects in *D. falleni*

We measured the effect of *Jaenimonas* on *D. falleni* by exposing a cohort of larvae to parasites, as above. Eggs were placed in vials (10 eggs per vial, 24 vials; N= 240), and exposed to either *Jaenimonas* or a mock infection in a balanced design. The cohort was followed as flies developed, with flies being transferred to solitary vials upon eclosion. Flies were frozen 7-days post eclosion. Females were dissected at 40× using a dissecting microscope and mature eggs counted. One wing per surviving fly was also photographed (Retiga 2000R camera (Q-imaging) and Image Pro Express software (Media Cybernetics)), and traced with wing area calculated in ImageJ (NIH). PCR to confirm trypanosomatid genotype used the reverse primer from the qPCR assay and an alternative forward primer to yield a longer amplicon: dtryp2F: 5'-ATGGGGGATATTTAAACCCATC-3', with an annealing temperature of 56°C. Amplicons were sequenced by Macrogen USA.

To test for effects of *Jaenimonas* exposure on fitness parameters, we included experimental fly vial as a random effect in mixed models using the *lme4* package of R [171]. These included egg-adult development time (Poisson GLMM), egg-adult survival (binomial GLMM), adult wing area (LMM), and fecundity (Poisson GLMM). Significance of trypanosomatid effects for GLMMs was assessed using likelihood-ratio tests.

Host range of *Jaenimonas*

We tested the host range of *Jaenimonas* in the laboratory by exposing three species of *Drosophila* to infective doses of the parasite as above. Eggs were collected from *D. falleni*, *D. neotestacea* (line originally collected in West Hartford CT, 2006; *Spiroplasma* and *Wolbachia* negative), and *D. melanogaster* (Oregon R) and exposed to parasites in groups of 20. Exposed larvae (5 days post-oviposition) and adult flies (5 days post-eclosion) were collected (16 of each species at each life stage; N = 96), and infection quantified by qPCR of whole larva or fly DNA extractions. Statistical tests of host and life stage effects on infection intensity (log-transformed) and infection frequency were conducted using a linear model and a binomial GLM, respectively.

mRNA-sequencing of *Jaenimonas* infected *D. melanogaster* larvae

We collected eggs oviposited by *D. melanogaster* on mushroom agar disks, transferred them to banana pieces, and exposed them to *Jaenimonas* or a negative control as above. Five days after placement on banana, we collected 10 larvae at random from trypanosomatid-exposed vials and screened them for infection by dissection; 7/10 larvae had obvious heavy infection, and free-swimming trypanosomatids could be observed in the guts of all exposed larvae. After this confirmation, we placed 8 larvae into each of 3 cryotubes per treatment at random (3 replicates per treatment, N = 6). Larvae were flash frozen in liquid nitrogen, and RNA extracted in 300 μL of Trizol (Invitrogen) according to the manufacturer's instructions. DNA was digested using DNaseI (Thermo Fisher) and RNA re-extracted using an RNeasy Kit (Qiagen).

Total RNA was shipped to Genome Quebec (Montreal, QC), which provided QC analysis (Agilent Bioanalyzer), library construction (Illumina TruSeq mRNA stranded), and 100-bp single-end Illumina sequencing. Sequencing generated an average of 29 $\pm$ 1.63 million (mean se) single-end reads per library. Raw reads have been deposited in the NCBI SRA under PRJNA277742.

To confirm that these infections consisted of only *Jaenimonas* trypanosomatids, we additionally aligned reads that did not align to the *D. melanogaster* genome (see below) to both *Jaenimonas* and *Crithidia fasciculata* SSU rRNA sequences using Geneious (Biomatters Ltd, default mapping settings). Reassembling mapped reads from these alignments consistently yielded a single genotype corresponding to *Jaenimonas*.

We conducted tests for differential expression by aligning reads to the *D. melanogaster* genome (release BDGP5) using Tophat v.2.0.13, followed by analysis of differential expression in Cufflinks v.2.2.1, with α of $Q < 0.05$. Upon initial analysis, we found that one library had dramatically divergent expression of a number of genes, particularly those of the Osiris and Tweedle gene families, which tended to be very highly expressed in this library and completely absent in the rest. We presume that this was due to one or more larvae in this sample being at a different developmental stage. We therefore repeated the analysis omitting this library; this final analysis of differential expression was qualitatively similar to the first but lacked identification of numerous Osiris and Tweedle-family genes as differentially expressed.

We tested for significant enrichment of Gene Ontology terms among up- and down-regulated genes separately using DAVID, assessing significance of GO terms based on Benjamini-Hochberg corrected P-values.

Drosophila melanogaster genotypes for functional immunity

Drosophila melanogaster lines w1118 and Oregon R were used as wild type controls. The RelishE20 line *Hedengren1999* was used as immune-deficient control. Resistance to infection of a line with a loss-of-function mutation in the drosocrystallin gene (CG16963, Dcy) was compared to that of a rescue line created by precise excision of the Minos element (Dcy-rescue line), both described in [154].

Systemic infection of flies by microinjection

We challenged flies by injecting 13.8nL of parasite or bacteria suspension in PBS in the thorax using the Nanoject apparatus (Drummond Scientific) and pulled glass needles. Cultured parasites were washed in PBS and adjusted to a final concentration of 10^9 parasites/mL (corresponding to ∼14 000 parasites injected/fly). Overnight cultures of *Micrococcus luteus* and *Escherichia coli* were concentrated and resuspended in PBS to final OD_{600} of 5 and mixed in a 1:1 ratio. PBS-only injected flies were used as clean-injury controls.

RT-qPCR of immune effectors

We isolated RNA from pooled samples of 15 females (2-6 days old) collected at the indicated time points (Figs. 5.8D and E). RNA extraction, reverse-transcription and

qPCR were performed as described by [150]. Expression of antimicrobial peptides diptericin (CG12763) and drosomycin (CG10810) was normalized to that of ribosomal protein L32 (CG7939). Experiments were repeated at least twice: the results of a representative experiment are shown.

Supplement: Interactions between trypanosomatids and other parasites in *Drosophila*

Introduction

In addition to trypanosomatids, *Drosophila* also host a number of ecologically important parasites and endosymbiotic bacteria, which often affect each other in important manners [19, 172]. The high prevalence of trypanosomatids often observed in wild fly populations suggests that they might have important interactions with other prevalent parasites. We used *Jaenimonas* as a model to investigate the interplay between trypanosomatids, endosymbiotic bacteria, and parasitic nematodes in the mushroom-feeding host *Drosophila neotestacea*. These infections have been studied in detail in *D. neotestacea*, as *Spiroplasma* provides a strong degree of protection against the parasitic nematode *Howardula aoronymphium*. Through screens of wild flies, we find that there is a strong association between trypanosomatid infection and nematode infection in the wild; however, these effects were not evident in controlled infection experiments using *Jaenimonas*, suggesting that this association is due to a shared transmission pressure rather than parasite interactions within the host.

Results and Discussion

We tested for associations between trypanosomatids and the prevalent and virulent nematode parasite *Howardula aoronymphium* by screening of wild *D. neotestacea* collected in British Columbia in 2011 [1], using diagnostic PCR sensitive to a broad diversity of monoxenous trypanosomatids [96]; these flies had previously been diagnosed for *Howardula* infection by dissection [1]. Trypanosomatids were detected in 28% of flies overall; however considering *Howardula* infection status revealed that 43 % of *Howardula*-infected flies also had a trypanosomtid infection whereas only 20 % of uninfected flies did (Figure 4.7; χ^2_1=5.71, P = 0.017; N = 116), indicating ∼2-fold greater likelihood of trypanosomatid infection in the presence of *Howardula*.

We tested for evidence of facilitation or interference between *Jaenimonas*, *Howardula*, and the bacterial endosymbiont *Spiroplasma* in *D. neotestacea* using a factorial infection experiment. In this host *Spiroplasma* plays a defensive role, decreasing the size of reproductive *Howardula* 'motherworms' and rescuing the effects of *Howardula* infection that normally sterilizes the host [19]. This experiment was motivated

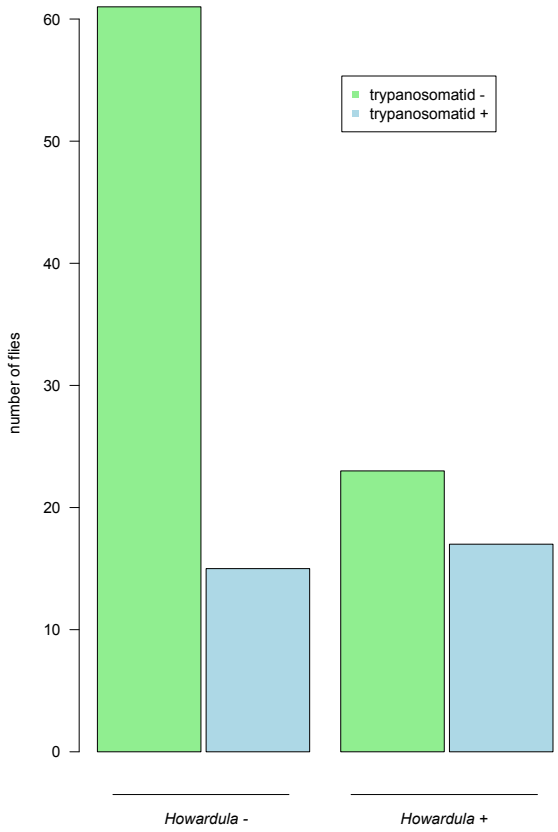

Figure 4.7: Trypansomatid infection is associated with *Howardula aoronymphium* nematode infection in wild *D. neotestacea* (P = 0.017; N = 116)

.

by multiple factors: the non-random association between *Howardula* and trypanosomatids observed in the wild (above), our previous observation that *Spiroplasma* infected flies appear to have heavier *Jaenimonas* burden [65], and additional potential for indirect effects of *Spiroplasma* on *Jaenimonas* through *Spiroplasma*'s effect on *Howardula*. These controlled infections showed trypanosomatid exposure to have little effect on either the infection prevalence of *Howardula* (χ^2_1=0.36, P = 0.55), or the size of motherworms infecting flies (Figure 4.8 - only motherworm sizes shown for brevity; ANOVA; $F_{1,53} = 1.78$, P = 0.12), although as expected *Spiroplasma* caused a dramatic reduction in worm size ($F_{1,53} = 18.10$, P < 0.001). Similarly, *Spiroplasma* had no detectable effect on *Jaenimonas* infection prevalence or intensity, either as a main effect or through an interaction with *Spiroplasma* (LM and binomial GLM respectively; P > 0.4 for all effects; N = 95). However, because of the destructive sampling of these flies, we did not diagnose all infections in all individuals, instead

relying on exposure as a proxy for infection, potentially obscuring more subtle effects.

Collectively, these data suggest there to be a clear positive association between trypanosomatid and *Howardula* infection in the wild, but that this is not readily reproducible under controlled conditions in the laboratory. The observed pattern might therefore be due to a shared infection pressure rather than interactions within the host. We also found, however, that beyond *J. drosophilae* there is a substantial diversity of trypanosomatids infecting D. neotestacea, and it remains possible that other trypanosomatid species could interact more strongly with *Howardula* or *Spiroplasma* within the host.

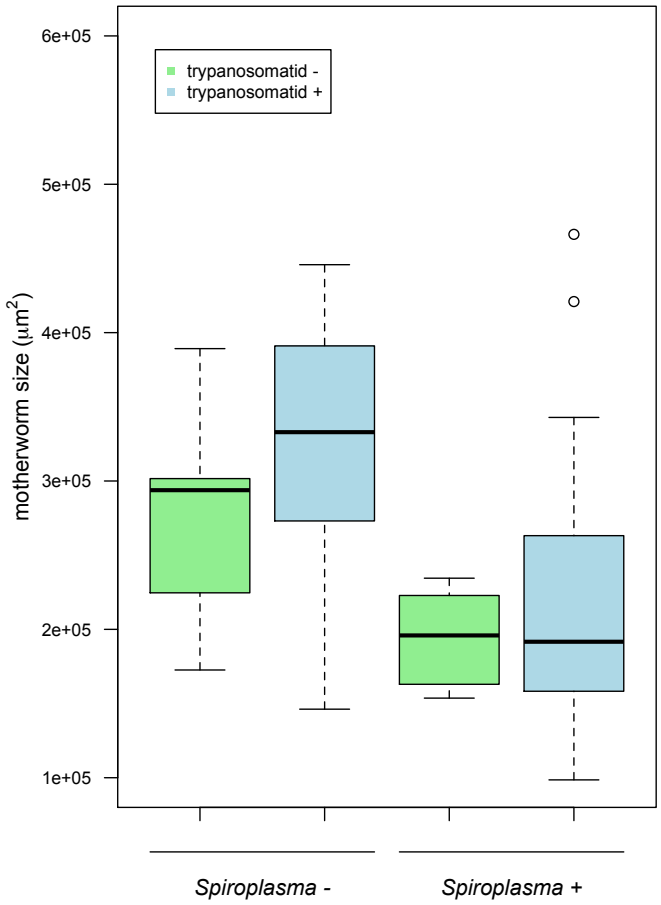

Figure 4.8: *Jaenimonas* exposure does not detectably affect the size of *Howardula* motherworms ($P = 0.12$), while *Spiroplasma* substantially decreases size ($P < 0.001$).

Methods

Wild flies were screened for trypanosomatid infection using primers specific to the spliced-leader RNA (SL RNA, above). This assay detects a diversity of monoxenous trypanosomatids [96]. Further analysis of a subset of these samples using qPCR followed by high-resolution melt curve analysis with the above *Jaenimonas* primer set, in addition to sequencing ∼800 bp of the 18S rRNA, suggested that the majority of infections in this cohort of *D. neotestacea* were from other uncharacterized trypanosomatids, not *Jaenimonas*.

Infection with *Spiroplasma* and *Howardula* combined the above oral *Jaenimonas* infection protocol with nematode and symbiont infections, following the methods described by [65]. This *Jaenimonas* × *Spiroplasma* × *Howardula* factorial design included 7 vials for each of the 8 treatments, with eggs in groups of 20 in each vial (N = 1120 eggs). Survivors to adulthood were frozen as 5-day old adults and sexed. Males were DNA-extracted and *Jaenimonas* infection quantified via qPCR, whereas females were dissected and average motherworm size calculated as per [1]. Tests of effects of exposure/infection status on measured response variables were done using LMs or GLMs as appropriate.

Chapter 5

Symbiosis in ecological networks

Introduction

Symbiosis is now known to be central to the development of evolutionary novelty and complexity [173], and is increasingly understood to play a critical role in the ecology of multicellular organisms [116]. Many examples now underscore the key importance of symbioses in diverse systems, from coral reefs to grasslands [116, 174]. Yet, by and large, symbiosis receives little attention as a process that shapes the structure and function of ecosystems. This is particularly true in the study of ecological networks and especially food webs - the most widely used network approaches to understand the relationships between energy flow, structure, and stability in natural complex systems. This is no doubt for many reasons, both historical and practical, in that symbioses have in many cases escaped detection, often relying on modern molecular methods to detect and characterize [76], while simultaneously adding a high degree of complexity or intractability to common network and food web analyses (e.g. [175]). A growing awareness of the pervasiveness and importance of symbioses in diverse ecosystems that parallels an ongoing revolution in molecular biology [116], however, now requires that we update our understanding of ecosystem structure and function in ways that recognize their contributions.

Representing biological systems as networks has yielded insight into many aspects of ecology and evolution. In ecology, this has contributed to our understanding of processes as diverse and important as keystone predation, trophic cascades, and relationships between ecosystem complexity and stability, among many others [176–179]. The most common representation of natural networks is undoubtedly the food web. This is a map of who eats whom within an ecosystem or community, and a framework

that represents energy flow that has become a central concept in ecology. Alongside other ecological networks, food webs are also increasingly employed as predictive tools. In theory, by understanding the important linkages among species in an ecosystem, the outcome of perturbations or additions to species might be predicted - for instance in the forms of species invasions, extinctions, or population crashes (e.g., [180–182]). Despite the obvious utility of such predictive frameworks, progress has been hampered by the complexity of real systems, which food web studies must confront on multiple levels [183–185].

On one hand, the observations of species interactions that form the basis of these networks are laborious and subject to strong biases from sampling effort and study scope - an issue that has plagued food web and other network studies since their inception [179, 186]. More conceptually, food webs, both empirical and theoretical, explicitly exclude non-trophic interactions: these are species interactions that are non-consumptive, and may be direct or indirect, but are increasingly understood to have important roles in determining the structure of the real systems that comprise food webs [187]. Symbioses affect food webs in both of these capacities, as trophic actors [36, 188], and as modifiers of other trophic interactions [3, 30, 189], but are rarely considered in network approaches. Recognizing the ways in which symbioses fit - or do not fit - within a broader food web framework allows an opportunity to test the comprehensiveness of such approaches to understanding ecosystem structure and function, while also clarifying the relative importance of symbiosis in real ecosystems.

What is symbiosis?

Defining symbiosis is necessary to meaningfully discuss its ecological implications. First presented as the 'living together of unlike organisms' well over a century ago (de Bary 1879, cited in [76]), symbiosis has since acquired a more nuanced meaning. Now, the term is often reserved for associations that are jointly beneficial for the involved partners [76], although some authors consider any physically intimate association to be symbiotic - a definition that considers parasites and pathogens to be symbionts (e.g., [190, 191]). This definition may be useful as it recognizes that symbiotic associations lie along a continuum of effects on the host from harmful to helpful. For clarity and to distinguish work on symbiotic mutualisms from a vast literature on parasites and pathogens, though, I restrict my definition of symbiosis here to refer to mutualistic interactions.

Likewise, not all mutualisms should be considered symbioses; plant-pollinator mutualisms, for example are of great ecological importance and have been intensively studied from a network perspective [192], but do not fulfill the physical intimacy that may be argued to be required of a symbiosis - a distinction that becomes more important when compared to antagonistic strategies. Antagonistic consumer-resource interactions have been delineated using a series of criteria that distinguishes predators from micropredators from parasites, etc. [193], and these differences can be important in modelling interactions appropriately and understanding species roles in food webs [184, 194]. Although likely to be informative, so far no such framework appears to have been applied to mutualistic interactions. Symbioses, for the most part, also involve disparities in size, with smaller symbionts associating with larger hosts. I will use the terms symbiont and host in this capacity, but it is also interesting to note that ecological and evolutionary causes and consequences of these size disparities are mostly uninvestigated relative to parasites [193]. Thus, for now, I consider symbioses to be jointly beneficial relationships possessing a high degree of physical intimacy, but recognize that this definition is somewhat subjective and that benefits to the host can be cryptic or context dependent.

Within recognized symbioses diverse mutualistic strategies have evolved [76]. Symbioses result from different symbiont transmission processes, and share different degrees of host intimacy, sometimes being limited to the exterior of the host, possibly in compartmentalized structures such as the host gut or specially evolved crypts [195, 196], or endosymbiotic, in intercellular locations, intracellular locations, or even in specialized organs [36, 197]. The localization of symbioses often suggests the importance of different transmission routes, as well as the co-evolutionary history of symbiont and host [198, 199].

Symbionts are acquired by the host through a combination of vertical and horizontal transmission between infected hosts, but may also be acquired from the environment [76,198]. Vertical transmission is the passage of the symbiosis from parent to offspring, and is typically matrilineal when it occurs [76]. Insects are the best known as the hosts of vertically transmitted symbioses, and have diverse associations with microbes that are passed from the mother to her offspring in the germ line [35, 36], but other examples are well known such as the transmission of fungal endophytes in grasses [30]. The evolutionary consequences of this transmission strategy, and the degree of host-symbiont interrelationship it entails, have been the focus of much research [28, 37, 39, 200]. As a consequence, insect symbioses are better understood

than most others, and thus are the basis of many of the examples I discuss. More recently, vertical transmission has been argued to be important in diverse symbioses and other infectious processes [191] - even where no germ-line transmission occurs (e.g. 'pseudo-vertical' transmission) providing a component of heritability upon which selection might act [201]. The environment also may play a role in the evolution of symbiont transmission strategies: for instance vertical transmission appears to be less common in marine environments, even among obligate symbioses [198], possibly because greater opportunity for horizontal and environmental transmission through the water column make this a more sound strategy; the same may be said of plant root symbioses in the rhizosphere that are critically required by the host but also readily acquired from the environment [202, 203].

Finally, symbioses may be obligate or facultative, for either the host or the symbiont. This means that some symbioses are strictly required for the host to survive, whereas others are not, and that some symbionts may have free-living stages. In general, though, I refer to obligate and facultative symbioses in terms of the requirement of the host. Obligate symbioses demonstrate an evolutionary commitment (or enslavement) to a symbiotic lifestyle [199]. In some associations, obligate symbioses display strict partner fidelity, whereas in others different lineages fulfill the role of host or symbiont [198]. In these cases, studying the outcome of genotype $\times$ genotype host-symbiont interaction can provide intriguing insight into the effects of symbionts on ecologically relevant host traits in many cases, potentially leading to ecologically important outcomes [198, 202, 204, 205].

Why would symbioses affect food webs?

Food webs fundamentally represent the flow of energy flow through ecosystems, and symbioses are typically underpinned by exchange of energy, often in the form of otherwise limiting resources. In this respect many symbioses influence energy flux in their respective ecosystems, and have long been known to be of key ecological importance [188]. Well-known examples include associations between photosynthetic autotrophs and the heterotrophs that house them, as are observed in lichens, coral, and anemones; fungi and nitrogen fixing bacteria that associate with plant roots; and obligate nutritional symbioses of diverse insects, among many others [76, 116, 188, 202, 206]. At an evolutionary level, the acquisition of novel metabolic function by host cells in the form of plastids and mitochondria represents the extreme end

of integration in this exchange of goods, and has been essential to the evolution of complex life [207].

From an ecological perspective, the question is not therefore if symbioses are important, but rather how they are important, and if there is sufficient dynamism in host-symbiont associations to influence our prevailing understanding of ecological dynamics. For instance, it might be argued that obligate symbioses are subsumed in a host phenotype that behaves as a single ecological unit. Indeed, this is how they are nearly always represented: by omission. I argue that this simplification obscures the substantial dynamism of many host-symbiont relationships, including in affecting key host traits that can influence food web topology and function. Indeed, of the multitude of insect food webs studied, probably all are heavily influenced by the complement of symbioses in insects as demonstrated for aphids [208]. Resolving if - and how - to meaningfully incorporate these diverse effects into existing food web architectures remains a major challenge.

Defining food webs and other ecological networks

Traditional food webs document predator-prey relationships: who eats whom [179]. More recently, food web approaches have been expanded to include other consumer-resource relationships such as those of parasites with their hosts [209, 210]. More broadly, different network types have been used to represent other interactions such as those of plant and pollinator [192], and in some cases, host and symbiont [211].

Food webs are typically represented by a square S × S matrix, with S species under consideration which are represented as nodes in food web graphs [179]. Cells of the matrix can represent the presence or absence of predation by S_i on S_j, or alternatively, in better resolved webs, the relative strength ('weight') of such an interaction, often in form of biomass transfer (Figure 5.1) [179]. These, in turn, are the links, or edges, of the food web graph. This representation is known as an adjacency matrix in graph theory, with nodes connected by an edge being 'adjacent' [179, 212].

Within this framework, webs may be further divided into sub-webs, with axes ordered according to the type of consumer strategy (eg., predators vs. micropredators vs. parasites) [209] that better distinguish interaction types. From such matrices many summary parameters are often computed to represent topology. Common parameters include species richness (S), the number of links (populated cells) in the web (L), the directed connectance (the proportion of possible links realized (L/S^2),

	A	B	C	D	E
A	0	1	1	0	0
B	0	0	0	1	0
C	0	0	0	1	1
D	0	0	0	0	0
E	0	0	0	0	0

	1	2	3
A	1	0	0
B	1	1	0
C	1	1	1
D	0	0	1

Figure 5.1: Conventional adjacency matrix (A) and bipartite network (B), such as are commonly used to represent food webs and plant-pollinator networks. Note that the links in the food web are directed, with arrows representing direction of energy flow.

the link density (L/S), and many others (see [179, 213] for review). Recognizing that some of the possible links (S^2) are actually biologically impossible - aka forbidden links - has in some cases required substantial revision of our understanding of food webs, as the denominator for connectance and related calculations can radically change [209]. At any rate, a majority of food web studies compare food web structure using these and related topological descriptors, and a major early but ongoing research focus has been on understanding the relationship of these parameters with food web dynamics - such as the tendency toward or away from stability, in its many definitions [176, 178, 179, 214].

Theoretical food web studies studies commonly take other approaches. One is to study small portions of webs in which simple species assemblages - typically 2 or 3 species or levels (often called modules or motifs) - can be tractably modelled with a series of differential equations (e.g. [189, 215]). Such approaches typically employ variations of bioenergetic models pioneered by Yodzis and Innes [215], and test the

influence of specific perturbations or modifications to modules on system dynamics. Alternatively, simulations may be employed to examine more complex networks, often using a model of network assembly to generate a network based on given starting parameters (e.g. the niche model; [216]); this allows iterative assembly and perturbations for hypothesis testing (e.g., 'bootstrapping'). There is a vast literature on food webs that is well reviewed elsewhere [178, 179, 217], and it is not my intent to review it here. Instead, I consider in what ways our understanding of food web structure and function can be improved by considering symbioses, given the history and limitations of the above-described approaches.

It is necessary to mention that mutualistic interactions - such as plant-pollinator [218] networks as well as, by my definition, some symbiotic plant-mycorrhizae and coral-zooxanthellae networks - have been extensively investigated using bipartite network approaches [192, 211, 219]. These approaches have principally been applied to plant-pollinator mutualisms [192], where pollinators interact with a subset of plants, and direct plant-plant or pollinator-pollinator, as well as exploitative (consumer-resource) interactions are disallowed. While suggesting intriguing properties of such networks [192], these analyses are removed from the broader ecological arena of predation and competition in which all these interactions must be embedded [74]. Integrating symbiotic and other mutualistic interactions into a framework that recognizes both mutualistic and antagonistic interactions is clearly necessary for a holisitc understanding of real ecosystems [185]. To this end, recent studies have begun to explore hybrid webs or networked networks to merge interaction types, and this may be a promising approach [74, 75, 175, 182, 220] - although it is so far rarely used.

One curious outcome of divergent approaches to modelling exploitative and mutualistic relationships arises when considering parasites. It is now well understood that symbionts lie on a continuum with parasites, and in many cases are parasites under certain conditions (i.e., have a net fitness cost [78,221]), but they have received a completely different focus in network studies. Symbionts are nearly always excluded from food webs, while parasites have achieved a status as legitimate trophic actors, with status equal to or even exceeding predators in multiple studies now [184,209,210,222]. How did this happen? First, though many symbioses represent trophic links between species, they are jointly positive. In simple food web models inclusion of mutualisms generates a positive and destabilizing feedback loop that violates conservation of mass within the system [7], and general solutions to this are not well developed or widely implemented [220]. Second, symbioses are often cryptic; it is only with

the advent of sophisticated molecular techniques that we are gaining an appreciation of their importance across systems, and their historic exclusion from food webs is understandable. Last, many symbioses can function in food webs indirectly influencing other consumer-resource relationships. Generally, indirect effects such as these are now believed to be essential to the persistence and function of real food webs [187, 223], but theory to allow their inclusion in empirical and more complex model food webs has not kept pace [217]. Because of these considerations, producing unified network models that include all types of species interactions may laudable goal, for now I focus on specific ways in which symbioses have been demonstrated to impact important food web properties.

Symbiosis allows new trophic links

The organization of food web links determines many putative emergent properties within an ecosystem. For example, a common approach in both food web and plant-pollinator networks is to systematically delete nodes and their associated links from empirical webs to simulate extinctions [179,224]. When all the links joining a remaining node are gone, that species suffers a secondary extinction, simplistically indicative of the 'robustness' of the system. This approach clearly relies on deep sampling of the community, as unrecognized links will invalidate the analysis, and also demonstrates that the pattern of links has deep importance for predictions we make from food webs. Other aspects of conductance are also important. For instance the degree distribution (P_i that a node has degree (number of links) i) is particularly important to the dynamical properties of networks: this best intuited when considering epidemiological networks where the frequency of highly connected nodes (potential superspreaders) can critically determine outbreak dynamics [225], but also applies to food webs [180].

The ability of one organism to consume another depends on a complex set of traits that differ between interactions and ecosystems (e.g. [226]), but many well-studied trophic interactions rely on symbiosis. In the case of insects, numerous nutritional endosymbioses have allowed the exploitation of nutrient-poor resources, such as plant sap, and enabled the existence and radiation of many thousands of sap-feeding insect species and their associated parasites and predators [199]. Similarly, many blood-feeding insects also rely on nutritional symbioses, including bed-bugs [197] and tse-tse flies, [227] as well as non-insect parasites that include filarial nematodes and leeches [228,229]. Wood-feeders like termites also rely on protist gut symbionts, which

themselves require bacterial symbionts, to digest cellulose in their diet [230]. While these nutritional symbioses are often more-or-less fixed, one consequence is that host success relies on the symbiont such that suboptimal conditions can be indirectly fatal to the host, as seen in catastrophic coral bleaching events [231]. More generally, other animals are also rely on their microbiota. Recently *Daphnia magna*, keystone grazers in aquatic systems, were found to obligately rely on the presence of bacteria [232].

Experimental tests of the ecological effects of microbe free conditions are essentially impossible under realistic conditions, although evidence ranging from the outcome of fecal transplant experiments in mice [233] to metagenomic experiments of human gut communities [234] demonstrates that a substantial portion of host metabolism is attributable to a microbiome that is highly malleable. It is clear that many trophic interactions observed in food web studies would not exist but for symbiosis, raising the possibility that these symbioses dictate patterns of trophic links.

In this regard symbioses can demonstrably dictate the prey that can be exploited by different individuals, even within the same species. This is best demonstrated in aphids, where host plant use can be constrained by the presence of particular symbionts [235,236]. Phylogenetic studies suggest that the expansion of aphid lineages into novel niches is also coincident with the acquisition of specific symbionts [237]. Acquiring a symbiont can thus create a new links in the food web beyond the symbiosis itself, and contribute to variation within species for these links. Such effects would increase connectance and possibly the shape of the degree distribution with symbiont-affected species having greater degree. Again the magnitude of these effects depends on how common this may be - a question awaiting study.

Symbiosis weakens existing trophic links

In addition to enabling the exploitation of novel or otherwise suboptimal resources, symbioses can decrease the strength of existing trophic links or eliminate them altogether. This is clearly evident from the rapidly accumulating literature on defensive symbioses that protect their hosts from exploitation - symbiotic associations that protect the host from natural enemies [33]. These, by definition, distort existing trophic links, either weakening them or severing them altogether. In the few direct experiments conducted these drove a consumer extinct in a tritrophic microcosm experiment [3], and fundamentally altered many of the community-level properties of parasitoid-aphid communities feeding on plants defended by fungal endophytes [238].

Many other examples of defense exist. Certain ants, for example have long been understood to serve defensive roles, protecting acacia trees and other tropical plants from herbivory, with speculated attendant effects on food web structure [239] (On a related note the successful invasion of *Acacia* spp. into the South African fynbos has been ascribed to their symbioses with nitrogen-fixing bacteria [240]). Recently, more cryptic examples of defense have been uncovered. Bacterial associates of marine organisms that include bryozoans, crustaceans, and corals, among others, produce potent toxins or antibiotics that appear to play defensive roles [31, 33, 241, 242]. Multiple insect endosymbionts also produce toxins that might target parasites or predators [61, 66, 123]. Insect symbionts might also directly affect host defense and decrease transmission by increasing crypsis - as has been argued for symbioses as diverse as *Rickettsiella* symbionts of aphids [243] to *Vibrio* bacteria in squid [32].

In general, while food webs now increasingly incorporate macroparasites [209, 210], few include pathogens (e.g. microparasites), which are difficult to detect and quantify. Defensive symbioses that interfere with pathogens would thus seem to be operating at too fine a resolution for most food web approaches to date. There is, however, a dramatic increase in the perceived importance of disease processes in ecosystems [8]. The ecological effects of protective symbioses if they interfere with highly virulent and otherwise catastrophic pathogens - as has been proposed in the case of amphibian chytridiomycosis [244] - might be potentially very large. Recently, network approaches have been employed alongside high-throughput DNA sequencing to simultaneously resolve the dynamics of host, predator, and pathogen in certain food web compartments [245]. It is reasonable to expect such approaches to be applied to more conventional food webs in the near future, as molecular techniques become more affordable and more sophisticated analyses are developed. This might help to clarify the contributions of symbioses that protect against pathogens, as well as the predators, parasites, and parasitoids that are now increasingly included in networks.

A consequence of symbiosis enabling new food web links or weakening others is that the energy flow through existing links can be decrease, although the consistency of this effect has not been investigated. However, decreased interaction strength in response to symbiosis has been demonstrated empirically in at least one experiment [238]. Theoretical work now suggests food webs characterized by a greater number of links with lower per-capita and longer tailed interaction strength distributions are more stable [246]. Plausible mechanisms thus exist to make symbiosis a generally stabilizing phenomenon, although this prediction requires further investigation.

Symbiosis increases the growth rates of primary producers

The success of primary producers is a key factor in determining ecosystem properties. In terrestrial systems, most primary productivity results from plant growth, which again is largely dependent on well-studied mycorrhizal and rhizobial partners [240]. Intriguingly, marine phytoplankton at the base of oceanic food webs also now appear to associate with diverse bacteria that increase their growth in microcosms [247]. Such studies in non-model systems are only beginning, and it seems likely that the scope of microbial symbioses with primary producers extends well beyond what has been previously recognized.

In many cases, these symbioses are now known to mutable, and responsive to environmental change. For example, the breakdown of nutritional symbioses on ecological timescales can lead to ecosystem collapse, again as seen in coral bleaching [248]. Given the cryptic nature of many symbioses, and the ways in which their benefits now appear largely dependent on the combination of host and symbiont genotype [202, 204], it appears that unobserved host-symbiont dynamics could influence the productivity of diverse ecosystems [240].

At higher trophic levels, symbioses that supplement energy have the potential to disrupt the outcome of species interactions and interspecific competition. Classic Lotka-Volterra competition models demonstrate that the competitor with positive growth at the lowest resource density (R^*) excludes the other [7]. Symbioses that decrease the prey necessary for positive growth can thus reverse the outcome of competitive interactions. This was elegantly demonstrated in experiments in which a *Chlorella* symbiosis reversed the outcome of competition between *Paramecium* species in experimental microcosms [249]. Again, such effects are probably common, but have not yet been studied in any detail.

Symbiosis increases intraspecific variation in ecologically important traits

A major outcome of symbiosis is that there is often dramatic intraspecific variation among hosts that are polymorphic for symbioses [19, 204, 240, 250]. Again, this is particularly well-known in insects, where species and/or populations are composed of individuals with differing symbiont associations that might affect key traits [250–252]. In symbioses involving a single symbiont lineage, this effect will be binary as a result of symbiont presence or absence, and can result in a bimodal trait distribution [19,77].

In other symbioses in which the host can associate with different symbiont genotypes or a consortium of symbionts - such as in the gut - traits might be expected to follow more of a bell curve analogous to a host-encoded trait with a polygenic basis, depending on infection frequencies (see for instance [251]). Although not well tested, the additive effects of symbiont and host-encoded variance in a trait would nonetheless be *a priori* expected to increase the variance in that trait.

The effect of intraspecific variation has recently gained substantial attention in food web and community ecology research, and trait variation is expected to affect species interactions in multiple ways [253, 254]. A recent review highlighted mechanisms through which trait variation can affect species interactions and ecological dynamics - these included through Jensen's inequality [255], phenotypic subsidies, and portfolio effects, among others (see [253] for a review of these terms) - but all of which can demonstrably be affected by symbioses. The intraspecific variance of a trait with a haploid basis (a given symbiont is present or absent) - such as those of hosts typically affected by facultative symbioses - will be maximized at intermediate symbiont frequencies (Figure 5.2): thus symbioses that are not fixed in host populations may be expected to contribute greatly to intraspecifc trait variation.

For symbioses at higher frequencies or at fixation, variation in partner quality [204,256] or the composition of the symbiotic microbiome [233] has been demonstrated to contribute greatly to host phenotype, increasing trait variation.

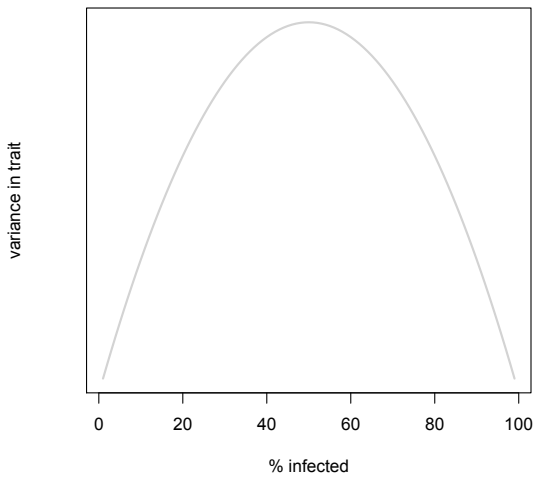

Figure 5.2: Intraspecific variation in a trait conferred solely by a symbiont is maximized at intermediate symbiont infection frequencies.

Symbiosis facilitates rapid evolutionary change

The growing field of eco-evolutionary dynamics seeks to understand evolutionary feedbacks on short timescales, particularly as they relate to food webs and other ecological networks [257], and will need to consider symbioses as dynamic sources of phenotypic change. As discussed, symbiont transmission may be vertical, horizontal, or environmental (see [77,173,198]). While vertical transmission of symbioses provides a component of heritability that has parallels with more conventional inheritance that may selected upon [77], horizontal and environmental transmission can allow for the extremely rapid dissemination of symbiont-encoded traits that benefit the host.

Vertically transmitted symbioses can be considered using population genetics frameworks that consider symbiont frequency analogous to allele frequency, but as a haploid state [77]. Simple models of the deterministic spread of these symbionts demonstrate that they will spread through a population at rates comparable to those of dominant nuclear alleles under equal selective pressures. Vertically transmitted symbionts, however, often suffer loss from imperfect vertical transmission, meaning that even under constant positive selection such symbionts will not fix in populations, maintaining polymorphism for infection and associated traits [77] - which as discussed will be expected to have additional consequences in food webs [253]. Further, the effective population size of vertically transmitted lineages is substantially less than their nuclear analogues, and frequent bottlenecks within their hosts increase the influence of drift and the opportunity for rapid but unpredictable frequency changes, even for beneficial symbionts [77]. Indeed, recent surveys of ecologically important aphid symbionts suggest extremely rapid changes in frequency, which have been argued as responses to selective pressure [251].

While some symbioses are clearly entirely vertically transmitted on ecological timescales, many are partly or entirely horizontally acquired [173, 198]. Relative to an inherited trait, horizontal acquisition of novel symbiont-encoded traits allows extremely rapid response to changing environmental or ecological conditions, accelerating the response of the host and the likelihood of affecting ecological dynamics, analogous in ways to lateral gene transfer in bacteria [258]; a likely example of this is the horizontal acquisition of pesticide resistance in Japanese beanbugs through their symbionts, although the rate of spread of this trait has not been quantified [259]. Furthermore, beneficial traits of symbionts may themselves be encoded by elements that allow and facilitate horizontal transfer - for instance an aphid defensive symbiont

relies on mobile phage-encoded toxins [62] that appear to move horizontally often.

Symbioses are trophic and non-trophic actors in food webs

An unresolved issue in developing conceptual frameworks that consider symbioses (or other mutualisms) is deciding how similar or dissimilar they are to conventional free-living species as nodes in food webs. For instance, Polis and Strong argued for the importance of symbiotic mutualisms in the dynamics of complex communities but also that they do not constitute trophic interactions, presumably because no predation per se occurs [223]. Similar considerations arise when considering parasites in food webs, which are now conventionally assigned their own nodes within the network alongside free-living species [184,210]. In reality many symbionts - and parasites - are never free living, and may only affect one host, straining the convention that they deserve nodes of equal weight to free living species [12]. In fact, while some parasites and pathogens clearly influence ecosystem energetics by directly consuming their hosts [260] - most seem to influence real systems by altering existing trophic interactions [223, 261] or otherwise modulating nutrient fluxes - a notable example being the phage-mediated lysis of bacterioplankton [262] at the base of oceanic food webs [263]. This is not to say that parasites are unimportant, but rather that the balance of their effects might be less direct than implied by their inclusion as independent nodes in food web models. The same is also likely to be true of many symbioses.

In this vein symbioses have been included in simple food web models as 'rheagogies': non-trophic actors that exert their effects indirectly, mediating the properties of other links [189,264]. These approaches have not been widely adopted though, and incorporating the many ways that such effects influence food web dynamics would seem to introduce an intractable level of complexity and contingency to real systems. This same issue arises with other ecologically important traits of organisms that influence food web and community dynamics - so called trait-mediated indirect interactions - and has not yet been adequately resolved [185, 187].

At the same time, many - if not a majority of - symbioses unequivocally rely on the reciprocal exchange of energy between host and symbiont, and should thus be considered trophic interactions. Past food webs have subsumed this joint host-symbiont relationships into food web nodes that constitute a single species, but it is now clear that variation in host-symbiont associations can introduce a degree of variation in these nodes that can affect food webs through diverse mechanisms [238,

265].

Case Study: *Spiroplasma* symbiosis in woodland flies

Spiroplasma is an arthropod associated bacterium that can be parasitic, commensal, or mutualistic in insects [46], including in flies of the genus *Drosophila* [46]. Numerous *Drosophila* species are infected by *Spiroplasma* strains, which tend to be vertically transmitted [46]. These associations have been studied because they can both manipulate host reproduction by killing males (the non-transmitting sex) [37], and provide benefits by protecting hosts against virulent natural enemies that include parasitic nematodes and parasitoid wasps [19, 44].

Drosophila neotestacea is a mushroom feeding fly that is widespread across North America. In the wild, its major exploiter appears to be *Howardula aoronymphium*, a nematode parasite that infects 25% of flies across the continent, though like most *Drosophila*, *D. neotestacea's* predators are not known. *Howardula* infection normally sterilizes flies but a vertically transmitted strain of *Spiroplasma* rescues parasite-induced sterility and decreases the intensity of nematode infection. Several studies have now examined the ecological and evolutionary consequences of this association, allowing insight into the effect of this symbiosis on the local species interaction network.

First, *Spiroplasma* is spreading extremely rapidly through host populations across North America [1, 19]. Simple population genetics models demonstrate high selection coefficients (s) for *Spiroplasma*, on the order of 0.1-0.2 [19]. This is very high, and approximates or exceeds selection coefficients for the spread of insecticide resistance in mosquitoes [266], which are among the highest known and in direct response to human intervention.

It has been argued that the abrupt acquisition of an additional genotype in symbiosis is a source of 'megamutations', allowing for differences in s that greatly exceed those likely to arise by chance mutation of existing genes that on average, have very small s [77]. In any case, this example demonstrates an extremely rapid evolutionary change as a consequence of symbiosis, with evolution occurring on largely ecological timescales with large expected consequences for the community ecology of *Drosophila* and *Howardula* [3]. Additional horizontal components of transmission could make this change even more drastic: conceptual models of the spread of such symbionts suggest that, as the frequency of infection increases in a focal species, increased op-

portunities for horizontal transmission might allow symbionts to jump to additional hosts, in this case conferring resistance [2, 77], and altering the ecological landscape for *Howardula* even more drastically.

Last, this symbiosis dramatically increases the intraspecific variation in the traits of both resistance and tolerance to the parasite. As discussed, this variance can have profound consequences: nematode motherworms are 20% smaller in the presence of *Spiroplasma*(Figure 5.3). Parasite fecundity is typically a non-linear function of size, and in this case *Spiroplasma* may additionally disrupt the parasite [65], but the net result is that *Spiroplasma* decreases the reproductive number of the parasite (R^0) such that it goes rapidly extinct in microcosm experiments [3]. Further, because *Spiroplasma* is not vertically transmitted to 100% of offspring, it will not fix in host populations, maintaining polymorphism for this resistance in *D. neotestacea* to decrease the likelihood of widespread extinction of *Howardula* [1].

Collectively, this case study provides clear examples of symbiont-mediated effects that strongly influence *D. neotestacea* and its principal consumer. Understanding the generality of such effects across diverse symbioses remains a major challenge.

Future directions and conclusions

In many ways ecology has so far escaped a need to reckon with symbiosis, but this is beginning to change. A burgeoning literature on disease ecology now advocates the importance of parasites to the structure and dynamics of ecosystems [8,9,184,267], but a commensurate focus on symbiosis is lacking. Is this simply because symbioses are unimportant, or does it reflect past biases and a focus on predation and competition as the primary drivers of ecosystem structure and function? I have so far argued the latter, and below focus on advancements that will help to resolve these issues.

Recent work has proposed a unified framework for all consumer-resource interactions [194], a goal that could allow the inclusion of diverse consumer strategies in networks under a common general model. So far, this framework has not been expanded to include mutualistic interactions, but the possibility of achieving realistic functional relationships between hosts and symbionts in a trophic context is intriguing. So far, though there has been no overall consensus or consistency in how to build simple models of symbiotic networks outside of widely used bipartite frameworks, precluding easy synthesis, and more general models are clearly needed [74, 175, 189, 220, 268]. Unfortunately even better models do not resolve the importance of symbiont-mediated

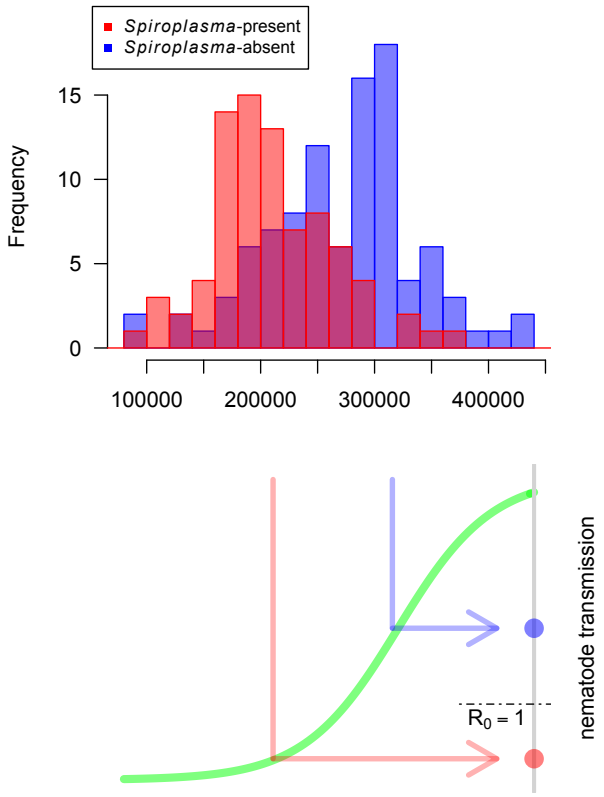

Figure 5.3: *Spiroplasma* infection induces a bimodal distribution in resistance to *Howardula* (top), with variance $\propto$ *Spiroplasma* infection frequency (data collated from [1, 2]). Parasite success is a non-linear function of motherworm size (hypothesized relationship shown). This causes *Howardula* to go rapidly extinct in microcosm experiments ($R_0 < 1$) [3]; because R_0 is a per-capita measure, it will be strongly affected by intermediate *Spiroplasma* frequences through Jensen's inequality.

indirect effects, which appear to be pervasive and important, but the same may be said of the ubiquity of other indirect effects routinely ignored by food web models.

Other avenues, however, might provide promising ways forward. Network complexity is not unique to ecosystems, and confronts researchers in diverse fields. A recent surge of interest in the use of so-called multilayer networks may provide insight into many different systems [212], including ecosystems. In many systems represented by networks, the nodes can in reality interact in a multiplicity of ways. Consider, for example, the many ways that people interact with each other in the course of a day within their social network. In food webs, which are classically described by an S^2 adjacency matrix, all links necessarily represent the same interaction type

(feeding), forcing any non-feeding interactions to be discounted. Multilayer networks allow increased 'dimensions' with additional layers of adjacency matrices, each with the possibility of representing different type of interaction, from which the topological properties of these multilayer networks may be extracted. With respect to ecosystems, adding layers to species interactions that could represent mutualisms and direct non-consumptive antagonism (such as interference competition) might be a critical step to resolving ecologically realistic network structure. Interestingly, such approaches were proposed in broad strokes decades ago for food webs [269], yet have not apparently been attempted. Although ecological studies merging different network types has been explored [74,75,175,182], it has been done outside of this framework. Now, with the explosion of data amenable to network analysis - ranging from epidemiological networks modelling disease spread, to interactions on social media, to gene regulatory networks - this field is rapidly developing, and ecological network studies may stand to gain from the tools developed in other fields [270].

Beyond these conceptual advances, cursory investigations make it quickly apparent that empirical datasets that could populate such models are not easily found, if they exist at all (but see a notable exception; [182]). While such networks could potentially be assembled from merging existing networks, such as exist for some communities, there appear to be few datasets that measure different interaction types synchronously to avoid temporal and spatial heterogeneities that can strongly bias network approaches [186](but see [271] for well-resolved networks that include parasitic interactions). Progress might be made in this respect by focusing on smaller communities or networks where the majority of occurring interaction types can be observed contemporaneously. This is not trivial: to achieve this on a more reductionist level, some recent studies have measured the numerical responses of all community members simultaneously to infer interaction types and strengths through repeated DNA sequencing of the metagenome of planktonic marine systems [245].

Ultimately, a comprehensive understanding of ecological networks will have to confront the complexity of real ecosystems, and include symbioses and other species interactions [182,185,187]. For now, consideration of the many ways in which symbiosis influences important aspects of food webs should encourage caution when interpreting networks of single interaction types, and underscore the importance of symbiosis as an ecological process. Future work can help to resolve which aspects of symbiosis are most important in determining network structure and dynamics; I hope that this synthesis can inform future research that may help to resolve these complexities.

Appendix A

Supplementary Information

Table A.1: Targets, primer sequences, and reaction conditions for PCR and qPCR reactions of Chapter 2

Diagnostic Primers			
name	**Target**	**Primer Sequence**	
P58	*Spiroplasma*	GTTGGTTGAATAATATCTGTTG	
	putative adhesin	GATGGTGCTAAATTATATTGAC	
aor	*Howardula*	TTCGTTTGGAGCTTTCCAAACCTGG	
	cytochrome C	AACACTYGAACCCACATGACCCAA	
tpi	*Drosophila*	CAACTGGAAGATGAAYGGICACC	
	triose phosphate isomerase	TTCTTGGCATAGGCGCACATYTG	
Quantitative PCR Primers			
name	**Target**	**Primer Sequence**	**Efficiency**
lysX	Lysozyme X	CACATAATCCGCAATGAACG	103%
	(FBgn000431)	CTCTGCTGCAGTTGTTCCTG	(58°C)
SpdS	Spermidine synthase	CATTTTCCCAAAGTGGCCTA	87%
	(FBgn0037723)	CTTTTCCACCCAACGAGGTA	(55°C)
Cp1	Cysteine proteinase 1	CCATTGAGCATGACATACCG	104%
	(FBgn0013770)	ATTGATTGGCGTGAAAAAGG	(55°C)
CG14500	c-type lectin X	GTGTGTGCAGGCAATCAACT	99%
	(FBgn0034318)	TCCCGAAGTCCAGACGTATC	(55°C)
dptB	Diptericin-B	GTCCACTAGATGGGGCTTGA	96%
	(FBgn0034407))	GGAGAAGGATTGGGATAGGC	(55°C)
attC	attacin-C	TGGCCAACTGATTCTGTGAG	93%
	(FBgn0041579)	CCGCGGGTAATACACAATCT	(55°C)
Rpl28	60S ribosomal protein	TACCTGGGTCAGATCCTTGC	97%
	L28 (FBgn0035422)	CGTGCCGTTAAGAACACCAC	(60°C)
SpiroRIP	Putative RIP	GGCCGCAAGTTCTTATCAAA	99%
		TGGCCCTACCACCACAT	(55°C)
dnaA	*Spiroplasma* dnaA	TTAAGAGCAGTTTCAAAATCGGG	90%
		TGAAAAAAACAAACAAATTGTTATTACTTC	(55°C)

Table A.2: Selected Gene Ontology categories significantly enriched in transcripts responding to *Howardula* and *Spiroplasma* infection. *** $P_{adj} < 0.01$, ** $P_{adj} < 0.05$, * $P_{adj} < 0.1$.

GO term	Ontology	No. of Genes	P-value
up-regulated in response to *Howardula*			
carbohydrate binding (GO:0030246)	MF	14	* * *
extracellular region (GO:0005576)	CC	39	* * *
nutrient reservoir activity (GO:0045735)	MF	4	* * *
diazepam binding (GO:0050809)	MF	4	**
extracellular matrix (GO:0031012)	CC	9	* * *
phosphatidylcholine 1-acylhydrolase activity (GO:0008970)	MF	5	* * *
sphingomyelin catabolic process (GO:0006685)	MF	3	* * *
structural constituent of chitin-based cuticle (GO:0005214)	MF	23	* * *
enzyme inhibitor activity (GO:0004857)	MF	4	**
down-regulated in response to *Howardula*			
chorion (GO:0042600)	CC	13	* * *
chorion containing eggshell formation (GO:0007304)	MF	10	* * *
mitotic sister chromatid segregration (GO:0000070)	BP	5	* * *
positive regulation of translation (GO:0045727)	BP	4	**
tube development (GO:0000982)	MF	2	*
egg activation (GO:0007343)	BP	3	*
down-regulated in response to *Spiroplasma*			
n.a.			
up-regulated in response to *Spiroplasma*			
chorion (GO:0042600)	CC	6	* * *
extracellular matrix structural constituent (GO:0005201)	MF	5	* * *
myosin light chain kinase activity (GO:0004687)	MF	3	* * *
long chain fatty acid transporter activity (GO:0005324)	MF	4	**
***Spiroplasma* × *Howardula* interaction**			
integral to plasma membrane (GO:0005887)	CC	16	* * *
chitin-based cuticle development (GO:0040003)	BP	9	* * *
extracellular region (GO:0005576) (GO:0000070)	CC	24	* * *

Table A.3: Efficiencies of primers used for all assays in Chapter 3. Efficiency and specificity were validated on standard curves of 5 × 10-fold serial dilutions of synthetic DNA (IDT gBlocks) for all primer sets, except for the primer pair for the *Howardula* normalizing primer set, which was tested using a dilution series of cDNA reverse transcribed from *Howardula*-infected flies. Bases specific to sites of depurination are in bold, and deliberate mismatches underlined.

Primer Set	Primer Sequences	R^2	Efficiency
	Drosophila		
Intact	CGACAGCATTCCTGCGTAGTAAGA ACAATGCAAATTGCCCCTTA	0.995	106.6%
Depurinated	CGACAGCATTCCTGCGTAGTAAGT ACAATGCAAATTGCCCCTTA	0.997	101.4%
Normalizer	CGACAGCATTCCTGCGTAGTAAGA CAAGGACATTGCCAGGTAGG	0.997	102.7%
	Howardula		
Intact	TGATAGTAATCCTGCTTAGTAAGA CACCGGAGAGCAACGATATT	0.997	98.0%
Depurinated	TGATAGTAATCCTGCTTAGTAAGT CACCGGAGAGCAACGATATT	0.998	105.4%
Normailzer	CAAATGCCTCGTCGGATG GCCAAAGCCTCCCACTTATAC	0.991	92.1%
	Rabbit		
Intact	GGGTTTAGACCGTCGTGAGA AGTGGAACCGCAGGTTCAGA	0.998	79.6%
Depurinated	GGGTTTAGACCGTCGTGAGA TGTGGAACCGCAGGTTCAGA	0.997	78.9%
Normalizer	CGTTGGATTGTTCACCCACT CATACACCAAATGTCTGAACCTG	0.999	96.4%

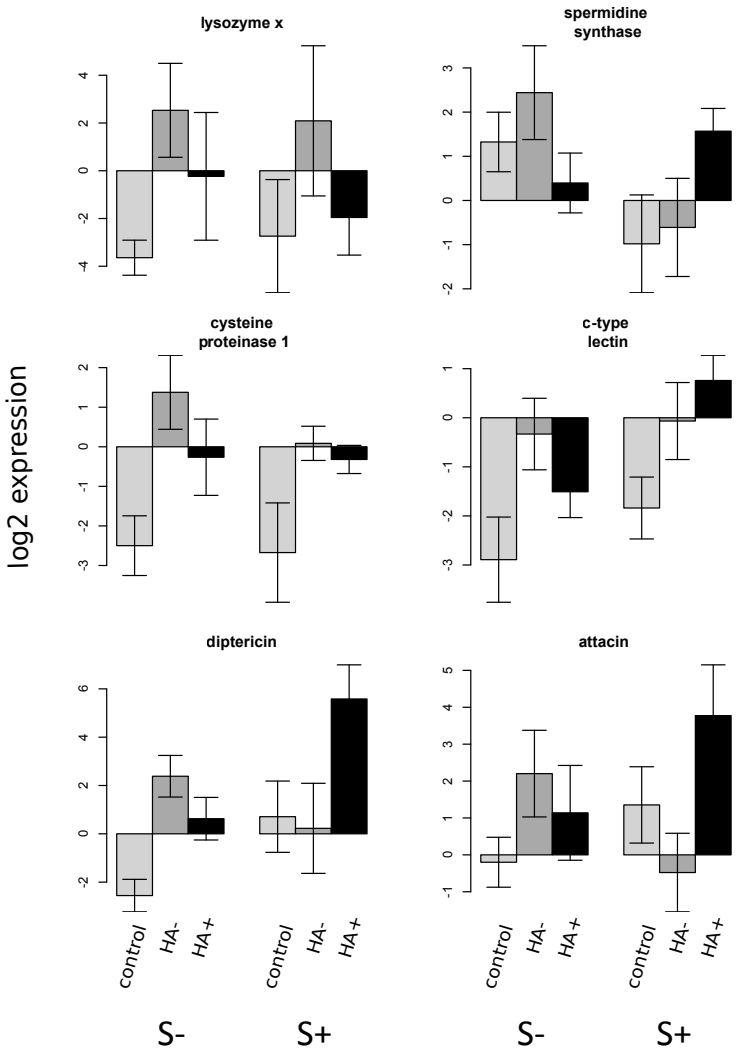

Figure A.1: Results of RT-qPCR of selected *Drosophila neotestacea* genes, in response to *Spiroplasma* infection (S) and *Howardula* exposure and infection (HA). Nematode exposed flies were diagnosed as infected or uninfected using PCR (HA+ and HA-, respectively).

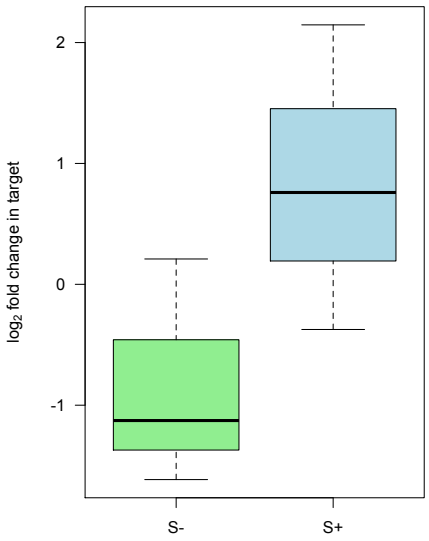

Figure A.2: Ribosomal RNA in *D. neotestacea* ovaries is not strongly depurinated at the site of RIP attack in the presence of *Spiroplasma* (S+) (N = 6; P = 0.14). Abundance of depurinated template normalized to upstream rRNA presented. Ovaries were dissected from 1-2 week old gravid females

.

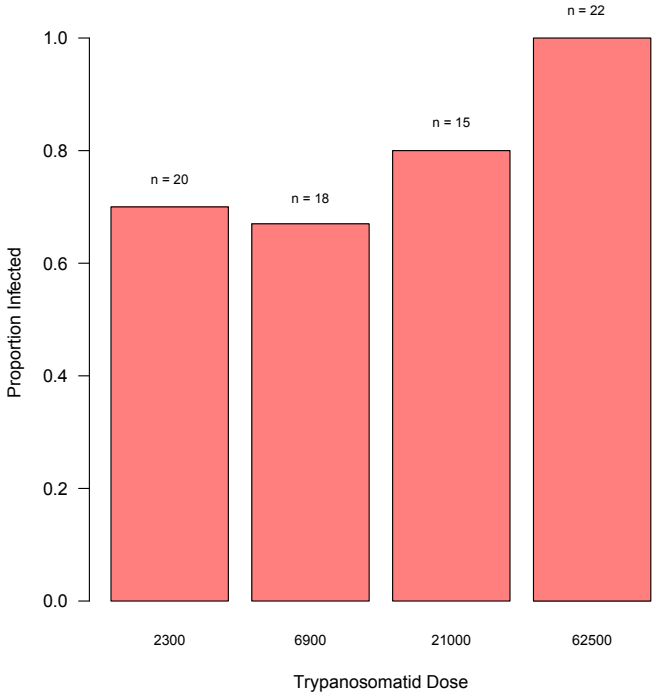

Figure A.3: *Jaenimonas* infects *D. falleni* larvae at high rates following oral exposure to a range of doses. Infection rates measured in larvae by dissection at 5 dpo following exposure at 1 dpo, to *Jaenimonas* diluted to ∼2300 cells per exposure

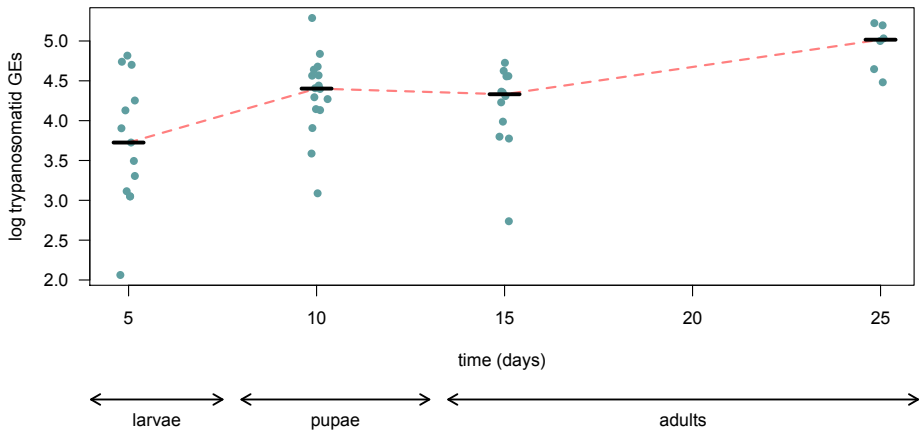

Figure A.4: Intensity of *Jaenimonas* infection in *D. falleni* over time. Larvae were exposed to *Jaenimonas* at 1 dpo and sampled at shown time points using qPCR. Black bars denote median intensity; intensity increases significantly over time ($P < 0.001$).

Bibliography

[1] Cockburn SN, et al. (2013) Dynamics of the continent-wide spread of a *Drosophila* defensive symbiont. *Ecology letters* 16:609–16.

[2] Haselkorn TS, Cockburn SN, Hamilton PT, Perlman SJ, Jaenike J (2013) Infectious adaptation: Potential host range of a defensive endosymbiont in *Drosophila*. *Evolution* 67:934–945.

[3] Jaenike J, Brekke TD (2011) Defensive endosymbionts: a cryptic trophic level in community ecology. *Ecology letters* 14:150–5.

[4] Anagnostakis S (1987) Chestnut blight: the classical problem of an introduced pathogen. *Mycologia* 79:23–37.

[5] Sumption K, Flowerdew J (1985) The ecological effects of the decline in rabbits (*Oryctolagus cuniculus* L.) due to myxomatosis. *Mammal Review* 15:151–186.

[6] Dobson AP, Hudson P (1986) Parasites, disease and the structure of ecological communities. *Trends in Ecology & Evolution* 1:11–15.

[7] Mittelbach G (2012) *Community Ecology* (Sinauer Associates).

[8] Daszak P (2000) Emerging infectious diseases of wildlife - threats to tiodiversity and human health. *Science* 287:443–449.

[9] Harvell CD, et al. (2002) Climate warming and disease risk for terrestrial and marine biota. *Science* 296:2158–2162.

[10] De Castro F, Bolker B (2004) Mechanisms of disease-induced extinction. *Ecology Letters* 8:117–126.

[11] Smith KF, Sax DF, Lafferty KD (2006) Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology* 20:1349–1357.

[12] Selakovic S, de Ruiter PC, Heesterbeek H (2014) Infectious disease agents mediate interaction in food webs and ecosystems. *Proceedings of the Royal Society B: Biological Sciences* 281:20132709–20132709.

[13] Johnson PTJ, de Roode JC, Fenton A (2015) Why infectious disease research needs community ecology. *Science* 349:1259504–1259504.

[14] Bull J (1994) Perspective: Virulence. *Evolution* 48:1423–1437.

[15] Petney TN, Andrews RH (1998) Multiparasite communities in animals and humans: frequency, structure and pathogenic significance. *International journal for parasitology* 28:377–93.

[16] Cox FE (2001) Concomitant infections, parasites and immune responses. *Parasitology* 122 Suppl:S23–38.

[17] Barton ES, et al. (2007) Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature* 447:326–9.

[18] Telfer S, et al. (2010) Species interactions in a parasite community drive infection risk in a wildlife population. *Science* 330:243–246.

[19] Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ (2010) Adaptation via Symbiosis: Recent Spread of a *Drosophila* Defensive Symbiont. *Science* 329:212–215.

[20] Lawn SD, Bekker LG, Middelkoop K, Myer L, Wood R (2006) Impact of HIV infection on the epidemiology of tuberculosis in a peri-urban community in South Africa: The need for age-specific interventions. *Clinical Infectious Diseases* 42:1040–1047.

[21] Blackwell AD, Martin M, Kaplan H, Gurven M (2013) Antagonism between two intestinal parasites in humans: the importance of co-infection for infection risk and recovery dynamics. *Proceedings of the Royal Society B: Biological Sciences* 280:20131671–20131671.

[22] Teixeira L, Ferreira A, Ashburner M (2008) The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS biology* 6:e2.

[23] Roossinck MJ (2011) The good viruses: viral mutualistic symbioses. *Nature Reviews Microbiology* 9:99–108.

[24] Johnson PTJ, Hoverman JT (2012) Parasite diversity and coinfection determine pathogen infection success and host fitness. *Proceedings of the National Academy of Sciences of the United States of America* 109:9006–11.

[25] Alizon S, de Roode JC, Michalakis Y (2013) Multiple infections and the evolution of virulence. *Ecology Letters* 16:556–567.

[26] Frank SA (1996) Models of parasite virulence. *The Quarterly Review of Biology* 71:37.

[27] de Roode JC, et al. (2005) Virulence and competitive ability in genetically diverse malaria infections. *Proceedings of the National Academy of Sciences of the United States of America* 102:7624–8.

[28] Lipsitch M, Siller S, Nowak M (1996) The evolution of virulence in pathogens with vertical and horizontal transmission. *Evolution* 50:245–59.

[29] Hechinger RF, Wood AC, Kuris AM (2011) Social organization in a flatworm: trematode parasites form soldier and reproductive castes. *Proceedings of the Royal Society B: Biological Sciences* 278:656–665.

[30] Clay K (1988) Fungal endophytes of grasses: a defensive mutualisim between plants and fungi. *Ecology* 69:10–16.

[31] Gil-Turnes MS, Hay ME, Fenical W (1989) Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* 246:116–118.

[32] Ruby EG (1996) Lesson from a cooperative bacterial animal association: The *Vibrio fischeri–Euprymna scolopes* light organ symbiosis. *Annual Review of Microbiology* 50:591–624.

[33] Haine ER (2008) Symbiont-mediated protection. *Proceedings of the Royal Society B: Biological Sciences* 275:353–361.

[34] Zug R, Hammerstein P (2012) Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PloS one* 7:e38544.

[35] Russell JA, et al. (2012) A veritable menagerie of heritable bacteria from ants, butterflies, and beyond: broad molecular surveys and a systematic review. *PLoS ONE* 7:e51027.

[36] Baumann P, et al. (1995) Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annual review of microbiology* 49:55–94.

[37] Hurst GDD, Jiggins FM (2000) Male-killing bacteria in insects: Mechanisms, incidence, and implications. *Emerging Infectious Diseases* 6:329–336.

[38] Hunter MS, Perlman SJ, Kelly SE (2003) A bacterial symbiont in the *Bacteroidetes* induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proceedings of the Royal Society B: Biological Sciences* 270:2185–90.

[39] Werren J, Baldo L, Clark M (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology* 6:741–751.

[40] Kellner RLL, Dettner K (1996) Differential efficacy of toxic pederin in deterring potential arthropod predators of *Paederus* (Coleoptera: Staphylinidae) offspring. *Oecologia* 107:293–300.

[41] Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* 100:1803–7.

[42] Scarborough C, Ferrari J, Godfray H (2005) Aphid protected from pathogen by endosymbiont. *Science* 310:1781.

[43] Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) *Wolbachia* and virus protection in insects. *Science* 322:702.

[44] Xie J, Vilchez I, Mateos M (2010) *Spiroplasma* bacteria enhance survival of *Drosophila hydei* ttacked by the parasitic wasp *Leptopilina heterotoma*. *PLoS one* 5:e12149.

[45] Sachs JL, Mueller UG, Wilcox TP, Bull JJ (2004) The Evolution of Cooperation. *The Quarterly Review of Biology* 79:135–160.

[46] Anbutsu H, Fukatsu T (2011) *Spiroplasma* as a model insect endosymbiont. *Environmental microbiology reports* 3:144–53.

[47] Bian G, Xu Y, Lu P, Xie Y, Xi Z (2010) The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS pathogens* 6:e1000833.

[48] Walker T, et al. (2011) The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* 476:450–3.

[49] Hoffmann AA, et al. (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 476:454–7.

[50] Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL (2011) *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS pathogens* 7:e1002043.

[51] Bian G, et al. (2013) *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium infection*. *Science* 340:748–51.

[52] Turelli M, Hoffmann AA (1991) Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature* 353:440–442.

[53] Oliver KM, Smith AH, Russell JA (2013) Defensive symbiosis in the real world - advancing ecological studies of heritable, protective bacteria in aphids and beyond. *Functional Ecology* 28:341–355.

[54] Hamilton PT, Perlman SJ (2013) Host defense via symbiosis in *Drosophila*. *PLoS Pathogens* 9:1–4.

[55] Jaenike J (1992) Mycophagous *Drosophila* and their nematode parasites.

[56] Jaenike J, Perlman SJ (2002) Ecology and evolution of host-parasite associations: mycophagous *Drosophila* and their parasitic nematodes. *The American naturalist* 160 Suppl:S23–39.

[57] Moreira LA, et al. (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell* 139:1268–78.

[58] Pan X, Zhou G, Wu J, Bian G (2012) *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences* 109.

[59] Rancès E, Ye YH, Woolfit M, McGraw Ea, O'Neill SL (2012) The relative importance of innate immune priming in *Wolbachia*-mediated dengue interference. *PLoS pathogens* 8:e1002548.

[60] Caragata EP, et al. (2013) Dietary cholesterol modulates pathogen blocking by *Wolbachia*. *PLoS Pathogens* 9:e1003459.

[61] Piel J (2002) A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. *Proceedings of the National Academy of Sciences of the United States of America* 99:14002–7.

[62] Oliver KM, Degnan PH, Hunter MS, Moran NA (2009) Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* 325:992–4.

[63] Dobson AP (1985) The population dynamics of competition between parasites. *Parasitology* 91:317–47.

[64] Mideo N, Alizon S, Day T (2008) Linking within- and between-host dynamics in the evolutionary epidemiology of infectious diseases. *Trends in Ecology and Evolution* 23:511–517.

[65] Hamilton PT, Leong JS, Koop BF, Perlman SJ (2014) Transcriptional responses in a *Drosophila* defensive symbiosis. *Molecular Ecology* 23:1558–1570.

[66] Nakabachi A, et al. (2013) Defensive bacteriome symbiont with a drastically reduced genome. *Current Biology* pp 1–7.

[67] Stirpe F (2004) Ribosome-inactivating proteins. *Toxicon* 44:371–383.

[68] de Virgilio M, Lombardi A, Caliandro R, Fabbrini MS (2010) Ribosome-inactivating proteins: from plant defense to tumor attack. *Toxins* 2:2699–737.

[69] Melchior WB, Tolleson WH (2010) A functional quantitative polymerase chain reaction assay for ricin, Shiga toxin, and related ribosome-inactivating proteins. *Analytical Biochemistry* 396:204–211.

[70] Pierce M, Kahn JN, Chiou J, Tumer NE (2011) Development of a quantitative RT-PCR assay to examine the kinetics of ribosome depurination by ribosome inactivating proteins using Saccharomyces cerevisiae as a model. *RNA* 17:201–210.

[71] Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. *Annual Review of Immunology* 25:697–743.

[72] Rowton ED, McGhee RB (1978) Population dynamics of *Herpetomonas ampelophilae*, with a note on the systematics of *Herpetomonas* from *Drosophila* spp. *Journal of Protozoology* 25:232–235.

[73] Hamilton PT, et al. (2015) Infection dynamics and immune response in a newly described *Drosophila* - trypanosomatid association. *mBio* 6:1–11.

[74] Fontaine C, et al. (2011) The ecological and evolutionary implications of merging different types of networks. *Ecology Letters* 14:1170–1181.

[75] Sauve AMC, Fontaine C, Thébault E (2014) Structure-stability relationships in networks combining mutualistic and antagonistic interactions. *Oikos* 123:378–384.

[76] Moran NA (2006) Symbiosis. *Current Biology* 16:R866–71.

[77] Jaenike J (2012) Population genetics of beneficial heritable symbionts. *Trends in Ecology & Evolution* 27:226–232.

[78] Oliver KM, Campos J, Moran NA, Hunter MS (2008) Population dynamics of defensive symbionts in aphids. *Proceedings of the Royal Society B: Biological Sciences* 275:293–299.

[79] Brownlie JC, Johnson KN (2009) Symbiont-mediated protection in insect hosts. *Trends in Microbiology* 17:348–354.

[80] Lukasik P, van Asch M, Guo H, Ferrari J, Godfray HCJ (2013) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters* 16:214–8.

[81] Miller R (1967) Pattern and process in competition. *Advances in Ecological Research* pp 1–74.

[82] Holt RD (1977) Predation, apparent competition, and the structure of prey communities. *Theoretical Population Biology* 12:197–229.

[83] Read AF, Taylor LS (2001) The ecology of genetically diverse infections. *Science* 292:1099–1102.

[84] Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* 326:134–6.

[85] Pedersen AB, Fenton A (2007) Emphasizing the ecology in parasite community ecology. *Trends in Ecology and Evolution* 22:133–139.

[86] Riley MA, Wertz JE (2002) Bacteriocins: Evolution, ecology, and application. *Annual Review of Microbiology* 56:117–137.

[87] Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23:1289–91.

[88] Anbutsu H, Fukatsu T (2003) Population Dynamics of Male-Killing and Non-Male-Killing *Spiroplasma*s in *Drosophila melanogaster*. *Applied and Environmental Microbiology* 69:1428–1434.

[89] Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29:e45.

[90] Grabherr MG, et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature biotechnology* 29:644–52.

[91] Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25:3389–402.

[92] Leong JS, et al. (2010) *Salmo salar* and *Esox lucius* full-length cDNA sequences reveal changes in evolutionary pressures on a post-tetraploidization genome. *BMC Genomics* 11:279.

[93] Anders S, Huber W (2010) Differential expression analysis for sequence count data. *Genome Biology* 11:R106.

[94] R Core Team (2014) R: A Language and Environment for Statistical Computing.

[95] Young MD, Wakefield MJ, Smyth GK, Oshlack A (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology* 11:R14.

[96] Westenberger SJ, et al. (2004) Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the spliced leader RNA gene. *Parasitology* 129:537–547.

[97] Nair MG, et al. (2005) Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infection and Immunity* 73:385–94.

[98] Castillo JC, Reynolds SE, Eleftherianos I (2011) Insect immune responses to nematode parasites. *Trends in Parasitology* 27:537–47.

[99] Castillo JC, Shokal U, Eleftherianos I (2013) Immune gene transcription in *Drosophila* adult flies infected by entomopathogenic nematodes and their mutualistic bacteria. *Journal of insect physiology* 59:179–85.

[100] Koch H, Schmid-Hempel P (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences of the United States of America* 108:19288–92.

[101] Hutchence KJ, Fischer B, Paterson S, Hurst GDD (2011) How do insects react to novel inherited symbionts? A microarray analysis of *Drosophila melanogaster* response to the presence of natural and introduced *Spiroplasma*. *Molecular Ecology* 20:950–8.

[102] Anbutsu H, Fukatsu T (2010) Evasion, suppression and tolerance of *Drosophila* innate immunity by a male-killing *Spiroplasma* endosymbiont. *Insect Molecular Biology* 19:481–488.

[103] Herren JK, Lemaitre B (2011) *Spiroplasma* and host immunity: activation of humoral immune responses increases endosymbiont load and susceptibility to certain Gram-negative bacterial pathogens in *Drosophila melanogaster*. *Cellular Microbiology* 13:1385–96.

[104] Hurst GDD, Anbutsu H, Kutsukake M, Fukatsu T (2003) Hidden from the host: *Spiroplasma* bacteria infecting *Drosophila* do not cause an immune response, but are suppressed by ectopic immune activation. *Insect Molecular Biology* 12:93–97.

[105] Wong ZS, Hedges LM, Brownlie JC, Johnson KN (2011) *Wolbachia*-mediated antibacterial protection and immune gene regulation in *Drosophila*. *PLoS ONE* 6:e25430.

[106] Cirimotich CM, Ramirez JL, Dimopoulos G (2011) Native microbiota shape insect vector competence for human pathogens. *Cell Host & Microbe* 10:307–310.

[107] Hyrsl P, et al. (2011) Clotting factors and eicosanoids protect against nematode infections. *Journal of Innate Immunity* 3:65–70.

[108] Ebbert MA, Burkholder JJ, Marlowe JL (2001) Trypanosomatid prevalence and host habitat choice in woodland *Drosophila*. *Journal of Invertebrate Pathology* 77:27–32.

[109] Wilfert L, Longdon B, Ferreira AGA, Bayer F, Jiggins FM (2011) Trypanosomatids are common and diverse parasites of *Drosophila*. *Parasitology* 138:858–865.

[110] Maslov DA, Votýpka J, Yurchenko V, Lukeš J (2013) Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed. *Trends in Parasitology* 29:43–52.

[111] Boulanger N, et al. (2001) Immune response of *Drosophila melanogaster* to infection with the flagellate parasite *Crithidia* spp. *Insect Biochemistry and Molecular Biology* 31:129–137.

[112] Johannes L, Römer W (2010) Shiga toxins — from cell biology to biomedical applications. *Nature Reviews Microbiology* 8:105–16.

[113] Degnan PH, Moran NA (2008) Diverse phage-encoded toxins in a protective insect endosymbiont. *Applied and Environmental Microbiology* 74:6782–6791.

[114] van der Wilk F, Dullemans AM, Verbeek M, van den Heuvel JF (1999) Isolation and characterization of APSE-1, a bacteriophage infecting the secondary endosymbiont of *Acyrthosiphon pisum*. *Virology* 262:104–13.

[115] Moran NA (2007) Symbiosis as an adaptive process and source of phenotypic complexity. *Proceedings of the National Academy of Sciences of the United States of America* 104 Suppl:8627–8633.

[116] McFall-Ngai M, et al. (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences* 110:3229–3236.

[117] Kaltenpoth M, Gotter W, Hurzner G, Strohm E (2005) Symbiotic bacteria protect wasp larvae from fungal infection. *Current Biology* 15:475–479.

[118] Jaenike J (1995) Interactions between and their mycophagous nematode *Drosophila* to community parasites: from physiological to community ecology. *Oikos* 72:235–244.

[119] Xie J, Butler S, Sanchez G, Mateos M (2014) Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps. *Heredity* 112:399–408.

[120] Bergan J, Dyve Lingelem AB, Simm R, Skotland T, Sandvig K (2012) Shiga toxins. *Toxicon* 60:1085–1107.

[121] Paredes JC, et al. (2015) Genome sequence of the *Drosophila melanogaster* male-killing *Spiroplasma* strain MSRO endosymbiont. *mBio* 6:1–12.

[122] Iordanov MS, et al. (1997) Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. *Molecular and Cellular Biology* 17:3373–3381.

[123] Kroiss J, et al. (2010) Symbiotic *Streptomycetes* provide antibiotic combination prophylaxis for wasp offspring. *Nature Chemical Biology* 6:261–263.

[124] Harumoto T, Anbutsu H, Fukatsu T (2014) Male-killing *Spiroplasma* induces sex-specific cell death via host apoptotic pathway. *PLoS Pathogens* 10.

[125] Haselkorn TS, Jaenike J (2015) Macroevolutionary persistence of heritable endosymbionts: acquisition, retention, and expression of adaptive phenotypes in *Spiroplasma*. *Molecular Ecology* 24:3752–3765.

[126] Nakayama S, et al. (2015) Can maternally inherited endosymbionts adapt to a novel host? Direct costs of *Spiroplasma* infection, but not vertical transmission efficiency, evolve rapidly after horizontal transfer into *D. melanogaster*. *Heredity* 114:539–543.

[127] Lassmann T, Sonnhammer ELL (2005) Kalign–an accurate and fast multiple sequence alignment algorithm. *BMC Bioinformatics* 6:298.

[128] Price MN, Dehal PS, Arkin AP (2009) Fasttree: Computing large minimum evolution trees with profiles instead of a distance matrix. *Molecular Biology and Evolution* 26:1641–1650.

[129] Tamura K, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28:2731–2739.

[130] Hao Z, et al. (2001) Tsetse immune responses and trypanosome transmission: implications for the development of tsetse-based strategies to reduce trypanosomiasis. *Proceedings of the National Academy of Sciences of the United States of America* 98:12648–12653.

[131] Podlipaev S (2001) The more insect trypanosomatids under study-the more diverse Trypanosomatidae appears. *International Journal for Parasitology* 31:648–652.

[132] Lukeš J, Skalický T, Týč J, Votýpka J, Yurchenko V (2014) Evolution of parasitism in kinetoplastid flagellates. *Molecular and Biochemical Parasitology* 195:115–122.

[133] Chatton E, Alilaire E (1908) Coexistence d'un *Leptomonas* (Herpetomonas) et d'un *Trypanosoma* chez un muscide non vulnerant, *Drosophila confusa* Steger. *CR Societe de Biologie* pp 1004–1006.

[134] Ebbert MA, Marlowe JL, Burkholder JJ (2003) Protozoan and intracellular fungal gut endosymbionts in Drosophila: prevalence and fitness effects of single and dual infections. *Journal of Invertebrate Pathology* 83:37–45.

[135] Wheeler RJ, Gluenz E, Gull K (2013) The limits on trypanosomatid morphological diversity. *PLoS ONE* 8:e79581.

[136] Yurchenko V, et al. (2014) Ultrastructure and molecular phylogeny of four new species of monoxenous trypanosomatids from flies (Diptera: Brachycera) with redefinition of the genus *Wallaceina*. *Folia Parasitologica* 61:97–112.

[137] Votýpka J, et al. (2014) *Kentomonas* gen. n., a new genus of endosymbiont-containing trypanosomatids of Strigomonadinae subfam. n. *Protist* 165:825–838.

[138] Yurchenko VY, Lukeš J, Jirků M, Maslov DA (2009) Selective recovery of the cultivation-prone components from mixed trypanosomatid infections: A case of several novel species isolated from Neotropical Heteroptera. *International Journal of Systematic and Evolutionary Microbiology* 59:893–909.

[139] Chandler JA, James PM (2013) Discovery of trypanosomatid parasites in globally distributed *Drosophila* species. *PloS one* 8:e61937.

[140] Yurchenko V, Lukes J, Xu X, Maslov DA (2006) An integrated morphological and molecular approach to a new species description in the trypanosomatidae: The case of *Leptomonas podlipaevi* n. sp., a parasite of *Boisea rubrolineata* (Hemiptera: Rhopalidae). *The Journal of Eukaryotic Microbiology* 53:103–111.

[141] Maslov DA, Westenberger SJ, Xu X, Campbell DA, Sturm NR (2007) Discovery and barcoding by analysis of spliced leader RNA gene sequences of new isolates of trypanosomatidae from Heteroptera in Costa Rica and Ecuador. *Journal of Eukaryotic Microbiology* 54:57–65.

[142] Brener Z (1973) Biology of *Trypanosoma cruzi*. *Annual Review of Microbiology* 27:347–382.

[143] Takashima S, Younossi-Hartenstein A, Ortiz PA, Hartenstein V (2011) A novel tissue in an established model system: the *Drosophila* pupal midgut. *Development Genes and Evolution* 221:69–81.

[144] Markow TA, O'Grady P (2008) Reproductive ecology of *Drosophila*. *Functional Ecology* 22:747–759.

[145] Trapnell C, et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols* 7:562–78.

[146] Huang DW, Sherman BT, Lempicki Ra (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* 4:44–57.

[147] Wertheim B, et al. (2005) Genome-wide gene expression in response to parasitoid attack in *Drosophila*. *Genome biology* 6:R94.

[148] Salazar-Jaramillo L, et al. (2014) Evolution of a cellular immune response in *Drosophila*: A phenotypic and genomic comparative analysis. *Genome Biology and Evolution* 6:273–289.

[149] Karlsson C, et al. (2004) Proteomic analysis of the *Drosophila* larval hemolymph clot. *Journal of Biological Chemistry* 279:52033–52041.

[150] Binggeli O, Neyen C, Poidevin M, Lemaitre B (2014) Prophenoloxidase activation Is required for survival to microbial infections in *Drosophila*. *PLoS Pathogens* 10.

[151] Buchon N, Broderick Na, Poidevin M, Pradervand S, Lemaitre B (2009) *Drosophila* intestinal response to bacterial infection: Activation of host defense and stem cell proliferation. *Cell Host and Microbe* 5:200–211.

[152] Hedengren M, et al. (1999) Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Molecular Cell* 4:827–837.

[153] Dostálová A, Volf P (2012) *Leishmania* development in sand flies: parasite-vector interactions overview. *Parasites & vectors* 5:276.

[154] Kuraishi T, Binggeli O, Opota O, Buchon N, Lemaitre B (2011) Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* 108:15966–15971.

[155] Broderick N, Lemaitre B (2012) Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes* pp 307–321.

[156] Thomson C, Clark TB, Kellen WR, Lindegren JE, Smith TA (1964) The transmission of *Crithidia fasciculata* Leger 1902 in *Culiseta incidens* (Thomson). *Journal of Protozoology* 11:400–402.

[157] Corwin RM (1960) A study of trypanosomatidae in *Drosophila*. *Masters thesis, University of Georgia*.

[158] Janssen A (1989) Optimal host selection by Drosophila parasitoids in the field. *Functional Ecology* 3:469–479.

[159] Brown MJF, Loosli R, Schmid-Hempel P (2000) Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos* 91:421–427.

[160] Hu C, Aksoy S (2006) Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. *Molecular microbiology* 60:1194–204.

[161] Weiss BL, Wang J, Maltz Ma, Wu Y, Aksoy S (2013) Trypanosome infection establishment in the tsetse fly gut is influenced by microbiome-regulated host immune barriers. *PLoS Pathogens* 9.

[162] Weiss B, Aksoy S (2011) Microbiome influences on insect host vector competence. *Trends in Parasitology* 27:514–522.

[163] Jirků M, Yurchenko VY, Lukeš J, Maslov DA (2012) New species of insect trypanosomatids from Costa Rica and the proposal for a new subfamily within the Trypanosomatidae. *Journal of Eukaryotic Microbiology* 59:537–547.

[164] Maslov D, Lukes J, Jirku M, Simpson L (1996) Phylogeny of trypanosomes as inferred from the small and large subunits rRNAs: implications for the evolution of paratsitism in the trypanosomatid protozoa. *Molecular and Biochemical Parasitolgy* 75:197–205.

[165] Carruthers VB, Cross GA (1992) High-efficiency clonal growth of bloodstream- and insect-form *Trypanosoma brucei* on agarose plates. *Proceedings of the National Academy of Sciences of the United States of America* 89:8818–8821.

[166] Kohl L, Sherwin T, Gull K (1999) Assembly of the paraflagellar rod and the flagellum attachment zone complex during the *Trypanosoma brucei* cell cycle. *The Journal of eukaryotic microbiology* 46:105–109.

[167] Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41:95–98.

[168] Ronquist F, et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61:539–542.

[169] Guindon S, et al. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology* 59:307–321.

[170] Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* 9:772–772.

[171] Bates D, Maechler M, Bolker B, Walker S (2014) {lme4}: Linear mixed-effects models using Eigen and S4.

[172] Jaenike J, Stahlhut JK, Boelio LM, Unckless RL (2010) Association between *Wolbachia* and *Spiroplasma* within *Drosophila neotestacea*: an emerging symbiotic mutualism? *Molecular Ecology* 19:414–25.

[173] Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics* 42:165–190.

[174] Richardson DM, Allsopp N, D'Antonio CM, Milton SJ, Rejmánek M (2000) Plant invasions–the role of mutualisms. *Biological reviews of the Cambridge Philosophical Society* 75:65–93.

[175] Mougi A, Kondoh M (2012) Diversity of interaction types and ecological community stability. *Science* 337:349–351.

[176] May RM (1972) Will a large complex system be stable? *Nature* 238:413–414.

[177] Paine RT (1980) Food Webs: linkage, interaction strength and community infrastructure. *The Journal of Animal Ecology* 49:666.

[178] Mccann KS (2000) The diversity–stability debate. *Nature* 405:228–233.

[179] Dunne JA (2006) The network structure of food webs. *Ecological networks: linking structure to dynamics in food webs* pp 27–86.

[180] Dunne JA, Williams RJ, Martinez ND (2002) Network structure and biodiversity loss in food webs: Robustness increases with connectance. *Ecology Letters* 5:558–567.

[181] Romanuk TN, et al. (2009) Predicting invasion success in complex ecological networks. *Philosophical Transactions of the Royal Society B: Biological Sciences* 364:1743–1754.

[182] Pocock MJO, Evans DM, Memmott J (2012) The robustness and restoration of a network of ecological networks. *Science* 335:973–977.

[183] Thompson RM, Hemberg M, Starzomski BM, Shurin JB (2007) Trophic levels and trophic tangles: The prevalence of omnivory in real food webs. *Ecology* 88:612–617.

[184] Lafferty KD, et al. (2008) Parasites in food webs: the ultimate missing links. *Ecology Letters* 11:533–546.

[185] Kéfi S, et al. (2012) More than a meal... integrating non-feeding interactions into food webs. *Ecology Letters* 15:291–300.

[186] Blüthgen N (2010) Why network analysis is often disconnected from community ecology: A critique and an ecologist's guide. *Basic and Applied Ecology* 11:185–195.

[187] Werner EE, Peacor SD (2003) A review of trait-mediated indirect interactions in ecological communities. *Ecology* 84:1083–1100.

[188] Vitousek PM, Walker LR (1989) Biological invasion by *Myrica faya* in Hawai'i : plant demography, nitrogen fixation, ecosystem effects. *Ecological Monographs* 59:247–265.

[189] Arditi R, Michalski J, Hirzel AH (2005) Rheagogies: Modelling non-trophic effects in food webs. *Ecological Complexity* 2:249–258.

[190] Mihaljevic JR (2012) Linking metacommunity theory and symbiont evolutionary ecology. *Trends in Ecology & Evolution* 27:323–329.

[191] Ebert D (2013) The epidemiology and evolution of symbionts with mixed-mode transmission. *Annual Review of Ecology, Evolution, and Systematics* 44:623–643.

[192] Bascompte J, Jordano P (2007) Mutualistic networks: The architecture of biodiversity. *Annual Review of Ecology, Evolution, and Systematics* 38:567–593.

[193] Lafferty KD, Kuris AM (2002) Trophic strategies, animal diversity and body size. *Trends in Ecology & Evolution* 17:507–513.

[194] Lafferty KD, et al. (2015) A general consumer-resource population model. *Science* 349:854–857.

[195] Fraune S, Bosch TCG (2007) Long-term maintenance of species-specific bacterial microbiota in the basal metazoan Hydra. *Proceedings of the National Academy of Sciences of the United States of America* 104:13146–13151.

[196] Engel P, Moran NA (2013) The gut microbiota of insects - diversity in structure and function. *FEMS microbiology reviews*.

[197] Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America* 107:769–74.

[198] Dubilier N, Bergin C, Lott C (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology* 6:725–740.

[199] Bennett GM, Moran NA (2015) Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. *Proceedings of the National Academy of Sciences* 112:10169–10176.

[200] Bull JJ, Molineux IJ, Rice WR (1991) Selection of benevolence in a host parasite system. *Evolution* 45:875–882.

[201] Opstal BEJV, Bordenstein SR (2015) Rethinking heritability of the microbiome. *Science*.

[202] Sanders IR, et al. (1998) Mycorrhzal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* 396:69–72.

[203] Wardle DA, et al. (2004) Ecological linkages between aboveground and belowground biota. *Science* 304:1629–1633.

[204] Sampayo EM, Ridgway T, Bongaerts P, Hoegh-Guldberg O (2008) Bleaching susceptibility and mortality of corals are determined by fine-scale differences in symbiont type. *Proceedings of the National Academy of Sciences of the United States of America* 105:10444–10449.

[205] Moran NA, Yun Y (2015) Experimental replacement of an obligate insect symbiont. *Proceedings of the National Academy of Sciences* 112:2093–2096.

[206] Baker AC (2003) Flexibility and specificity in coral-algal symbiosis: diversity, ecology, and biogeography of Symbiodinium. *Annual Review of Ecology, Evolution, and Systematics* 34:661–689.

[207] Keeling PJ, McCutcheon JP, Doolittle WF (2015) Symbiosis becoming permanent: Survival of the luckiest. *Proceedings of the National Academy of Sciences* 112:10101–10103.

[208] Harmon JP, Moran NA, Ives AR (2009) Species response to environmental change: impacts of food web interactions and evolution. *Science* 323:1347–1350.

[209] Lafferty KD, Dobson AP, Kuris AM (2006) Parasites dominate food web links. *Proceedings of the National Academy of Sciences of the United States of America* 103:11211–11216.

[210] Dunne JA, et al. (2013) Parasites affect food web structure primarily through increased diversity and complexity. *PLoS Biology* 11:e1001579.

[211] Fabina NS, Putnam HM, Franklin EC, Stat M, Gates RD (2013) Symbiotic specificity, association patterns, and function determine community responses to global changes: Defining critical research areas for coral-Symbiodinium symbioses. *Global Change Biology* 19:3306–3316.

[212] Kivelä M, et al. (2014) Multilayer networks. *Journal of Complex Networks* 2:203–271.

[213] Bersier LF, Banašek-Richter C, Cattin MF (2002) Quantitative descriptors of food-web matrices. *Ecology* 83:2394–2407.

[214] Rooney N, McCann KS (2012) Integrating food web diversity, structure and stability. *Trends in Ecology & Evolution* 27:40–46.

[215] Yodzis AP, Innes S (1992) Body size and consumer-resource dynamics. *The American Naturalist* 139:1151–1175.

[216] Williams RJ, Martinez ND (2000) Simple rules yield complex food webs. *Nature* 404:180–183.

[217] Thompson RM, et al. (2012) Food webs : reconciling the structure and function of biodiversity. *Trends in Ecology & Evolution* 27:689–697.

[218] Bascompte J, Jordano P, Melián CJ, Olesen JM (2003) The nested assembly of plant-animal mutualistic networks. *Proceedings of the National Academy of Sciences of the United States of America* 100:9383–9387.

[219] Bahram M, Harend H, Tedersoo L (2014) Network perspectives of ectomycorrhizal associations. *Fungal Ecology* 7:70–77.

[220] Kondoh M, Mougi A (2015) Interaction-type diversity hypothesis and interaction strength: the condition for the positive complexity-stability effect to arise. *Population Ecology* pp 21–27.

[221] Thrall PH, Hochberg ME, Burdon JJ, Bever JD (2007) Coevolution of symbiotic mutualists and parasites in a community context. *Trends in Ecology & Evolution* 22:120–126.

[222] Poulin R (2010) Network analysis shining light on parasite ecology and diversity. *Trends in parasitology* 26:492–8.

[223] Polis GA, Strong DR (1996) Food web complexity and community dynamics. *The American Naturalist* 147:813.

[224] Rezende EL, Lavabre JE, Guimarães PR, Jordano P, Bascompte J (2007) Non-random coextinctions in phylogenetically structured mutualistic networks. *Nature* 448:925–928.

[225] May RM (2006) Network structure and the biology of populations. *Trends in Ecology & Evolution* 21:394–399.

[226] Petchey OL, Beckerman AP, Riede JO, Warren PH (2008) Size, foraging, and food web structure. *Proceedings of the National Academy of Sciences of the United States of America* 105:4191–4196.

[227] Aksoy S (1995) *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. *International Journal of Systematic Bacteriology* 45:848–851.

[228] Bandi C, Anderson TJC, Genchi C, Blaxter ML (1998) Phylogeny of *Wolbachia* in filarial nematodes. *Proceedings of the Royal Society B: Biological Sciences* 265:2407–2413.

[229] Graf J, Kikuchi Y, Rio RVM (2006) Leeches and their microbiota: naturally simple symbiosis models. *Trends in Microbiology* 14:365–371.

[230] Ohkuma M (2008) Symbioses of flagellates and prokaryotes in the gut of lower termites. *Trends in Microbiology* 16:345–352.

[231] Douglas AE (2003) Coral bleaching - How and why? *Marine Pollution Bulletin* 46:385–392.

[232] Sison-Mangus MP, Mushegian AA, Ebert D (2014) Water fleas require microbiota for survival, growth and reproduction. *The ISME Journal* 9:59–67.

[233] Turnbaugh PJ, et al. (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444:1027–31.

[234] David LA, et al. (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505:559–63.

[235] Tsuchida T, Koga R, Fukatsu T (2004) Host plant specialization governed by facultative symbiont. *Science* 303:1989.

[236] Wagner SM, et al. (2015) Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. *Functional Ecology* In Press.

[237] Henry LM, et al. (2013) Horizontally transmitted symbionts and host colonization of ecological niches. *Current Biology* 23:1713–1717.

[238] Omacini M, Chaneton EJ, Ghersa CM, Müller CB (2001) Symbiotic fungal endophytes control insect host-parasite interaction webs. *Nature* 409:78–81.

[239] Heil M, McKey D (2003) Protective ant-plant interactions as model systems in ecology and evolutionary research. *Annual Review of Ecology, Evolution, and Systematics* 34:425–553.

[240] van der Heijden MGA, Bardgett RD, van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11:296–310.

[241] Lopanik N, Lindquist N, Targett N (2004) Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia* 139:131–139.

[242] Ritchie K (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Marine Ecology Progress Series* 322:1–14.

[243] Tsuchida T, et al. (2010) Symbiotic bacterium modifies aphid body color. *Science* 330:1102–1104.

[244] Bletz MC, et al. (2013) Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters* 16:807–820.

[245] Chow CET, Kim DY, Sachdeva R, Caron DA, Fuhrman JA (2014) Top-down controls on bacterial community structure: microbial network analysis of bacteria, T4-like viruses and protists. *The ISME Journal* 8:816–829.

[246] McCann K, Hastings A, Huxel GR (1998) Weak trophic interactions and the balance of nature. *Nature* 395:794–798.

[247] Amin SA, et al. (2015) Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. *Nature* 522:98–101.

[248] Brown BE (1997) Coral bleaching: causes and consequences. *Coral Reefs* 16:S129–S138.

[249] Müller JP, Hauzy C, Hulot FD (2012) Ingredients for protist coexistence: Competition, endosymbiosis and a pinch of biochemical interactions. *Journal of Animal Ecology* 81:222–232.

[250] Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annual Review of Entomology* 55:247–266.

[251] Smith AH, et al. (2015) Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. *Molecular Ecology* 24:1135–1149.

[252] Chiel E, et al. (2007) Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bulletin of Entomological Research* 97:407.

[253] Bolnick DI, et al. (2011) Why intraspecific trait variation matters in community ecology. *Trends in Ecology and Evolution* 26:183–192.

[254] Violle C, et al. (2012) The return of the variance: Intraspecific variability in community ecology. *Trends in Ecology and Evolution* 27:244–252.

[255] Ruel JJ, Ayres MP (1999) Jensen's inequality predicts effects of environmental variation. *Trends in Ecology and Evolution* 14:361–366.

[256] Lesser MP, Stat M, Gates RD (2013) The endosymbiotic dinoflagellates (*Symbiodinium* sp.) of corals are parasites and mutualists. *Coral Reefs* 32:603–611.

[257] Fussmann GF, Loreau M, Abrams PA (2007) Eco-evolutionary dynamics of communities and ecosystems. *Functional Ecology* 21:465–477.

[258] Ochman H, Lawrence JG, Groisman EA (2000) Lateral gene transfer and the nature of bacterial innovation. *Nature* 405:299–304.

[259] Kikuchi Y, et al. (2012) Symbiont-mediated insecticide resistance. *Proceedings of the National Academy of Sciences of the United States of America* 109:8618–22.

[260] Kuris AM, et al. (2008) Ecosystem energetic implications of parasite and free-living biomass in three estuaries. *Nature* 454:515–518.

[261] Hatcher MJ, Dick JTA, Dunn AM (2006) How parasites affect interactions between competitors and predators. *Ecology Letters* 9:1253–1271.

[262] Middelboe M, Riemann L, Steward G, Hansen V, Nybroe O (2003) Virus-induced transfer of organic carbon between marine bacteria in a model community. *Aquatic Microbial Ecology* 33:1–10.

[263] Suttle CA (2005) Viruses in the sea. *Nature* 437:356–361.

[264] Goudard A, Loreau M (2008) Nontrophic interactions, biodiversity, and ecosystem functioning: an interaction web model. *The American naturalist* 171:91–106.

[265] Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proceedings of the Royal Society B: Biological Sciences* 273:603–610.

[266] Barbosa S, Black IV WC, Hastings I (2011) Challenges in estimating insecticide selection pressures from mosquito field data. *PLoS Neglected Tropical Diseases* 5.

[267] McCallum H, Dobson A (1995) Detecting disease and parasite threats to endangered species and ecosystems. *Trends in Ecology & Evolution* 10:190–194.

[268] Allesina S, Tang S (2011) Stability criteria for complex ecosystems. *Nature* 483:12.

[269] Cohen JE, et al. (1993) Improving Food Webs. *Ecology* 74:252.

[270] De Domenico M, Porter MA, Arenas A (2015) MuxViz: a tool for multilayer analysis and visualization of networks. *Journal of Complex Networks* 3:159–176.

[271] Hechinger R, Lafferty K (2011) Food webs including parasites, biomass, body sizes, and life stages for three California/Baja California estuaries. *Ecology* 92:2011.