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Density Dynamics of Endosymbiotic Wolbachia Bacteria in the Drosophila Host

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DENSITY DYNAMICS OF ENDOSYMBIOTIC *WOLBACHIA* BACTERIA IN THE *DROSOPHILA* HOST

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To: Dean Michael R. Heithaus
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This dissertation, written by Zinat Sharmin, and entitled Density Dynamics of Endosymbiotic *Wolbachia* Bacteria in the *Drosophila* Host, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2021

DEDICATION

I dedicate this dissertation to my beloved husband, Dr. Mahmud Pavel, and my beautiful daughter, Arfa Gazi Zaheen

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ABSTRACT OF THE DISSERTATION

DENSITY DYNAMICS OF ENDOSYMBIOTIC *WOLBACHIA* BACTERIA IN THE *DROSOPHILA* HOST

by

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Wolbachia pipientis is one of the most widespread bacterial endosymbionts, infecting mites, crustaceans and filarial nematodes as well as about half of all insect species. These bacteria cause many neglected human diseases that include African river blindness and lymphatic filariasis affecting over 100 million people worldwide. Interestingly, *Wolbachia* also suppress the transmission of viruses such as Dengue, Chikungunya and Zika. In most reported cases, *Wolbachia* antiviral activity is a density-dependent effect, with high *Wolbachia* density conferring the strongest viral suppression. However, little is currently known about how *Wolbachia* load is controlled within the insect host. A small number of studies have suggested that germline *Wolbachia* abundance is responsive to host dietary signaling and other factors. It remains an open question whether the germline mechanisms that affect *Wolbachia* colonization are also shared by the soma.

The work of my dissertation focuses on identifying the cellular and molecular mechanisms that affect the abundance of *Wolbachia* carried within the insect host. To this end, we have optimized a whole body qPCR screening assay to facilitate identification of candidate pathways. Employing this technique, we have demonstrated that host diet affects *Wolbachia* abundance in reproductive tissues, but not whole body

Wolbachia loads. To identify more generalized mechanisms that affect *Wolbachia* load, we tested candidate cellular pathways for their impact on body-wide *Wolbachia* abundance. The analysis revealed 5 candidate host processes as major regulators of *Wolbachia* abundance in *D. melanogaster* and *D. simulans* hosts. Inhibition of the ubiquitin-proteasome pathway decreased body-wide *Wolbachia* loads, whereas, inhibition of the IMD pathway, Calcium signaling, the Ras/mTOR pathway and Wnt signaling increased body-wide loads. Genetic manipulation further confirmed the impact of Ras/mTOR and Wnt pathways on *Wolbachia* load in vivo. Taken together, these findings provide new insight into how bacterial endosymbiont loads are specified by the host.

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CHAPTER I: INTRODUCTION

1.1 Symbiosis

Symbiosis is a dynamic association that plays a vital role in the emergence of biological diversity (Margulis, 2004; Moran, 2006; Paracer & Ahmadjian, 2000). In its simplest form, symbiosis is an intimate long-term association between two distinct biological species, such as the relationships between multicellular organisms and microorganisms. The microbial associates are known as the symbionts (Archibald, John, 2014; Moran, 2006). According to the spatial localization of the symbionts involved, symbiosis can be classified as ecto- or endosymbiosis (Das & Varma, 2009). In ectosymbiosis, the symbiont remains on the exterior of the host's body, such as on the exposed surface of the digestive tract, while in endosymbiosis, the symbiont resides inside the tissues and cells of its host (Das & Varma, 2009). Symbiotic associations greatly affect ecosystem processes such as carbon cycling, biodiversity, animal development, and productivity (Hooper et al., 2005; Wall & Moore, 1999). More than half of all animal phyla, particularly in arthropods which account for about 80% of animal diversity, have long-term intimate symbiotic associations (McFall-Ngai et al., 2013).

The consequences of symbiotic interactions differ depending on their association, described in terms of mutualism, commensalism, or parasitism (Moran, 2006). In mutualistic relationships, both species benefit from each other in terms of nourishment, survival, protection and/or reproduction. Symbiotic associations in which one organism benefits without causing any harm or benefit to the other referred to as commensalistic symbiosis. In parasitism, one species is harmed by the other (Leung & Poulin, 2008; Moran et al., 2008; Oliver et al., 2006). This series of successive changes from mutualism to parasitism cannot be absolute, and the existence of the same symbiotic partner can be either good, neutral, or negative, depending on the

circumstances. For instance, *Salmonella* bacteria have a commensalistic association with poultry, in contrast to their pathogenic relationship with humans (Jajere, 2019; White et al., 1997).

Symbiotic interactions are widespread and have broad significance in biology. Numerous symbiotic associations are facilitated by natural selection (O'Malley, 2015). The "endosymbiont hypothesis" is a well-known example of an interaction that began more than 1.5 billion years ago, with uptake of free-living alpha-proteobacteria by the ancestor eukaryotic cells. The event resulted in the modern-day mitochondria of eukaryotes (Dimijian, 2000; Lang et al., 1999; Margulis, 1981). Subsequent uptake of cyanobacteria culminated in modern-day chloroplasts, and thus eukaryotic benefit from the process of photosynthesis (Margulis, 1981; Raven & Allen, 2003). As science progresses, researchers continue to explore novel endosymbiotic relationships between bacteria and insects.

Bacteria-insect endosymbioses are widely prevalent in nature. Many of the bacterial endosymbionts are vertically transmitted, and generally distinguished as obligate (primary) or facultative (secondary) endosymbionts (Moran et al., 2008). In obligate endosymbiosis, neither partner can live independent of the other. For example, many plant sap-feeding insects harbor bacterial endosymbionts that provide essential nutrients that are lacking in the host diet (Douglas, 1998; Moran et al., 2008). In return, the insect body provides the microbes with a stable, habitable niche (Hansen & Moran, 2011; Mandrioli, 2009). Alternatively, in facultative endosymbiosis, interacting species are not exclusively reliant upon each other to survive or reproduction (Baumann, 2005; Ferrari & Vavre, 2011; Moran et al., 2008). For instance, the algal and cyanobacterial symbionts that comprise lichens can also live independently, classifying their relationship as facultative (Armaleo et al., 2019). A broad array of host effects can be elicited by

facultative symbionts, ranging from mutualistic to parasitic (Bäckhed et al., 2005; Baumann, 2005; Braendle et al., 2003; Hurst & Werren, 2001; Koropatnick et al., 2004; Macdonald & Monteleone, 2005). Despite the growing interest in bacteria-insect relationships, the basic knowledge of how endosymbionts are maintained within host is still lacking. The focal point of my research is to explore the symbiotic interactions between insects and microorganisms, particularly, the endosymbiotic relationship between *Wolbachia* and *Drosophila*.

1.2 The fascinating endosymbiont, *Wolbachia*: a brief background

Wolbachia, a type of gram-negative intracellular bacteria, are among the most widespread and well-studied endosymbionts. *Wolbachia* bacteria were first identified in reproductive tissues of the mosquito *Culex pipiens* by Hertig and Wolbach in 1924. These endosymbionts were initially described as a "*Rickettsia*-like microorganism," because they are most closely related to *Ehrlichia, Anaplasma,* and *Rickettsia* species. Later renamed *Wolbachia pipientis* (Hertig, 1936; Hertig & Wolbach, 1924), "*Wolbachia"* bacteria are now regarded as a distinct genus of alpha-proteobacteria under the order of *Rickettsiales* (Werren, 1997). As per field usage, the term "*Wolbachia"* is used when referring to multiple endosymbionts, whereas "*Wolbachion"* is the singular form of the word.

Wolbachia infection is widespread in nature. Unlike *Rickettsiales* relatives, *Wolbachia* do not infect vertebrates directly but are known to be associated with mites, crustaceans, and a broad range of arthropods (Bourtzis & Miller, 2003; Serbus et al., 2008; Weinert et al., 2015; Zug & Hammerstein, 2012). *Wolbachia* are also carried by many filarial nematodes, including, *Brugia malayi, Brugia timori, Wuchereria bancrofti, Onchocerciasis* volvulus (Punkosdy et al., 2003; Saint André et al., 2002) and certain plant-parasite nematodes, such as, *Pratylenchus* and *Radopholus* (Wasala et al., 2019).

Recent studies have estimated that 52%-60% of terrestrial arthropods carry *Wolbachia* (Sazama et al., 2017; Weinert et al., 2015). In most cases however, the infection frequency within a given species is below 50% (Sazama et al., 2019). These estimates suggest that *Wolbachia* are widespread among insect species, however, not necessarily within the same species (Sazama et al., 2019). An important abiotic factor temperature is often correlated with the variation in infection frequencies. High temperature has been reported to eliminate *Wolbachia* from the host (Jia et al., 2009). Another study demonstrated how climate change leads to a decline in the prevalence of *Wolbachia* (Charlesworth et al., 2019). In wild communities of the butterfly *Zizeeria Maha*, *Wolbachia* densities have been shown to vary seasonally (Sumi et al., 2017).

Wolbachia are genetically diverse, and until recently, the genus *Wolbachia* was taxonomically divided into six supergroups, A through F (Baldo et al., 2006; Casiraghi et al., 2005; Lo et al., 2002). The first and most widely identified supergroups are super groups A and B. These groups correspond to *Wolbachia* that infect arthropods (Breeuwer et al., 1992; Werren et al., 1995a; Werren et al., 1995b). Three major arthropod subphyla under these supergroups are Hexapoda, Chelicerata, and Crustacea (Ros et al., 2009). The endosymbionts infecting filarial nematodes belong to the super groups C and D (Bandi et al., 1998). These supergroups are restricted only to the Nematoda (Ros et al., 2009). Supergroup F *Wolbachia* exhibit less genetic variation compared to the others, and until now, this the only group that is carried by both nematodes and arthropod hosts, involving the phylum Nematoda and arthropod subphyla Hexapoda and Chelicerata (Casiraghi et al., 2005; Dunn & Stabb, 2005; Rasgon & Scott, 2004; Ros et al., 2009; Vaishampayan et al., 2007). Supergroup E is less widespread compared to the other supergroups, and includes endosymbionts carried by the springtail *Folsomia candida* (Vandekerckhove et al., 1999). The diversity

and geographical distribution of *Wolbachia*-infected host species is increasingly expanding. Currently, sequencing data suggests that there are 16 monophyletic lineage groups, with the most recent one being the super group S (Lefoulon et al., 2020). The discovery of *Wolbachia* (or, *Wolbachia*-like organisms) in plant nematodes is largely responsible for the radical expansion of the supergroup range (Brown et al., 2016; Wasala et al., 2019). The basis for assigning species names to these different groups and determining where "*Wolbachia"* stops and other genera begin, is currently under discussion by the experts in the field.

1.3 *Wolbachia* as a way of examining insect-bacterial symbiosis

Wolbachia endosymbiosis serves as an excellent model to study host-microbe interactions at cellular and molecular levels. *Wolbachia* are widespread in nature and span the range of parasitic, mutualistic, and commensalistic association with the host (Min & Benzer, 1997; Serbus et al., 2008; Weeks et al., 2007; Werren et al., 2008; Zug & Hammerstein, 2012). For example, *Wolbachia* are capable of provisioning their host filarial nematodes with riboflavin, ATP, and flavin adenine dinucleotide (Foster et al., 2005; Grote et al., 2017). *Wolbachia* are also known for their important contributions in heme biosynthesis, which place them in an obligate mutualistic relationship with the host (Foster et al., 2005; Ghedin et al., 2007; Slatko et al., 2010). *Wolbachia* within certain dipterans is also necessary to facilitate host reproduction (Dedeine et al., 2005; Starr & Cline, 2002). Conversely, *Wolbachia* serve as reproductive parasites in most of arthropod-*Wolbachia* associations by exploiting host reproduction (Taylor et al., 2005). *Wolbachia* in blue moon butterflies have been reported to skew the sex ratio towards female by killing nearly all the males before they even hatch (Charlat et al., 2009; Dyson & Hurst, 2004; Hopkins, G. H. E., 1926). In variant scenarios, *Wolbachia* have also been shown to cause tissue degeneration, ultimately shortening the host lifespan (Min &

Benzer, 1997). The growing interest in *Wolbachia* is largely as a result of the diversity of their effects on host.

1.4 Advantage of studying *Wolbachia* within *Drosophila* species

One of the most informative model systems for studying *Wolbachia*-insect endosymbiosis is the well-established research model organism, *Drosophila melanogaster*. *Drosophila* species have the advantage of a naturally occurring *Wolbachia* infection, referred to as the "*w*Mel" strain (Yamaguchi & Yoshida, 2018). The species *Drosophila simulans* is naturally infected with the "*w*Ri" strain of *Wolbachia*, which shares the supergroup A designation along with *w*Mel (Wu et al., 2004). *Drosophila melanogaster* (fruit flies) have been used as a powerful genetic model organism since Thomas Hunt Morgan and his collaborators had made pioneering discoveries with them almost 100 years ago. The availability of numerous mutant strains of fruit flies allows for the study of diverse pathways relevant in biological and biomedical research (Alberts et al., 2002; Jennings, 2011; Lodish et al., 2000; Wangler et al., 2015). The advent of fluorescently labeled transgenic fusion proteins (Chalfie et al., 1994; Clyne et al., 2003; Morin et al., 2001), inducible expression system (Barwell et al., 2017; Nicholson et al., 2008; Osterwalder et al., 2001; Roman et al., 2001) and near genome-wide availability of specific RNAi knockouts (Hu et al., 2021; Kuttenkeuler, 2004), have made *Drosophila* genetics very powerful. Using these tools, *Drosophila* research has made dramatic strides in explaining genetics, molecular and cell biology, among other areas (Hales et al., 2015; Jennings, 2011). Thus, all of these established resources can be employed to investigate *Wolbachia*-host interactions at the cellular, tissue and organismal level.

1.5 *Wolbachia* colonization in *Drosophila* host cells/tissues

Wolbachia are broadly distributed in specific somatic tissues in *Drosophila,* as demonstrated by recent studies using PCR and fluorescent cytological techniques (Pietri et al., 2016). In adult *D. melanogaster, Wolbachia* were found to localize in central brain, retina, optic lobe, ganglia, somatic cyst cells, and somatic stem cells (Albertson et al., 2013; Casper-Lindley et al., 2011; Strunov et al., 2013; Toomey et al., 2013; Veneti et al., 2003). In adult *D. simulans,* the presence of *Wolbachia* was documented in head, muscle, midgut, Malpighian tubules, wings, and hemolymph (Dobson et al., 1999; Osborne et al., 2009). The density of *Wolbachia*, however, differed depending on the type of tissue (Bian et al., 2010; Dobson et al., 1999; Moreira et al., 2009).

Wolbachia are very well known for their prevalence in germline cells (Clark & Karr, 2002; McGraw et al., 2002; Sacchi et al., 2010; Veneti et al., 2004). Abundance of *Wolbachia* within the germline is fundamentally important to support maternal transmission of the bacteria through female hosts (Serbus et al., 2008; Werren et al., 2008). During the transmission process, *Wolbachia* carried by maternal germline stem cells are passed on to differentiating daughter cells, called cystoblasts (Fig 1). Each cystoblast divides to create a 16-cell germline cyst that is encapsulated by a layer of somatic cells. These encapsulated germline cells are referred to as egg chambers. The egg chamber ultimately converts into a single egg cell that carries *Wolbachia.* Most *Wolbachia* are distributed symmetrically throughout the embryo. A subset of *Wolbachia* localize to the embryo posterior pole, positioning the bacteria for inclusion into the next generation of germline cells during cellularization (Fig 1) (Hadfield & Axton, 1999; Serbus et al., 2008; Serbus & Sullivan, 2007; Veneti et al., 2004).

Wolbachia segregation within the sperm is not very well understood. During spermatogenesis, the germline stem cells divide asymmetrically to create gonial blast

cells, which eventually give rise to 16 interconnected spermatogonial cells with uneven distribution of *Wolbachia*. The spermatagonia soon develop into spermatocytes carrying proliferating *Wolbachia*. During spermiogenesis, the spermatocytes develop into 64 interconnected spermatids. After spermeiogenesis, *Wolbachia* are carried by many of the resulting spermatids. However, during the individualization process, *Wolbachia* are eliminated from the sperm along with the excess cytoplasmic components in a so-called waste bag structure (Riparbelli et al., 2007; Serbus et al., 2008; Zabalou et al., 2004). The exclusion of *Wolbachia* from sperm prevents paternal transmission of *Wolbachia.*

Thus, *Wolbachia* must ensure their abundance in the maternal germline to facilitate their transmission at the cellular level in embryogenesis. Once *Wolbachia* are successfully taken up into embryonic cells, it is not clear how the bacteria are maintained within the body, nor how their density is regulated. To date, reports informing *Wolbachia* titer control in insects are very limited.

1.6 Significance of *Wolbachia* titer (density) for disease management

Basic understanding of how *Wolbachia* titer is regulated within insects and worms has practical importance for disease suppression strategies. *Wolbachia* are the causative agent of African river blindness (onchocerciasis) (Saint André et al., 2002) and a major factor in induction of lymphatic filariasis (Cross et al., 2001; Punkosdy et al., 2003). More than 150 million people around the world are afflicted by filarial nematodes (Daehnel et al., 2007; Slatko et al., 2014). For instance, lymphatic filariasis are caused by *Brugia malayi* and *Wuchereria bancrofti*, while onchocerciasis is caused by *Onchocerca volvulus*. *Wolbachia* are present throughout the nematode life cycle, and colonization increases during the host adult stage (Fenn & Blaxter, 2004; McGarry et al., 2004; Taylor et al., 1999). As an obligate mutualist, *Wolbachia* are required for various aspects of filarial nematode biology (mentioned earlier), including larval development

and production of offspring. Efficient survival of these filarial nematodes also depends greatly on *Wolbachia* infection. Reduction of *Wolbachia* titer by the antibiotics Rifampicin and Doxycycline have been found to exert fatal impact on nematodes (Hoerauf et al., 2001; Townson et al., 2000). *Wolbachia* as a contributor to the pathogenesis of filariasis makes this bacterium an attractive target for treatment of filarial disease (Johnston & Taylor, 2007; Slatko et al., 2010; Supali et al., 2008).

In 2008, it was found that *Wolbachia*-infected fruit flies were resistant to plus strand RNA viruses, including Flock House Virus and *Drosophila* C Virus (Hedges et al., 2008; Teixeira et al., 2008). Follow-up work quickly revealed this same viral suppression trait carries through to *Wolbachia*-infected *Aedes* mosquitoes, suppressing replication and transmission of plus-strand RNA viruses including Zika, Dengue, and Chikungunya that affect millions of people worldwide every year (Paixão et al., 2018; Xue et al., 2018). Importantly, the blocking of virus is usually stronger in insect hosts carrying higher *Wolbachia* titers (Lu et al., 2012; Moreira et al., 2009). Thus, understanding the underlying mechanisms that naturally regulate *Wolbachia* titer in vivo can potentially contribute to development of practical tools for disease suppression. The major contribution of *Wolbachia* to the progression of filarial disease, as well as, suppression of viral transmission, has given rise to exceptional interest in pursuing host mechanisms that regulate *Wolbachia* titer in vivo.

1.7 How is *Wolbachia* titer maintained inside the host? To what extent the density regulatory mechanisms are known

As of today, very limited information is available regarding the mechanisms of *Wolbachia* colonization within host. Symbiont density can be controlled by means of the host or the symbiont (intrinsic factors). Symbionts may alter their growth or development by altering their replication rate. Hosts can also regulate the growth of symbionts by

limiting nutrients, producing toxins, as well as redirecting the microbes to lysosome inducing their destruction (Scherr et al., 2007). Environmental factors (extrinsic factors) have also been shown to influence *Wolbachia* titer (Bordenstein & Bordenstein, 2011; Hughes et al., 2014; Serbus et al., 2015). Here we discussed the factors reported to date as affecting *Wolbachia* titer within insect hosts.

1.7.1 Extrinsic factors

Temperature: Environmental factors, such as temperature, have a great influence on *Wolbachia* titer. For instance, embryos from both *D. bifasciata* and *D. simulans* have been shown to carry fewer bacteria at elevated temperature than at lower temperature (Clancy & Hoffmann, 1998; Hurst et al., 2000). The wasp *Nasonia vitripennis* has also been reported to have reduced bacterial load at higher temperature compared to cold temperature, as a consequence of increased densities of its endogenous phage (Bordenstein & Bordenstein, 2011). Conversely, in *D. melanogaster*, higher temperature was shown to elevate *Wolbachia* titer in adult brain tissue (Strunov et al., 2013). A further variation on this outcome was reported in a study of *Drosophila sechellia, and Drosophila teissieri*. Male flies had an elevated *Wolbachia* titer at temperature cooler than 23°C and 31°C, whereas females did not exhibit any titer variation with the temperature shift (Hague et al., 2020). Host background might be contributing to this differential effect. These data suggest that there is an ideal middle range for *Wolbachia* replication and/or survival. Any deviation in optimal conditions may alter that range.

Host microbiota: *Wolbachia* titer has been reported to be influenced by the host microbiome. Since *Wolbachia* inhabit a within-host niche, co-infection of multiple bacterial lineages is a possible outcome, which would have resulted in a fight for resources among bacterial groups (Caragata et al., 2014; Geoghegan et al., 2017;

Jiménez et al., 2019). Co-infection with *Spiroplasma,* for example, was shown to reduce *Wolbachia* load in *Drosophila* (Goto et al., 2006). The gut microbiome of *Anopheles* mosquitoes have been shown to hinder vertical transmission of a transinfected *Wolbachia* strain (Hughes et al., 2014). The mechanisms that drive the gut microbiota-mediated impact on *Wolbachia* is unclear, but their continued existence is perhaps one explanation for why certain mosquito populations cannot be stably infected with *Wolbachia* strains (Hughes et al., 2014; Rossi et al., 2015). Alternatively, the presence of *Wolbachia* has shown to reduce the abundance of gut microbe *Acetobacter* in *Drosophila melanogaster* (Simhadri et al., 2017). The mosquito *Aedes aegypti* has also shown a dramatic alteration in resident bacterial abundance when transinfected with *Wolbachia* (Audsley et al., 2018). These findings suggest a complex interaction between the endosymbiont *Wolbachia* and the resident microbiome.

Host diet: Most recently, cytological assessments have stipulated that germline *Wolbachia* titer in *Drosophila* is responsive to host diet. For instance, certain forms of sugars, such as, lactose, maltose, trehalose, and sucrose, increase *Wolbachia* load in developing oocytes (Camacho et al., 2017). On the contrary, exposure of flies to yeast-enriched diet causes a dramatic reduction of bacterial load in the oocytes (Serbus et al., 2015). However, whether the impact of yeast-enriched diet is tissue-dependent or, generalized to the whole body is not known.

1.7.2 Intrinsic factors

Our knowledge on the nature and function of different intrinsic elements in regulating *Wolbachia* colonization is rudimentary. Transinfection-based approaches (transfer of *Wolbachia* into heterologous host) have demonstrated the importance of host genetic background on *Wolbachia* titer (Chafee et al., 2011; Christensen et al., 2016; Poinsot et al., 1998). Host developmental dynamics have also been reported to influence

Wolbachia load. For instance, during oogenesis, *Wolbachia* appears to concentrate at the polar follicle cells, although not in the lateral follicle cells (Kamath et al., 2018). The ovarian somatic stem cell niche also contributes to *Wolbachia* abundance in germline cells (Toomey et al., 2013). To date, the undestanding of *Wolbachia* titer regulations are limited mostly to germlines and cell-culture studies. Here we discussed a few intrinsic host factors that are known to have an impact on *Wolbachia* abundance.

1.7.2.1 Germline based studies

The majority of studies informing the molecular basis for *Wolbachia* titer control to date have been conducted in *Drosophila* maternal germline cells. Mutations in the host germline cells, such as disruption of the *Drosophila gurken* (*grk*) gene, have been shown to significantly reduce *Wolbachia* titer in the oocytes (Serbus et al., 2011). Because *grk* is important to reorganize the oocyte cytoskeleton, the impact of *grk* on *Wolbachia* titer invokes a connection between cytoskeletal proteins and germline *Wolbachia* titer, which has been supported by other studies. Chemical disruption of microtubule stability has been shown to disrupt *Wolbachia* localization as well as titer regulation (Ferree et al., 2005; Russell et al., 2018; Serbus et al., 2011; Serbus & Sullivan, 2007). Disruption of the actin cytoskeleton with mutant actin-binding proteins, chickadee, and quail, also led to reduction of *Wolbachia* titer in fly germaria and early egg chambers, followed ultimately by loss of *Wolbachia* from the mutant lines (Newton et al., 2015).

A separate set of factors central to prototeostasis have also been found to affect germline *Wolbachia* titers. Knockdowns of the *Ubc6* gene, which encodes the E2 conjugating enzyme of the ubiquitin pathway reduced bacterial load in oocytes (White et al., 2017a). Another study carried out by Grobler and colleagues in 2018, however, have reported a different outcome. They showed that RNAi inhibition of proteasome core

components increases *Wolbachia* titer in *Drosophila* germline (Grobler et al., 2018). Because the Grobler study did not assess the impact of ubiquitin-mediated components on germline *Wolbachia* titer, the final interpretation on the discrepancy regarding the impact of ubiquitin-proteasome pathway cannot be made at this time.

Host ribosomal components have also been shown to affect germline *Wolbachia* titer. RNAi mediated disruption of host ribosome complex increased *Wolbachia* titer in the ovary (Grobler et al., 2018). This suggests that reducing protein loads within the cell benefits *Wolbachia* to some extent. *Wolbachia* are thought to rely upon amino acid metabolism as an energy source (Foster et al., 2005; Wu et al., 2004), so one possibility is that *Wolbachia* access these resources more easily when host protein abundance is reduced. Another possibility is that reduced host protein loads also mean lower amounts of factors that are suppressive or toxic to *Wolbachia.* It remains further unclear whether these and other mechanisms that affect germline *Wolbachia* titers represent developmentally specific or generalized impacts.

1.7.2.2 RNAi-based cell culture studies

Host mechanisms that drive *Wolbachia* colonization within somatic tissues are less well known. The very first comprehensive screens conducted by White et al. 2017 and Grobler et al. 2018 have shed light onto host genes that might be relevant for somatic titer regulation. Both studies used RNAi to screen *Wolbachia*-infected somatic tissue culture cells for host pathways implicated in titer regulation. In 2017, White and colleagues demonstrated an association between host metabolic pathways and *Wolbachia* density. The importance of host lipid metabolism and mitochondrial components was implicated in controlling *Wolbachia* titer in cells. Most strikingly, the study demonstrated that RNAi knockdown of ubiquitin related genes from the ERAD ubiquitin ligase (endoplasmic reticulum associated protein degradation pathways),

reduced bacterial load. This suggests that *Wolbachia* require a functioning ERAD pathway to uphold a regular number of bacteria within the cells. The results also more generally support the expectation that *Wolbachia* depend on their host cells for energy and other metabolic factors (White et al., 2017a).

Another high-throughput RNAi screen, carried out by Grobler et al. 2018, showed a wide array of host systems and complexes that affected *Wolbachia* titer in *Drosophila* cells (Grobler et al., 2018). The major finding by Grobler and colleagues was that host ribosomal factors, specifically the translation initiation complex, are essential to *Wolbachia* infections. It was shown that host translation is directly hindered by the presence of *Wolbachia*, which possibly occurs at the post-translational level. They also showed effects for the cytoskeleton components on somatic *Wolbachia* titer that were consistent with prior germline *Wolbachia* studies (Ferree et al., 2005; Serbus et al., 2011; Serbus & Sullivan, 2007), suggesting that somatic and germline *Wolbachia* titer may be regulated similarly. Another finding from the study was that knockdown of proteasomal core components increase titer in cell culture. However, knockdown of several ubiquitin related components was shown to reduce titer consistent with the ubiquitin-proteasome related arguments from White et al. 2017a. Additional somatic titer effects were identified for host factors involved in cell adhesion & extracellular matrix, metabolism & transporters, cell signaling, membrane dynamics and vesicular trafficking (Grobler et al., 2018). The involvement of multiple host factors suggests that a wide range of host processes may affect *Wolbachia* titer. It is also notable that the success of both RNAi screens demonstrates that RNAi effects are visible within the timescale of such knockdowns. This opens the possibility of pursuing future RNAi knockdowns in whole fly systems that fall within the same time range.

1.7.2.3 Biological concerns about quantification of *Wolbachia* titer in cell cultures

As explained above, the knowledge we have so far about somatic titer regulation is mainly extrapolated from tissue culture studies. The limitations with tissue culture studies leaves open whether the observed titer shift in cell cultures is caused by altered bacterial life cycle dynamics, or by a change in invasion dynamics. It has been shown that *Wolbachia* can be horizontally transmitted from infected to uninfected tissue culture cells, without the requirements of direct cell-to-cell contact. *Wolbachia* horizontal transmission is accomplished through a host endocytic mechanism resembling phagocytosis (White et al., 2017b). In tissue culture format, many bacteria are floating on the medium as a result of cell death (egress), which might cause horizontal invasion of the remaining viable cells. Therefore, the basis for *Wolbachia* titer changes in response to certain treatment/RNAi manipulations remains unclear. Because of the possible concerns associated with the interpretations from tissue culture studies, the molecular mechanisms of how hosts regulate the density of their resident endosymbionts overall remain elusive.

1.8 What is known about density regulation of other microbes?

There are hints from other systems that consensus host mechanisms may significantly affect *Wolbachia* colonization of somatic cells. The presence of bacteria, and their density within host cells, has been extensively studied for many disease-related microbes, including *Ehrlichia chaffeensis, Listeria monocytogenes, Chlamydia caviae, Mycobacterium fortuitum* and *Francisella tularensis* (Agaisse et al., 2005; Derré et al., 2007; Levenhagen et al., 2012; Philips et al., 2005; Rikihisa et al., 1994). It has been reported that the restriction of host signaling pathways, such as the MAP kinase signaling pathway, significantly decreases the number of *Anaplasma* and *Coxiella* carried by infected host cells (Czyż et al., 2014; Xiong et al., 2009). The ubiquitin

pathway has been shown to regulate survival and replication of *Ehrlichia* and *Anaplasma* (Severo et al., 2013; Zhu et al., 2017). The cytoskeleton has also been documented by several studies to maintain the density of *Ehrlichia, Listeria monocytogenes, Chlamydia caviae, Mycobacterium fortuitum* (Agaisse et al., 2005; Derré et al., 2007; Levenhagen et al., 2012; Philips et al., 2005; Rikihisa et al., 1994).

Studies of bacterial titer control in other systems are not entirely free of complications. Some host processes, such as mTOR signaling, have been reported to have conflicting effects. An RNAi screen for host genes that affect *Chlamydia* and *Listeria* showed that knockdown of *Tor* from the mTOR signaling suppress the infection (Derré et al., 2007), whereas, another study reported an increase in *Anaplasma* growth upon mTOR inhibition (Niu et al., 2008). A more generalized concern about such studies is that many of those were also conducted in tissue culture. Therefore, the concerns regarding the inability to distinguish replication from invasion apply to these other systems as well. However, since some of the host mechanisms implicated in titer control for bacterial pathogens are consistent with results from germline *Wolbachia* studies, it remains possible that processes affecting colonization in other systems are also applicable to *Wolbachia*. We will use literature as a guide to identify possible density-regulating mechanisms that affect *Wolbachia* loads carried within whole host organisms.

1.9 Objective of this dissertation and organization

Despite growing evidence of host-symbiont interplay, knowledge of how bacterial density is regulated within the host is limited, for nearly all endosymbionts including *Wolbachia*. My overall research goal is to inform the cellular basis of endosymbiont-host interactions, pursuing the *Wolbachia-Drosophila* endosymbiotic system as a model. I am particularly interested in systematic identification of host pathways and processes that affect *Wolbachia* titer at the level of whole host organisms.

To elucidate *Wolbachia* colonization, my Chapter II describes how a whole fly model system was used to search for prospective pathways of endosymbiont density regulation. The study was built upon the as-yet unconventional use of adult insects in a validated, plate-format assays to substantiate results using high statistical power. Here, I addressed the lack of empirical tools to elucidate the biology of titer regulation. We developed a qPCR-based protocol that determines the absolute count of the *Wolbachia* surface protein (*wsp*) gene as a proxy for whole body *Wolbachia* titers. With thorough optimization, we maximized detection of *Wolbachia* within whole host organisms, which was not possible earlier with conventional methods.

Chapter III demonstrates how the optimized protocol can efficiently be used to detect titer changes within the whole organism. Using the optimized protocol, we have illustrated whether germline titer control mechanisms serve as a proxy for body-wide effects. Previous studies have revealed the dramatic effect of yeast-rich diets on germline *Wolbachia* titer (Serbus et al., 2015). Here we show what is required to reliably detect differences by absolute qPCR counts, and test whether the impact of dietary yeast extrapolates to the whole organism. The results indicated that dietary yeast drives *Wolbachia* depletion in oogenesis, but not in whole body samples. The yeast is also an excellent illustration of how relative qPCR can distort outputs. The absolute count method also showed that the non-antibiotic compound bortezomib reduces ovarian, as well as, whole-body titers, indicating that generalized effects are possible.

In Chapter IV, I explored host-endosymbiont interactions by analyzing various host processes that might be relevant to the maintenance of *Wolbachia.* We conducted a candidate drug screen to identify host factors that either support or suppress *Wolbachia* titer within whole organism. To our knowledge, this is the first study to systematically assess titer controlling mechanisms using the whole organism. The outcomes from the

drug screen were further supported by genetic analyses. Results from Chapter IV begin to fill the knowledge gap regarding the influence of hosts on whole body colonization by *Wolbachia* endosymbionts.

This dissertation is intended to contribute to the understanding of *Wolbachia*-host interactions by using validated approaches that are rooted in empirical data. The thesis bridges important holes in understanding *Wolbachia* regulation within the host and offers new insights and methods from which future studies can be designed.

The data/findings derived from chapter IV are combined efforts of myself and my mentee Hani Samarah, who worked very closely with me carrying out the experiments.

Figures

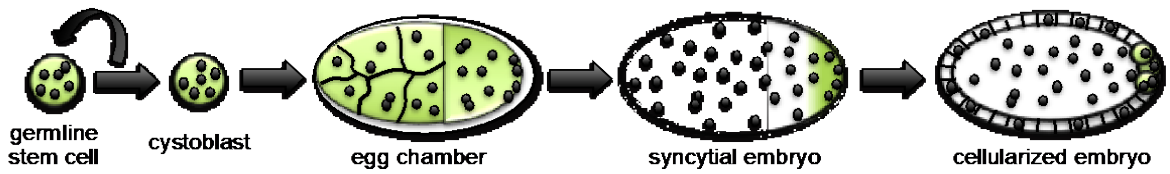

Figure 1: Maternal transmission of *Wolbachia.* Green: germline.
White: soma. Grey dots: *Wolbachia.*

References

Agaisse, H., Burrack, L. S., Philips, J. A., Rubin, E. J., Perrimon, N., & Higgins, D. E. (2005). Genome-wide RNAi screen for host factors required for intracellular bacterial infection. *Science (New York, N.Y.)*, *309*(5738), 1248–1251. https://doi.org/10.1126/science.1116008

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). *Drosophila* and the molecular genetics of pattern formation: Genesis of the body plan. *Molecular Biology of the Cell. 4th Edition*. https://www.ncbi.nlm.nih.gov/books/NBK26906/

Albertson, R., Tan, V., Leads, R. R., Reyes, M., Sullivan, W., & Casper-Lindley, C. (2013). Mapping *Wolbachia* distributions in the adult *Drosophila* brain. *Cellular Microbiology*, *15*(9), 1527–1544. https://doi.org/10.1111/cmi.12136

Archibald, John. (2014). One Plus One Equals One: Symbiosis and the evolution of complex life. https://www.goodreads.com/book/show/19523133-one-plus-one-equals-one

Armaleo, D., Müller, O., Lutzoni, F., Andrésson, Ó. S., Blanc, G., Bode, H. B., Collart, F. R., Dal Grande, F., Dietrich, F., Grigoriev, I. V., Joneson, S., Kuo, A., Larsen, P. E., Logsdon, J. M., Lopez, D., Martin, F., May, S. P., McDonald, T. R., Merchant, S. S., … Xavier, B. B. (2019). The lichen symbiosis re-viewed through the genomes of *Cladonia grayi* and its algal partner *Asterochloris glomerata*. *BMC Genomics*, 20(1), 605. https://doi.org/10.1186/s12864-019-5629-x

Audsley, M. D., Seleznev, A., Joubert, D. A., Woolfit, M., O'Neill, S. L., & McGraw, E. A. (2018). *Wolbachia* infection alters the relative abundance of resident bacteria in adult *Aedes aegypti* mosquitoes, but not larvae. *Molecular Ecology, 27*(1), 297–309. https://doi.org/10.1111/mec.14436

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science (New York, N.Y.),* 307(5717), 1915–1920. https://doi.org/10.1126/science.1104816

Baldo, L., Dunning Hotopp, J. C., Jolley, K. A., Bordenstein, S. R., Biber, S. A., Choudhury, R. R., Hayashi, C., Maiden, M. C. J., Tettelin, H., & Werren, J. H. (2006). Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Applied and Environmental Microbiology, 72(11)*, 7098–7110. https://doi.org/10.1128/AEM.00731-06

Bandi, C., Anderson, T. J., Genchi, C., & Blaxter, M. L. (1998). Phylogeny of *Wolbachia* in filarial nematodes. *Proceedings of the Royal Society B: Biological Sciences, 265*(1413), 2407–2413.

Barwell, T., DeVeale, B., Poirier, L., Zheng, J., Seroude, F., & Seroude, L. (2017). Regulating the UAS/GAL4 system in adult *Drosophila* with Tet-off GAL80 transgenes. *BioRxiv*, 167056. https://doi.org/10.1101/167056

Baumann, P. (2005). Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review of Microbiology, 59*, 155–189. https://doi.org/10.1146/annurev.micro.59.030804.121041

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(4*), e1000833. https://doi.org/10.1371/journal.ppat.1000833

Bordenstein, S. R., & Bordenstein, S. R. (2011). Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia,* and cytoplasmic incompatibility. *PloS One*, *6*(12), e29106. https://doi.org/10.1371/journal.pone.0029106

Bourtzis, K., & Miller, T. A. (2003). *Insect Symbiosis*. CRC Press.

Braendle, C., Miura, T., Bickel, R., Shingleton, A. W., Kambhampati, S., & Stern, D. L. (2003). Developmental origin and evolution of bacteriocytes in the *aphid-Buchnera* symbiosis. *PLoS Biology*, *1*(1), E21. https://doi.org/10.1371/journal.pbio.0000021

Breeuwer, J. A., Stouthamer, R., Barns, S. M., Pelletier, D. A., Weisburg, W. G., & Werren, J. H. (1992). Phylogeny of cytoplasmic incompatibility micro-organisms in the parasitoid wasp genus Nasonia (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. *Insect Molecular Biology*, *1*(1), 25–36. https://doi.org/10.1111/j.1365-2583.1993.tb00074.x

Brown, A. M. V., Wasala, S. K., Howe, D. K., Peetz, A. B., Zasada, I. A., & Denver, D. R. (2016). Genomic evidence for plant-parasitic nematodes as the earliest *Wolbachia* hosts. *Scientific Reports, 6.* https://doi.org/10.1038/srep34955

Camacho, M., Oliva, M., & Serbus, L. R. (2017). Dietary saccharides and sweet tastants have differential effects on colonization of *Drosophila* oocytes by *Wolbachia* endosymbionts. *Biology Open*, *6*(7), 1074–1083. https://doi.org/10.1242/bio.023895

Caragata, E. P., Rancès, E., O'Neill, S. L., & McGraw, E. A. (2014). Competition for amino acids between *Wolbachia* and the mosquito host, *Aedes aegypti*. *Microbial Ecology, 67*(1), 205–218. https://doi.org/10.1007/s00248-013-0339-4

Casiraghi, M., Bordenstein, S. R., Baldo, L., Lo, N., Beninati, T., Wernegreen, J. J., Werren, J. H., & Bandi, C. (2005). Phylogeny of *Wolbachia pipientis* based on gltA, groEL and ftsZ gene sequences: Clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. *Microbiology (Reading, England)*, *151*(Pt 12), 4015–4022. https://doi.org/10.1099/mic.0.28313-0

Casper-Lindley, C., Kimura, S., Saxton, D. S., Essaw, Y., Simpson, I., Tan, V., & Sullivan, W. (2011). Rapid fluorescence-based screening for Wolbachia endosymbionts in *Drosophila* germ line and somatic tissues ▿. *Applied and*

Environmental Microbiology, *77*(14), 4788–4794. https://doi.org/10.1128/AEM.00215-11

Chafee, M. E., Zecher, C. N., Gourley, M. L., Schmidt, V. T., Chen, J. H., Bordenstein, S. R., Clark, M. E., & Bordenstein, S. R. (2011). Decoupling of host–symbiont–phage coadaptations following transfer between insect species. *Genetics*, *187*(1), 203–215. https://doi.org/10.1534/genetics.110.120675

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, *263*(5148), 802–805. https://doi.org/10.1126/science.8303295

Charlat, S., Duplouy, A., Hornett, E. A., Dyson, E. A., Davies, N., Roderick, G. K., Wedell, N., & Hurst, G. D. (2009). The joint evolutionary histories of *Wolbachia* and mitochondria in *Hypolimnas bolina*. *BMC Evolutionary Biology*, *9*, 64. https://doi.org/10.1186/1471-2148-9-64

Charlesworth, J., Weinert, L. A., Araujo, E. V., & Welch, J. J. (2019). *Wolbachia*, *Cardinium* and climate: An analysis of global data. *Biology Letters*, *15*(8). https://doi.org/10.1098/rsbl.2019.0273

Christensen, S., Pérez Dulzaides, R., Hedrick, V. E., Momtaz, A. J. M. Z., Nakayasu, E. S., Paul, L. N., & Serbus, L. R. (2016). *Wolbachia* endosymbionts modify *Drosophila* ovary protein levels in a context-dependent manner. *Applied and Environmental Microbiology*, *82*(17), 5354–5363. https://doi.org/10.1128/AEM.01255-16

Clancy, D. J., & Hoffmann, A. A. (1998). Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans*. *Entomologia Experimentalis et Applicata*, *86*(1), 13–24. https://doi.org/10.1046/j.1570-7458.1998.00261.x

Clark, M. E., & Karr, T. L. (2002). Distribution of *Wolbachia* within *Drosophila* reproductive tissue: Implications for the expression of cytoplasmic incompatibility. *Integrative and Comparative Biology*, *42*(2), 332–339. https://doi.org/10.1093/icb/42.2.332

Clyne, P. J., Brotman, J. S., Sweeney, S. T., & Davis, G. (2003). Green fluorescent protein tagging *Drosophila* Proteins at their native genomic loci with small p elements. *Genetics*, *165*(3), 1433–1441.

Cross, H. F., Haarbrink, M., Egerton, G., Yazdanbakhsh, M., & Taylor, M. J. (2001). Severe reactions to filarial chemotherapy and release of *Wolbachia* endosymbionts into blood. *Lancet (London, England)*, *358*(9296), 1873–1875. https://doi.org/10.1016/S0140-6736(01)06899-4

Czyż, D. M., Potluri, L.-P., Jain-Gupta, N., Riley, S. P., Martinez, J. J., Steck, T. L., Crosson, S., Shuman, H. A., & Gabay, J. E. (2014). Host-directed antimicrobial

drugs with broad-spectrum efficacy against intracellular bacterial pathogens. *MBio*, *5*(4). https://doi.org/10.1128/mBio.01534-14

Daehnel, K., Hise, A. G., Gillette-Ferguson, I., & Pearlman, E. (2007). *Wolbachia* and *Onchocerca volvulus*: pathogenesis of river blindness. *Wolbachia: A Bug's Life in Another Bug*, *5*, 133–145. https://doi.org/10.1159/000104404

Das, A., & Varma, A. (2009). Symbiosis: The art of living. In A. Varma & A. C. Kharkwal (Eds.), *Symbiotic Fungi: Principles and Practice* (pp. 1–28). Springer. https://doi.org/10.1007/978-3-540-95894-9_1

Dedeine, F., Boulétreau, M., & Vavre, F. (2005). Wolbachia requirement for oogenesis: Occurrence within the genus *Asobara* (*Hymenoptera, Braconidae)* and evidence for intraspecific variation in *A. tabida*. *Heredity*, *95*(5), 394–400. https://doi.org/10.1038/sj.hdy.6800739

Derré, I., Pypaert, M., Dautry-Varsat, A., & Agaisse, H. (2007). RNAi screen in *Drosophila* cells reveals the involvement of the tom complex in *Chlamydia* infection. *PLoS Pathogens*, *3*(10). https://doi.org/10.1371/journal.ppat.0030155

Dimijian, G. G. (2000). Evolving together: The biology of symbiosis, part 2. *Proceedings (Baylor University. Medical Center)*, *13*(4), 381–390.

Dobson, S. L., Bourtzis, K., Braig, H. R., Jones, B. F., Zhou, W., Rousset, F., & O'Neill, S. L. (1999). *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochemistry and Molecular Biology*, *29*(2), 153–160. https://doi.org/10.1016/s0965-1748(98)00119-2

Douglas, A. E. (1998). Nutritional interactions in insect-microbial symbioses: *Aphids* and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology*, *43*, 17–37. https://doi.org/10.1146/annurev.ento.43.1.17

Dunn, A. K., & Stabb, E. V. (2005). Culture-independent characterization of the microbiota of the ant lion *Myrmeleon mobilis (Neuroptera: Myrmeleontidae*). *Applied and Environmental Microbiology*, *71*(12), 8784–8794. https://doi.org/10.1128/AEM.71.12.8784-8794.2005

Dyson, E. A., & Hurst, G. D. D. (2004). Persistence of an extreme sex-ratio bias in a natural population. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(17), 6520–6523. https://doi.org/10.1073/pnas.0304068101

Fenn, K., & Blaxter, M. (2004). Quantification of *Wolbachia* bacteria in *Brugia malayi* through the nematode lifecycle. *Molecular and Biochemical Parasitology*, *137*(2), 361–364. https://doi.org/10.1016/j.molbiopara.2004.06.012

Ferrari, J., & Vavre, F. (2011). Bacterial symbionts in insects or the story of communities affecting communities. *Philosophical Transactions of the Royal Society B:*

Biological Sciences, *366*(1569), 1389–1400. https://doi.org/10.1098/rstb.2010.0226

Ferree, P. M., Frydman, H. M., Li, J. M., Cao, J., Wieschaus, E., & Sullivan, W. (2005). Wolbachia utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte. *PLoS Pathogens*, *1*(2). https://doi.org/10.1371/journal.ppat.0010014

Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., Kyrpides, N., Ghedin, E., Wang, S., Goltsman, E., … Slatko, B. (2005). The *Wolbachia* genome of *Brugia malayi:* Endosymbiont evolution within a human pathogenic nematode. *PLoS Biology*, *3*(4). https://doi.org/10.1371/journal.pbio.0030121

Geoghegan, V., Stainton, K., Rainey, S. M., Ant, T. H., Dowle, A. A., Larson, T., Hester, S., Charles, P. D., Thomas, B., & Sinkins, S. P. (2017). Perturbed cholesterol and vesicular trafficking associated with dengue blocking in *Wolbachia*-infected *Aedes aegypti* cells. *Nature Communications*, *8*(1), 526. https://doi.org/10.1038/s41467-017-00610-8

Ghedin, E., Wang, S., Spiro, D., Caler, E., Zhao, Q., Crabtree, J., Allen, J. E., Delcher, A. L., Guiliano, D. B., Miranda-Saavedra, D., Angiuoli, S. V., Creasy, T., Amedeo, P., Haas, B., El-Sayed, N. M., Wortman, J. R., Feldblyum, T., Tallon, L., Schatz, M., … Scott, A. L. (2007). Draft genome of the filarial nematode parasite *Brugia malayi*. *Science (New York, N.Y.)*, *317*(5845), 1756–1760. https://doi.org/10.1126/science.1145406

Goto, S., Anbutsu, H., & Fukatsu, T. (2006). Asymmetrical Interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. *Applied and Environmental Microbiology*, *72*(7), 4805–4810. https://doi.org/10.1128/AEM.00416-06

Grobler, Y., Yun, C. Y., Kahler, D. J., Bergman, C. M., Lee, H., Oliver, B., & Lehmann, R. (2018). Whole genome screen reveals a novel relationship between *Wolbachia* levels and *Drosophila* host translation. *PLoS Pathogens*, *14*(11), e1007445. https://doi.org/10.1371/journal.ppat.1007445

Grote, A., Voronin, D., Ding, T., Twaddle, A., Unnasch, T. R., Lustigman, S., & Ghedin, E. (2017). Defining *Brugia malayi* and *Wolbachia* symbiosis by stage-specific dual RNA-seq. *PLoS Neglected Tropical Diseases*, *11*(3). https://doi.org/10.1371/journal.pntd.0005357

Hadfield, S. J., & Axton, J. M. (1999). Germ cells colonized by endosymbiotic bacteria. *Nature*, *402*(6761), 482. https://doi.org/10.1038/45002

Hague, M. T. J., Caldwell, C. N., & Cooper, B. S. (2020). Pervasive effects of *Wolbachia* on host temperature preference. *MBio*, *11*(5). https://doi.org/10.1128/mBio.01768-20

Hales, K. G., Korey, C. A., Larracuente, A. M., & Roberts, D. M. (2015). Genetics on the Fly: A primer on the *Drosophila* model system. *Genetics*, *201*(3), 815–842. https://doi.org/10.1534/genetics.115.183392

Hansen, A. K., & Moran, N. A. (2011). Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(7), 2849–2854. https://doi.org/10.1073/pnas.1013465108

Hedges, L. M., Brownlie, J. C., O'Neill, S. L., & Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science (New York, N.Y.)*, *322*(5902), 702. https://doi.org/10.1126/science.1162418

Hertig, M. (1936). The *Rickettsia, Wolbachia pipientis* (gen. Et sp.n.) and associated inclusions of the mosquito, *Culex pipiens*. *Parasitology*, *28*(4), 453–486. https://doi.org/10.1017/S0031182000022666

Hertig, M., & Wolbach, S. B. (1924). Studies on *Rickettsia*-like micro-organisms in insects. *The Journal of Medical Research*, *44*(3), 329-374.7.

Hoerauf, A., Mand, S., Adjei, O., Fleischer, B., & Büttner, D. W. (2001). Depletion of *Wolbachia* endobacteria in *Onchocerca volvulus* by doxycycline and microfilaridermia after ivermectin treatment. *Lancet (London, England)*, *357*(9266), 1415–1416. https://doi.org/10.1016/S0140-6736(00)04581-5

Hooper, D. U., Chapin, F. S., Ewel, J. J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J. H., Lodge, D. M., Loreau, M., Naeem, S., Schmid, B., Setälä, H., Symstad, A. J., Vandermeer, J., & Wardle, D. A. (2005). Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecological Monographs*, *75*(1), 3–35. https://doi.org/10.1890/04-0922

Hopkins, G. H. E. (1926). Insects of *Samoa* and other *Samoan* terrestrial arthropoda (pp. 1–20). https://www.biodiversitylibrary.org/bibliography/68619

Hu, Y., Comjean, A., Rodiger, J., Liu, Y., Gao, Y., Chung, V., Zirin, J., Perrimon, N., & Mohr, S. E. (2021). FlyRNAi.org—the database of the *Drosophila* RNAi screening center and transgenic RNAi project: 2021 update. *Nucleic Acids Research*, *49*(D1), D908–D915. https://doi.org/10.1093/nar/gkaa936

Hughes, G. L., Dodson, B. L., Johnson, R. M., Murdock, C. C., Tsujimoto, H., Suzuki, Y., Patt, A. A., Cui, L., Nossa, C. W., Barry, R. M., Sakamoto, J. M., Hornett, E. A., & Rasgon, J. L. (2014). Native microbiome impedes vertical transmission of *Wolbachia* in *Anopheles* mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(34), 12498–12503. https://doi.org/10.1073/pnas.1408888111

Hurst, G. D., Johnson, A. P., Schulenburg, J. H., & Fuyama, Y. (2000). Male-killing *Wolbachia* in *Drosophila*: A temperature-sensitive trait with a threshold bacterial density. *Genetics*, *156*(2), 699–709.

Hurst, G. D., & Werren, J. H. (2001). The role of selfish genetic elements in eukaryotic evolution. *Nature Reviews. Genetics*, *2*(8), 597–606. https://doi.org/10.1038/35084545

Jajere, S. M. (2019). A review of *Salmonella enterica* with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. *Veterinary World*, *12*(4), 504–521. https://doi.org/10.14202/vetworld.2019.504-521

Jennings, B. H. (2011). Drosophila – a versatile model in biology & medicine. *Materials Today*, *14*(5), 190–195. https://doi.org/10.1016/S1369-7021(11)70113-4

Jia, F.-X., Yang, M.-S., Yang, W.-J., & Wang, J.-J. (2009). Influence of continuous high temperature conditions on *Wolbachia* infection frequency and the fitness of *Liposcelis tricolor* (Psocoptera: Liposcelididae). *Environmental Entomology*, *38*(5), 1365–1372. https://doi.org/10.1603/022.038.0503

Jiménez, N. E., Gerdtzen, Z. P., Olivera-Nappa, Á., Salgado, J. C., & Conca, C. (2019). A systems biology approach for studying *Wolbachia* metabolism reveals points of interaction with its host in the context of arboviral infection. *PLoS Neglected Tropical Diseases*, *13*(8), e0007678. https://doi.org/10.1371/journal.pntd.0007678

Johnston, K. L., & Taylor, M. J. (2007). *Wolbachia* in filarial parasites: Targets for filarial infection and disease control. *Current Infectious Disease Reports*, *9*(1), 55–59. https://doi.org/10.1007/s11908-007-0023-2

Kamath, A. D., Deehan, M. A., & Frydman, H. M. (2018). Polar cell fate stimulates *Wolbachia* intracellular growth. *Development*, *145*(6). https://doi.org/10.1242/dev.158097

Koropatnick, T. A., Engle, J. T., Apicella, M. A., Stabb, E. V., Goldman, W. E., & McFall-Ngai, M. J. (2004). Microbial factor-mediated development in a host-bacterial mutualism. *Science (New York, N.Y.)*, *306*(5699), 1186–1188. https://doi.org/10.1126/science.1102218

Kuttenkeuler, D. (2004). Genome-wide RNAi as a route to gene function in *Drosophila*. *Briefings in Functional Genomics and Proteomics*, *3*(2), 168–176. https://doi.org/10.1093/bfgp/3.2.168

Lang, B. F., Gray, M. W., & Burger, G. (1999). Mitochondrial genome evolution and the origin of eukaryotes. *Annual Review of Genetics*, *33*, 351–397. https://doi.org/10.1146/annurev.genet.33.1.351

Lefoulon, E., Clark, T., Borveto, F., Perriat-Sanguinet, M., Moulia, C., Slatko, B. E., & Gavotte, L. (2020). Pseudoscorpion *Wolbachia* symbionts: Diversity and evidence for a new supergroup S. *BMC Microbiology*, *20*(1), 188. https://doi.org/10.1186/s12866-020-01863-y

Leung, T. L. F., & Poulin, R. (2008). Parasitism, commensalism, and mutualism: Exploring the many shades of symbioses.

Levenhagen, M. A., Alves, R. N., Rieck, S. E., Labruna, M. B., & Beletti, M. E. (2012). The role of cytoskeleton, components of inositol phospholipid signaling pathway and iron in <Emphasis Type="Italic">Ehrlichia canis</Emphasis> in vitro proliferation. *Veterinary Research Communications*, *36*(3), 195–199. https://doi.org/10.1007/s11259-012-9525-y

Lo, N., Casiraghi, M., Salati, E., Bazzocchi, C., & Bandi, C. (2002). How many *Wolbachia* supergroups exist? *Molecular Biology and Evolution*, *19*(3), 341–346. https://doi.org/10.1093/oxfordjournals.molbev.a004087

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., & Darnell, J. (2000). Isolation and analysis of mutants. *Molecular Cell Biology. 4th Edition*. https://www.ncbi.nlm.nih.gov/books/NBK21735/

Lu, P., Bian, G., Pan, X., & Xi, Z. (2012). Wolbachia induces density-dependent inhibition to Dengue virus in mosquito cells. *PLoS Neglected Tropical Diseases*, *6*(7). https://doi.org/10.1371/journal.pntd.0001754

Macdonald, T. T., & Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science (New York, N.Y.)*, *307*(5717), 1920–1925. https://doi.org/10.1126/science.1106442

Mandrioli, M. (2009). Insect-symbiont: The key relationship to get in-depth insight on the host choice of bacteria. *Invertebrate Survival Journal*, *6*(2), 98–101.

Margulis, L. (1981). Symbiosis in cell evolution: Life and its environment on the early Earth. W. H. Freeman.

Margulis, L. (2004). Serial endosymbiotic theory (SET) and composite individuality. Microbiology Today, 31(4), 172-175.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences*, *110*(9), 3229–3236. https://doi.org/10.1073/pnas.1218525110

McGarry, H. F., Egerton, G. L., & Taylor, M. J. (2004). Population dynamics of *Wolbachia* bacterial endosymbionts in *Brugia malayi*. *Molecular and Biochemical Parasitology*, *135*(1), 57–67. https://doi.org/10.1016/j.molbiopara.2004.01.006

McGraw, E. A., Merritt, D. J., Droller, J. N., & O'Neill, S. L. (2002). *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proceedings of the*

National Academy of Sciences, *99*(5), 2918–2923. https://doi.org/10.1073/pnas.052466499

Min, K.-T., & Benzer, S. (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proceedings of the National Academy of Sciences*, *94*(20), 10792–10796. https://doi.org/10.1073/pnas.94.20.10792

Moran, N. A. (2006). Symbiosis. *Current Biology: CB*, *16*(20), R866-871. https://doi.org/10.1016/j.cub.2006.09.019

Moran, N. A., McCutcheon, J. P., & Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, *42*, 165–190. https://doi.org/10.1146/annurev.genet.41.110306.130119

Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G., Pyke, A. T., Hedges, L. M., Rocha, B. C., Hall-Mendelin, S., Day, A., Riegler, M., Hugo, L. E., Johnson, K. N., Kay, B. H., McGraw, E. A., van den Hurk, A. F., Ryan, P. A., & O'Neill, S. L. (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and Plasmodium. *Cell*, *139*(7), 1268–1278. https://doi.org/10.1016/j.cell.2009.11.042

Morin, X., Daneman, R., Zavortink, M., & Chia, W. (2001). A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *Proceedings of the National Academy of Sciences*, *98*(26), 15050–15055. https://doi.org/10.1073/pnas.261408198

Newton, I. L. G., Savytskyy, O., & Sheehan, K. B. (2015). *Wolbachia* utilize host actin for efficient maternal transmission in *Drosophila melanogaster. PLoS Pathogens*, *11*(4). https://doi.org/10.1371/journal.ppat.1004798

Nicholson, L., Singh, G. K., Osterwalder, T., Roman, G. W., Davis, R. L., & Keshishian, H. (2008). Spatial and temporal control of gene expression in *Drosophila* using the inducible geneswitch GAL4 system. I. Screen for larval nervous system drivers. *Genetics*, *178*(1), 215–234. https://doi.org/10.1534/genetics.107.081968

Niu, H., Yamaguchi, M., & Rikihisa, Y. (2008). Subversion of cellular autophagy by *Anaplasma phagocytophilum*. *Cellular Microbiology*, *10*(3), 593–605. https://doi.org/10.1111/j.1462-5822.2007.01068.x

Oliver, K. M., Moran, N. A., & Hunter, M. S. (2006). Costs and benefits of a superinfection of facultative symbionts in aphids. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1591), 1273–1280. https://doi.org/10.1098/rspb.2005.3436

O'Malley, M. A. (2015). Endosymbiosis and its implications for evolutionary theory. *Proceedings of the National Academy of Sciences*, *112*(33), 10270–10277. https://doi.org/10.1073/pnas.1421389112

Osborne, S. E., Leong, Y. S., O'Neill, S. L., & Johnson, K. N. (2009). Variation in Antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathogens*, *5*(11). https://doi.org/10.1371/journal.ppat.1000656

Osterwalder, T., Yoon, K. S., White, B. H., & Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. *Proceedings of the National Academy of Sciences*, *98*(22), 12596–12601. https://doi.org/10.1073/pnas.221303298

Paixão, E. S., Teixeira, M. G., & Rodrigues, L. C. (2018). Zika, chikungunya and dengue: The causes and threats of new and re-emerging arboviral diseases. *BMJ Global Health*, *3*(Suppl 1), e000530. https://doi.org/10.1136/bmjgh-2017-000530

Paracer, S., & Ahmadjian, V. (2000). Symbiosis: An Introduction to Biological Associations. *Oxford University Press*.

Philips, J. A., Rubin, E. J., & Perrimon, N. (2005). Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. *Science (New York, N.Y.)*, *309*(5738), 1251–1253. https://doi.org/10.1126/science.1116006

Pietri, J. E., DeBruhl, H., & Sullivan, W. (2016). The rich somatic life of *Wolbachia*. *MicrobiologyOpen*, *5*(6), 923–936. https://doi.org/10.1002/mbo3.390

Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C., & Merçot, H. (1998). *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: Host effect and cytoplasmic incompatibility relationships. *Genetics*, *150*(1), 227–237.

Punkosdy, G. A., Addiss, D. G., & Lammie, P. J. (2003). Characterization of antibody responses to *Wolbachia* surface protein in humans with lymphatic filariasis. *Infection and Immunity*, *71*(9), 5104–5114. https://doi.org/10.1128/IAI.71.9.5104-5114.2003

Rasgon, J. L., & Scott, T. W. (2004). Phylogenetic characterization of *Wolbachia* symbionts infecting *Cimex lectularius L.* and *Oeciacus vicarius Horvath (Hemiptera: Cimicidae)*. *Journal of Medical Entomology*, *41*(6), 1175–1178. https://doi.org/10.1603/0022-2585-41.6.1175

Raven, J. A., & Allen, J. F. (2003). Genomics and chloroplast evolution: What did cyanobacteria do for plants? *Genome Biology*, *4*(3), 209.

Rikihisa, Y., Zhang, Y., & Park, J. (1994). Inhibition of infection of macrophages with *Ehrlichia risticii* by cytochalasins, monodansylcadaverine, and taxol. *Infection and Immunity*, *62*(11), 5126–5132.

Riparbelli, M. G., Giordano, R., & Callaini, G. (2007). Effects of *Wolbachia* on sperm maturation and architecture in *Drosophila simulans Riverside*. *Mechanisms of Development*, *124*(9), 699–714. https://doi.org/10.1016/j.mod.2007.07.001

Roman, G., Endo, K., Zong, L., & Davis, R. L. (2001). P{Switch}, a system for spatial and temporal control of gene expression in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, *98*(22), 12602–12607. https://doi.org/10.1073/pnas.221303998

Ros, V. I. D., Fleming, V. M., Feil, E. J., & Breeuwer, J. A. J. (2009). How diverse is the genus *Wolbachia*? multiple-gene sequencing reveals a putatively new *Wolbachia* Supergroup recovered from spider mites (*Acari: Tetranychidae)*. *Applied and Environmental Microbiology*, *75*(4), 1036–1043. https://doi.org/10.1128/AEM.01109-08

Rossi, P., Ricci, I., Cappelli, A., Damiani, C., Ulissi, U., Mancini, M. V., Valzano, M., Capone, A., Epis, S., Crotti, E., Chouaia, B., Scuppa, P., Joshi, D., Xi, Z., Mandrioli, M., Sacchi, L., O'Neill, S. L., & Favia, G. (2015). Mutual exclusion of *Asaia* and *Wolbachia* in the reproductive organs of mosquito vectors. *Parasites & Vectors*, *8*, 278. https://doi.org/10.1186/s13071-015-0888-0

Russell, S. L., Lemseffer, N., & Sullivan, W. T. (2018). *Wolbachia* and host germline components compete for kinesin-mediated transport to the posterior pole of the *Drosophila* oocyte. *PLoS Pathogens*, *14*(8). https://doi.org/10.1371/journal.ppat.1007216

Sacchi, L., Genchi, M., Clementi, E., Negri, I., Alma, A., Ohler, S., Sassera, D., Bourtzis, K., & Bandi, C. (2010). Bacteriocyte-like cells harbour *Wolbachia* in the ovary of *Drosophila melanogaster* (*Insecta, Diptera)* and *Zyginidia pullula (Insecta, Hemiptera*). *Tissue & Cell*, *42*(5), 328–333. https://doi.org/10.1016/j.tice.2010.07.009

Saint André, A. v, Blackwell, N. M., Hall, L. R., Hoerauf, A., Brattig, N. W., Volkmann, L., Taylor, M. J., Ford, L., Hise, A. G., Lass, J. H., Diaconu, E., & Pearlman, E. (2002). The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. *Science (New York, N.Y.)*, *295*(5561), 1892–1895. https://doi.org/10.1126/science.1068732

Sazama, E. J., Bosch, M. J., Shouldis, C. S., Ouellette, S. P., & Wesner, J. S. (2017). Incidence of *Wolbachia* in aquatic insects. *Ecology and Evolution*, *7*(4), 1165–1169. https://doi.org/10.1002/ece3.2742

Sazama, E. J., Ouellette, S. P., & Wesner, J. S. (2019). Bacterial endosymbionts are common among, but not necessarily within, insect species. *Environmental Entomology*, *48*(1), 127–133. https://doi.org/10.1093/ee/nvy188

Scherr, N., Honnappa, S., Kunz, G., Mueller, P., Jayachandran, R., Winkler, F., Pieters, J., & Steinmetz, M. O. (2007). Structural basis for the specific inhibition of protein kinase G, a virulence factor of *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences*, *104*(29), 12151–12156. https://doi.org/10.1073/pnas.0702842104

Serbus, L. R., Casper-Lindley, C., Landmann, F., & Sullivan, W. (2008). The genetics and cell biology of *Wolbachia*-host interactions. *Annual Review of Genetics*, *42*, 683–707.

Serbus, L. R., Ferreccio, A., Zhukova, M., McMorris, C. L., Kiseleva, E., & Sullivan, W. (2011). A feedback loop between *Wolbachia* and the *Drosophila* gurken mRNP complex influences *Wolbachia* titer. *Journal of Cell Science*, *124*(Pt 24), 4299–4308. https://doi.org/10.1242/jcs.092510

Serbus, L. R., & Sullivan, W. (2007). A cellular basis for *Wolbachia* recruitment to the host germline. *PLoS Pathogens*, *3*(12). https://doi.org/10.1371/journal.ppat.0030190

Serbus, L. R., White, P. M., Silva, J. P., Rabe, A., Teixeira, L., Albertson, R., & Sullivan, W. (2015). The impact of host diet on *Wolbachia* titer in *Drosophila. PLoS Pathogens*, *11*(3). https://doi.org/10.1371/journal.ppat.1004777

Severo, M. S., Choy, A., Stephens, K. D., Sakhon, O. S., Chen, G., Chung, D.-W. D., Le Roch, K. G., Blaha, G., & Pedra, J. H. F. (2013). The E3 ubiquitin ligase xiap restricts *Anaplasma phagocytophilum* colonization of *Ixodes scapularis* ticks. *The Journal of Infectious Diseases*, *208*(11), 1830–1840. https://doi.org/10.1093/infdis/jit380

Simhadri, R. K., Fast, E. M., Guo, R., Schultz, M. J., Vaisman, N., Ortiz, L., Bybee, J., Slatko, B. E., & Frydman, H. M. (2017). The gut commensal microbiome of *Drosophila melanogaster* is modified by the endosymbiont *Wolbachia. MSphere*, *2*(5). https://doi.org/10.1128/mSphere.00287-17

Slatko, B. E., Luck, A. N., Dobson, S. L., & Foster, J. M. (2014). *Wolbachia* endosymbionts and human disease control. *Molecular and Biochemical Parasitology*, *195*(2), 88–95. https://doi.org/10.1016/j.molbiopara.2014.07.004

Slatko, B. E., Taylor, M. J., & Foster, J. M. (2010). The *Wolbachia* endosymbiont as an anti-filarial nematode target. *Symbiosis (Philadelphia, Pa.)*, *51*(1), 55–65. https://doi.org/10.1007/s13199-010-0067-1

Starr, D. J., & Cline, T. W. (2002). A host parasite interaction rescues *Drosophila* oogenesis defects. *Nature*, *418*(6893), 76–79. https://doi.org/10.1038/nature00843

Strunov, A., Kiseleva, E., & Gottlieb, Y. (2013). Spatial and temporal distribution of pathogenic *Wolbachia* strain *wMelPop* in *Drosophila melanogaster* central nervous system under different temperature conditions. *Journal of Invertebrate Pathology*, *114*(1), 22–30. https://doi.org/10.1016/j.jip.2013.05.001

Sumi, T., Miura, K., & Miyatake, T. (2017). *Wolbachia* density changes seasonally amongst populations of the pale grass blue butterfly, *Zizeeria maha* (Lepidoptera: Lycaenidae). *PloS One*, *12*(4), e0175373. https://doi.org/10.1371/journal.pone.0175373

Supali, T., Djuardi, Y., Pfarr, K. M., Wibowo, H., Taylor, M. J., Hoerauf, A., Houwing-Duistermaat, J. J., Yazdanbakhsh, M., & Sartono, E. (2008). Doxycycline treatment of *Brugia malayi*-infected persons reduces microfilaremia and adverse reactions after diethylcarbamazine and albendazole treatment. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *46*(9), 1385–1393. https://doi.org/10.1086/586753

Taylor, M. J., Bilo, K., Cross, H. F., Archer, J. P., & Underwood, A. P. (1999). 16S rDNA phylogeny and ultrastructural characterization of *Wolbachia* intracellular bacteria of the filarial nematodes *Brugia malayi, B. pahangi,* and *Wuchereria bancrofti. Experimental Parasitology*, *91*(4), 356–361. https://doi.org/10.1006/expr.1998.4383

Taylor, Mark J., Bandi, C., & Hoerauf, A. (2005). *Wolbachia* bacterial endosymbionts of filarial nematodes. *Advances in Parasitology*, *60*, 245–284. https://doi.org/10.1016/S0065-308X(05)60004-8

Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, *6*(12). https://doi.org/10.1371/journal.pbio.1000002

Toomey, M. E., Panaram, K., Fast, E. M., Beatty, C., & Frydman, H. M. (2013). Evolutionarily conserved *Wolbachia*-encoded factors control pattern of stem-cell niche tropism in *Drosophila* ovaries and favor infection. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(26), 10788–10793. https://doi.org/10.1073/pnas.1301524110

Townson, S., Hutton, D., Siemienska, J., Hollick, L., Scanlon, T., Tagboto, S. K., & Taylor, M. J. (2000). Antibiotics and *Wolbachia* in filarial nematodes: Antifilarial activity of rifampicin, oxytetracycline and chloramphenicol against *Onchocerca gutturosa, Onchocerca lienalis* and *Brugia pahang*i. *Annals of Tropical Medicine and Parasitology*, *94*(8), 801–816.

Vaishampayan, P. A., Dhotre, D. P., Gupta, R. P., Lalwani, P., Ghate, H., Patole, M. S., & Shouche, Y. S. (2007). Molecular evidence and phylogenetic affiliations of *Wolbachia* in cockroaches. *Molecular Phylogenetics and Evolution*, *44*(3), 1346–1351. https://doi.org/10.1016/j.ympev.2007.01.003

Vandekerckhove, T. T., Watteyne, S., Willems, A., Swings, J. G., Mertens, J., & Gillis, M. (1999). Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (*Hexapoda, Collembola*) and its implications for Wolbachial taxonomy. *FEMS Microbiology Letters*, *180*(2), 279–286. https://doi.org/10.1111/j.1574-6968.1999.tb08807.x

Veneti, Z., Clark, M. E., Karr, T. L., Savakis, C., & Bourtzis, K. (2004). Heads or tails: Host-parasite interactions in the *Drosophila-Wolbachia* system. *Applied and Environmental Microbiology*, *70*(9), 5366–5372. https://doi.org/10.1128/AEM.70.9.5366-5372.2004

Veneti, Z., Clark, M. E., Zabalou, S., Karr, T. L., Savakis, C., & Bourtzis, K. (2003). Cytoplasmic incompatibility and sperm cyst infection in different *Drosophila-Wolbachia* associations*. Genetics, 164(2),* 545–552.

Wall, D. H., & Moore, J. C. (1999). Interactions underground: Soil biodiversity, mutualism, and ecosystem processes. *BioScience*, *49*(2), 109–117. https://doi.org/10.2307/1313536

Wangler, M. F., Yamamoto, S., & Bellen, H. J. (2015). Fruit flies in biomedical research. *Genetics*, *199*(3), 639–653. https://doi.org/10.1534/genetics.114.171785

Wasala, S. K., Brown, A. M. V., Kang, J., Howe, D. K., Peetz, A. B., Zasada, I. A., & Denver, D. R. (2019). Variable abundance and distribution of *Wolbachia* and *Cardinium* endosymbionts in plant-parasitic nematode field populations. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00964

Weeks, A. R., Turelli, M., Harcombe, W. R., Reynolds, K. T., & Hoffmann, A. A. (2007). From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLOS Biology*, *5*(5), e114. https://doi.org/10.1371/journal.pbio.0050114

Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z., & Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1807), 20150249. https://doi.org/10.1098/rspb.2015.0249

Werren, J. H., Zhang, W., & Guo, L. R. (1995a). Evolution and phylogeny of *Wolbachia*: Reproductive parasites of arthropods. *Proceedings. Biological Sciences*, *261*(1360), 55–63. https://doi.org/10.1098/rspb.1995.0117

Werren, John H. (1997). Biology of *Wolbachia*. *Annual Review of Entomology*, *42*(1), 587–609. https://doi.org/10.1146/annurev.ento.42.1.587

Werren, John H, Baldo, L., & Clark, M. E. (2008). *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751.

Werren, John H., Windsor, D., & Guo, L. R. (1995b). Distribution of *Wolbachia* among neotropical arthropods. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *262*(1364), 197–204. https://doi.org/10.1098/rspb.1995.0196

White, P. L., Baker, A. R., & James, W. O. (1997). Strategies to control *Salmonella* and *Campylobacter* in raw poultry product*s*. 17.

White, P. M., Serbus, L. R., Debec, A., Codina, A., Bray, W., Guichet, A., Lokey, R. S., & Sullivan, W. (2017a). Reliance of *Wolbachia* on high rates of host proteolysis revealed by a genome-wide RNAi screen of *Drosophila* cells. *Genetics*, *205*(4), 1473–1488. https://doi.org/10.1534/genetics.116.198903

Wu, M., Sun, L. V., Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J. C., McGraw, E. A., Martin, W., Esser, C., Ahmadinejad, N., Wiegand, C., Madupu, R., Beanan, M. J., Brinkac, L. M., Daugherty, S. C., Durkin, A. S., Kolonay, J. F., Nelson, W. C., Mohamoud, Y., … Eisen, J. A. (2004). Phylogenomics of the reproductive parasite *Wolbachia pipientis wMel*: A streamlined genome overrun by mobile genetic elements. *PLoS Biology*, *2*(3), E69. https://doi.org/10.1371/journal.pbio.0020069

Xiong, Q., Lin, M., & Rikihisa, Y. (2009). Cholesterol-dependent *Anaplasma phagocytophilum* exploits the low-density lipoprotein uptake pathway. *PLoS Pathogens*, *5*(3). https://doi.org/10.1371/journal.ppat.1000329

Xue, L., Fang, X., & Hyman, J. M. (2018). Comparing the effectiveness of different strains of *Wolbachia* for controlling chikungunya, dengue fever, and zika. *PLoS Neglected Tropical Diseases*, *12*(7), e0006666. https://doi.org/10.1371/journal.pntd.0006666

Yamaguchi, M., & Yoshida, H. (2018). Drosophila as a model organism. In M. Yamaguchi (Ed.), *Drosophila Models for Human Diseases* (pp. 1–10). Springer Singapore. https://doi.org/10.1007/978-981-13-0529-0_1

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C., & Bourtzis, K. (2004). *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the National Academy of Sciences*, *101*(42), 15042–15045. https://doi.org/10.1073/pnas.0403853101

Zhu, B., Das, S., Mitra, S., Farris, T. R., & McBride, J. W. (2017). *Ehrlichia chaffeensis* TRP120 moonlights as a hect e3 ligase involved in self- and host ubiquitination to influence protein interactions and stability for intracellular survival. *Infection and Immunity*, *85*(9). https://doi.org/10.1128/IAI.00290-17

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*(6), e38544. https://doi.org/10.1371/journal.pone.0038544

CHAPTER II: QUANTITATIVE APPROACH FOR ASSESSING *WOLBACHIA* TITER WITHIN WHOLE INSECTS

2.1 Abstract

Endosymbiotic *Wolbachia* bacteria are associated with a wide range of arthropods and filarial nematodes. The occurrence of these vertically transmitted endosymbiont is linked to a variety of reproductive manipulations, including cytoplasmic incompatibility, parthenogenesis, male killing, and feminization. *Wolbachia* abundance plays a significant role in maintaining the *Wolbachia*-host relationship by influencing the success of vertical transmission of *Wolbachia* and induction of host reproductive phenotypes. However, our understanding of *Wolbachia* density dynamics of *Wolbachia* within the host is limited. The lack of agreed-upon methods for quantifying abundance within whole organisms has created challenges in data interpretation to date. Chapter II showcases a standardized absolute count approach, using real-time PCR to quantify *Wolbachia* within whole insects. Using *Wolbachia*-infected *Drosophila melanogaster* as a model, we demonstrate the accuracy and precision of the protocol in determining *Wolbachia* abundance. By maximizing resolution of *Wolbachia*, this approach may shed new insight into the relationship between *Wolbachia* endosymbionts and their hosts, by informing bacterial distribution and abundance within whole organisms.

2.2 Introduction

Symbiotic relationships exist among different types of organisms (Margulis et al., 1991), including noteworthy examples of symbiosis between insect and microbes (Bourtzis & Miller, 2003). Symbiotic microorganisms are found in the gut, body cavity and/or cells of most insects. Some symbionts are mutually beneficial and contribute to their host's fitness, while others are parasitic and detrimental to their hosts (Bourtzis & Miller, 2003; Zchori-Fein & Bourtzis, 2011). The term symbiont is applied to bacterial

species which form symbiotic association with other organisms. Endosymbionts are distinctive in that they physically occupy the cells and tissues of their host organism (Kikuchi, 2009).

Wolbachia are intracellular, gram-negative alpha-proteobacteria. These bacterial endosymbionts are prevalent in nature (Saridaki & Bourtzis, 2010). *Wolbachia* were first detected in mosquitoes (Hertig, 1936; Hertig & Wolbach, 1924), and since then, have been found in a myriad of arthropods including the research model organism *Drosophila melanogaster* (Werren et al., 2008). Recent estimates report the prevalence of *Wolbachia* infection in terrestrial arthropods to be between 52% to 60% (Sazama et al., 2017; Weinert et al., 2015). The frequency of infected individuals within species may range widely from one species to the next (Sazama et al., 2019).

The ability of *Wolbachia* to persist and spread within the host population is dependent on vertical transmission from one generation to next, specifically by female hosts (Landmann, 2019; Werren et al., 2008). As such, *Wolbachia* actively manipulate the host reproductive system to enhance maternal transmission efficacy. Four widely-discussed reproductive phenotypes caused by *Wolbachia* are parthenogenesis, (female reproduction without males) (Stouthamer et al., 1999), male killing (death of infected male embryos) (Hurst et al., 1999), feminization (conversion of genetic males into functional females) (Rousset et al., 1992) and cytoplasmic incompatibility (elimination of uninfected embryos) (Yen & Barr, 1971). *Wolbachia* need to maintain an increased density to induce these reproductive manipulations (Boyle et al., 1993; Unckless et al., 2009). Interaction with the host cytoskeleton also ensures high concentrations of *Wolbachia* at the posterior pole of an egg during late oogenesis, which results in a heavily infected germline, assuring the successful transmission of *Wolbachia* in the next generations (Hadfield & Axton, 1999; Serbus & Sullivan, 2007; Veneti et al., 2004).

The abundance of *Wolbachia* within the host has biomedical implications. *Wolbachia* have gained much interest in recent years because of their potential role to suppress vector-borne disease. Introduction of *Wolbachia* in the mosquito *Aedes aegypti,* a vector for the Dengue fever virus (DENV), has been shown to dramatically reduce DENV load (Moreira et al., 2009; Walker et al., 2011), as well as Yellow fever, Chikungunya and Zika (Caragata et al., 2016; Hurk et al., 2012; Moreira et al., 2009). In *Drosophila, Wolbachia* was shown to provide protection from Drosophila C virus and Flock House virus (Hedges et al., 2008; Teixeira et al., 2008). Furthermore, many filarial nematodes host *Wolbachia,* including *Onchocerca volvulus*, which causes river blindness and *Wuchereria bancrofti* and *Brugia malayi,* which cause lymphatic filariasis (Taylor & Hoerauf, 1999). These nematodes are dependent on their obligate mutualist *Wolbachia* for survival and reproduction (Makepeace et al., 2006; Townson et al., 2000). Tissue pathology associated with these diseases have been demonstrated as responsive to the presence of *Wolbachia* (Punkosdy et al., 2003; Saint André et al., 2002). Despite the tremendous biological and biomedical implications of *Wolbachia* abundance, it is not yet well understood how *Wolbachia* are regulated and distributed within the host.

Precise quantification of *Wolbachia* titer is a fundamental prerequisite to analyze bacterial replication and distribution within the body. Quantification may reveal critical aspects of host-microbe interactions. Previous experiments addressing *Wolbachia* colonization have employed an array of techniques across arthropods and nematode host systems. Cytology-based approaches have been widely used to quantify *Wolbachia* within germline cells (Casper-Lindley et al., 2011; Christensen et al., 2019; Newton et al., 2015; Serbus & Sullivan, 2007). Tissue culture-based RNAi screens have also been carried out, which were coupled with fluorescent DNA stains (White et al., 2017a) and

fluorescence in situ hybridization techniques (Grobler et al., 2018) to quantify *Wolbachia* in somatic cells. As the significance of bacterial abundance is becoming more evident, researchers are also employing quantitative PCR (qPCR) to estimate *Wolbachia* abundance in whole insects or nematodes (Simoncini et al., 2001). Researchers have predominantly used relative qPCR to quantify *Wolbachia* in different biological contexts (Chrostek et al., 2013; Duarte et al., 2020; Mee et al., 2015; Moreira et al., 2009; Voronin et al., 2012; Zha et al., 2014). The absolute qPCR approach has also been used in many studies to quantify *Wolbachia* titer (Ant & Sinkins, 2018; Frentiu et al., 2010; Lu et al., 2019; Zhou & Li, 2016). Absolute qPCR determines the copy number of a candidate *Wolbachia* gene by comparing the data against standard curves of known DNA concentration. The candidate *Wolbachia* gene is interpreted as a proxy for absolute bacterial chromosome abundance, and thus bacterial titer within the sample. The diversity of techniques used to measure *Wolbachia* quantity in vivo has hindered comparative data interpretation up until now.

Chapter II presents a standardized and validated *Wolbachia* quantification assay, that can be used as a valuable tool in *Wolbachia* research community as well as for our own work. Utilizing the *Drosophila* model system, we optimized sample preparation for amplification by qPCR, enabling efficiently quantification of *Wolbachia* titer within whole host organisms. With thorough optimization and validation, the results showed maximum sensitivity and accuracy of the protocol in determining *Wolbachia* titer. Our approach is unique in that whole body counts allow us to inform bacterial abundance and distribution patterns within the host system, as well as how genetic and environmental factors specify such patterns.

2.3 Methods and materials

2.3.1 Fly stocks & maintenance

All the flies were maintained with standard fly food. The recipe is derived from Bloomington Drosophila stock center (*http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm*) (Camacho et al., 2017; Christensen et al., 2016). Briefly, the food is cooked in large batches that contain vital nutrients, for example, 337 grams of yeast, 96 grams of agar, 1325 grams of yellow corn meal, 190 grams of soy flour, 1.5 liters of Karo light corn syrup, 94 ml of propionic acid and 20 liters of water. The food is typically stored at 4 degrees and melted before use.

The study was performed using fruit flies (*Drosophila melanogaster*) of genotype *w; Sp/Cyo; Sb/Tm6B* carrying the *w*Mel *Wolbachia* strain (Christensen et al., 2016). Uninfected control flies of this same genotype represent the original parental strain, prior to addition of *w*Mel. All the flies were raised in plastic bottles containing ~25ml of standard fly food and were maintained in 12h:12h light-dark cycles at a constant 25 °C temperature. The stock bottles, containing both male and female flies, were seeded for 3-days under this environment and then were discarded. The new progeny starts eclosing after 10- to 12-days. Progeny were collected within 0 hr to 24 hrs post eclosure. Newly eclosed, adult flies were aged for 5 days in the controlled, 25 °C environment. The first 2 days of rearing is done on standard food. For the next 3-days of rearing, adult flies were exposed to appropriate food conditions for the experiment.

2.3.2 Optimizing plate assay format

A 24-well plate format (Corning 24-well plates (Cat# 3738) was used to raise 2-day old fruit flies. The amount of food and the number of flies that can go in each well were systematically optimized. To determine the appropriate amount of food, 12 wells of the screening plate were filled with 1.5 mL and the other 12-wells were filled with 1 mL of

melted standard food. Because of the space restrictions, we could not add a higher amount. The food is usually melted in a small glass beaker and dispensed quickly before cooling and solidifying. After transferring food into each well, the whole plate is solidified under a fume hood with proper covering to prevent condensation.

To determine the survival rate of the flies, 9 females and 3 males or (7 females and 3 males) were transferred in each well with the varying amount of food (1 mL and 1.5 mL). Flies were put to sleep using CO2 gas and transferred into a perfusion-friendly empty 24-well plate. The feeding plate was then carefully placed on top of the perfusion-friendly lid. Bench tape was applied to seal the 2 sides of the plates, which help securing the perfusion-friendly lid onto the feeding plate. The plate was then flipped over once the flies are awake, in order to prevent flies from getting stuck in the food. The flies were then incubated in this plate set up for 3- to 5-days, during which their survival rate was recorded.

2.3.3 Primer efficiency test

Primer efficiency tests were conducted on serially diluted DNA samples. The DNA was extracted using the optimized protocol, so that it could be used as a template to generate standard curves. The undiluted DNA was the reference point from where a series of 10-fold dilution was created: undiluted, 1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000. We performed qPCR on these serially diluted samples using 5 µM of *wsp* primers as used for the other experiments. Samples were amplified in triplicate and the average Ct (Cycle threshold) values of the technical replicates was determined. Log concentration of each sample dilution was calculated using the log formula in excel and then plotted against the average Ct values followed by generation of a linear regression curve. The slope of the trendline was obtained, which was used to calculate primer efficiency using a qPCR efficiency calculator available on the ThermoFisher website.

2.3.4 DNA extraction and body-wide qPCR of *Wolbachia* titer

For total body-wide counts from each sample, a group of 5 female flies was homogenized together in 200 µL of buffer containing 10 mM Tris HCl (pH 8.0), 1 mM EDTA and 25 mM NaCl, with or without 1% SDS. Additionally, samples were processed with or without the addition of 2 µL of 20 mg/ml of proteinase K, followed by incubation at either 56°C or 70°C. After incubation for 1 h, samples treated with proteinase K were inactivated by heating the samples at 95°C for 3 min. Samples were then centrifuged at 17,970 x g for 15 minutes at 4°C Avoiding the pellet, 100 µL of supernatant was collected and DNA was either used directly for qPCR, diluted in TE, or was concentrated by ethanol precipitation. For precipitation, 1/10 volume of 3 M Na-acetate and 250 µL of absolute ethanol was added to 100 µL of the supernatant. Samples were mixed gently and kept at − 20°C for > 2 h, then centrifuged at 17,970 x g for 15 minutes at 4°C. Resulting pellets were washed with 500 µL of 70% ethanol, and re-centrifuged at 17,970 x g for 15 minutes at 4°C. The DNA pellet was air dried and re-suspended in 100 µL of TE buffer. The DNA samples were then used directly, or serially diluted for qPCR.

The absolute quantification of *Wolbachia* was determined using the standard curves of known DNA concentrations. Here, a PGEMT plasmid vector carrying 160bp of PCR-amplified *wsp* fragment was used as a reference. A QIAprep Miniprep package was used to purify the cloned plasmid carrying the *wsp* sequence. The concentration of plasmids was then determined using Qubit® 2.0 Fluorometer. A 10-fold serial dilution series of the purified plasmid, ranging from 1 × 10^3 to 1 × 108^ copies/2 µL, was used to construct the standard curves. Cycle threshold (Ct) values for each dilution were assessed in triplicates utilizing the real-time QPCR (Bio-Rad CFX96 Connect Optics Module Real-Time System). Absolute copy number of *Wolbachia* in the unknown samples were then obtained by relating the Ct values to the standard curves generated

in real time qPCR. The primers used to target *wsp* gene were: Forward 5' CATTGGTGTTGGTGTTGGTG 3', reverse 5' ACCGAAATAACGAGCTCCAG 3' primers with a concentration of 5 µM.

An additional plasmid standard was also prepared in parallel, from *D. melanogaster ribosomal protein L32* (*rpl32*) to standardize sample loading in *Wolbachia* (−) samples. These plasmids were prepared by cloning a 194 bp fragment of *rpl32* using forward (5′-CCGCTTCAAGGGACAGTATC) and reverse (5′-CAATCTCCTTGCGCTTCTTG) primers.

2.3.5 Spike-in control to test extraction yield and presence of qPCR inhibitor in samples

A spike-in control was used to test the efficiency of our DNA extraction protocol. To test for DNA retention (or material loss) during the extraction process, 4979pg of lambda DNA was added to 200 µL of squishing buffer along with the flies prior DNA extraction. The nucleic acid was extracted and purified together with the lambda spiking DNA, using our protocol optimized as described above. It is important to note here that half of the original volume was collected during the extraction process to obtain the final amount of DNA for qPCR. For control samples, 4979pg of lambda DNA was added to TE buffer (pH 8.0), where the total volume was equivalent to amount of buffer used for DNA extraction. The samples were then mixed well, followed by transfer of 100 µL of the mixture to a different tube, to match handling of the control with experimental samples that used half of the original volume. qPCR was then performed on experimental samples and control samples using lambda DNA specific primers: forward (5'-CAGGTAGCGCAGATCATCAA 3') and reverse (5'-GCGTTAACCTGTTCCATCGT 3'). Once the Ct values of lambda DNA were obtained for both experimental and control

samples, the percent of yield (DNA retention) was calculated using the following formula: Yield (%) = (½^(Ct of experimental sample-Ct of control sample)) * 100.

2.3.6 Statistical analyses

All primary data collected in this study were matched with appropriate statistical analyses, as per a standard decision tree (Fig 2.1). Data were analyzed for consistency with a normal distribution using the Shapiro-Wilk test, and for homogeneity of variances using Levene's test (Lim & Loh, 1996; Mohd Razali & Yap, 2011; Shapiro & Wilk, 1965). For normal data, distributions showing homogenous variances were compared by T-test. Distributions with unequal variances were compared by Welch's T-test (Vargha & Delaney, 1998; Wasserman, 1994). For non-normal data, distributions with homogeneous variances were compared using the Mann-Whitney U test (Rietveld & van Hout, 2015; Vargha & Delaney, 1998). For non-normal distributions with unequal variances, significance was estimated using randomization based T-tests with bootstrapping, as recommended by field literature (de Cuevas & Spradling, 1998; Lim & Loh, 1996; Rietveld & van Hout, 2015; Shapiro & Wilk, 1965; Wasserman, 1994). The IBM SPSS v.23 analysis package was used for all statistical tests performed in this study (Field, 2013).

We were unsure how many samples would suffice to reliably identify *Wolbachia* titer differences across conditions. Having collected 18 samples per subject group, we conducted power analysis to determine the smallest number of samples that would likely be needed to reveal a significant difference. To assess the power of different sample sizes, we used a procedure programmed by Dr. Philip K. Stoddard in MATLAB™ (Mathworks, Natick MA) that sampled randomly with replacement from *Wolbachia* titer datasets being compared. The script (Wol_power) tested for titer differences between the control and treatment conditions for each sub-sample set. Sub-samples ranged from

2 to 18 data points, with 10,000 sample iterations per sample size. Significance was assessed in accordance with the normality of data being analyzed, using T-tests (ttest2, with variance settings adjusted to match the data) and Mann-Whitney U (ranksum) (Plonsky, 2015; Preacher & Hayes, 2004). The α-value was set at 0.01, 2-tailed. A summary graphic for each analysis indicates the proportion of significant results obtained for each sub-sample size. This power analysis of reduced datasets informs the level of certainty associated with observed *Wolbachia* titer differences.

2.4 Results

2.4.1 Assay development to detect body-wide *Wolbachia* titer differences in *Drosophila* species

A broad technical goal of this work is to identify treatments that alter whole-body titers, which involves exposing replicate populations of flies to specific feeding conditions for a limited period of time. Standardizing rearing conditions is a prerequisite to carry out large scale screening of whole flies. To this end, we first established a fly rearing protocol in a 24-well plate format. We then tested the optimal number of flies that can survive in each well with minimal food by measuring fly survival time. Two different volumes of food were tested, either 1 mL or 1.5 mL, with wells each carrying 12 flies in these initial tests. These conditions balance the possible benefit of stabilizing food hydration for the larger volumes vs. having more space for movement in the case of lesser food volumes. We found that for both food amounts, at least 6 flies survived in 100% of the wells, at least 7 or more survived in 92% of the wells after 3 days of feeding (Table 2.1). The data suggested that fly survival was somewhat more variable in the 1.5 mL-containing food wells, with an average survival per well of 10.6 flies in the first replicate, and 9.75 flies in the second replicate. By comparison, the average survival per well for the 1.0 mL food condition was 10.25 for the first replicate, and 10.5 for the

second replicate (Table 2.1). In the interest of consistency across experiments, we proceeded with the standard food volume of 1 mL per well, giving the flies ample room to travel within the well.

To determine whether the survival rate improves any further with fewer flies, we placed 10 flies in wells containing 1 mL of food and monitored fly survival rates for up to 5 days. At least nine or more flies survived in 100% of the wells at day 3, while at least seven or more flies survived at day 5 (Table 2.2). The data exhibited an average survival rate per well of 9.58 flies after 3 days of feeding in both replicates. The average survival rate reduced to 8.25 flies per well after 5 days of feeding (Table 2.2). It is noticeable here that many of the flies that died were trapped in the food at the end of the 5-day incubation because larval offspring had gradually made the food media stickier. The outcome from this test indicates certainty that we can get at least 5 flies from each well up to a 5-day range of time as needed. This would be sufficient for qPCR sample preparation. Subsequent experiments were carried out using 3-days feedings in accord with past germline-based feeding assays (Camacho et al., 2017; Serbus et al., 2015). It can be concluded from here that the empirically optimized plate-rearing conditions enable harvest of enough flies to sustain qPCR-based analyses in a consistent, reproducible manner.

2.4.2 Successful amplifications of template DNA as indicated by efficiency testing

Determining qPCR amplification efficiency is crucial when setting up a qPCR assay. Suboptimal quality of template and primers contributes to lower amplification efficiencies, leading to inaccurate data interpretations (Beckmann & Fallon, 2012; Falckenhayn et al., 2016; Sreedharan et al., 2018). To evaluate the extent to which template DNA can accurately be amplified, we conducted a primer efficiency test. The *Wolbachia surface protein* gene (*wsp*), a reference gene for *Wolbachia*, was selected as

a template for the assessment (Chrostek et al., 2013). The primer efficiency test was carried out by diluting the target in series and then obtaining the Ct (Cycle threshold) values through qPCR. The resulting Ct values of the dilution series were 2.9-3.8 cycles apart, which produced a linear regression over the 7 orders of magnitude from undiluted DNA to 1/10^6 dilution (n= 2 experimental replicates r^2 = 0.995-0.999). This resulted in a qPCR efficiency of 98.92%-99.01% (−3.346 ≥ slope ≥ −3.348), regarded as an indicator of accuracy (Robledo et al., 2014) (Fig 2.2). The findings combined indicate that the active amplification of *wsp* gene at various concentrations support the *wsp* quantification as a proxy for *Wolbachia* abundance.

2.4.3 Optimized methodology increases the quality of *wsp* absolute counts

As detection of whole-body titer differences is central to this project, it is imperative to maximize the sensitivity of *wsp* detection by qPCR, which serves as a proxy for *Wolbachia* titer in vivo. The absolute quantification method was used in this assay, in which *wsp* copy number amplified from experimental samples is compared against known concentrations of a plasmid standard (Fig 2.3) (Ant & Sinkins, 2018; Christensen et al., 2016; Frentiu et al., 2010; Specht et al., 2018; Whelan et al., 2003; Zhou & Li, 2016).

To obtain quality-controlled absolute quantification, sample preparation was empirically optimized to maximize resolution of *wsp* abundance. Use of detergent, proteinase K, specific temperatures, ethanol precipitation and a range of sample dilutions were systematically tested (see method for details). Briefly, the data indicated that SDS/Proteinase K based extraction (Goldenberger et al., 1995) is more efficient compared to non-SDS/non-Proteinase K–based extraction (Fig 2.4). Incubation of homogenized fly samples, in combination with SDS and proteinase K, at 56°C resulted in median 3-8-fold increase in *wsp* detection. Ethanol-based precipitation further

increased the *wsp* abundance 1.14-1.28-fold compared to the non-precipitated samples. Combining all these conditions, we were able to amplify *wsp* from non-diluted DNA samples, which was previously unattainable due to sample contaminants and thus, obtain significantly higher *wsp* absolute count compared to serially diluted DNA samples. The outcome indicates that the optimized methodology substantially improved DNA quality, formalized by increased sensitivity of *wsp* detection. Consequently, this increased resolution enhances our ability to identify future treatments that alter *wsp* counts as a reflection of *Wolbachia* titer change in vivo (Fig 2.3).

2.4.4 Evaluation of the optimized qPCR assay to facilitate correct interpretation of system-wide analysis.

Although the methodology for sample preparation is now well optimized, testing for material loss during DNA isolation is also critical for accurate data interpretation. To test the extent of DNA retention during sample preparation, we used spike-in controls for the assay. For that, fly samples were spiked with bacteriophage lambda DNA prior to extraction, along with a set of controls in parallel. Technically, if DNA was accurately retained throughout the extraction process, both the control and experimental samples should have similar Ct values. Loss of DNA would be indicated by higher Ct values. Our first replicate produced an average Ct values of 15.15 for the experimental samples, which was not significantly distinct from the controls with an average Ct of 15.25 (t-test $p = 0.335$, $n = 9$) (Fig 2.5A). Comparable finding was obtained for the second replicate as well (t-test $p = 0.749$, $n = 9$) (Fig 2.5A). Both replicates achieve an efficiency of 100%-107% for DNA retention. As such, the data suggest that we do not inadvertently lose sample content during the extraction process.

2.4.5 Specificity of *wsp* amplification has been confirmed through the optimized absolute qPCR methods.

Having optimized detection of the *Wolbachia wsp* gene by qPCR, we next turned to direct demonstration of *Wolbachia* detection by qPCR. Though uncommon for qPCR experiments, demonstration of positive and negative controls is a standard for publishing Western blots in scientific literature (Sheehan et al., 2016; Tamarozzi et al., 2014). To validate the fact that body-wide *wsp* amplification by qPCR corresponds to *Wolbachia* infection, specificity of template amplification was tested by examining the abundance of *wsp* from fly stocks previously confirmed as *Wolbachia* (−) and *Wolbachia* (+) through staining and microbiome profiling (Christensen et al., 2019). Real-time qPCR was able to amplify *wsp* gene only from the *Wolbachia* (+) samples. No *wsp* signal was detected from *Wolbachia* (−) control samples (n = 18, $p \leq 0.001$ with appropriate statistics based on each experimental data type) (Fig 2.6A). To verify that the DNA was not unintentionally lost from *Wolbachia* (−) samples, we performed qPCR on the host fruit fly gene *rpl32,* as a loading control for the assay. We detected successful amplifications of *rpl32* from both *Wolbachia* (−) and *Wolbachia* (+) samples (Fig 2.6A).

Sub-sampling of the data further supports the certainty of these interpretations. For sub-sampling comparisons of *wsp* abundance between uninfected and *Wolbachia*-infected conditions (Fig 2.6B), significance was achieved at an α-value of 0.01 by analyzing as few as 3 samples. This outcome confirms that the differential abundance of *wsp* across conditions shown is not an artifact of technical handling errors, but instead provides accurate as well as sensitive detection of *Wolbachia* infection from whole insect samples.

2.5 Discussion

The development of an assay to determine absolute *Wolbachia* count is a reasonable initial step towards investigating *Wolbachia* colonization in insect hosts. Technical limits on resolution of *Wolbachia* titer have impeded comparative analyses of body-wide colonization to date. The methods presented here represent a major advance in providing clear detection of *Wolbachia* abundance from whole body samples. We believe that this methodology provides systematic internal controls for consistent and accurate *Wolbachia* quantification across a range of conditions, including dietary manipulations, drug treatments and genetic function disruption experiments. In enabling pursuit of mechanistic hypotheses, this methodology opens the possibility of modeling host colonization as an integrated process.

In this report, we have described development of a plate-based platform to raise flies with the ultimate goal of unraveling body-wide *Wolbachia* abundance, and further validating it as an effective and economical tool for large-scale fly screening in future. While plate-based assays are still not commonly reported for adult insects, a few researchers have taken a similar approach to raise flies for various purposes (Markstein et al., 2014; Seong et al., 2020; Willoughby et al., 2013). Our approach was somewhat distinct in that our assay involves manual distribution of food and flies in individual wells, and the female flies are collected from each well after a certain period followed by processing for qPCR. Therefore, the time required to sort and disperse *Drosophila* manually in 24 well plate format is the first and minor limiting factor for our method. This can be quickly circumvented with adequate practice. Secondly, manual homogenization of flies from individual wells takes time and can trigger technical errors, although regular practice and future use of an automatic homogenizer can solve this bottleneck.

This study also documented the use of absolute qPCR to quantify body-wide *Wolbachia*. Relative qPCR has been used during the past decade to determine *Wolbachia* amount in this field (Chrostek et al., 2013; Herran et al., 2020; Voronin et al., 2012). This approach describes *Wolbachia* density in terms of a ratio of a *Wolbachia*-specific gene, such as *wsp*, versus a host reference gene. The method assumes that the abundance of host gene remains stable. The reason for this assumption is likely because after insect growth during larval stages, adult body size does not change much afterwards (Church & Robertson, 1966). However, the external appearance of constancy prevents visibility of dynamic processes ongoing in the host that could potentially change abundance measurements of reference genes that occur during such analyses. An example of how host reference gene abundance changes across conditions is described in Chapter III. The absolute-count methodology we present can be generalized to any treatment condition in future studies.

It is well known in microbial research that the quality of DNA extraction is closely linked to the efficiency of microorganism detection. In low biomass samples, such as fruit fly collections, requirements for higher DNA concentrations and DNA quality are especially important. In this regard, selecting the appropriate protocol for DNA extraction ensures the consistency of DNA quality as well as efficient retrieval of *Wolbachia*-derived template present in the sample. In accord with prior studies, we infer that the best practice to improve identification of *Wolbachia* by absolute qPCR is to apply sodium dodecyl sulfate (SDS) and proteinase K to fly samples (Goldenberger et al., 1995; Natarajan et al., 2016; Qamar et al., 2017), further enhanced with ethanol precipitation (Lever et al., 2015). The empirical data yielded by these procedural modifications speak to the benefit of improved *Wolbachia* detection.

It is important to note that DNA extraction and amplification can differ considerably between host taxa, tissue and endosymbiont type (Thonar et al., 2012; Tkacz et al., 2018). The results may also be affected by the degradation of DNA during sampling, transportation and other processes as well as by inhibitory contaminants in the samples. The presence of PCR inhibitors in tissues has previously been reported to hinder *Wolbachia* detection in mosquito *Culex pipientis,* for example (Beckmann & Fallon, 2012). It is therefore crucial to test for DNA retention of the experimental samples. We attempted to use a bacteriophage lambda spike-in control to assess DNA extraction efficiency with the optimized protocol. Our initial attempt resulted in DNA retention yield of 100%-107%, as indicated by the comparable Ct values of the experimental samples (with fly tissue) compared to the control (without fly tissue). This supports the efficacy of the validated protocol to obtain maximum resolution of *Wolbachia* and therefore, provides the ability to accurately interpret data in comparative analyses. For example, the data presented in Fig 2.6, when extrapolated back to source material, would appear to imply that individual flies raised on control food carry approximately 36 million *Wolbachia*.

Altogether, our optimized sample preparation approach maximized the detection of *Wolbachia* by real-time qPCR. Successful amplification of the *wsp* gene in *Wolbachia*-infected samples over uninfected samples demonstrated the target-specific sensitivity of the optimized protocol. The average absolute *wsp* copy numbers per fly notably varies somewhat across replicates. This is in agreement with published findings that body-wide *Wolbachia* titer can vary up to 180,000-fold and 20,000-fold in mosquitoes and wild-caught *Drosophila innubila*, respectively (Ahantarig et al., 2008; Unckless et al., 2009). Environmental as well as internal factors might drive such titer variation, and the mechanistic basis for this variation is not yet understood.

This experimental methodology was also presented here with a thorough and systematic approach to statistical analysis. Experimental data has been traditionally accompanied by power analysis, informing the sufficiency of sample size. As our datasets are frequently non-parametric, existing power analysis approaches would not yield accurate results. We outlined a methodology for selecting appropriate statistical tests and then applying sub-sampling analyses to empirically determine appropriate sample sizes. An α-value of 0.05 is considered standard in many disciplines as sufficient to reject the null hypothesis and conclude that there is a statistically meaningful difference between comparison groups (Morrison & Henkel, 2006; Skipper et al., 1967). However, sub-sampling data to identify the "n" required for significance at $\alpha = 0.01$ further informs the scale of the differences observed between conditions, such as between *Wolbachia*-infected vs. uninfected samples. This analysis showed that a minimum sample size of 3 is required for significance in comparing those groups. However, going forward, sub-sampling analyses need to be repeated to determine appropriate sample size when testing the effects of host diet, specific small molecules, and host genetic changes. This will help ensure that interpretations are based on reliable and repeatable effects of host processes on *Wolbachia* titer, and avoid artifacts due to misinterpretation of statistical certainty.

To summarize, the sample preparation methods presented here can be generally applied to other fundamental studies of *Wolbachia* titer control in vivo. One area of interest is determining the absolute number of *Wolbachia* retained by other *Wolbachia*/host strain combinations, and testing for conserved sensitivity to candidate host mechanisms. This method can also be used to process data from drug- and RNAi-based screens that investigate host effects on *Wolbachia* titer, as well as to investigate the basis for generalized variation in *Wolbachia* levels in vivo. We believe that our

methodology will translate readily across *Wolbachia*/host combinations and may be adapted to other insect tissues or possibly endosymbiont/host models.

Figures and Tables

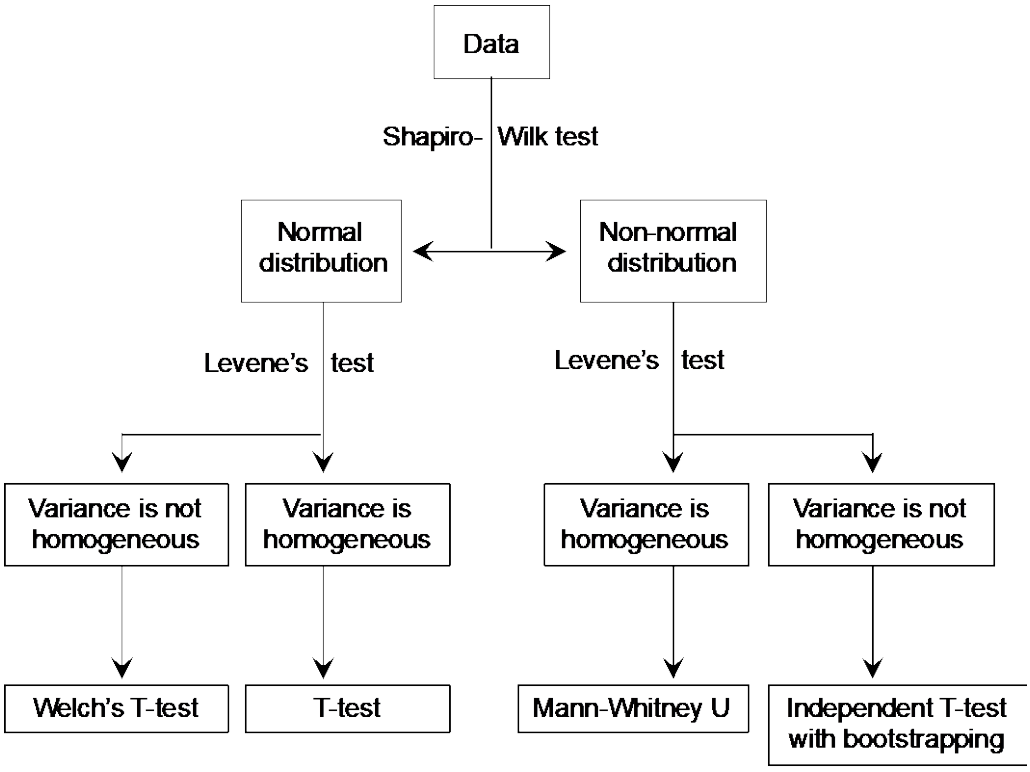

Fig. 2.1. Selection of statistical methods for pairwise data comparisons. Data normality was assessed by the Shapiro-Wilk test, and homogeneity of variance was assessed by Levene's test. Statistical analyses were subsequently performed were directed by the outcome of those tests as outlined here.

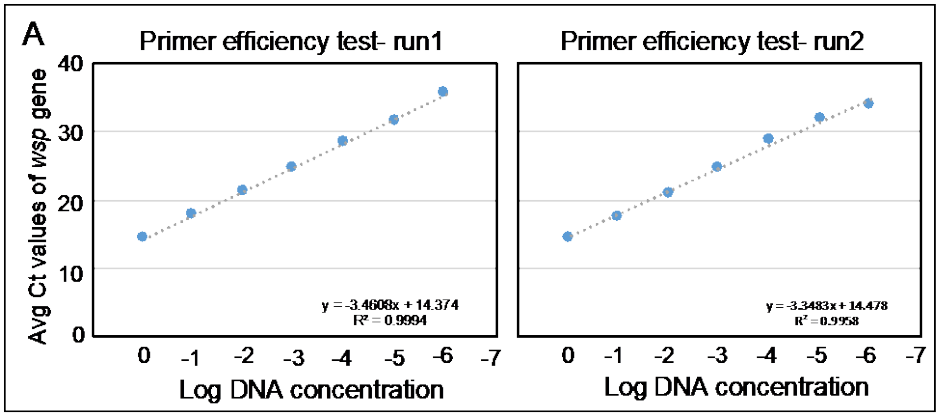

Fig 2.2. Primer efficiency test. Data shows *wsp* amplification of serially diluted samples, as obtained by qPCR. Cycle threshold (Ct) values of DNA samples, which had 1:10 dilution covering 7 dilution points are plotted. The X-axis represents the log concentration of those diluted DNA samples. The Y-axis represents the Ct values of each serial dilutions. Each dot represents the average of 3 amplifications per dilution. This test was run in 2 separate biological replicates.

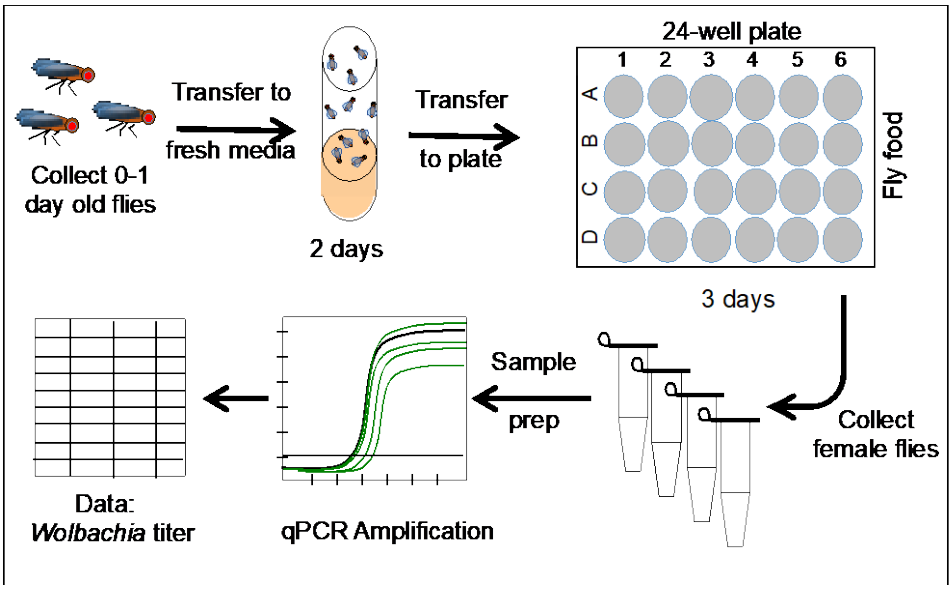

Fig. 2.3. Approach used for real-time quantitative PCR analysis of *Wolbachia* titer in whole *D. melanogaster* flies. The workflow used for fly preparation, sample preparation and qPCR analysis are shown. See Methods for further details.

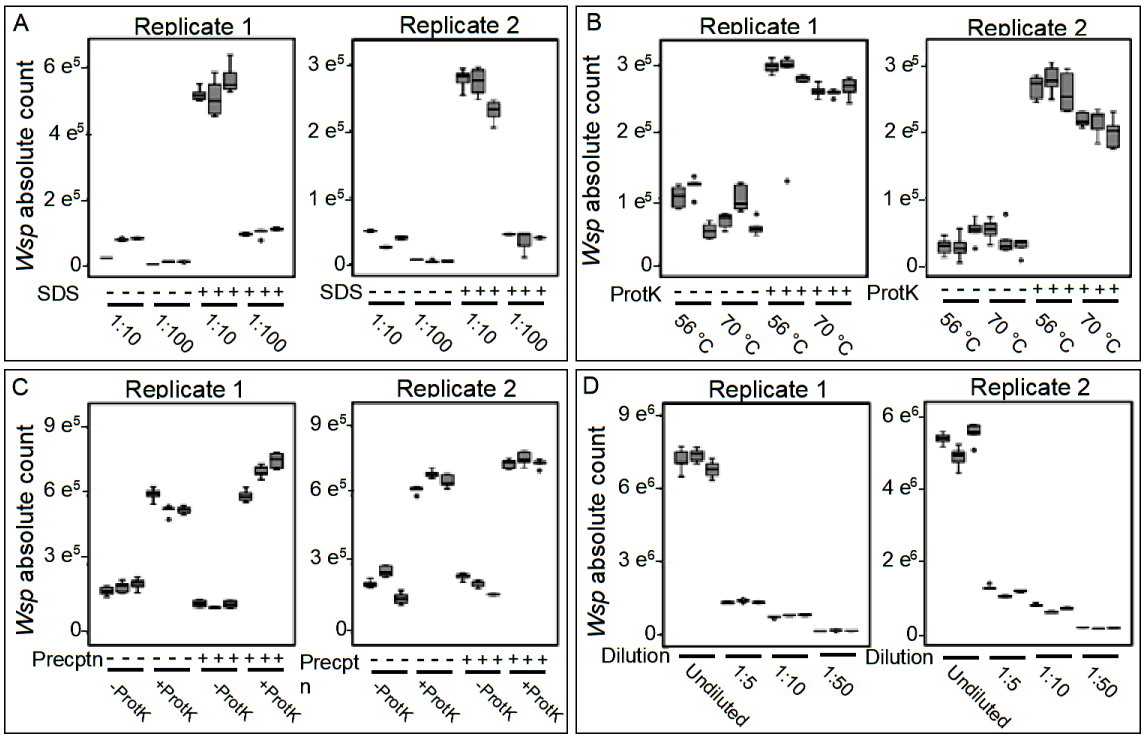

Fig 2.4. Optimization of sample prep for determining absolute counts by real-time qPCR. Systematic testing was performed to optimize A) use of SDS, B) proteinase K and incubation temperature, C) ethanol precipitation, and D) sample dilution. n = 18 for each boxplot shown, representing 6 technical replicates from each of 3 sample tubes.

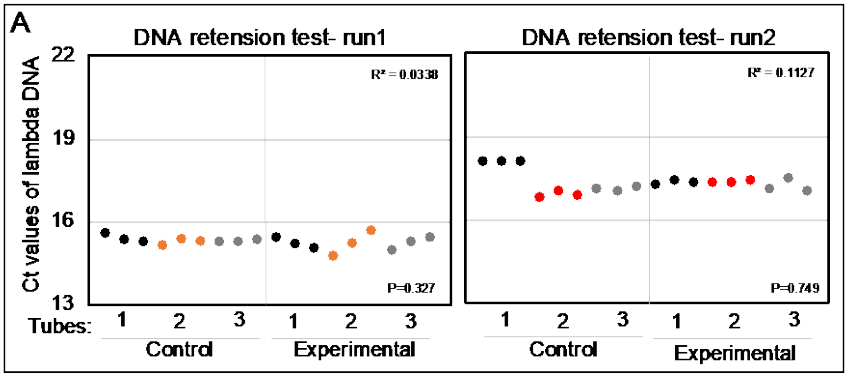

Fig. 2.5. Evaluation of qPCR assay using bacteriophage lambda spiking DNA. Lambda DNA amplifications as indicated by cycle threshold (Ct) values. Data represents λ-DNA amplifications from control samples and experimental samples A) DNA retention test: spiked samples pre-extraction. “n” represents 9 technical replicates from each of 3 sample tubes.

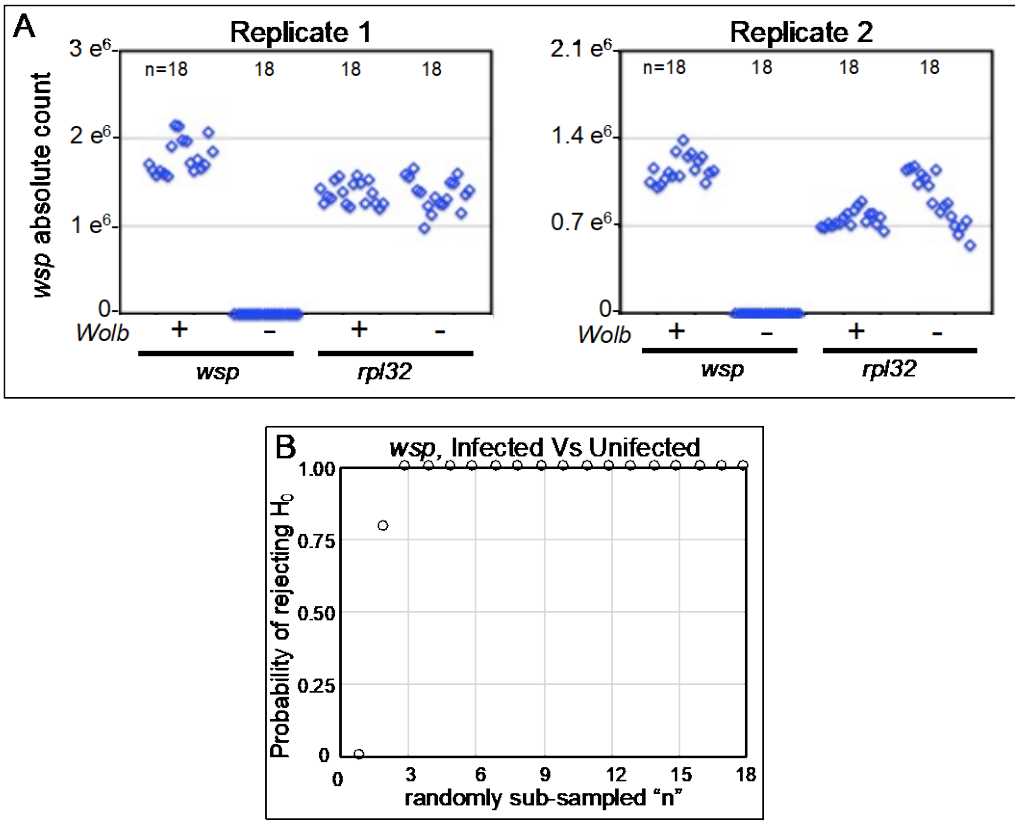

Fig. 2.6. Absolute *wsp* abundance as indicated by real-time qPCR. Data from each sample/well represent 5 female flies. A.) Validation that body-wide *wsp* amplification by qPCR corresponds to *Wolbachia* infection. "n" represents 6 technical replicates from each of 3 sample tubes. B) Comparisons of randomly sub-sampled data from *Wolbachia* (−) and *Wolbachia* (+) conditions when performing analysis of *wsp* copy number, with alpha set at 0.01, n = 18. Power analyses represent copy numbers from experimental replicate 2, test used Welch's T-test. Data from replicate 1 could not be analyzed due to the requirement of Independent bootstrap t-test.

Number of flies survived in each well after 3-days of feeding, replicate#1		
# Well	Amount of food in each well	
	1.5 mL	1 mL
Well 1	10	12
Well 2	12	10
Well 3	12	10
Well 4	9	12
Well 5	11	7
Well 6	8	11
Well 7	11	8
Well 8	12	11
Well 9	12	10
Well 10	8	10
Well 11	10	12
Well 12	12	10
Average	10.58	10.25
Standard deviation	1.56	1.54

Number of flies survived in each well after 3-days of feeding, replicate#2		
# Well	Amount of food in each well	
	1.5 mL	1 mL
Well 1	8	12
Well 2	12	9
Well 3	9	11
Well 4	10	10
Well 5	9	10
Well 6	10	11
Well 7	10	11
Well 8	9	11
Well 9	11	9
Well 10	12	10
Well 11	7	12
Well 12	10	10
Average	9.75	10.50
Standard deviation	1.48	1.00

Table 2.1 Testing the amount of food and number of flies that survive in a 24-well plate format: 12 wells were prepared per food amount, with each carrying 12 flies. The survival rate was recorded after 3-days of feeding in the plate assay format. The experiment was run in 2 biological plate replicates. The average number of surviving flies, and the standard deviation per food amount are displayed at the bottom of each column.

	Number of flies survived in each well, replicate#1				
# Well	Day 1	Day 2	Day 3	Day 4	Day 5
Well 1	10	10	10	9	9
Well 2	10	10	9	9	8
Well 3	10	10	10	10	9
Well 4	10	10	10	9	7
Well 5	10	10	9	9	9
Well 6	10	10	9	9	7
Well 7	10	10	10	10	9
Well 8	10	10	10	10	9
Well 9	10	10	9	9	8
Well 10	10	10	9	7	7
Well 11	10	10	10	10	9
Well 12	10	10	10	8	8
Average	10.00	10.00	9.58	9.08	8.25
Standard deviation	0.00	0.00	0.51	0.90	0.87

	Number of flies survived in each well, replicate#2				
# Well	Day 1	Day 2	Day 3	Day 4	Day 5
Well 1	10	10	10	9	8
Well 2	10	10	10	10	7
Well 3	10	10	9	9	8
Well 4	10	10	10	10	8
Well 5	10	10	10	9	9
Well 6	10	10	9	9	9
Well 7	10	10	9	9	9
Well 8	10	10	10	9	9
Well 9	10	10	9	9	8
Well 10	10	10	10	10	9
Well 11	10	10	10	10	8
Well 12	10	10	9	7	7
Average	10.00	10.00	9.58	9.17	8.25
Standard deviation	0.00	0.00	0.51	0.83	0.75

Table 2.2 Monitoring fly survival rate up to 5 days in a 24-well plate format: Each well contains 1 mL of standard food each carrying 10 flies. The survival rate was recorded for 5 days in the plate-based assay. The experiment was run in 2 biological plate replicates. The average number of flies survived and standard deviation per day are indicated at the bottom of each column.

References

Ahantarig, A., Trinachartvanit, W., & Kittayapong, P. (2008). Relative *Wolbachia* density of field-collected *Aedes albopictus* mosquitoes in Thailand. *Journal of Vector Ecology: Journal of the Society for Vector Ecology*, *33*(1), 173–177.

Ant, T. H., & Sinkins, S. P. (2018). A *Wolbachia* triple-strain infection generates self-incompatibility in *Aedes albopictus* and transmission instability in *Aedes aegypti*. *Parasites & Vectors*, *11*. https://doi.org/10.1186/s13071-018-2870-0

Beckmann, J. F., & Fallon, A. M. (2012). Decapitation improves detection of *Wolbachia pipientis* (*Rickettsiales: Anaplasmataceae*) in *Culex pipiens* (*Diptera: Culicidae)* mosquitoes by the polymerase chain reaction. *Journal of Medical Entomology*, *49*(5), 1103–1108.

Bourtzis, K., & Miller, T. A. (2003). Insect Symbiosis. CRC Press.

Boyle, L., O'Neill, S. L., Robertson, H. M., & Karr, T. L. (1993). Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science (New York, N.Y.)*, *260*(5115), 1796–1799.

Camacho, M., Oliva, M., & Serbus, L. R. (2017). Dietary saccharides and sweet tastants have differential effects on colonization of *Drosophila* oocytes by *Wolbachia* endosymbionts. *Biology Open*, *6*(7), 1074–1083. https://doi.org/10.1242/bio.023895

Caragata, E. P., Dutra, H. L. C., & Moreira, L. A. (2016). Inhibition of Zika virus by *Wolbachia* in *Aedes aegypti*. *Microbial Cell (Graz, Austria)*, *3*(7), 293–295. https://doi.org/10.15698/mic2016.07.513

Casper-Lindley, C., Kimura, S., Saxton, D. S., Essaw, Y., Simpson, I., Tan, V., & Sullivan, W. (2011). Rapid fluorescence-based screening for *Wolbachia* endosymbionts in *Drosophila* germ line and somatic tissues ▿. *Applied and Environmental Microbiology*, *77*(14), 4788–4794. https://doi.org/10.1128/AEM.00215-11

Christensen, S., Camacho, M., Sharmin, Z., Momtaz, A. J. M. Z., Perez, L., Navarro, G., Triana, J., Samarah, H., Turelli, M., & Serbus, L. R. (2019). Quantitative methods for assessing local and bodywide contributions to *Wolbachia* titer in maternal germline cells of *Drosophila*. *BMC Microbiology*, *19*. https://doi.org/10.1186/s12866-019-1579-3

Christensen, S., Pérez Dulzaides, R., Hedrick, V. E., Momtaz, A. J. M. Z., Nakayasu, E. S., Paul, L. N., & Serbus, L. R. (2016). *Wolbachia* endosymbionts modify *Drosophila* ovary protein levels in a context-dependent manner. *Applied and Environmental Microbiology*, *82*(17), 5354–5363. https://doi.org/10.1128/AEM.01255-16

Chrostek, E., Marialva, M. S. P., Esteves, S. S., Weinert, L. A., Martinez, J., Jiggins, F. M., & Teixeira, L. (2013). Wolbachia variants induce differential protection to viruses in *Drosophila melanogaster*: A phenotypic and phylogenomic analysis. *PLoS Genetics*, *9*(12), e1003896. https://doi.org/10.1371/journal.pgen.1003896

Church, R. B., & Robertson, F. W. (1966). A biochemical study of the growth of *Drosophila melanogaster*. *Journal of Experimental Zoology*, *162*(3), 337–351. https://doi.org/10.1002/jez.1401620309

de Cuevas, M., & Spradling, A. C. (1998). Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification. *Development (Cambridge, England)*, *125*(15), 2781–2789.

Duarte, E. H., Carvalho, A., López-Madrigal, S., & Teixeira, L. (2020). Regulation of *Wolbachia* proliferation by the amplification and deletion of an addictive genomic island. *BioRxiv*, 2020.09.08.288217. https://doi.org/10.1101/2020.09.08.288217

Falckenhayn, C., Carneiro, V. C., de Mendonça Amarante, A., Schmid, K., Hanna, K., Kang, S., Helm, M., Dimopoulos, G., Fantappié, M. R., & Lyko, F. (2016). Comprehensive DNA methylation analysis of the *Aedes aegypti* genome. *Scientific Reports*, *6*, 36444. https://doi.org/10.1038/srep36444

Field, A. (2013). Discovering Statistics Using IBM SPSS Statistics, 4th Edition (4th edition). SAGE Publications Ltd.

Frentiu, F. D., Robinson, J., Young, P. R., McGraw, E. A., & O'Neill, S. L. (2010). *Wolbachia*-mediated resistance to Dengue virus infection and death at the cellular Level. *PLoS ONE*, *5*(10). https://doi.org/10.1371/journal.pone.0013398

Goldenberger, D., Perschil, I., Ritzler, M., & Altwegg, M. (1995). A simple "universal" DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. *PCR Methods and Applications*, *4*(6), 368–370.

Grobler, Y., Yun, C. Y., Kahler, D. J., Bergman, C. M., Lee, H., Oliver, B., & Lehmann, R. (2018). Whole genome screen reveals a novel relationship between *Wolbachia* levels and *Drosophila* host translation. *PLoS Pathogens*, *14*(11), e1007445. https://doi.org/10.1371/journal.ppat.1007445

Hadfield, S. J., & Axton, J. M. (1999). Germ cells colonized by endosymbiotic bacteria. *Nature*, *402*(6761), 482. https://doi.org/10.1038/45002

Hedges, L. M., Brownlie, J. C., O'Neill, S. L., & Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science (New York, N.Y.)*, *322*(5902), 702. https://doi.org/10.1126/science.1162418

Herran, B., Geniez, S., Delaunay, C., Raimond, M., Lesobre, J., Bertaux, J., Slatko, B., & Grève, P. (2020). The shutting down of the insulin pathway: A developmental window for *Wolbachia* load and feminization. *Scientific Reports*, *10*(1), 10551. https://doi.org/10.1038/s41598-020-67428-1

Hertig, M. (1936). The *Rickettsia, Wolbachia pipientis* (gen. Et sp.n.) and associated inclusions of the mosquito, *Culex pipiens*. *Parasitology*, *28*(4), 453–486. https://doi.org/10.1017/S0031182000022666

Hertig, M., & Wolbach, S. B. (1924). Studies on *Rickettsia*-like micro-organisms in insects. *The Journal of Medical Research*, *44*(3), 329-374.7.

Hurk, A. F. van den, Hall-Mendelin, S., Pyke, A. T., Frentiu, F. D., McElroy, K., Day, A., Higgs, S., & O'Neill, S. L. (2012). Impact of *Wolbachia* on infection with *Chikungunya* and yellow fever viruses in the mosquito vector *Aedes aegypti*. *PLOS Neglected Tropical Diseases*, *6*(11), e1892. https://doi.org/10.1371/journal.pntd.0001892

Hurst, G. D. D., Jiggins, F. M., Hinrich Graf von der Schulenburg, J., Bertrand, D., West, S. A., Goriacheva, I. I., Zakharov, I. A., Werren, J. H., Stouthamer, R., & Majerus, M. E. N. (1999). Male–killing *Wolbachia* in two species of insect. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *266*(1420), 735–740. https://doi.org/10.1098/rspb.1999.0698

Kikuchi, Y. (2009). Endosymbiotic bacteria in insects: Their diversity and culturability. *Microbes and Environments*, *24*(3), 195–204. https://doi.org/10.1264/jsme2.me09140s

Landmann, F. (2019). The *Wolbachia* endosymbionts. *Microbiology Spectrum*, *7*(2). https://doi.org/10.1128/microbiolspec.BAI-0018-2019

Lever, M. A., Torti, A., Eickenbusch, P., Michaud, A. B., Šantl-Temkiv, T., & Jørgensen, B. B. (2015). A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.00476

Lim, T.-S., & Loh, W.-Y. (1996). A comparison of tests of equality of variances. *Computational Statistics & Data Analysis*, *22*(3), 287–301. https://doi.org/10.1016/0167-9473(95)00054-2

Lu, Y., Miao, S., Wang, Z., & Wang, S. (2019). Prevalence of *Wolbachia* in 10 *Tenebrionidae* stored-product insects and spatiotemporal infection dynamics in *Tribolium confusum* (Jaquelin Du Val) (*Coleoptera: Tenebrionidae)*. *Grain & Oil Science and Technology*, *2*(4), 85–90. https://doi.org/10.1016/j.gaost.2019.11.002

Makepeace, B. L., Rodgers, L., & Trees, A. J. (2006). Rate of elimination of *Wolbachia pipientis* by Doxycycline in vitro increases following drug withdrawal. *Antimicrobial Agents and Chemotherapy*, *50*(3), 922–927. https://doi.org/10.1128/AAC.50.3.922-927.2006

Margulis, U. of M. A. M. L., Margulis, L., & Fester, R. (1991). Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis. MIT Press.

Markstein, M., Dettorre, S., Cho, J., Neumuller, R. A., Craig-Muller, S., & Perrimon, N. (2014). Systematic screen of chemotherapeutics in *Drosophila* stem cell tumors. *Proceedings of the National Academy of Sciences*, *111*(12), 4530–4535. https://doi.org/10.1073/pnas.1401160111

Mee, P. T., Weeks, A. R., Walker, P. J., Hoffmann, A. A., & Duchemin, J.-B. (2015). Detection of low-level *Cardinium* and *Wolbachia* Infections in Culicoides. *Applied and Environmental Microbiology*, *81*(18), 6177–6188. https://doi.org/10.1128/AEM.01239-15

Mohd Razali, N., & Yap, B. (2011). Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *J. Stat. Model. Analytics*, *2*.

Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G., Pyke, A. T., Hedges, L. M., Rocha, B. C., Hall-Mendelin, S., Day, A., Riegler, M., Hugo, L. E., Johnson, K. N., Kay, B. H., McGraw, E. A., van den Hurk, A. F., Ryan, P. A., & O'Neill, S. L. (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and Plasmodium. *Cell*, *139*(7), 1268–1278. https://doi.org/10.1016/j.cell.2009.11.042

Morrison, D. E., & Henkel, R. E. (2006). The significance test controversy: A reader. Transaction Publishers.

Natarajan, V. P., Zhang, X., Morono, Y., Inagaki, F., & Wang, F. (2016). A Modified SDS-based DNA extraction method for high quality environmental DNA from seafloor environments. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00986

Newton, I. L. G., Savytskyy, O., & Sheehan, K. B. (2015). *Wolbachia* utilize host actin for efficient maternal transmission in *Drosophila melanogaster*. *PLoS Pathogens*, *11*(4). https://doi.org/10.1371/journal.ppat.1004798

Plonsky, L. (2015). Advancing Quantitative Methods in Second Language Research. Routledge.

Preacher, K. J., & Hayes, A. F. (2004). SPSS and SAS procedures for estimating indirect effects in simple mediation models. *Behavior Research Methods, Instruments, & Computers*, *36*(4), 717–731. https://doi.org/10.3758/BF03206553

Punkosdy, G. A., Addiss, D. G., & Lammie, P. J. (2003). Characterization of Antibody Responses to *Wolbachia* surface protein in humans with lymphatic filariasis. *Infection and Immunity*, *71*(9), 5104–5114. https://doi.org/10.1128/IAI.71.9.5104-5114.2003

Qamar, W., Khan, M. R., & Arafah, A. (2017). Optimization of conditions to extract high quality DNA for PCR analysis from whole blood using SDS-proteinase K method. *Saudi Journal of Biological Sciences*, *24*(7), 1465–1469. https://doi.org/10.1016/j.sjbs.2016.09.016

Rietveld, T., & van Hout, R. (2015). The t test and beyond: Recommendations for testing the central tendencies of two independent samples in research on speech, language and hearing pathology. *Journal of Communication Disorders*, *58*, 158–168. https://doi.org/10.1016/j.jcomdis.2015.08.002

Robledo, D., Hernández-Urcera, J., Cal, R. M., Pardo, B. G., Sánchez, L., Martínez, P., & Viñas, A. (2014). Analysis of qPCR reference gene stability determination methods and a practical approach for efficiency calculation on a turbot (*Scophthalmus maximus*) gonad dataset. *BMC Genomics*, *15*, 648. https://doi.org/10.1186/1471-2164-15-648

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P., & Solignac, M. (1992). *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings. Biological Sciences*, *250*(1328), 91–98. https://doi.org/10.1098/rspb.1992.0135

Saint André, A. v, Blackwell, N. M., Hall, L. R., Hoerauf, A., Brattig, N. W., Volkmann, L., Taylor, M. J., Ford, L., Hise, A. G., Lass, J. H., Diaconu, E., & Pearlman, E. (2002). The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. *Science (New York, N.Y.)*, *295*(5561), 1892–1895. https://doi.org/10.1126/science.1068732

Saridaki, A., & Bourtzis, K. (2010). *Wolbachia*: More than just a bug in insects' genitals. *Current Opinion in Microbiology*, *13*(1), 67–72. https://doi.org/10.1016/j.mib.2009.11.005

Sazama, E. J., Bosch, M. J., Shouldis, C. S., Ouellette, S. P., & Wesner, J. S. (2017). Incidence of *Wolbachia* in aquatic insects. *Ecology and Evolution*, *7*(4), 1165–1169. https://doi.org/10.1002/ece3.2742

Sazama, E. J., Ouellette, S. P., & Wesner, J. S. (2019). Bacterial endosymbionts are common among, but not necessarily within, insect species. *Environmental Entomology*, *48*(1), 127–133. https://doi.org/10.1093/ee/nvy188

Schmidt, T. L., Barton, N. H., Rašić, G., Turley, A. P., Montgomery, B. L., Iturbe-Ormaetxe, I., Cook, P. E., Ryan, P. A., Ritchie, S. A., Hoffmann, A. A., O'Neill, S. L., & Turelli, M. (2017). Local introduction and heterogeneous spatial spread of dengue-suppressing *Wolbachia* through an urban population of *Aedes aegypti*. *PLoS Biology*, *15*(5). https://doi.org/10.1371/journal.pbio.2001894

Seong, K.-H., Matsumura, T., Shimada-Niwa, Y., Niwa, R., & Kang, S. (2020). The Drosophila individual activity monitoring and detection system (DIAMonDS). *ELife*, *9*, e58630. https://doi.org/10.7554/eLife.58630

Serbus, L. R., & Sullivan, W. (2007). A cellular basis for *Wolbachia* recruitment to the host germline. *PLoS Pathogens*, *3*(12). https://doi.org/10.1371/journal.ppat.0030190

Serbus, L. R., White, P. M., Silva, J. P., Rabe, A., Teixeira, L., Albertson, R., & Sullivan, W. (2015). The impact of host diet on *Wolbachia* titer in *Drosophila*. *PLoS Pathogens*, *11*(3). https://doi.org/10.1371/journal.ppat.1004777

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (Complete samples). *Biometrika*, *52*(3/4), 591–611. https://doi.org/10.2307/2333709

Sheehan, K. B., Martin, M., Lesser, C. F., Isberg, R. R., & Newton, I. L. G. (2016). Identification and characterization of a candidate *Wolbachia pipientis* Type IV Effector That Interacts with the Actin Cytoskeleton. *MBio*, *7*(4). https://doi.org/10.1128/mBio.00622-16

Simoncini, L., Casiraghi, M., Bazzocchi, C., Sacchi, L., Bandi, C., & Genchi, C. (2001). Real-time PCR for quantification of the bacterial endosymbionts (*Wolbachia*) of filarial nematodes. *Parassitologia*, *43*(4), 173–178.

Skipper, J. K., Guenther, A. L., & Nass, G. (1967). The Sacredness of .05: A Note concerning the Uses of Statistical Levels of Significance in Social Science. *The American Sociologist*, *2*(1), 16–18.

Specht, S., Pfarr, K. M., Arriens, S., Hübner, M. P., Klarmann-Schulz, U., Koschel, M., Sternberg, S., Martin, C., Ford, L., Taylor, M. J., & Hoerauf, A. (2018). Combinations of registered drugs reduce treatment times required to deplete *Wolbachia* in the *Litomosoides sigmodontis* mouse model. *PLoS Neglected Tropical Diseases*, *12*(1). https://doi.org/10.1371/journal.pntd.0006116

Sreedharan, S. P., Kumar, A., & Giridhar, P. (2018). Primer design and amplification efficiencies are crucial for reliability of quantitative PCR studies of caffeine biosynthetic N-methyltransferases in coffee. *3 Biotech*, *8*(11), 467. https://doi.org/10.1007/s13205-018-1487-5

Stouthamer, R., Breeuwer, J. A., & Hurst, G. D. (1999). *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annual Review of Microbiology*, *53*, 71–102. https://doi.org/10.1146/annurev.micro.53.1.71

Tamarozzi, F., Wright, H. L., Thomas, H. B., Edwards, S. W., & Taylor, M. J. (2014). A lack of confirmation with alternative assays questions the validity of IL-17A expression in human neutrophils using immunohistochemistry. *Immunology Letters*, *162*(2, Part B), 194–198. https://doi.org/10.1016/j.imlet.2014.10.025

Taylor, M. J., & Hoerauf, A. (1999). Wolbachia bacteria of filarial nematodes. *Parasitology Today (Personal Ed.)*, *15*(11), 437–442. https://doi.org/10.1016/s0169-4758(99)01533-1

Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, *6*(12). https://doi.org/10.1371/journal.pbio.1000002

Thonar, C., Erb, A., & Jansa, J. (2012). Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communities—Marker design, verification,

calibration and field validation. *Molecular Ecology Resources*, *12*(2), 219–232. https://doi.org/10.1111/j.1755-0998.2011.03086.x

Tkacz, A., Hortala, M., & Poole, P. S. (2018). Absolute quantitation of microbiota abundance in environmental samples. *Microbiome*, *6*(1), 110. https://doi.org/10.1186/s40168-018-0491-7

Townson, S., Hutton, D., Siemienska, J., Hollick, L., Scanlon, T., Tagboto, S. K., & Taylor, M. J. (2000). Antibiotics and *Wolbachia* in filarial nematodes: Antifilarial activity of rifampicin, oxytetracycline and chloramphenicol against *Onchocerca gutturosa, Onchocerca lienalis* and *Brugia pahangi*. *Annals of Tropical Medicine and Parasitology*, *94*(8), 801–816.

Unckless, R. L., Boelio, L. M., Herren, J. K., & Jaenike, J. (2009). *Wolbachia* as populations within individual insects: Causes and consequences of density variation in natural populations. *Proceedings. Biological Sciences*, *276*(1668), 2805–2811. https://doi.org/10.1098/rspb.2009.0287

Vargha, A., & Delaney, H. D. (1998). The Kruskal-Wallis test and stochastic homogeneity. *Journal of Educational and Behavioral Statistics*, *23*(2), 170–192. https://doi.org/10.3102/10769986023002170

Veneti, Z., Clark, M. E., Karr, T. L., Savakis, C., & Bourtzis, K. (2004). Heads or tails: Host-parasite interactions in the *Drosophila-Wolbachia* system. *Applied and Environmental Microbiology*, *70*(9), 5366–5372. https://doi.org/10.1128/AEM.70.9.5366-5372.2004

Voronin, D., Cook, D. A. N., Steven, A., & Taylor, M. J. (2012). Autophagy regulates *Wolbachia* populations across diverse symbiotic associations. *Proceedings of the National Academy of Sciences*, *109*(25), E1638–E1646. https://doi.org/10.1073/pnas.1203519109

Walker, T., Johnson, P. H., Moreira, L. A., Iturbe-Ormaetxe, I., Frentiu, F. D., McMeniman, C. J., Leong, Y. S., Dong, Y., Axford, J., Kriesner, P., Lloyd, A. L., Ritchie, S. A., O'Neill, S. L., & Hoffmann, A. A. (2011). The *wMel Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature*, *476*(7361), 450–453. https://doi.org/10.1038/nature10355

Wasserman, S. (1994). Review of A Handbook for Data Analysis in the Behavioral Sciences; (Vol. 1) Statistical Issues; (Vol. 2) Methodological Issues. [Review of *Review of A Handbook for Data Analysis in the Behavioral Sciences; (Vol. 1) Statistical Issues; (Vol. 2) Methodological Issues.*, by G. Keren & C. Lewis]. *Journal of the American Statistical Association*, *89*(426), 715–717. https://doi.org/10.2307/2290884

Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z., & Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1807), 20150249. https://doi.org/10.1098/rspb.2015.0249

Werren, J. H., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751. https://doi.org/10.1038/nrmicro1969

Whelan, J. A., Russell, N. B., & Whelan, M. A. (2003). A method for the absolute quantification of cDNA using real-time PCR. *Journal of Immunological Methods*, *278*(1–2), 261–269. https://doi.org/10.1016/s0022-1759(03)00223-0

White, P. M., Serbus, L. R., Debec, A., Codina, A., Bray, W., Guichet, A., Lokey, R. S., & Sullivan, W. (2017a). Reliance of *Wolbachia* on high rates of host proteolysis revealed by a genome-wide rnai screen of *Drosophila* Cells. *Genetics*, *205*(4), 1473–1488. https://doi.org/10.1534/genetics.116.198903

Willoughby, L. F., Schlosser, T., Manning, S. A., Parisot, J. P., Street, I. P., Richardson, H. E., Humbert, P. O., & Brumby, A. M. (2013). An in vivo large-scale chemical screening platform using *Drosophila* for anti-cancer drug discovery. *Disease Models & Mechanisms*, *6*(2), 521–529. https://doi.org/10.1242/dmm.009985

Yen, J. H., & Barr, A. R. (1971). New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. *Nature*, *232*(5313), 657–658. https://doi.org/10.1038/232657a0

Zchori-Fein, E., & Bourtzis, K. (2011). *Manipulative Tenants: Bacteria Associated with Arthropods*. CRC Press.

Zha, X., Zhang, W., Zhou, C., Zhang, L., Xiang, Z., & Xia, Q. (2014). Detection and characterization of *Wolbachia* infection in silkworm. *Genetics and Molecular Biology*, *37*(3), 573–580.

Zhou, X.-F., & Li, Z.-X. (2016). Establishment of the cytoplasmic incompatibility-inducing *Wolbachia* strain *wMel* in an important agricultural pest insect. *Scientific Reports*, *6*, 39200. https://doi.org/10.1038/srep39200

CHAPTER III: CHARACTERIZING LOCAL VERSUS BODY-WIDE *WOLBACHIA* ABUNDANCE IN *DROSOPHILA MELANOGASTER*

3.1 Abstract

The bacterial endosymbiont *Wolbachia* can have varying impact on the host, ranging from mutualistic to parasitic. Because of their infection dynamics, *Wolbachia*-insect associations are considered as a great model for investigating molecular mechanisms that regulate endosymbiont density. While the host factors governing germline *Wolbachia* colonization have been investigated in recent years, little is known about how *Wolbachia* are regulated within somatic tissues. Here we examined whether germline titer controlling mechanisms contribute to body-wide response. By making use of absolute qPCR to quantify bacterial abundance within whole body, we demonstrated that germline titer control mechanisms do not necessarily correspond to the whole insect. We found that nutrient-altered host diets, which reduced *Wolbachia* titer in germline, exerted no significant effect on body-wide *Wolbachia* titer. Notably, relative qPCR methods distorted apparent *Wolbachia* abundance, due to altered host DNA copy number in nutrient-altered conditions. This highlights the importance of absolute quantification data for formulating and testing mechanistic hypotheses. Another major outcome from this study is that non-antibiotic, host-targeting compounds alter *Wolbachia* abundance, with a candidate drug depleting *Wolbachia* from both whole body and ovarian tissue samples. This finding suggests that some host functions affect *Wolbachia* in a tissue-specific manner, whereas other functions exert a more generalized impact on *Wolbachia* abundance in vivo.

3.2 Introduction

Heritable symbiosis is widespread in nature and is particularly associated with the insect community (Bennett & Moran, 2015). Many endosymbiotic relationships have

been illustrated as part of insect symbiosis. The interactions between bacteria and insect cells have a significant influence on different physiological functions of the host (Eleftherianos et al., 2013). *Wolbachia* are obligate, intracellular endosymbionts. These gram negative alpha-proteobacteria are pervasive, carried by 52%-60% of terrestrial arthropods, including *Drosophila melanogaster* (Sazama et al., 2017; Weinert et al., 2015). *Wolbachia* live in host cell cytoplasm and are thought to rely upon nutrients from host cells for proliferation and replication (Serbus et al., 2017; Voronin et al., 2019; Wu et al., 2004).

Wolbachia occupy a wide range of the symbiotic spectrum in arthropods, spanning from parasitism to mutualism. *Wolbachia* are commonly referred to as reproductive parasites. Because *Wolbachia* are maternally transmitted, it is in the interest of the bacteria to favor infected females. *Wolbachia* achieve this by inducing cytoplasmic incompatibility, feminization, parthenogenesis and male killing, all of which favor *Wolbachia*-infected females. In other cases, *Wolbachia* are necessary for viability and fertility of the host organisms (Dedeine et al., 2005; Townson et al., 2000). In most host insect types, *Wolbachia* are assumed to act as commensals. The bacteria colonize a diverse set of cells and tissues in *Drosophila,* representing somatic as well as reproductive organs, but the infection shows no obvious adverse effects on the host. The *D. melanogaster* model has the further advantage of a naturally occurring *Wolbachia* infection (Yamaguchi & Yoshida, 2018), positioning it as a prime system for modeling endosymbiont infection (Frydman et al., 2006; Newton & Sheehan, 2015; Pietri et al., 2016; Serbus & Sullivan, 2007; Sheehan et al., 2016).

Wolbachia are transmitted maternally. Their persistence in the germline is crucial for successful propagation. In certain systems, like *D. melanogaster, Wolbachia* utilizes host microtubules and pole plasm to enrich the posterior pole of a late stage oocytes

(Ferree et al., 2005; Serbus & Sullivan, 2007). This concentration gradient at the posterior pole ensures their efficient transmission to the next generation of germline cells, which are destined to form at that same posterior site (Lasko & Ashburner, 1990).

Interestingly, *Wolbachia* have also been reported to show horizontal transmission within and between species (Baldo et al., 2008; Cordaux et al., 2011; Huigens et al., 2000; Raychoudhury et al., 2009; Werren et al., 1995a). Microinjection of *Drosophila* with *Wolbachia*-infected hemolymph demonstrated the propensity of *Wolbachia* to penetrate the somatic stem cell niche and eventually germline cells as well (Frydman et al., 2006). Other examples of horizontal transmission include late-stage invasion of *Drosophila simulans* egg chambers by variant *Wolbachia* strains, as well as *Wolbachia* invasion of uninfected tissue culture cells when cocultured (Casper-Lindley et al., 2011; White et al., 2017b). Once *Wolbachia* are successfully transmitted through these mechanisms, it is not clear how the bacteria are maintained within the body, nor how bacterial density is regulated.

Several studies have documented the importance of different intrinsic host factors on germline *Wolbachia* titer. For instance, disruption of the fly axis determinant gene *gurken*, reduced bacterial load during *Drosophila* oogenesis (Serbus et al., 2011). Cytoskeletal components, such as, actin and microtubules, have also been reported to positively regulate germline titer (Ferree et al., 2005; Newton et al., 2015; Serbus et al., 2011). The significance of host ubiqutin-proteasome pathway has been documented recently in maintaining germline titer. RNAi knockdown of several ubiquitin related genes decreased titer (Grobler et al., 2018; White et al., 2017a), where as, knockdown of proteasomal core components increased titer (Grobler et al., 2018). Host ribosomal components have also been reported to restrict *Wolbachia* titer in the germline (Grobler et al., 2018).

Recent studies have demontrated that external factors, such as host diet, can influence germline *Wolbachia* titer (Serbus et al., 2015). *Drosophila melanogaster* fed with sugars, such as lactose, galactose, trehalose, and maltose, exhibited higher *Wolbachia* titer in germline (Camacho et al., 2017). In contrast, yeast-enriched diets induced a remarkable depletion of oocyte *Wolbachia* titer. Yeast-rich diets are known to trigger release of insulin-like peptides from neural insulin-producing cells (Géminard et al., 2009). Notably, ablation of these insulin-producing cells eliminates yeast-based suppression of germline *Wolbachia* titer (Serbus et al., 2015). Because of this, the current model is that insulin release, triggered by dietary yeast, is responsible for suppressing germline colonization. As insulin release is a systemic response, it is unclear whether a systemic *Wolbachia* titer response also occurred.

Many questions about *Wolbachia* titer regulation remain open: How is *Wolbachia* infection maintained within the whole organism? Is it possible to detect a titer change within a specified time frame? To what extent does germline *Wolbachia* titer represent titer of the entire body? The systematic analyses presented in this chapter address those questions. Here we demonstrated with antibiotic rifampicin, that body-wide *Wolbachia* titer changes are detectable in *D. melanogaster* within a 3-day time frame, using the absolute qPCR assay optimized in Chapter II. Furthermore, absolute quantification of *Wolbachia* enabled tracking of *Wolbachia* titers across whole fly and ovarian samples. The data demonstrated that ovarian *Wolbachia* titers are diet-sensitive, whereas whole-body *Wolbachia* titers are not. Notably, we also found that body-wide as well as germline *Wolbachia* titer changes are responsive to the non-antibiotic compound bortezomib, a known inhibitor of ubiquitin-proteasome system. Technical and statistical validation support the mechanistic insights yielded by this study: the interpretation that host mechanisms affecting germline titer can act locally and/or systemically.

3.3 Methods and materials:

3.3.1 *Drosophila* stocks and maintenance

The study was conducted using *Drosophila melanogaster* of the genotype *w*; *Sp/Cyo*; *Sb/TM6B* carrying endogenous *w*Mel strain (Christensen et al., 2016). The flies were maintained in the Invictus *Drosophila* incubator at 25°C, under a standard 12/12h light-dark cycle. To collect progeny for each experiment, bottles carrying standard food were seeded by parent flies (a mix of both male and female) for 3-days. The adults were then discarded to prevent overcrowding of embryos. After 10 to 12 days, the new offspring emerge, and the “0-day-old” flies are collected. These flies were aged for 2 days on standard food until they were used for experiments.

3.3.2 Fly food preparation

Flies are usually maintained in plastic bottles/vials containing standard fly food media. The recipe is derived from Bloomington stock center as described previously (Christensen et al., 2016) (also see Chapter II).

3.3.2.1 For diet-related experiments:

Each well of “yeast-enriched” food wells in the 24-well plate format represented 0.3 mL heat-inactivated yeast paste stirred into 0.7 µL melted standard food, stirred until homogeneous and smooth. The “un-enriched” food used in parallel represents 0.7 mL melted standard food mixed with 0.3 mL water (Camacho et al., 2017). These food mixtures were prepared in bulk each time, then dispensed into individual wells. The nutritional profile associated with these foods was determined by Medallion labs (Minneapolis, MN) (Christensen et al., 2019). There were 12-wells allocated for “yeast-enriched” food, while the other 12 wells were for “un-enriched” food in the 24-well plate. Once the food is solidified, 7 female flies and 3 males flies were transferred in each well.

3.3.2.2 Chemical food preparation: Plate assay experiments contained 1 mL of fly food per well. For control antibiotic experiments in the plate assay format, 200 µL of DMSO or 10 mM rifampicin-DMSO stock solution were stirred into 20 mL of melted standard food and dispensed into plate wells. This resulted in a final background DMSO concentration of 1% in all wells, and a 100 µM rifampicin in the antibiotic condition. To prepare food with a 1 µM final concentration of bortezomib, 2 µL of a 10 mM bortezomib stock were added in 20 mL of melted standard food. For the standard dose of 100 µM, 100 µL of 10 mM stock were added in 20 mL of food. For the controls, an equivalent amount of DMSO was applied to make the control with similar final concentrations.

3.3.3 DNA extraction and qPCR for whole body *Wolbachia* quantification

For whole body titer measurements, a group of 5 female flies was homogenized in 200 µL of squishing buffer, with addition of 2 µL of 20 mg/mL of proteinase K. The squishing buffer contains 10 mM Tris HCl (pH 8.0), 1 mM EDTA, 25 mM NaCl, and 1% SDS (Christensen et al., 2019). The homogenized mixtures were subsequently incubated in a water bath for 1 hour at 56 °C and heated at 95 °C for 3 mins to inactivate the proteinase K. Samples were then centrifuged at 17,970 x g for 15 minutes at 4°C (Beckman Microfuge 22R). 100 µL of supernatant was collected for ethanol precipitation while carefully avoiding the pellet. For the ethanol-based precipitation, 1/10 volume of 3M Na-acetate and 250 µL of absolute ethanol was added to the 100 µL of supernatant. The mixtures were gently mixed and incubated at -20°C for minimum 1-hour (or, overnight) and centrifuged afterwards at 17,970 x g for 15 minutes at 4°C. The supernatant was discarded very carefully from each tube, and the pellets were washed with 500 µL of 70% ethanol. Samples were re-centrifuged for 15 mins at 4°C using the same settings. Supernatants were removed and the DNA pellets were air dried followed

by resuspension in 100 µL of TE buffer (pH 8.0). DNA suspensions were then directly used for qPCR.

Absolute quantification of *Wolbachia* was carried out using reference plasmid standards that carry a 160 bp PCR-amplified fragment of the *Wolbachia* surface protein *(wsp*) gene (Christensen et al., 2016). Real-time PCR was carried out on a Bio-Rad CFX96 Connect Optics Module Real-Time System and absolute copy numbers for *Wolbachia* were obtained by comparing threshold cycle (Ct) values from *wsp* amplification against a standard curve generated from the plasmid standard, as in (Christensen et al., 2016). The primers used to target *wsp* gene were: Forward 5' CATTGGTGTTGGTGTTGGTG 3', reverse 5' ACCGAAATAACGAGCTCCAG 3', each at a concentration of 5 µM.

To obtain absolute host DNA copy numbers, a separate plasmid standard carrying insert of the *ribosomal protein L32* (*rpl32*) gene sequence was prepared. These plasmids were prepared by cloning a 194 bp fragment of *rpl32* using forward (5′-CCGCTTCAAGGGACAGTATC) and reverse (5′- CAATCTCCTTGCGCTTCTTG) primers. *rpl32* serves as a proxy of *D. melanogaster* host DNA copy number. *rpl32* copy numbers were used to generate the relative *Wolbachia* titer by calculating the ratio of *wsp/rpl32* per condition.

3.3.4 Statistical analysis

Statistical analyses were conducted on all the primary data collect from this study. Appropriate statistics were chosen per data set based on several parameters as described in Chapter II. Briefly, the Shapiro Wilk test was used to check for normality distribution and Levene's test was used to test for homogeneity variance. For normal data showing equal variance, an Independent t-test was used. Welch's t-test was used for data showing equal variance in the absence of a normally distribution. For non-

normal data showing unequal variance, the Mann-Whitney U test was used for statistical comparisons.

To determine sufficient sample size required to detect titer changes, we used the sub-sampling MATLAB program described in Chapter II. After selecting the appropriate type of statistical analysis above, the program performs 10,000 iterations of that analysis on random sub-samplings of the data for each "n" value, starting at n = 2, and ending at the maximal n for the experiment. The proportion of sub-samples meeting the set alpha value is displayed graphically, ultimately showing the sample size required to reach 99.5% certainty or higher for given p-values. For this, analysis, the alpha value was set at 0.01.

3.4 Results

3.4.1 Antibiotic-induced changes in body-wide *Wolbachia* titer are detected by our qPCR amplification approach.

The focal point of this study is to detect *Wolbachia* titer changes in whole host organisms in response to varying treatment conditions. A possible caveat of such analyses is that even if the host is exposed to a bactericidal treatment, endosymbiont loads may be a lagging indicator of that, as it takes time for the host to clear out dead bacteria. To determine whether body-wide qPCR can detect *Wolbachia* titer changes within the time frame of a 3-day feeding assay, we tested whether rifampicin, an antibiotic drug previously shown to target *Wolbachia* (Hermans et al., 2001; Specht et al., 2008; Townson et al., 2000), would reduce *wsp* abundance in *Wolbachia*-infected flies. Female flies were exposed to food supplemented with control DMSO or 100 μM DMSO-solubilized rifampicin in a 24-well plate (Fig 3.1). Absolute *wsp* counts were then determined for 5 female flies per well. The rifampicin-treated condition exhibited 29% of

the *wsp* abundance detected from DMSO control flies ($p \leq 0.001$ as per statistical tests appropriate to each plate replicate) (Fig 3.2A).

To determine whether adequate replication supported this conclusion, data subsets were selected at random and tested for significance. This analysis indicated that *wsp* absolute counts from 4 samples were sufficient to show a significant difference between rifampicin and control conditions (Fig 3.2B). The results show that rifampicin suppresses body-wide *Wolbachia* titer, verifying that the optimized qPCR assay is able to detect body-wide titer changes within the same 3-day treatment timespan used for previous cytological analyses of germline *Wolbachia* titer (Camacho et al., 2017; Christensen et al., 2019; Serbus et al., 2015).

3.4.2 Mechanisms that affect germline *Wolbachia* titer do not necessarily exert comparable effects on whole body *Wolbachia* titer.

Since body-wide titer changes were detectable with antibiotics, we asked whether other treatments, such as, nutrient-altered host diets, can also elicit a whole body titer response. Cytological analyses previously indicated that yeast-enriched host diets dramatically deplete germline *Wolbachia* titer in *Drosophila* (Camacho et al., 2017; Christensen et al., 2019). However, it is unclear whether this represents a local or systemic *Wolbachia* titer response to dietary yeast. To investigate this, we tested the effect of yeast-enriched host diets on body-wide *Wolbachia* titer. Female flies were fed un-enriched or yeast-enriched diets in a 24-well format for 3 days, then absolute *wsp* counts were measured via qPCR. This analysis found no significant difference in *wsp* abundance between un-enriched and yeast-enriched conditions (n = 12 wells per condition, 3 technical replicates per well) (Fig 3.3 A). Sub-sampling analyses indicated less than 25% likelihood of significance, with the α-value set conservatively at 0.01 (Fig 3.3B) (Efron, 1992; Rietveld & van Hout, 2015). Overall, the qPCR data

indicate that dietary yeast does not significantly affect body-wide *Wolbachia* titer. This suggests that the molecular mechanisms governing systemic *Wolbachia* loads are distinct from those that determine *Wolbachia* titer in maternal germline cells.

3.4.3 Absolute quantification of *wsp* by real-time qPCR shows that host diet affects *Wolbachia* distribution within the body

The results described above raise a critical mechanistic question: Why do cytological studies of germline titer show sensitivity to dietary yeast (Christensen et al., 2016), if absolute counts of *Wolbachia* from whole body samples do not? It is known that dietary yeast greatly increases ovary size (Camacho et al., 2017; Géminard et al., 2009; LaFever & Drummond-Barbosa, 2005). Is ovarian *Wolbachia* depletion an artifact of ovary size, with the same number of bacteria spread out within a greater volume? Or does *Wolbachia* depletion from maternal germline cells cause an overall reduction in ovarian titer? To distinguish between these possibilities, we quantified *Wolbachia* titer in whole flies and in dissected ovaries, prepared in parallel from shared populations of flies.

To first identify what represents a sufficient sample size for measuring ovarian *Wolbachia* loads, qPCR analyses were performed on rifampicin-treated ovarian samples. These results were consistent with the plate assay validation experiments performed above. Absolute quantification of *wsp* showed that rifampicin reduced whole body *Wolbachia* titers to 33–41% of the DMSO control (T-test, $p < 0.001$, n = 18) (Fig 3.4 A). Rifampicin effects on ovarian *Wolbachia* titer were even more exaggerated. Rifampicin-treated ovaries, dissected from the same population of flies, showed *Wolbachia* titers at 7–17% of control levels (Welch's T-test, $p < 0.001$, n = 18) (Fig 3.4A). This demonstrates that qPCR analyses of ovarian samples can show *Wolbachia* titer responses to feeding treatments within the 3-day time period of the assay. To confirm how many ovarian sample measurements are required to detect significant differences

across conditions, we carried out the sub-sampling analyses. With α set conservatively at 0.01, the data indicated that absolute *wsp* counts from 8 or more ovarian samples are sufficient to differentiate between rifampicin and control conditions (Fig 3.4B).

To determine how ovarian *Wolbachia* titers respond to a nutrient-altered diet, we next used qPCR to assay *Wolbachia* titer in yeast-fed flies. Consistent with the data above, absolute quantification of *wsp* from whole body samples showed no significant difference between un-enriched and yeast-enriched food conditions (various tests, $p = 0.203–0.265$, $n = 18$) (Fig 3.4C). Sub-sampling analyses confirmed that these conditions had only an 11% to 20% chance of satisfying statistical significance, using the criterion of $p < 0.01$. By contrast, absolute quantification of *wsp* from ovary samples indicated a marked *Wolbachia* depletion in response to dietary yeast. Ovary tissues from yeast-fed flies exhibited 31% to 43% of the *Wolbachia* titer detected in the un-enriched controls (various tests, $p < 0.001$, $n = 18$) (Fig 3.4C). Sub-sampling analyses revealed this outcome as robust, as $n = 6$ would have been sufficient to satisfy the criterion of $p < 0.01$ (Fig 3.4D). Thus, absolute counts indicate that *Wolbachia* titers are low in ovarian tissues of yeast-fed flies, even though whole body *Wolbachia* titers are stable. This suggests that yeast affects *Wolbachia* distribution within the body, through tissue-specific regulation of invasion or bacterial replication, and not by system-wide regulation.

3.4.4 Relative qPCR yields misleading results when assessing host dietary impacts on *Wolbachia* titer

Relative quantification using qPCR has been used to assess *Wolbachia* densities across diverse host systems (Chrostek et al., 2013; LePage et al., 2017; McGarry et al., 2004; Moreira et al., 2009; K. M. Richardson et al., 2019; Serbus et al., 2015). In this approach, *Wolbachia* titer is reported as a ratio of *wsp* versus a host reference gene,

such as *rpl32.* This method implicitly assumes that host DNA copy number remains stable across the conditions being tested. To date, studies using the relative quantification method to assess *Wolbachia* titers in vivo have not directly measured host DNA copy number over time, nor in response to varying experimental conditions. Thus, the assumption of host copy number as a stable baseline in such experiments is unverified.

To test the accuracy of relative quantification in assessment of *Wolbachia* titer*,* we measured the absolute copy number of the host *rpl32* gene in parallel with *wsp,* then calculated *wsp/rpl32* ratios from the absolute counts. In rifampicin control tests, *wsp/rpl32* ratios from rifampicin conditions were 30–36% of the ratios seen for control DMSO in whole body samples (various tests, $p < 0.001–0.043$, $n = 18$) and 9–15% of control DMSO in ovarian samples (various tests, $p < 0.001–0.001$, $n = 18$) (Fig. 3.5A). Thus, for rifampicin-related tests, the results from relative quantification paralleled those from the absolute quantification method (compare Figs 3.4A and 3.5A).

Next, we determined relative *Wolbachia* titer in ovarian samples under nutrient altered conditions. *wsp/rpl32* ratios were analyzed from fly ovaries exposed to either a yeast-enriched diet or an un-enriched diet. *wsp/rpl32* values were significantly lower in yeast-fed ovaries, exhibiting 31– 43% of the ratios observed in control ovaries (Welch's T-test, $p < 0.001$, $n = 18$) (Fig 3.5B). Thus, relative and absolute quantification methods so far indicated similar titer-reducing effects at the ovary level upon yeast treatment (compare Figs 3.4C and 3.5B). Sub-sampling analyses further supported this outcome, showing the requirement of n= 4 or 5 to meet significance (compare Figs 3.6 B & D).

To test the accuracy of relative quantification with respect to body-wide *Wolbachia* titer in response to yeast-enriched host diets, we measured the ratio of

wsp/rpl32 from whole body samples. Surprisingly, relative *Wolbachia* titers differed from the results yielded by absolute counts (compare Figs 3.4C and 3.5B). The *wsp/rpl32* values were significantly lower in yeast-fed flies at the level of the whole body, showing 54– 68% of the ratios observed in control whole body samples (T-test, $p < 0.001$, n = 18) (Fig 3.5B). Sub-sampling analyses were consistent with this outcome, indicating 4–18 samples as sufficient to satisfy $p < 0.01$ in 98.5–100% of cases (compare Figs 3.6 A & C).

Thus, outcomes using ratios (relative counts) suggest that dietary yeast suppresses body-wide *Wolbachia* titers, though absolute counts consistently show that body-wide titers are not yeast-sensitive. Ratios are misleading with respect to body-wide *Wolbachia* abundance because yeast-feeding induces a 1.5 to 1.9 fold median increase in absolute counts of *rpl32* in ovarian tissues, contradicting any assumption that host gene counts remain constant. Therefore, to avoid potential artifacts of host response to treatment conditions, the data indicate that the absolute quantification qPCR method must be used going forward, for accurate quantification of *Wolbachia*.

3.4.5 The non-antibiotic drug, bortezomib, suppresses whole-body *Wolbachia* titer

It has become evident that, unlike host antibiotic treatments, yeast-enriched host diets do not affect body-wide *Wolbachia* abundance in *D. melanogaster*. This raises an intriguing question: Can non-antibiotic compounds elicit a body-wide titer response? A comprehensive RNAi screen has recently reported that inhibition of ubiquitin-proteasome pathway decreases *Wolbachia* titer in tissue culture cells (Grobler et al., 2018; White et al., 2017a), as well as in *Drosophila* germlines (White et al., 2017a). A separate set of studies has shown that the drug bortezomib (also named as PS-341), acts as an inhibitor of ubiquitin proteasome pathway, specifically by targeting the eukaryotic 20S

proteasomal subunit (Adams et al., 1999; Richardson et al., 2003). We decided to test the impact of bortezomib on body-wide *Wolbachia* titer. In initial tests, flies were treated with a conservative 1 μM dose of bortezomib for 3 days, and absolute *wsp* counts were measured using real-time qPCR (n= 18, 6 wells per condition, 3 technical replicates each). The bortezomib treated flies exhibited 60% – 63% of *wsp* absolute counts detected in the DMSO control flies ($p < 0.001$; with appropriate statistics) (Fig 3.7A).

To test whether adequate samples were analyzed to support this interpretation, we compared and evaluated randomly sub-sampled data from DMSO and bortezomib treatments in MatLab. The first analysis included all the technical replicates in our analyses (n= 18; 6 wells with 3 technical replicates each), which showed the requirements of 6 or more data points for significance with the alpha set at 0.01 (Fig 3.8A). The analyses were repeated for different combinations of well replicates versus, technical replicates. All data samplings yielded comparable findings indicating requirement of 6 or more data points for significance (Fig 3.8 B-C). Thus, subsequent body-wide titer measurements after this point were collected from 3 wells, 2 technical replicates each.

To determine whether the impact of bortezomib will persist at an increased concentration, we exposed the flies to 100 μM bortezomib, a dose used in many fly and cell culture screen (Kim et al., 2008; Markstein et al., 2014; Serbus et al., 2012). After 3-days of feeding, the bortezomib reduced whole body *wsp* counts to 48% –50% of the DMSO controls (n= 6, $p < 0.001$) (Fig 3.7B). Subsampling analyses further confirmed that 6 samples per condition are sufficient at alpha 0.01 (Fig 3.8D). The confirmation that bortezomib decreases body-wide *Wolbachia* titer demonstrates the potential for non-antibiotic compounds to induce whole body titer response.

3.4.6 Bortezomib suppresses *Wolbachia* colonization of the *Drosophila* germline

We have seen above that yeast-enriched diets reduce ovarian *Wolbachia* titer, whereas the whole body remains unaffected. Since the drug bortezomib reduces *Wolbachia* titer in whole body samples, that raises questions about whether the impact of bortezomib is generalized. To test whether bortezomib affects ovarian *Wolbachia* titer, we measured *Wolbachia* absolute counts in whole fly extracts and ovarian samples, derived from populations raised in parallel. Flies were exposed to 100 μM bortezomib over a 3-day feeding period. Consistent with the data above, flies treated with 100 μM bortezomib for 3-days, showed a 49% to 52% of *wsp* counts observed in the whole body of DMSO control flies (Welch's t-test, $p < 0.001$, n = 18) (Fig. 3.9A). Sub-sampling analyses support these statistical interpretations, indicating that as few as 6 samples were sufficient to satisfy an alpha value of 0.01, regardless of what data analysis method was used (Fig. 3.10). Dissected ovary tissue from the same fly population also exhibited *Wolbachia* depletion. Ovary tissues from bortezomib-treated flies exhibited a 44-53% of *wsp* abundance observed in DMSO treated ovaries (various tests, $p < 0.001$, n = 18) (Fig. 3.9B). Sub-sampling analyses further support this outcome, showing the requirement of 8 or more samples for a consistent readout (Fig. 3.11).

To summarize, absolute counts indicate that the host proteasome inhibitor, bortezomib, exerts a generalized suppressive effect on body-wide *Wolbachia* titer. This is consistent with *Wolbachia* reliance upon the host ubiquitin-proteasome system as previously observed (White et al., 2017a). This work demonstrates that adult drug feedings can provide insight into host pathways that specify systemwide as well as tissue-level *Wolbachia* titers in vivo.

3.5 Discussion

Wolbachia endosymbionts must overcome difficulties shared by bacterial pathogens when colonizing host cells. The fundamental challenges are associated with circumventing host defense mechanisms, as well as replicating inside the host (Hentschel et al., 2000). Direct observation of bacterial titer carried by host cells over time, and under different treatment conditions, is critical to inform the mechanisms of colonization. Studies have been carried out to understand germline colonization by *Wolbachia*. However, it is not known how somatic *Wolbachia* titer is regulated in vivo. Technical limitations on determining *Wolbachia* titer have further impeded understanding of systemwide colonization to date. This chapter applied the DNA extraction and absolute quantification methods optimized in Chapter II to assess whole-body *Wolbachia* titer in response to different host treatment conditions. This chapter also demonstrated how to confirm *Wolbachia* titer responses to experimental conditions, with statistical certainty, at the scale of a whole insect and reproductive tissues.

The quantitative analyses showed the capacity of optimized sample preparation and qPCR techniques to detect body-wide titer changes within the time frame of a feeding assay. Utilizing the same strategies, the data showed that yeast-enriched diets reduced ovarian *Wolbachia* titer, which is in accordance with previous cytological studies of *D. melanogaster* maternal germline cells (Christensen et al., 2019; Serbus et al., 2015). The whole body titer, by comparison, did not change upon yeast treatment. One possible interpretation of these results is that yeast-driven insulin signaling triggers redistribution of *Wolbachia* within the body. This is in agreement with published findings that ovarectomized females exhibit higher somatic *Wolbachia* titers in yeast-enriched conditions (Serbus et al., 2015). A caveat of that study is its use of the relative count qPCR method, which leaves abundance comparisons ultimately unclear. An alternate

possibility is that insulin favors somatic replication while suppressing *Wolbachia* replication in the germline. A limitation of the current qPCR assay is that it does not inform replication or binary fission rates. We are currently trying to understand *Wolbachia* replication patterns at different *Drosophila* developmental stages as part of a separate study. Therefore, as of now, the question of how body-wide *Wolbachia* abundance is specified remains unanswered.

A major outcome from this study was that absolute counts showed equivalent *Wolbachia* titers across nutrient-altered diets, whereas relative quantification did not. The basis for this effect was an increase in baseline *rpl32* levels in yeast-fed flies. This makes sense considering the biology of reproduction. Advanced arthropods like *Drosophila* have meroistic, polytrophic ovaries, in which each oocyte has a dedicated set of 15 nurse cells that load the oocyte with all content needed for embryogenesis (Klowden, 2008). To support mass production, *Drosophila* nurse cell nuclei endoreplicate their DNA. This yields ploidy on the order of 2000+ for any given nurse cell (Dej & Spradling, 1999), and intrinsically increases *rpl32* copy number per host. As such, any treatment that affects nurse cell ploidy or ovary productivity will also affect *rpl32* abundance as a consequence. Relative measurements do not yield inherently "incorrect" numbers, but interpretations that can be made from such data are limited. This work makes clear: relative counts do not uniformly represent absolute *Wolbachia* abundance across experimental conditions. Thus, absolute quantification approaches are important to acquire data that support sound, testable models in complex biological systems.

Another notable outcome from this study was that bortezomib, a proteasome inhibitor, dramatically reduced *Wolbachia* titer in both whole body and ovary tissues. This finding is in line with previous study that disruption of ubiquitin-proteasome pathway depletes germline *Wolbachia* titer, in tissue culture cells as well as germline tissues

analyzed by confocal microscopy (White et al., 2017a). The hypothesis presented by that study was that *Wolbachia* requires amino acids derived from this protein degradation pathway for survival. In such case, blockage of this system would hinder the essential resources coming from it. Another possibility is that host proteins toxic to *Wolbachia* are normally eliminated by the proteasome, so loss of this enzymatic function adversely affects the bacteria. A second RNAi screen showed that knockdown of the proteasome complex increased titer in cell cultures, and in ovarian tissue (Grobler et al., 2018). The basis for this inconsistency between is not clear, because knockdown of several other ubiquitin related genes was shown to decrease titer in the same study, consistent with the White et al 2017 results. Perhaps each cell screening assay was differentially sensitized to aspects of proteasome function due to differences in cell rearing, experimental conditions and/or divergence of the cell lines themselves.

In summary, absolute qPCR technique enables accurate, reproducible and statistically rigorous measurement of endosymbiotic *Wolbachia* bacteria across tissue type. The study of nutrient-altered host diets demonstrates that somatic and germline *Wolbachia* titers are regulated differently to some extent. However, *Wolbachia* responses to ubiquitin pathway inhibition, in the whole body as well as reproductive tissue, indicates that some host processes do exert a generalized impact on *Wolbachia* titer in vivo. This work opens up the possibility to further explore the effect of other host processes on *Wolbachia* titer in vivo, thus, informing the mechanisms of endosymbiosis within a natural, physiological context.

Figures

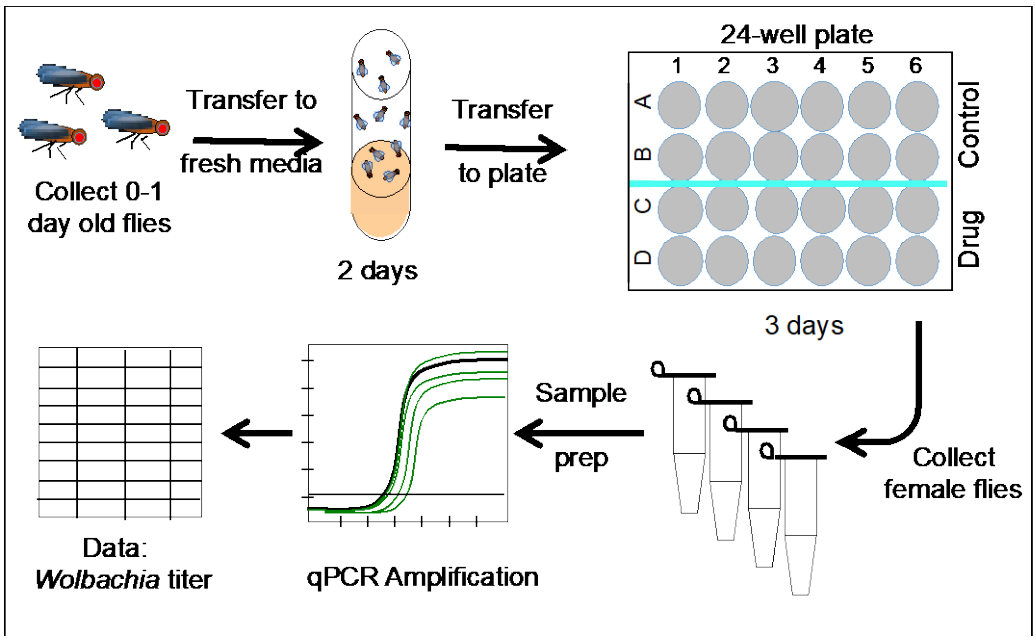

Fig. 3.1. Approach used for real-time quantitative PCR analysis of *Wolbachia* titer in whole *D. melanogaster* flies. The workflow used for fly preparation, drug treatment, sample preparation and qPCR analysis is shown. See Methods for further details.

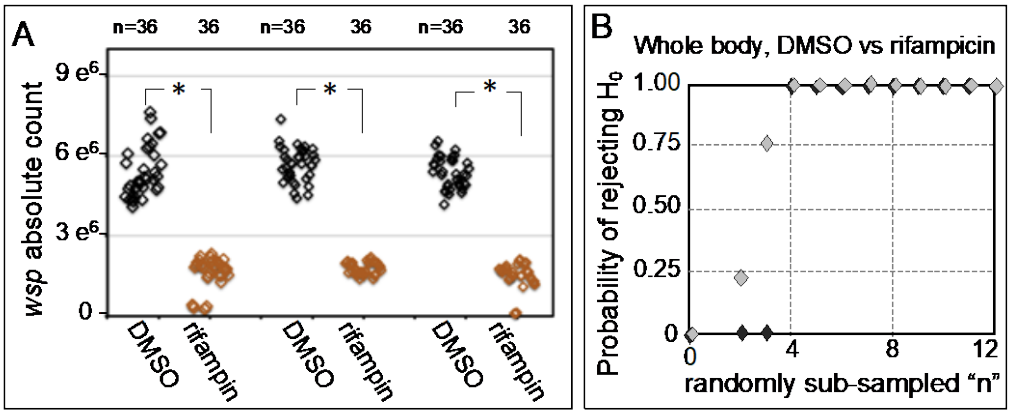

Fig. 3.2. Absolute *wsp* abundance of DMSO versus rifampicin-treated whole body samples as indicated by real-time qPCR. Data from each sample/well represent 5 female flies. A) Test for body-wide *wsp* abundance changes within assayed timespan. Carrier DMSO and rifampicin conditions are shown. Data from 3 plate replicates are shown in pairs, in terms of 3 technical replicates from each of 12 wells. B) Comparisons of randomly sub-sampled data from DMSO and rifampicin conditions, with alpha set at 0.01. n = 3 technical replicates from each of 12 wells. Tests used per replicate: Plate 2 (grey): Welch's T-test. Plate 3 (black): Mann-Whitney U. * indicates $p < 0.01$.

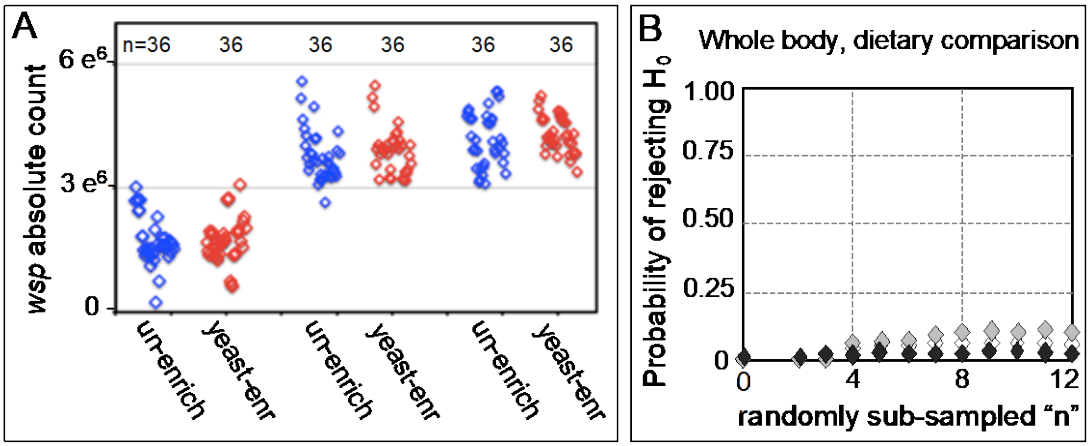

Fig. 3.3. Absolute *wsp* abundance in whole body samples from hosts subjected to yeast-enriched versus un-enriched dietary conditions. Data from each sample/well represent 5 female flies. A) Comparisons of body-wide *wsp* abundance in un-enriched versus yeast-enriched conditions. n = 3 technical replicates from 12 wells. B) Comparing randomly sub-sampled data from un-enriched and yeast-enriched conditions, with alpha set at 0.01. n = 3 technical replicates from 12 wells. Tests used per replicate: Plate 1 (white): Mann-Whitney U. Plate 2 (grey): Mann-Whitney U. Plate 3 (black): Welch's T-test.

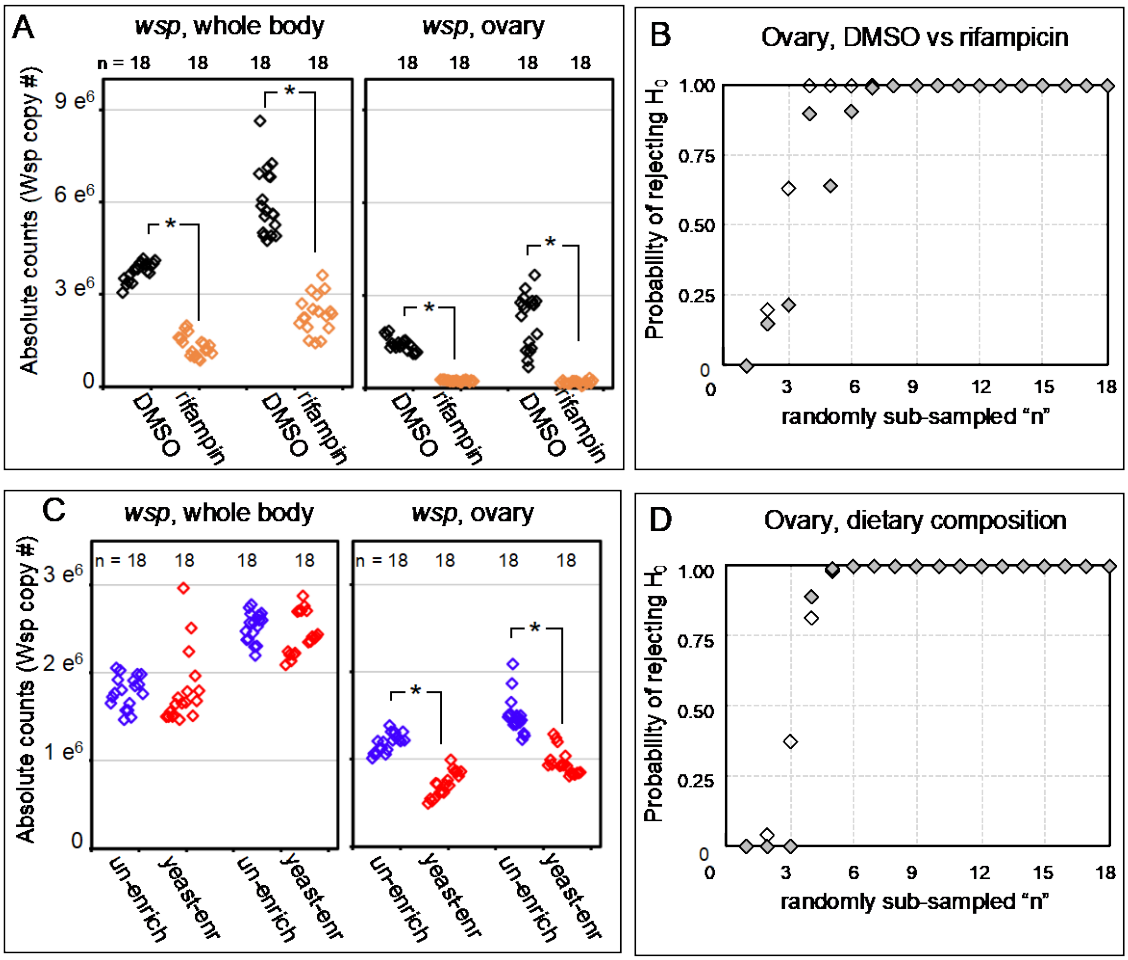

Fig. 3.4. *wsp* absolute counts as indicated by real-time qPCR. Whole fly extracts and ovarian extracts are compared in each experiment. Panels show data from 2 independent plate replicates. "n" represents 3 technical replicates from 6 sample tubes. Data from each sample/well represent material from 5 female flies. A) *wsp* abundance was compared in control DMSO vs. rifampicin treatment conditions. B) Sub-sampling analyses from DMSO vs Rifampicin ovary data, alpha=0.01. Plate replicate1: white diamonds, Plate replicate 2: grey diamonds. Statistics used: Welch's T-test. C) *wsp* abundance was compared in un-enriched vs. yeast-enriched treatment conditions. D) Sub-sampling analyses, comparing ovary data from un-enriched verses yeast-enriched dietary conditions. * indicates $p < 0.01$.

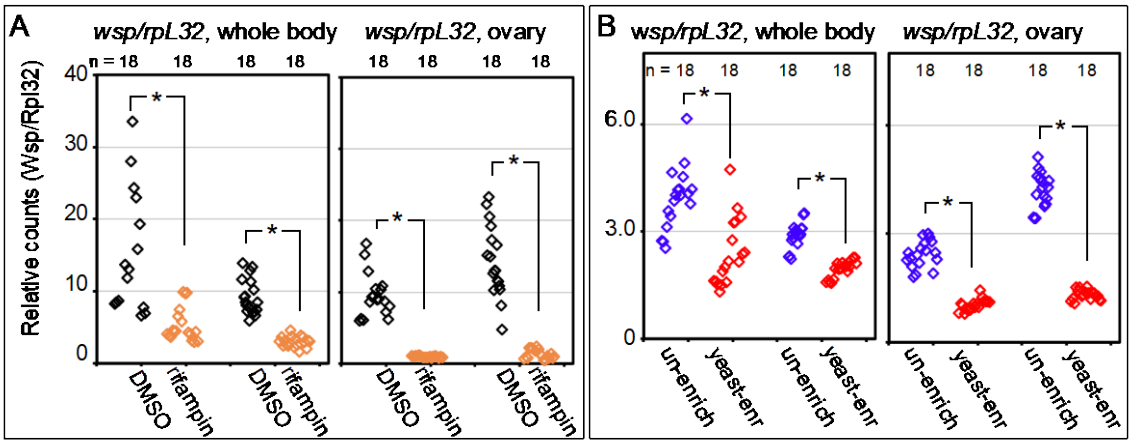

Fig. 3.5. Relative counts, showing a ratio of *wsp/rpl32* abundance in A) control DMSO vs. rifampicin treatment conditions, and B) un-enriched vs. yeast-enriched treatment conditions. Statistical tests were applied as appropriate to each dataset. * indicates $p < 0.05$.

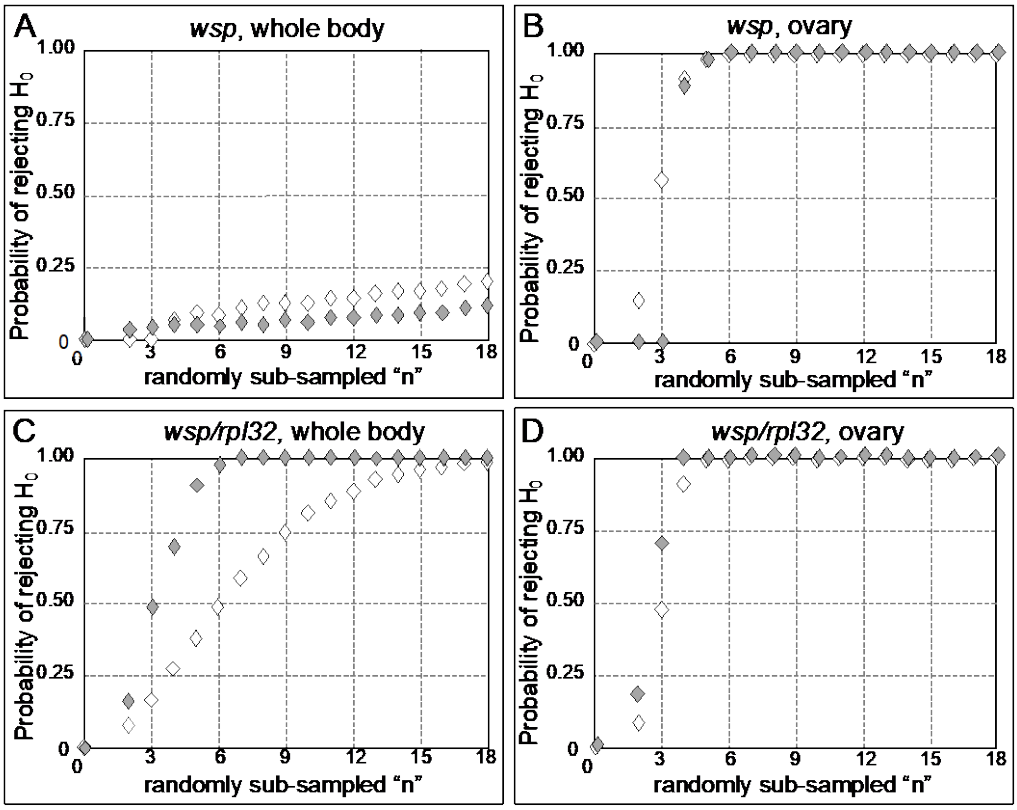

Fig. 3.6. Comparisons of randomly sub-sampled data for whole body versus ovarian samples under different dietary conditions. Un-enriched and yeast-enriched conditions are compared. Two different experimental replicates of each experiment were performed, overlaid in white and grey. All graphs display the likelihood of seeing a significant difference at $p < 0.01$ between food types when performing analysis of *wsp* copy number in A) whole body samples, and B) ovary samples, as well as relative quantification ratios of *wsp/rpl32* counts from C) whole body samples, and D) ovary samples.

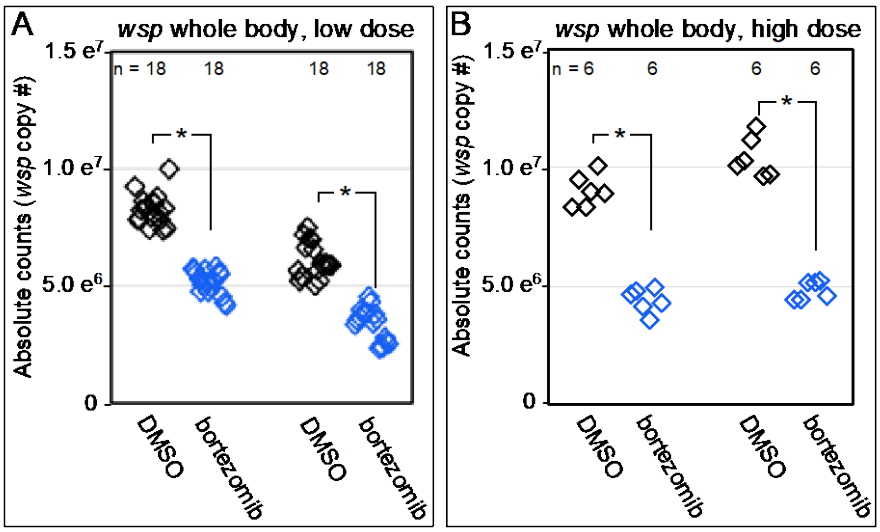

Figure 3.7 *wsp* abundance in whole body as indicated by real-time qPCR. Data shows DMSO versus bortezomib at 1 μM and 100 μM doses. A) Showing data for the conservative dose of 1 μM. n=18 amplifications, 6 wells with 3 technical replicates each; $p < 0.001$. B) Showing data for the standard dose of 100 μM. n=6 amplification, 3 wells 2 technical replicates each. Each experiment was run in 2 separate biological replicates.
* indicates $p < 0.01$.

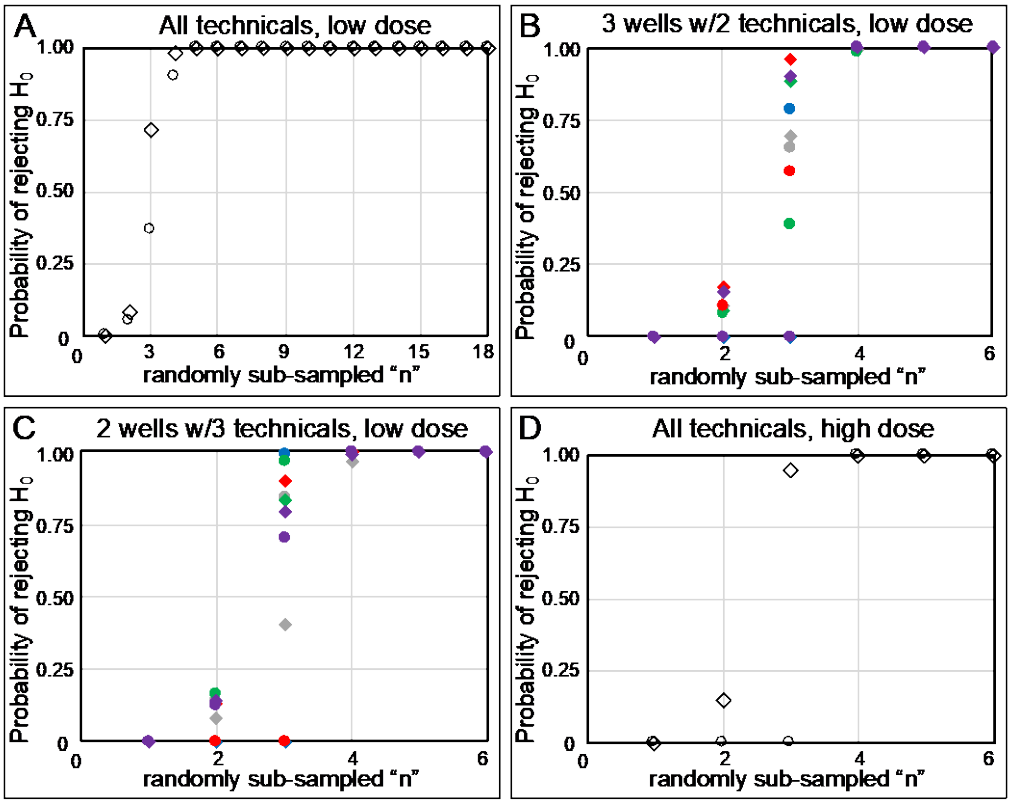

Figure 3.8 Comparisons of randomly sub-sampled data for DMSO versus bortezomib samples under 1 μM and 100 μM doses, with alpha set at 0.01. Each experiment was conducted in two separate biological replicates as overlapped in diamond and circle shapes. (A-C) Represent sub-sampling of data using a 1 μM dose of bortezomib. A) all technical replicates: 3 replicates in 6 wells, total n = 18. B) 3 randomly selected wells with 2 technical replicates each, n=6. C) 2 randomly selected wells with 3 technical replicates each, n=6. Five different combinations were used for sub-sampling in fig B and C. Blue: combination 1. Grey: combination 2. Green: combination 3. Red: combination 4. Purple: combination 5. (D) Sub-sampling of data using 100 μM dose of bortezomib, showing 3 wells with 2 technical each, n=6.

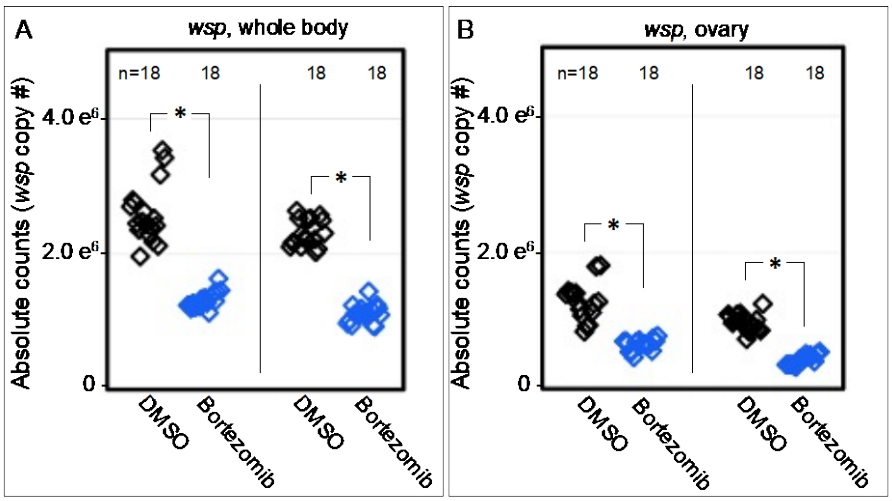

Figure 3.9 *wsp* abundance as indicated by real-time qPCR. Whole body and ovary samples are compared in parallel upon bortezomib treatment with a dose of 100 μM. Panels show data from 2 independent plate replicates. “n” represents 18 technical replicates from 6 wells. A) *wsp* abundance in whole body B) *wsp* abundance in ovarian samples. * indicates $p < 0.01$.

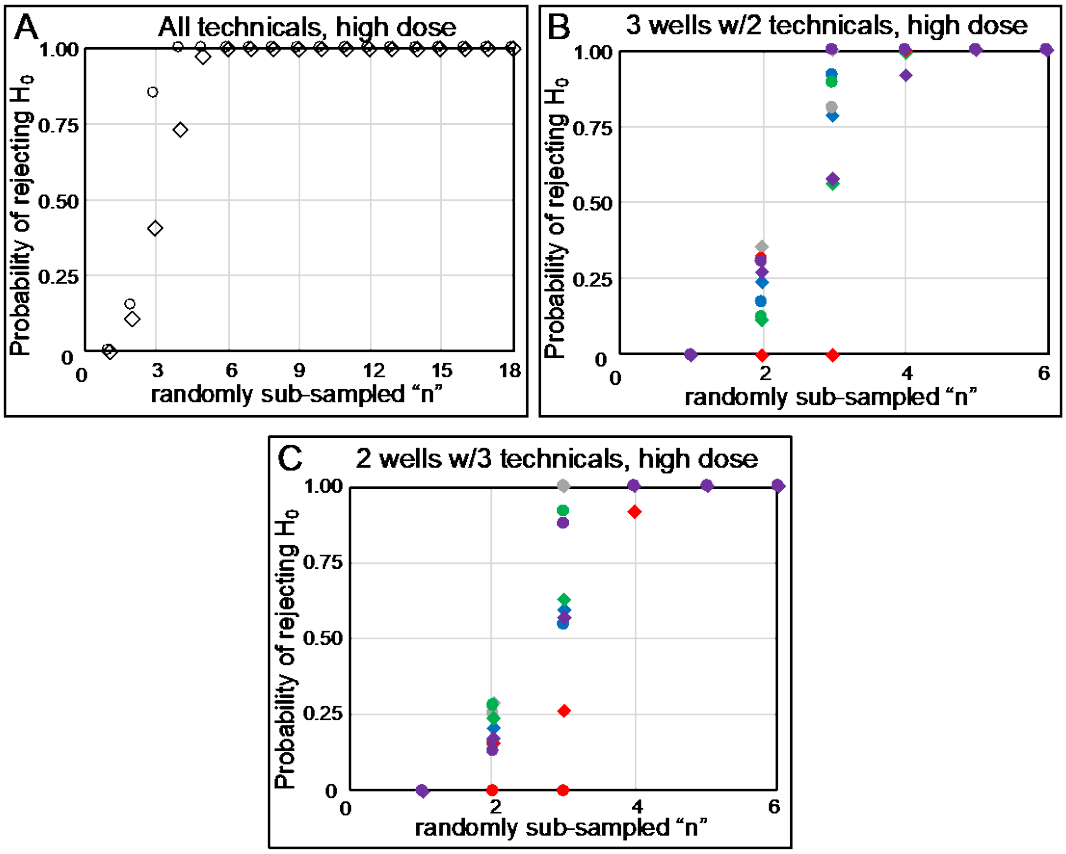

Figure 3.10 Comparisons of randomly sub-sampled data for DMSO versus bortezomib whole body samples, at the 100 μM dose, with alpha set at 0.01. Each experiment was conducted in two separate biological replicates as overlapped in diamond and circle shape. Figures represent sub-sampling of data using a 100 μM dose of bortezomib. A) All the technical replicates, n=18, consisting of 6 wells with 3 technical replicates each. B) 3 randomly selected wells with 2 technical replicates each, n=6. C) 2 random wells with 3 technical replicates each, n=6. Five different combinations were used for sub-sampling in fig B and C. Blue: combination 1. Grey: combination 2. Green: combination 3. Red: combination 4. Purple: combination 5.

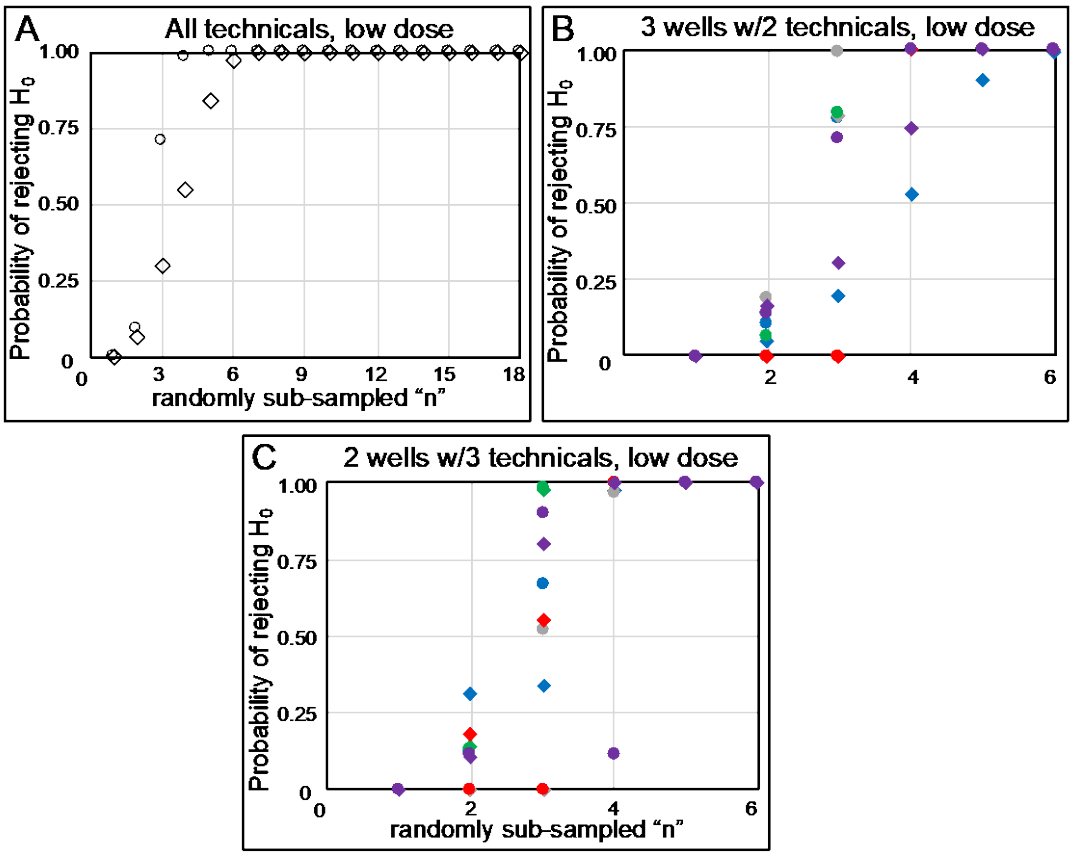

Figure 3.11 Comparisons of randomly sub-sampled data for DMSO versus bortezomib ovarian samples, at a 100 μM dose, with alpha set at 0.01. Each experiment was conducted in two separate biological replicates as overlapped in diamond and circle shape. Figures represent sub-sampling of data using a 100 μM dose of bortezomib. A) All the technical replicates, n=18, consisting of 6 wells with 3 technical replicates each. B) 3 randomly selected wells with 2 technical replicates each, n=6. C) 2 random wells with 3 technical replicates each, n=6. Five different combinations were used for sub-sampling in fig B and C. Blue: combination 1. Grey: combination 2. Green: combination 3. Red: combination 4. Purple: combination 5.

References

Adams, J., Palombella, V. J., Sausville, E. A., Johnson, J., Destree, A., Lazarus, D. D., Maas, J., Pien, C. S., Prakash, S., & Elliott, P. J. (1999). Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Research*, *59*(11), 2615–2622.

Baldo, L., Ayoub, N. A., Hayashi, C. Y., Russell, J. A., Stahlhut, J. K., & Werren, J. H. (2008). Insight into the routes of *Wolbachia* invasion: High levels of horizontal transfer in the spider genus Agelenopsis revealed by Wolbachia strain and mitochondrial DNA diversity. *Molecular Ecology*, *17*(2), 557–569. https://doi.org/10.1111/j.1365-294X.2007.03608.x

Bennett, G. M., & Moran, N. A. (2015). Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. *Proceedings of the National Academy of Sciences*, *112*(33), 10169–10176. https://doi.org/10.1073/pnas.1421388112

Camacho, M., Oliva, M., & Serbus, L. R. (2017). Dietary saccharides and sweet tastants have differential effects on colonization of *Drosophila* oocytes by *Wolbachia* endosymbionts. *Biology Open*, *6*(7), 1074–1083. https://doi.org/10.1242/bio.023895

Casper-Lindley, C., Kimura, S., Saxton, D. S., Essaw, Y., Simpson, I., Tan, V., & Sullivan, W. (2011). Rapid fluorescence-based screening for *Wolbachia* endosymbionts in *Dosophila* germ line and somatic tissues ▿ . *Applied and Environmental Microbiology*, *77*(14), 4788–4794. https://doi.org/10.1128/AEM.00215-11

Christensen, S., Camacho, M., Sharmin, Z., Momtaz, A. J. M. Z., Perez, L., Navarro, G., Triana, J., Samarah, H., Turelli, M., & Serbus, L. R. (2019). Quantitative methods for assessing local and bodywide contributions to *Wolbachia* titer in maternal germline cells of Drosophila. *BMC Microbiology*, *19*. https://doi.org/10.1186/s12866-019-1579-3

Christensen, S., Pérez Dulzaides, R., Hedrick, V. E., Momtaz, A. J. M. Z., Nakayasu, E. S., Paul, L. N., & Serbus, L. R. (2016). *Wolbachia* endosymbionts modify *Drosophila* ovary protein levels in a context-dependent Manner. *Applied and Environmental Microbiology*, *82*(17), 5354–5363. https://doi.org/10.1128/AEM.01255-16

Chrostek, E., Marialva, M. S. P., Esteves, S. S., Weinert, L. A., Martinez, J., Jiggins, F. M., & Teixeira, L. (2013). Wolbachia variants induce differential protection to viruses in *Drosophila melanogaster:* A phenotypic and phylogenomic analysis. *PLoS Genetics*, *9*(12). https://doi.org/10.1371/journal.pgen.1003896

Cordaux, R., Bouchon, D., & Grève, P. (2011). The impact of endosymbionts on the evolution of host sex-determination mechanisms. *Trends in Genetics: TIG*, *27*(8), 332–341. https://doi.org/10.1016/j.tig.2011.05.002

Dedeine, F., Boulétreau, M., & Vavre, F. (2005). *Wolbachia* requirement for oogenesis: Occurrence within the genus *Asobara (Hymenoptera, Braconidae)* and evidence for intraspecific variation in *A. tabida*. *Heredity*, *95*(5), 394–400. https://doi.org/10.1038/sj.hdy.6800739

Dej, K. J., & Spradling, A. C. (1999). The endocycle controls nurse cell polytene chromosome structure during *Drosophila* oogenesis. *Development (Cambridge, England)*, *126*(2), 293–303.

Efron, B. (1992). Bootstrap methods: Another look at the jackknife. In S. Kotz & N. L. Johnson (Eds.), *Breakthroughs in Statistics: Methodology and Distribution* (pp. 569–593). Springer. https://doi.org/10.1007/978-1-4612-4380-9_41

Eleftherianos, I., Atri, J., Accetta, J., & Castillo, J. C. (2013). Endosymbiotic bacteria in insects: Guardians of the immune system? *Frontiers in Physiology*, *4*. https://doi.org/10.3389/fphys.2013.00046

Ferree, P. M., Frydman, H. M., Li, J. M., Cao, J., Wieschaus, E., & Sullivan, W. (2005). Wolbachia utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte. *PLoS Pathogens*, *1*(2). https://doi.org/10.1371/journal.ppat.0010014

Frydman, H. M., Li, J. M., Robson, D. N., & Wieschaus, E. (2006). Somatic stem cell niche tropism in *Wolbachia*. *Nature*, *441*(7092), 509–512. https://doi.org/10.1038/nature04756

Géminard, C., Rulifson, E. J., & Léopold, P. (2009). Remote control of insulin secretion by fat cells in *Drosophila*. *Cell Metabolism*, *10*(3), 199–207. https://doi.org/10.1016/j.cmet.2009.08.002

Grobler, Y., Yun, C. Y., Kahler, D. J., Bergman, C. M., Lee, H., Oliver, B., & Lehmann, R. (2018). Whole genome screen reveals a novel relationship between *Wolbachia* levels and *Drosophila* host translation. *PLoS Pathogens*, *14*(11). https://doi.org/10.1371/journal.ppat.1007445

Hentschel, U., Steinert, M., & Hacker, J. (2000). Common molecular mechanisms of symbiosis and pathogenesis. *Trends in Microbiology*, *8*(5), 226–231. https://doi.org/10.1016/s0966-842x(00)01758-3

Hermans, P. G., Hart, C. A., & Trees, A. J. (2001). In vitro activity of antimicrobial agents against the endosymbiont *Wolbachia pipientis*. *The Journal of Antimicrobial Chemotherapy*, *47*(5), 659–663.

Huigens, M. E., Luck, R. F., Klaassen, R. H., Maas, M. F., Timmermans, M. J., & Stouthamer, R. (2000). Infectious parthenogenesis. *Nature*, *405*(6783), 178–179. https://doi.org/10.1038/35012066

Kim, B. H., Yin, C.-H., Guo, Q., Bach, E. A., Lee, H., Sandoval, C., Jayabose, S., Ulaczyk-Lesanko, A., Hall, D. G., & Baeg, G.-H. (2008). A small molecule

compound identified through a cell-based screening inhibits JAK/STAT pathway signaling in human cancer cells. *Molecular Cancer Therapeutics*, *7*(9), 2672–2680. https://doi.org/10.1158/1535-7163.MCT-08-0309

Klowden, M. J. (2008). Reproductive Systems. *Physiological Systems in Insects*, 181–238. https://doi.org/10.1016/B978-012369493-5.50005-5

LaFever, L., & Drummond-Barbosa, D. (2005). Direct control of germline stem cell division and cyst growth by neural insulin in *Drosophila*. *Science (New York, N.Y.)*, *309*(5737), 1071–1073. https://doi.org/10.1126/science.1111410

Lasko, P. F., & Ashburner, M. (1990). Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. *Genes & Development*, *4*(6), 905–921. https://doi.org/10.1101/gad.4.6.905

LePage, D. P., Metcalf, J. A., Bordenstein, S. R., On, J., Perlmutter, J. I., Shropshire, J. D., Layton, E. M., Funkhouser-Jones, L. J., Beckmann, J. F., & Bordenstein, S. R. (2017). Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature*, *543*(7644), 243–247. https://doi.org/10.1038/nature21391

Markstein, M., Dettorre, S., Cho, J., Neumuller, R. A., Craig-Muller, S., & Perrimon, N. (2014). Systematic screen of chemotherapeutics in *Drosophila* stem cell tumors. *Proceedings of the National Academy of Sciences*, *111*(12), 4530–4535. https://doi.org/10.1073/pnas.1401160111

McGarry, H. F., Egerton, G. L., & Taylor, M. J. (2004). Population dynamics of *Wolbachia* bacterial endosymbionts in *Brugia malayi*. *Molecular and Biochemical Parasitology*, *135*(1), 57–67. https://doi.org/10.1016/j.molbiopara.2004.01.006

Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G., Pyke, A. T., Hedges, L. M., Rocha, B. C., Hall-Mendelin, S., Day, A., Riegler, M., Hugo, L. E., Johnson, K. N., Kay, B. H., McGraw, E. A., van den Hurk, A. F., Ryan, P. A., & O'Neill, S. L. (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell*, *139*(7), 1268–1278. https://doi.org/10.1016/j.cell.2009.11.042

Newton, I. L. G., Savytskyy, O., & Sheehan, K. B. (2015). *Wolbachia* utilize host actin for efficient maternal transmission in *Drosophila melanogaster*. *PLoS Pathogens*, *11*(4). https://doi.org/10.1371/journal.ppat.1004798

Newton, I. L. G., & Sheehan, K. B. (2015). Passage of *Wolbachia pipientis* through mutant *Drosophila melanogaster* Induces phenotypic and genomic changes. *Applied and Environmental Microbiology*, *81*(3), 1032–1037. https://doi.org/10.1128/AEM.02987-14

Pietri, J. E., DeBruhl, H., & Sullivan, W. (2016). The rich somatic life of Wolbachia. *MicrobiologyOpen*, *5*(6), 923–936. https://doi.org/10.1002/mbo3.390

Raychoudhury, R., Baldo, L., Oliveira, D. C. S. G., & Werren, J. H. (2009). Modes of acquisition of *Wolbachia*: Horizontal transfer, hybrid introgression, and codivergence in the *Nasonia* species complex. *Evolution; International Journal of Organic Evolution*, *63*(1), 165–183. https://doi.org/10.1111/j.1558-5646.2008.00533.x

Richardson, K. M., Griffin, P. C., Lee, S. F., Ross, P. A., Endersby-Harshman, N. M., Schiffer, M., & Hoffmann, A. A. (2019). A *Wolbachia* infection from *Drosophila* that causes cytoplasmic incompatibility despite low prevalence and densities in males. *Heredity*, *122*(4), 428–440. https://doi.org/10.1038/s41437-018-0133-7

Richardson, P. G., Hideshima, T., & Anderson, K. C. (2003). Bortezomib (PS-341): A novel, first-in-class proteasome inhibitor for the treatment of multiple myeloma and other cancers. *Cancer Control: Journal of the Moffitt Cancer Center*, *10*(5), 361–369. https://doi.org/10.1177/107327480301000502

Rietveld, T., & van Hout, R. (2015). The t test and beyond: Recommendations for testing the central tendencies of two independent samples in research on speech, language and hearing pathology. *Journal of Communication Disorders*, *58*, 158–168. https://doi.org/10.1016/j.jcomdis.2015.08.002

Sazama, E. J., Bosch, M. J., Shouldis, C. S., Ouellette, S. P., & Wesner, J. S. (2017). Incidence of *Wolbachia* in aquatic insects. *Ecology and Evolution*, *7*(4), 1165–1169. https://doi.org/10.1002/ece3.2742

Serbus, L. R., Ferreccio, A., Zhukova, M., McMorris, C. L., Kiseleva, E., & Sullivan, W. (2011). A feedback loop between *Wolbachia* and the *Drosophila* gurken mRNP complex influences Wolbachia titer. *Journal of Cell Science*, *124*(Pt 24), 4299–4308. https://doi.org/10.1242/jcs.092510

Serbus, L. R., Landmann, F., Bray, W. M., White, P. M., Ruybal, J., Lokey, R. S., Debec, A., & Sullivan, W. (2012). A cell-based screen reveals that the albendazole metabolite, albendazole sulfone, targets *Wolbachia*. *PLoS Pathogens*, *8*(9), e1002922. https://doi.org/10.1371/journal.ppat.1002922

Serbus, L. R., Rodriguez, B. G., Sharmin, Z., Momtaz, A. J. M. Z., & Christensen, S. (2017). Predictive genomic analyses inform the basis for vitamin metabolism and provisioning in bacteria-arthropod endosymbioses. *G3 (Bethesda, Md.)*, *7*(6), 1887–1898. https://doi.org/10.1534/g3.117.042184

Serbus, L. R., & Sullivan, W. (2007). A cellular basis for *Wolbachia* recruitment to the host germline. *PLoS Pathogens*, *3*(12). https://doi.org/10.1371/journal.ppat.0030190

Serbus, L. R., White, P. M., Silva, J. P., Rabe, A., Teixeira, L., Albertson, R., & Sullivan, W. (2015). The impact of host diet on *Wolbachia* titer in *Drosophila*. *PLoS Pathogens*, *11*(3). https://doi.org/10.1371/journal.ppat.1004777

Sheehan, K. B., Martin, M., Lesser, C. F., Isberg, R. R., & Newton, I. L. G. (2016). Identification and characterization of a candidate *Wolbachia pipientis* Type IV effector that interacts with the actin cytoskeleton. *MBio*, *7*(4). https://doi.org/10.1128/mBio.00622-16

Specht, S., Mand, S., Marfo-Debrekyei, Y., Debrah, A. Y., Konadu, P., Adjei, O., Büttner, D. W., & Hoerauf, A. (2008). Efficacy of 2- and 4-week rifampicin treatment on the *Wolbachia* of *Onchocerca volvulus*. *Parasitology Research*, *103*(6), 1303–1309. https://doi.org/10.1007/s00436-008-1133-y

Townson, S., Hutton, D., Siemienska, J., Hollick, L., Scanlon, T., Tagboto, S. K., & Taylor, M. J. (2000). Antibiotics and *Wolbachia* in filarial nematodes: Antifilarial activity of rifampicin, oxytetracycline and chloramphenicol against *Onchocerca gutturosa, Onchocerca lienalis* and *Brugia pahangi. Annals of Tropical Medicine and Parasitology*, *94*(8), 801–816.

Voronin, D., Schnall, E., Grote, A., Jawahar, S., Ali, W., Unnasch, T. R., Ghedin, E., & Lustigman, S. (2019). Pyruvate produced by Brugia spp. Via glycolysis is essential for maintaining the mutualistic association between the parasite and its endosymbiont, *Wolbachia*. *PLoS Pathogens*, *15*(9). https://doi.org/10.1371/journal.ppat.1008085

Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z., & Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1807), 20150249. https://doi.org/10.1098/rspb.2015.0249

Werren, J. H., Zhang, W., & Guo, L. R. (1995). Evolution and phylogeny of *Wolbachia*: Reproductive parasites of arthropods. *Proceedings. Biological Sciences*, *261*(1360), 55–63. https://doi.org/10.1098/rspb.1995.0117

White, P. M., Pietri, J. E., Debec, A., Russell, S., Patel, B., & Sullivan, W. (2017b). Mechanisms of horizontal cell-to-cell transfer of *Wolbachia spp*. In *Drosophila melanogaster*. *Applied and Environmental Microbiology*, *83*(7). https://doi.org/10.1128/AEM.03425-16

White, P. M., Serbus, L. R., Debec, A., Codina, A., Bray, W., Guichet, A., Lokey, R. S., & Sullivan, W. (2017a). Reliance of *Wolbachia* on high rates of host proteolysis revealed by a genome-wide RNAi screen of *Drosophila* Cells. *Genetics*, *205*(4), 1473–1488. https://doi.org/10.1534/genetics.116.198903

Wu, M., Sun, L. V., Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J. C., McGraw, E. A., Martin, W., Esser, C., Ahmadinejad, N., Wiegand, C., Madupu, R., Beanan, M. J., Brinkac, L. M., Daugherty, S. C., Durkin, A. S., Kolonay, J. F., Nelson, W. C., Mohamoud, Y., … Eisen, J. A. (2004). Phylogenomics of the reproductive parasite *Wolbachia pipientis wMel*: A streamlined genome overrun by mobile genetic elements. *PLoS Biology*, *2*(3), E69. https://doi.org/10.1371/journal.pbio.0020069

Yamaguchi, M., & Yoshida, H. (2018). *Drosophila* as a Model Organism. In M. Yamaguchi (Ed.), *Drosophila Models for Human Diseases* (pp. 1–10). Springer Singapore. https://doi.org/10.1007/978-981-13-0529-0_1

CHAPTER IV: MECHANISMS OF WHOLE BODY COLONIZATION BY *WOLBACHIA,* AS INDICATED BY CHEMICAL AND GENETIC MANIPULATION OF THE *DROSOPHILA* HOST

4.1 Abstract

Wolbachia endosymbionts are widely prevalent in nature, infecting a diverse group of insect hosts. The density and distribution of *Wolbachia* have been considered to play a critical role for maintaining their symbiotic relationships. However, the cellular and molecular basis of *Wolbachia* titer control within the host is yet to be uncovered. To investigate host processes that control *Wolbachia* colonization and proliferation, we carried out a pilot drug screen, using 37 chemicals to target 14 consensus host processes implicated in titer control for other microbes. Absolute qPCR measurements indicated that 6 candidate compounds altered *Wolbachia* titer in both *D. melanogaster* and *D. simulans.* Drugs that increased titer spanned a wide array of biological functions, including the IMD pathway, Calcium signaling, the Ras/mTOR pathway, and the Wnt pathway. By contrast, the only drug to suppress titer was a Ubiquitin-proteasome pathway inhibitor. The implicated titer-altering host processes were retested using an inducible RNAi expression system. Genetic disruption of the mTOR and Wnt pathways resulted in increased bodywide *Wolbachia* titer, consistent with the drug treatment results. This work suggests that mTOR and Wnt pathways normally restrict *Wolbachia* abundance within the body, thus, moderating the *Wolbachia*-host endosymbiosis.

4.2 Introduction

Many insect species carry maternally transmitted bacterial endosymbionts that can greatly affect host's physiology (Duron et al., 2008; Hilgenboecker et al., 2008). Relying on their hosts for survival, some symbionts act as mutualists and confer fitness benefits, such as enhancing the fecundity and survival of their host (Turelli, 1994).

Alternatively, as parasites, many symbionts exploit host reproduction for their own transmission (Ehrman, 1998; Moran et al., 2008). Examples of such reproductive manipulation involves arresting the production of viable offspring, killing of males, parthenogenesis, and feminization of males (Moran et al., 2008). A vertically inherited symbiont must be able to proliferate in the host and be continuously passed across host generations for maintenance in the host population (Jaenike, 2009). Therefore, infection density and fitness effects are closely intertwined in the dynamics of vertical transmission. Reduced bacterial load, for instance, may lead to inefficient vertical transmission and subsequent loss of infection (McGraw et al., 2002; Newton et al., 2015). Excessive density may result in adverse impact on host fitness (Min & Benzer, 1997; Mouton et al., 2004). Given the selective pressure on both host and symbiont, the host cellular pathways would be expected to affect the maintenance of endosymbiont colonization.

Endosymbiotic *Wolbachia* bacteria are widespread in nature. This infection is known to be associated with mites, crustaceans and filarial nematodes, as well as about half of all insect species, including *Drosophila melanogaster* (Sazama et al., 2017; Weinert et al., 2015). *Wolbachia* are one of the most common intracellular endosymbionts, spanning the range of parasitic, mutualistic and commensalistic association with the host (Min & Benzer, 1997; Serbus et al., 2008; Weeks et al., 2007; Werren et al., 2008; Zug & Hammerstein, 2012). There is a growing interest in studying the biology of *Wolbachia*-host interactions. Analyses of *Wolbachia* in the *Drosophila melanogaster* research model organism has the advantage of a natural infection (Yamaguchi & Yoshida, 2018). Besides, the advancements of *Drosophila* genetics enable researchers to systematically examine cellular mechanisms within the host (Allocca et al., 2018; Jennings, 2011).

There is limited information on how insect hosts maintain *Wolbachia* within the body. To date, reports suggest that the density of *Wolbachia* is responsive to an array of intrinsic and extrinsic cues. The host cytoskeletal system (Ferree et al., 2005; Newton et al., 2015; Serbus et al., 2011), autophagy (Deehan et al., 2021; Voronin et al., 2012), host miRNAs, such as, aae-miR-2940 in mosquito (Hussain et al., 2011; Zhang et al., 2013), Insulin signaling (Serbus et al., 2015), the IMD pathway (Pan et al., 2018) have been documented to interact with *Wolbachia.* Host environmental factors, such as temperature, crowding (Wiwatanaratanabutr & Kittayapong, 2009), host diet (Camacho et al., 2017; Serbus et al., 2015), and host microbiota (Goto et al., 2006), have been shown to exert varying impact on *Wolbachia* titer. Recently, two comprehensive studies were carried out to identify host pathways implicated in titer control within somatic tissue culture cells (Grobler et al., 2018; White et al., 2017a). Using RNA interference, the screens demonstrated the importance of host lipid metabolism, mitochondrial components, vesicular trafficking, cytoskeletal components, cell signaling in controlling *Wolbachia* titer (Grobler et al., 2018; White et al., 2017a). Most importantly, significance was illustrated for the host endoplasmic reticulum (ER)-associated protein degradation pathway in supporting *Wolbachia* titer (White et al., 2017a), and host ribosomal components in restricting titer (Grobler et al., 2018).

The basis for interpretating *Wolbachia* infection levels from tissue culture studies is not very clear. In tissue culture wells, many bacteria can be floating on the medium due to cell death (egress), which might then horizontally re-invade other cells (White et al., 2017b). Furthermore, any treatment condition that alters *Wolbachia* titer in tissue culture cells could also do so by affecting the bacterial life cycle (replication vs. death). Cultured bacteria like *E. coli* are known to undergo rapid replication by binary fission during the exponential growth phase. Eventually, the bacteria shift over to a different

(non-replicative) stress management program when the population reaches the stationary phase. The implication is that replication cycles don't automatically occur at timed intervals, as much as they are a response to nutrient availability. Prolonged nutrient deprivation and toxin build up in the cultured bacterial population are responsible for bacterial death (Monod, 1949; Zwietering et al., 1990). Thus, bacterial population growth within tissue culture cells could exhibit titer changes as a reflection of an altered life cycle, positional effects, or some combination of the two. Therefore, detailed analyses to identify key processes of systemwide *Wolbachia* regulation are still required.

The cellular and molecular basis of titer control has been well-studied for disease-related microbes. Research studies over past decades have implicated a range of host processes in controlling density of other microbes. Disruption of the host cytoskeleton, for instance, has been shown to decrease the density of *Ehrlichia risticii* (Rikihisa et al., 1994), *Listeria monocytogenes* (Derré et al., 2007; Kühbacher et al., 2015), and *Mycobacterium fortuitum* (Philips et al., 2005), suggesting a requirement of cytoskeletal components for survival and replication of these microbes. Calcium and Wnt signaling have also been reported to facilitate growth of certain pathogenic bacteria (Czyż et al., 2014; Kessler et al., 2012; Luo et al., 2016; Rikihisa et al., 1995). Interestingly, the mTOR/autophagy pathway has a differential impact on density depending on the bacterial strain. Functional disruption of the mTOR pathway reduced load of *Ehrlichia chaffeensis* (Luo et al., 2017), *Chlamydia trachomatis, Listeria monocytogenes* (Derré et al., 2007), and *Salmonella typhimurium* (Birmingham et al., 2006). By contrast, inhibition of the same pathway elevated the titer of *Anaplasma phagocytophilum,* and *Rickettsia australis* infection within host cells (Bechelli et al., 2018; Niu et al., 2008). Redundant host effects on different bacterial species imply that a core set of host processes may exert generalized effects on bacterial colonization.

Because the best understood intracellular infection models involve pathogenic bacteria, however, it remains unclear to what extent host-side analyses are confounded by the stress of imminent mortality. It would be of interesting to understand how host cellular and molecular processes interact with mutualistic or commensalistic intracellular bacteria, like *Wolbachia*.

To identify host genes and processes that regulate *Wolbachia*, we carried out a candidate drug screen, targeting factors that affect host colonization by commonly studied microbes, and then examined the impact on systemwide *Wolbachia* titer. The use of adult flies avoids the limitations of tissue culture studies described above. By using optimized DNA extraction methods and absolute quantification by real-time qPCR (Christensen et al., 2019) (see also Chapter II), we screened 37 compounds for their impact on body-wide titer. 6 candidate drugs were identified that altered *Wolbachia* titer in both *Drosophila melanogaster* and *Drosophila simulans.* These drugs correspond to 5 different host cellular processes, including the host Ubiquitin-proteasome system, the IMD pathway, Calcium channel function, the mTOR/Ras pathway, and Wnt signaling. Follow up genetic tests confirmed a function for mTOR and Wnt signaling pathways in specifying whole-body *Wolbachia* load. To our knowledge, this is the first study to systematically test, identify and inform generalized mechanisms for *Wolbachia* titer control in adult insects. Future research on whether these processes are conserved across other *Wolbachia*-host combinations, and other endosymbiotic systems, may shed light on the molecular mechanisms of endosymbiont titer control as a core aspect of endosymbiosis in general.

4.3 Methods and materials:

4.3.1 *Drosophila* stocks and maintenance

Two fly strains were used in this study. Preliminary screening was performed using *Drosophila melanogaster* of the genotype *w*; *Sp/Cyo*; *Sb/TM6B* carrying the endogenous *w*Mel strain (Christensen et al., 2016). *Drosophila simulans* infected with the endogenous *Wolbachia riverside* (*w*Ri) strain was used for further analyses (Hoffmann et al., 1986). Flies are usually maintained in plastic bottles/vials containing standard fly food media. The recipe is derived from Bloomington stock center as described previously (Christensen et al., 2016). The flies were raised in the Invictus *Drosophila* incubator at 25°C, under standard 12/12h light-dark cycle. For the experiments, “0-day old” flies were collected and kept on standard fly food medium for 2 days. 2-days old flies were then used for drug screening. Only female flies were used for the screen, to reduce possible variation in population behavior per well.

4.3.2 Chemicals used for the whole-body screening

At least 2 chemicals were used to alter the functionality of each of the candidate host processes, comprising a total of 37 chemicals. The chemicals were purchased from different vendors (Table 4.1). All the drugs were dissolved in DMSO. Stock solutions were prepared in advance with a concentration of 10 mM and stored in -20°C. Light-sensitive drugs were kept in the dark. Rifampicin and DMSO were used as controls, with the concentration of DMSO capped at 1%.

4.3.3 Chemical screening in a plate-based format

The entire screening was performed in a 24-well plate format (Corning cat# 3738). 3 wells were assigned to DMSO controls, 3 wells were for rifampicin controls and the rest of the wells were used for testing unknown drug treatments with 3 wells each. To prepare the screening plate, first the standard food was melted in a regular glass

beaker. 10 mL of the warm food was transferred into a plastic vial and stirred to decrease the temperature. 100 µL of 10 mM chemical stock was then added into the food and stirred thoroughly. This resulted in a final drug concentration of 100 µM with 1% of DMSO. Rifampicin controls were prepared analogously with 100 µL of 10 mM rifampicin added to 10 mL of food. For the DMSO controls, equivalent amounts of DMSO was added to the food so that the final concentration does not exceed 1%. 1 mL of each the mixture was transferred into the respective wells, and the plate was loosely wrapped and stored under the fume hood. After the food cooled and solidified, 10 female flies were transferred to each well. Owing to the light sensitivity of certain drugs, the plate was coated with foil and incubated at 25°C. After 3-days of feeding, 5 female flies per well were processed for *wsp* quantification using body-wide qPCR.

4.3.4 *Wolbachia* infected GAL4 driver generation and genetic knockdown of genes

GAL4:UAS system was used in this study for gene knockdown by RNAi. The Actin5C-GAL4 driver of the genotype: *w; P{Act5C-GAL4-w}E1/Cyo* (stock# 25374) and the daughterless-GAL4 driver of the genotype: *w; P{w+, GMR12B08-GAL4}attP2* (stock# 48489) were used for body-wide expression. The original stocks were ordered from the Bloomington *Drosophila* stock center and later infected with *Wolbachia* from *Drosophila melanogaster* of the double balanced genotype *w*; *Sp/Cyo*; *Sb/TM6B* (White et al., 2017a), hereafter referred to as DB *w*Mel. To generate *Wolbachia*-infected driver lines, the males from each driver were crossed with virgin DB *w*Mel females. This process eventually established *Wolbachia*-infected Act5C-GAL4 and da-GAL4 lines.

Infected virgin females from each driver stocks were crossed to males from responder UAS (upstream activating sequence) RNAi lines listed in Table 4.2. The parent flies were removed from the vials after 3-4 days of mating. The F1 generation flies started eclosing after 10 days, and collected flies were aged for 5 days. Female flies

expressing dsRNA were identified by checking fly phenotypes. Non-expressing siblings were considered as a control group. A separate control set was also prepared in parallel with *Wolbachia*-infected virgin females from the driver stocks outcrossed to Oregon R (OreR) male flies. The RNAi-expressed group, the non-expressing controls, and OreR controls were then processed for *wsp* quantification.

4.3.5 DNA extraction and qPCR for whole body *Wolbachia* quantification

To quantify body-wide *Wolbachia* titer, DNA was extracted by homogenizing a group of 5 female flies in 200 µL of squishing buffer, with addition of 2 µL of 20 mg/ml of proteinase K. The squishing buffer is composed of 10 mM Tris HCl (pH 8.0), 1 mM EDTA, 25 mM NaCl, and 1% SDS (as per Chapter II) (Christensen et al., 2019). The homogenized mixtures were then incubated for 1 hour at 56°C. Afterwards, the proteinase K was inactivated by heating the samples at 95°C for 3 mins. Samples were then centrifuged at 17,970 x g for 15 minutes at 4°C (Beckman Microfuge 22R). 100 µL of supernatant was collected for ethanol precipitation while carefully avoiding the pellet. For the ethanol-based precipitation, 1/10 volume of 3 M Na-acetate and 250 µL of absolute ethanol was added to the 100 µL of supernatant. The mixtures were gently mixed and incubated at -20°C for minimum 1 hour (or, overnight) and centrifuged afterwards at 14,000 RPM and 18,000 RCF (G-force) for 15 minutes at 4°C, allowing the DNA to precipitate out at the bottom of the tubes as pellet. The supernatant was discarded very carefully from each tube and then pellets were washed with 500 µL of 70% ethanol. Samples were re-centrifuged for 15 mins at 4°C using the same settings. Supernatants were removed, and the DNA pellets were air dried followed by resuspension in 100 µL of TE buffer (pH 8.0). DNA suspensions were then directly used for qPCR.

To generate absolute count measurements of *Wolbachia* from the extracted DNA samples, absolute quantification of the *wolbachia surface protein* (*wsp*) gene was carried out using reference plasmid standards. PGEM-T vector carrying a 160 bp PCR-amplified fragment of the *Wolbachia* surface protein *(wsp*) gene was used as the reference (Christensen et al., 2016). Real-time PCR was carried out on a Bio-Rad CFX96 Connect Optics Module Real-Time System and absolute copy numbers for *Wolbachia* were obtained by comparing threshold cycle (Ct) values with a standard curve generated from the plasmid standard, as in (Christensen et al., 2016). The primers used to target the *wsp* gene were: Forward 5' CATTGGTGTTGGTGTTGGTG 3', reverse 5' ACCGAAATAACGAGCTCCAG 3' primers with a concentration of 5 µM.

4.3.6 Data Display for the drug screens and statistical analysis

Average *wsp* counts per drug treatment were normalized against their respective DMSO control and presented as scattered plot (White et al., 2017a). Statistical analyses were conducted on raw data. Appropriate statistics were used to compare data sets and make final interpretations. Suitable statistical tests were identified for each data set as per the decision tree outline in Chapter II (Fig. 2.1). Briefly, the normality distribution and homogeneity of variance for each dataset were evaluated by using the Shapiro Wilk test and by determining Levene's p-value, respectively. Data sets with normal distribution and homogeneous variation were compared using an Independent T-test. Data sets which are normally distributed, but have unequal variance, were compared by Welch's T-test. Non-normal data with homogeneous variation were compared using Mann-Whitney U test. For non-normal data with unequal variance, Independent T-test with bootstrapping was utilized (de Cuevas & Spradling, 1998; Lim & Loh, 1996; Rietveld & van Hout, 2015; Shapiro & Wilk, 1965; Wasserman, 1994). The IBM SPSS program was used to perform all statistical analyses.

4.4 Results

4.4.1 Selecting candidate drugs from cell-based screen to define *Wolbachia* density regulatory mechanisms

Our overall goal is to identify host pathways and processes that affect *Wolbachia* titer in vivo. We opted for a chemical disruption approach because chemicals can be retested for conserved effects across diverse *Wolbachia*-host systems, most of which involve study of non-model organisms. As this type of pilot screen has not been done before, we first faced the question of which small molecule inhibitors to test. One route is to take chemicals that previously altered *Wolbachia* infection in the context of tissue culture cells and retest their effects in vivo. A high-throughput, cell-based drug screen has previously identified compounds that significantly reduced *Wolbachia* infection in immortalized primary tissue culture cell lines (Serbus et al., 2012). The screen was rigorous and identified 270 different chemicals that reduced infection in 3/3 plate replicates, hereafter referred to as “hits”. The risk of pursuing these hits is the potential for bias in compound selection, as the chemical library used for the past tissue culture screen does not target host pathways equally or comprehensively (Serbus et al., 2012).

To assess the extent to which the profile of *Wolbachia*-suppressing chemicals reflects a possible chemical library bias, we classified the compounds by the function of their targets. This analysis partitioned the library into 48 categories of distinctive biological function, with antibacterial, cardiovascular agent, antineoplastic agent, and anti-inflammatory classifications including the largest numbers of compounds (Fig 4.1A). Then we asked how the *Wolbachia*-suppressing compounds associated with this library compare to the functional profile associated with the library. Of the chemical hits that suppressed *Wolbachia* infection across 3 plate replicates, the functional classes antineoplastic, antifungal, anthelmintic and antiparasitic, were somewhat

overrepresented as compared to the library composition overall (Fig 4.1B). However, the collective functional profile of the *Wolbachia*-suppressing hits generally resembled the category profile of the chemical library (Fig 4.1A-B). This outcome is consistent with the hit list as a reflection of library composition, and not as an indicator of biological functions with the highest relevance.

A further aspect of revisiting compounds from the prior cell screen is that it would be important to avoid toxic compounds. Our goal is to pursue biological foundations of endosymbiont titer control, not how impending host cell mortality affects endosymbiosis. To address this issue, we checked the toxicity of *Wolbachia*-suppressing hit compounds as individually reported per compound in the National Library of Medicine “PubChem” website. Our search data indicated that 25% of the library compounds were reported as toxic or moderately toxic (n = 2000) (Fig. 4.2A). By comparison, 51% of the *Wolbachia*-suppressing hit compounds were identified as similarly toxic (n = 270) (Fig. 4.2B). Therefore, it is possible that many of the compounds suppressed *Wolbachia* infection in tissue culture cells as an indirect effect of damaging the cells. 26% of the compounds identified as hits in the cell culture screen were classified as nontoxic, for a total of 70 candidate drugs. Considering the timeline of the PhD dissertation, as well as the issue of bias introduced by the library itself, we concluded that it would be best not to pursue this set of compounds for in vivo screening at this time.

4.4.2 Literature mining for candidate host processes to test against *Wolbachia*

An alternate route for a candidate chemical screen is to select compounds based upon the function of their molecular targets. The major strength of this approach is that the screen will be designed around biological predictions, based on existing mechanistic data. Host effects on the density of intracellular bacteria have been studied for a number

of bacterial pathogens. It is unclear whether host factors that influence the density of bacterial pathogens also generally affect bacterial colonization of eukaryotic cells.

To identify potentially conserved host effects on intracellular bacteria titer, we searched the literature for host factors that affect load/ survival of commonly studied microbes. After investigating 52 species from 17 genera, we found 26 bacterial species under all these genera for which involvement of host genes/pathways on density regulation has been discussed (Table 4.3). 12 organisms from our initial list were found to be under the class of alpha-proteobacteria (Table 4.3), because it is one of the largest and most widely studied groups of bacteria (Dworkin et al., 2006). The initial literature search with all the 26 bacterial strains, highlighted 14 host mechanisms as relevant to intracellular titer control for multiple bacterial endosymbionts (Table 4.4). Given heterogeneity in the approaches used to identify these pathways, a lack of reported redundancy for titer-altering pathways across prior studies is inconclusive. However, redundant reporting of pathway involvement across multiple host-microbe systems implies consensus host effects on intracellular bacteria. To determine whether a broad playbook for intracellular titer control extends to *Wolbachia,* host pathways highlighted by other systems were prioritized for pursuit.

To test the candidate host processes for *Wolbachia* titer effects, we identified candidate compounds that specifically target those. For each of the 14 redundant mechanisms (Table 4.4), the strategy was to test 2 or more compounds per host pathway/process, since compounds can fail outright for many reasons. When possible, compounds with opposite effects on the process of interest were included, such as the microtubule-depolymerizing drug, colchicine, and the microtubule-stabilizing drug, taxol, or, phospholipase C (PLC) inhibitor, U73122, and the PLC activator, 3-m3mfbs. This culminated in selection of 37 total candidate compounds (Table 4.1).

4.4.3 Host-directed drug screens revealed possible host factors that influence the density of *Wolbachia* within the body

The screening approach employed absolute quantification by real-time PCR (Christensen et al., 2019) to assess the impact of the candidate drugs on body-wide *Wolbachia* titer. *Drosophila melanogaster* flies, carrying the endogenous *w*Mel *Wolbachia* strain, were used for primary screening. Female flies were exposed to food supplemented with a 100 µM dose of DMSO-solubilized drugs or control food that carried equivalent amounts of DMSO. A DMSO-solubilized rifampicin control was also run on every qPCR plate to confirm ongoing susceptibility of *Wolbachia* titer to compound treatments.

In the initial screen, treatments showing a significant change in body-wide *Wolbachia* titer in both plate replicates, as compared to control DMSO, were classified as preliminary hits. Of 37 chemicals tested, the primary screen identified 16 compounds that met these criteria (Fig 4.3). The preliminary hit compounds were then re-tested for their reproducibility in a third plate replicate. Of the 16 chemicals retested, 11 of them were reconfirmed as hits during this step, representing a total of 9 host processes (Fig 4.4). The control rifampicin condition was shown to decrease titer throughout the screen, as well as the proteasome inhibitor bortezomib. By contrast, all other drugs classified as hits elicited an increase in body-wide *Wolbachia* titer, ranging from a 9-68% increase ($p < 0.001$-0.047, with appropriate statistics based on each data type, n=6 amplifications per condition) (Table 4.5). This suggests that the corresponding host pathways normally have a limiting impact on *Wolbachia*; and when these host factors are disrupted by the drugs, restrictions on whole body *Wolbachia* titer are diminished.

4.4.4 Testing candidate titer-altering processes in *Wolbachia*-infected *D. simulans*

If host mechanisms that affect intracellular bacteria exert conserved effects across species, we would expect for *Wolbachia* density regulation to be shared across *Wolbachia*-host combinations as well. To investigate a role for candidate processes across systems, we retested all the 11 compounds that affect titer in DB wMel against *D. simulans* (Dsim) flies that carry the endogenous *w*Ri *Wolbachia* strain. The Dsim *w*Ri secondary screen yielded 6 treatments that significantly affected body-wide titer (Figure 4.5) (Table 4.4). In all cases, the observed effects for Dsim *w*Ri were consistent with that seen for DB *w*Mel. Bortezomib depleted body-wide titer, whereas cay10512, nicardipine hcl, erlotinib, rapamycin and iwr-1 induced a consistent increase in body-wide *w*Ri titer, (*p*-value range < 0.001-0.041, using appropriate statistics, n=6 amplifications per condition) (Figure 4.5) (Table 4.4). These findings implicate the Ubiquitin-proteasome system, IMD signaling, calcium channel function, Ras/mTOR signaling, and Wnt signaling as generalized contributors to *Wolbachia* titer control across systems.

4.4.5 Genetic validation of the consensus host processes using GAL4-UAS system

To retest the involvement of processes implicated in body-wide titer regulation, we conducted directed genetic tests, using the GAL4:UAS system. GAL4 is a yeast-derived transcription factor, which activates transcription of upstream genes upon binding to the upstream activation sequence (UAS) (Brand & Perrimon, 1993). This system can be used to drive expression of an RNAi construct that can activate an RNAi response by introducing double stranded RNA (dsRNA). This dsRNA targets the corresponding RNA in vivo, and thus, effectively knocks down the expression of the target gene (Perrimon et al., 2010).

To drive dsRNA expression in *D. melanogaster*, we initially used a *Wolbachia*-infected Actin5c-GAL4 stock. This line is expected to show strong expression (Fig 4.6), and thus has the potential to best mimic the effect of high-dose small molecule inhibitor treatments. However, after crossing Actin5C-GAL4 to several UAS-RNAi lines, targeting essential components of the candidate processes, we found that that we were usually unable to retrieve the Actin5C-GAL4: UAS-RNAi progeny (Table 4.7). This indicates that the majority of those genetic combinations were lethal. For those crosses that did yield progeny, these were usually “escaper” (physiologically sick) organisms. Unlike the predicted progeny ratio of about 25% of the genotype, the escaper crosses yielded 5% or less of the expected genotype. It is possible that the low yield is due to the extended high expression level of Actin5C-GAL4 earlier in development (Fig 4.6), which may disrupt core developmental processes through extended expression of our selected RNAi constructs. Given the concerns about *Wolbachia* titer artifacts in escaper flies, use of the Actin5C-GAL4 driver was discontinued for RNAi function disruption tests.

To achieve efficient RNAi knockdown of critical genes and yet healthy progeny, we crossed the UAS-RNAi lines with daughterless-GAL4 (da-GAL4) driver stock that was already infected with *w*Mel (Serbus et al., 2015). The da-GAL4 driver is expected to be ubiquitously expressed in the soma, though with a milder expression level than the Actin5C driver, as per the endogenous daughterless gene (Fig. 4.6). As was expected, all of the crosses yielded viable offspring, and we were able to retrieve enough progeny of the da-GAL4: UAS-RNAi progeny class for subsequent titer analyses.

To experimentally assess the effect of da-GAL4: UAS-RNAi expression on body-wide *Wolbachia* titer, we performed absolute quantification by real-time qPCR. Offspring expressing the transgenic RNAi construct were analyzed in parallel with non-expressing siblings, as well as F1s created by outcrossing the da-GAL4 driver to OreR males. Using

this strategy, we tested the functional impact of 5 different host processes that had been implicated by drug screening as *Wolbachia*-altering pathways. We found that 3 of these processes did not alter body-wide titer upon disruption by da-GAL4: UAS-RNAi expression. These include the Ubiquitin-proteasome system, the IMD pathway and Calcium signaling. The genes chosen for Ubiquitin-proteasome system were Ubc6, a substrate for the proteasomal degradation pathway, and Prosalpha6, a 20S proteasomal subunit. Knockdown of these genes resulted in inconsistent impact on body-wide *Wolbachia* titer (Table 4.8). For the IMD pathway, knockdown of Relish, a culminating element of IMD cascade, and Tak1, involved in Relish activation, also led to inconsistent body-wide impact (Table 4.8). Functional disruption of Calcium signaling by knockdown of Ca1alphaD, an α subunit of an L-type voltage-gated Ca[2+], and Cacophony, a member of type II voltage gated calcium channel, did not change body-wide *Wolbachia* abundance (Table 4.8). Thought it remains possible that these host mechanisms have an important impact on *Wolbachia* titer in vivo, this experimental setup does not provide genetic support for such functions at this time.

The RNAi knockdown tests revealed 2 processes as contributors to body-wide titer control in vivo. One of these was Ras/mTOR signaling. Knockdown of Tor*,* a key component of the mTOR pathway, increased *Wolbachia* titer in the whole body across all replicates. Flies of the genotype da-GAL4: UAS-*tor* RNAi exhibited 12-37% higher *Wolbachia* titer compared to both non-expressed and OreR controls ($p < 0.001$- 0.028, Independent T-test, n=6 amplifications per condition) (Fig. 4.7) (Table 4.8). It is notable that knockdown of EGFR, the receptor that leads to activation of downstream kinases, such as, PI3, Akt, mTOR, showed an inconsistent titer impact. *EGFR* RNAi flies exhibited consistent titer increase of 31-44% when compared against the non-expressing sibling controls ($p < 0.001$-0.002, with appropriate statistics, n=6). However,

comparison with the OreR controls indicated an inconsistent change of titer, with 18% increase in one biological replicate but not in the other ($p < 0.001$-0.258, with appropriate statistics, n=6) (Table 4.8).

The RNAi knockdown tests suggested that Wnt signaling also moderates body-wide *Wolbachia* titer in vivo. Knockdown of *armadillo* gene*,* the fly homologue of β-catenin, increased *Wolbachia* titer. Flies of the genotype da-GAL4: UAS-*arm* RNAi exhibited a 7-22% increase in body-wide titer as compared to the controls ($p < 0.001$-0.034, Independent T-test, n=6) (Fig. 4.7) (Table 4.8). In a complementary test for Wnt pathway impact on body-wide *Wolbachia* titer, we also knocked down *shaggy* gene, a GSK-3, which is known to prevent induction of Wnt signaling by stabilizing β-catenin (Wu & Pan, 2010). In line with our observation with *armadillo* RNAi*,* we expected that a successful knockdown of *sgg* should result in either reduction or no change in *Wolbachia* titer. The results indicated inconsistent titer effects for *shaggy* RNAi flies (Table 4.8). Taken together, these data suggest that RNAi disruption of Ras/mTOR and Wnt signaling, even when driven by the mild da-GAL4 driver, can significantly affect *Wolbachia* load carried by whole insects.

A notable aspect of all RNAi knockdown tests conducted thus far is that the drivers used are specific to the soma. Thus, we would not expect to see significant changes to ovarian *Wolbachia* titer in response to da-GAL4 induced RNAi expression. As a control, we monitored *Wolbachia* abundance in ovary tissues in response to the same da-GAL4-induced RNAi knockdown conditions described above. The data showed that no stable change in ovary titer was elicited by the somatic RNAi knockdowns (Table 4.9). Thus, the primary, detectable effects at the whole-body level are somatic effects.

4.5 Discussion

In this study, we explored the relationships between *Wolbachia* and the *Drosophila* host by investigating the role of different host processes in maintaining *Wolbachia* titer. We performed a pilot drug screen coupled with absolute *Wolbachia* quantification from whole insect samples using real-time qPCR. We initially used drugs to screen for candidate titer-altering functions because of the advantages that we can retest immediately in other non-model organisms, as well as follow up with directed genetic tests of the same functions in *D. melanogaster*.

Our initial approach was to screen the spectrum drug library hits from a previous cell-based screening study (Serbus et al., 2012). However, it is evident that the chemical screening in cell culture had a few structural issues. The hit list strongly resembled the overall composition of the library, and half of the hits were toxic. This suggests that hits represent compound availability/toxicity more than indicating the most highly relevant pathways. To avoid these issues, we pursued host mechanisms that reportedly affect density of bacterial pathogens. Using these processes as a guide, we carried out a pilot drug screen to check if similar host pathways and processes generally affect *Wolbachia* titer. We cannot rule out the possibility that a drug can affect invasion patterns within the body. However, screening for *Wolbachia* titer changes at a whole body level provides a focused set of results to broadly inform the biology of titer control.

Our pilot screen successfully reported 6 compounds that substantially altered body-wide *Wolbachia* titer in both *D. melanogaster* and *D. simulans* hosts. Based upon the literature, these “hit” compounds are expected to correspond to 5 different biological processes, which are the Ubiquitin-proteasome pathway, the IMD pathway, Calcium signaling, the Ras/mTOR pathway, and the Wnt pathway (Table 4.6). Out of an initial 37 compounds tested, the hits retested positively across all 3 plate replicates in *D.*

melanogaster as well as *D. simulans,* indicating that the results are robust. Given the number and functional diversity of host mechanisms implicated in specifying body-wide bacterial loads, we expect that more host contributions remain to be discovered. The results presented here indicate that our pilot screen was nowhere near saturation on pathway identification.

As for any drug-based screen, there are some limits to data interpretation. Any instance of negative results could be due to a basic incompatibility of the experimental system with the drug, rather than the lack of target involvement in the process of interest. For positive hits, it remains possible that additional functional targets were affected, referred to as “off-target effects” (MacDonald et al., 2006). Though we cannot do anything about compound incompatibility with the host, it is possible to use genetics to test the efficacy of host pathway(s) that affect *Wolbachia* titer, as discussed below.

Genetic function-disruption tests have supported *Wolbachia* titer effects for a subset of host functions. The processes that were supported by chemical disruption tests, as well as da-GAL4: UAS-RNAi expression tests, are the Ras/mTOR and Wnt signaling pathways. The consistent *Wolbachia* titer response to these conditions, even when RNAi is expressed with the mild da-GAL4 driver, supports an in-vivo effect for these pathways. It is important to note that the current genetic analyses do not include robust power analyses for accurate sample size. Our preliminary sub-sampling tests of the RNAi data suggest that a sufficient "n" for high certainty of interpretation is likely to be in the range of 6-20 replicates. It is notable that statistical certainty for host RNAi effects on *Wolbachia* titer relies upon different sampling than used for the drug feeding experiments. Demonstrating statistical certainty remains an important aspect of developing a field standard for this work.

The literary context of our results is mixed. For example, our finding that functional disruption of mTOR pathway resulted in increased body-wide *Wolbachia* titer is consistent with our prior work. Cytological analyses of *Wolbachia* titer in late-stage oocytes indicated that the mTOR inhibitor rapamycin increases oocyte *Wolbachia* titer (Serbus et al., 2015). A study on *Anaplasma* also indicated that rapamycin treatment facilitates *Anaplasma* growth, which could be attributed to activation of host autophagy (Niu et al., 2008). The study showed that *Anaplasma* prevents the maturation of early autophagosomes to late autophagosomes, preventing subsequent fusion with the lysosome. Perhaps *Wolbachia* also avoid lysosomal degradation by sheltering within autophagosomes. However, several other studies have reported that disruption of the mTOR pathway decreased load of few other microbes including *Wolbachia* (see Table 4.10 for further details), presumably through indirect activation of host autophagy processes. The basis for this discrepancy may be due to use of different host/strain combinations, as well as possible distortions in data interpretation, due to use of tissue culture approach, or the “relative count” qPCR method (Christensen et al., 2019) (also see Chapter III). Future studies of autophagy and membrane trafficking impacts on *Wolbachia* titer will be required to test this hypothesis, particularly in light of conflicting work in the literature.

Our drug screen and genetic analyses have also indicated a role for the Wnt/β-catenin pathway. Inhibition of this pathway also enhanced body-wide *Wolbachia* load. This outcome is consistent with the autophagy related argument above, because Wnt/β-catenin signaling has previously been shown to negatively regulate autophagy (Petherick et al., 2013). Perhaps Wnt signaling enhances autophagosomal activity, helping to ultimately shield *Wolbachia* from lysosomal degradation. In contrast to this model, another study reported that silencing of GSK-3 increases *Wolbachia* titer in

Aedes fluviatilis embryos (da Rocha Fernandes et al., 2014). Because GSK-3 normally suppresses Wnt signaling (Caspi et al., 2008), it would be expected to activate autophagy, relief from such inhibition would be expected to decrease bacterial load as per our model. Possible explanations for this discrepancy might be use of the relative count qPCR method in this study, or regulatory differences across host systems (da Rocha Fernandes et al., 2014). However, there is also evidence that GSK-3 can inhibit autophagy through suppression of mTOR complex (Azoulay-Alfaguter et al., 2015), in which case, GSK-3 inhibition would be expected to increase bacterial load.

Several of the host processes implicated in *Wolbachia* titer control by chemical screening were not supported by results from in vivo RNAi testing. These were the Ubiquitin-proteasome pathway, the IMD pathway and Calcium signaling. One possible explanation is because the RNAi knockdown was carried out using daughterless-GAL4 driver, which has a mild expression across somatic tissues, unlike chemical tests that cause strong function disruptions throughout the body (Fig 4.6). We used the daughterless driver to avoid the unintended lethal effect of the extended RNAi expression in host development, as likely happened with the strong Actin5C-GAL4 driver. Another possibility is that RNAi disruption of the selected genes was compensated for by other, functionally redundant proteins. Thus, a final interpretation of how these processes affect *Wolbachia* titer in vivo cannot yet be made.

In the future, we will test the drug-inducible GeneSwitch (GS) GAL4 driver to test the titer impact of candidate host genes. A merit of this approach is that drug induction of GS-GAL4 can be restricted to adults only, thus avoiding potentially lethal effects of a strong gene knockdown during early development. Implementation of the GS-GAL4 tool has been demonstrated as effective in adults (Ford et al., 2007; Scialo et al., 2016; Serbus et al., 2015). A caveat of this approach is that activation of the GeneSwitch

GAL4 protein requires exposure to a specific estrogen analog, mifepristone, which is fed to the flies for up to 10 days. We don't know yet whether mifepristone affects *Wolbachia* titer on its own, but prior cell screening did identify 7 estrogen analogues as suppressors of *Wolbachia* infection (Serbus et al., 2012). It will be important to monitor if mifepristone itself affects whole body titers, and to what extent, so that the context for RNAi induction phenotypes is clear. The ability to compare data from GeneSwitch-GAL4: UAS-RNAi tests against results yielded by milder da-GAL4: UAS-RNAi tests will be important in this context.

One of the largest overall lessons from this work is its implications for the basis of endosymbiosis. Unlike bacterial pathogens, commensal endosymbionts like *w*Mel *Wolbachia* persist without obvious host injury. How is this balance negotiated? Our data imply this is achieved by active host suppression of the endosymbiont. Though proteasome inhibition reduced body-wide *Wolbachia* titer, every single other treatment of significance led to a whole body *Wolbachia* titer increase. This suggests that the terms of this endosymbiosis are directed by active host limitation of *Wolbachia* infection, with a restrictive role for mTOR and Wnt pathways on *Wolbachia* under normal circumstances. Future work on how these processes affect other *Wolbachia*-host systems, such as *Nasonia* or mosquitoes would be interesting to explore.

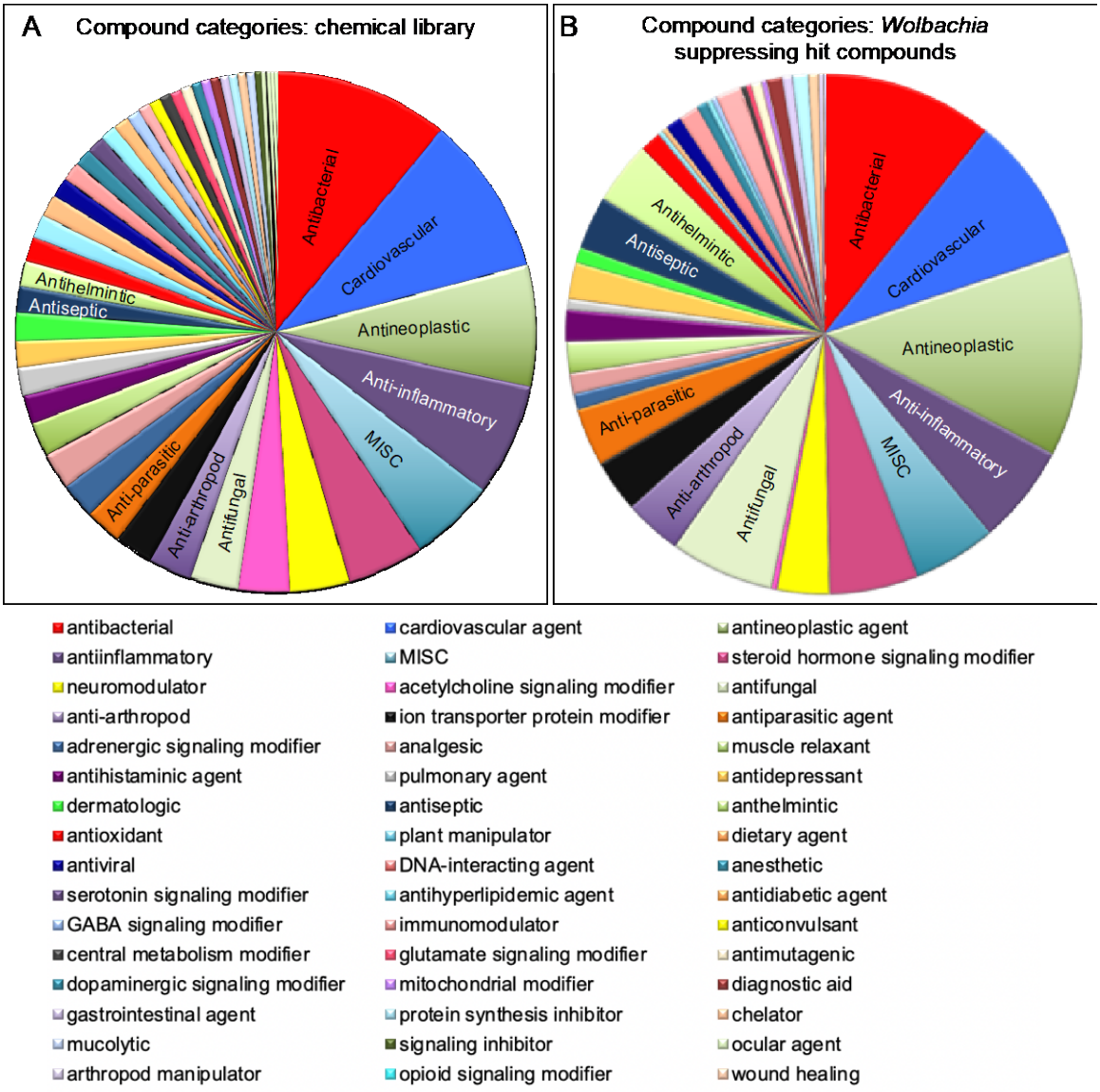

Figure 4.1 Functional analyses of Spectrum chemical library are presented in the pie charts. Each slice represents a different functional category, as indicated by the colors above A) Functional composition of the chemical library. B) Functional composition of the *Wolbachia*-suppressing hit compounds.

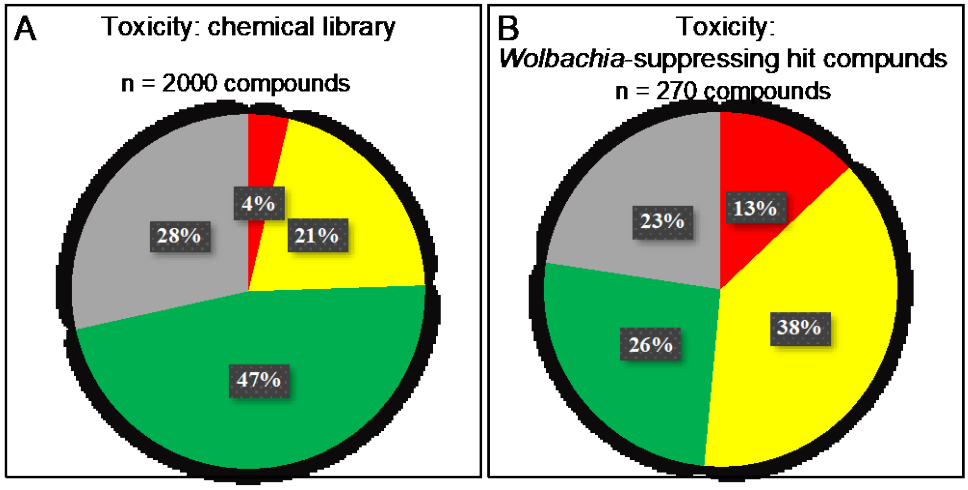

Figure 4.2 Toxicity analyses associated with the spectrum library compounds. Toxicity reports were obtained through publicly available PubChem database (Kim, 2016). Toxicity levels are represented by colors. Red: toxic compounds. Yellow: moderately toxic compounds. Green: non-toxic compounds. Grey: compound whose toxicity was not available through PubChem. A) Toxicity of compounds comprising the chemical library, with total n = 2000. B) Toxicity of the *Wolbachia*-suppressing hit compounds, with total n = 270.

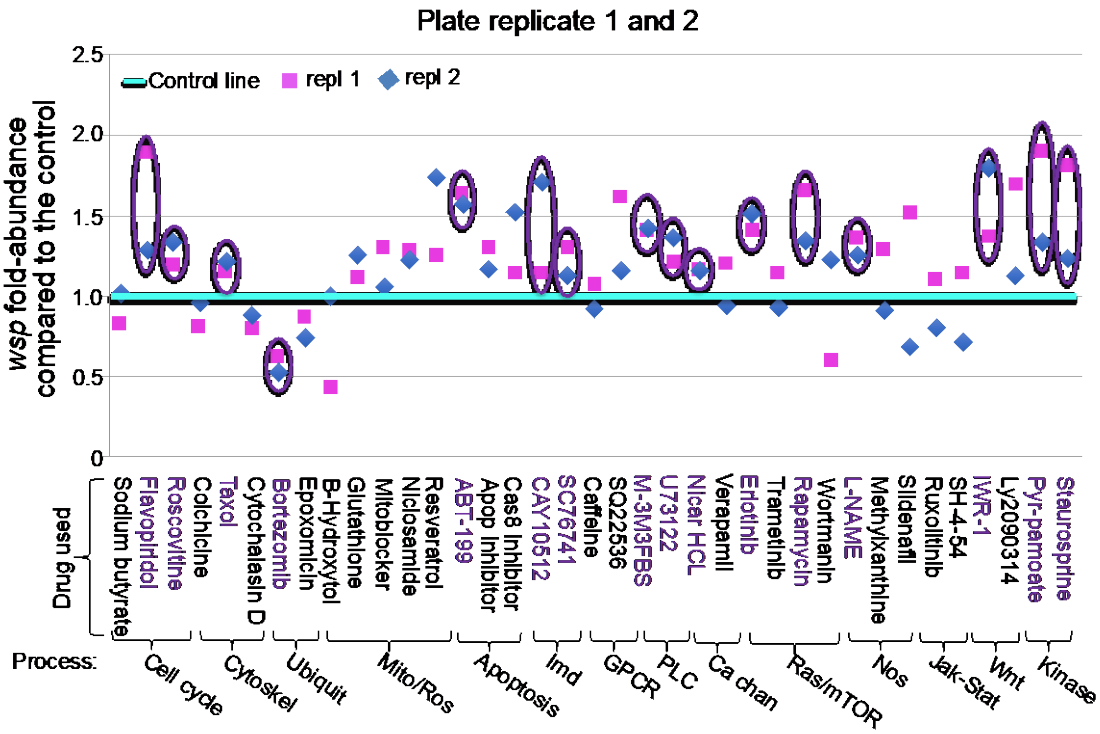

Figure 4.3. Preliminary results of whole body-based drug screening with DB *w*Mel. Graph shows the average absolute copy number for *wsp* in response to each treatment, normalized to respective DMSO controls. X-axis shows: 37 chemicals tested, representing14 different cellular processes. Circled: 16 compounds identified as “hits,” corresponding to 11 different biological processes. Purple squares: data from plate replicate 1. Blue diamonds: data from plate replicate 2. Cyan line: baseline titer, DMSO control. Appropriate statistics were applied on the raw data. Significance was set at * $p < 0.05$.

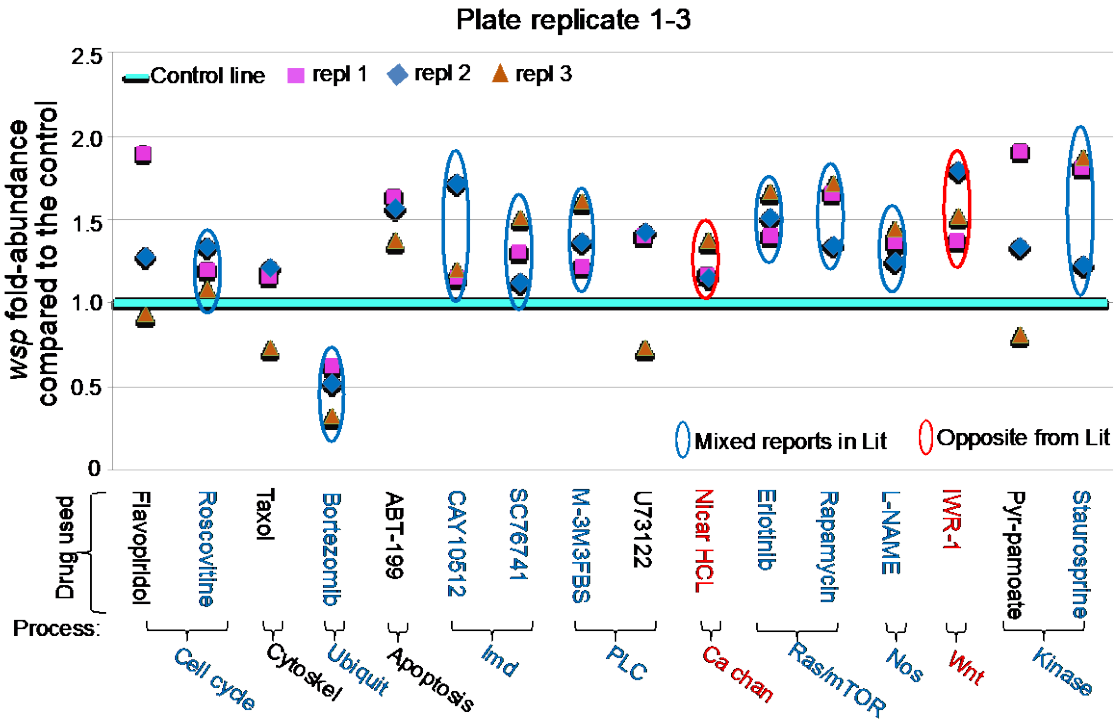

Figure 4.4. Compounds retested on DB *w*Mel in a third plate replicate are plotted. Each dot represents average absolute copy number of *wsp,* normalized to respective DMSO controls. X-axis shows 16 chemicals tested corresponding to 11 different biological processes. Circled: 11 compounds reconfirmed as "hits", corresponding to 9 different host cellular processes. Red circles: outcomes opposite of the literature. Blue circles: mixed records in the literature. Purple squares: data from plate replicate 1. Blue diamonds: data from plate replicate 2. Orange triangles: data from plate replicate 3. Cyan line: baseline titer, DMSO control. Appropriate statistics were applied on the raw data. Significance was set at * $p < 0.05$.

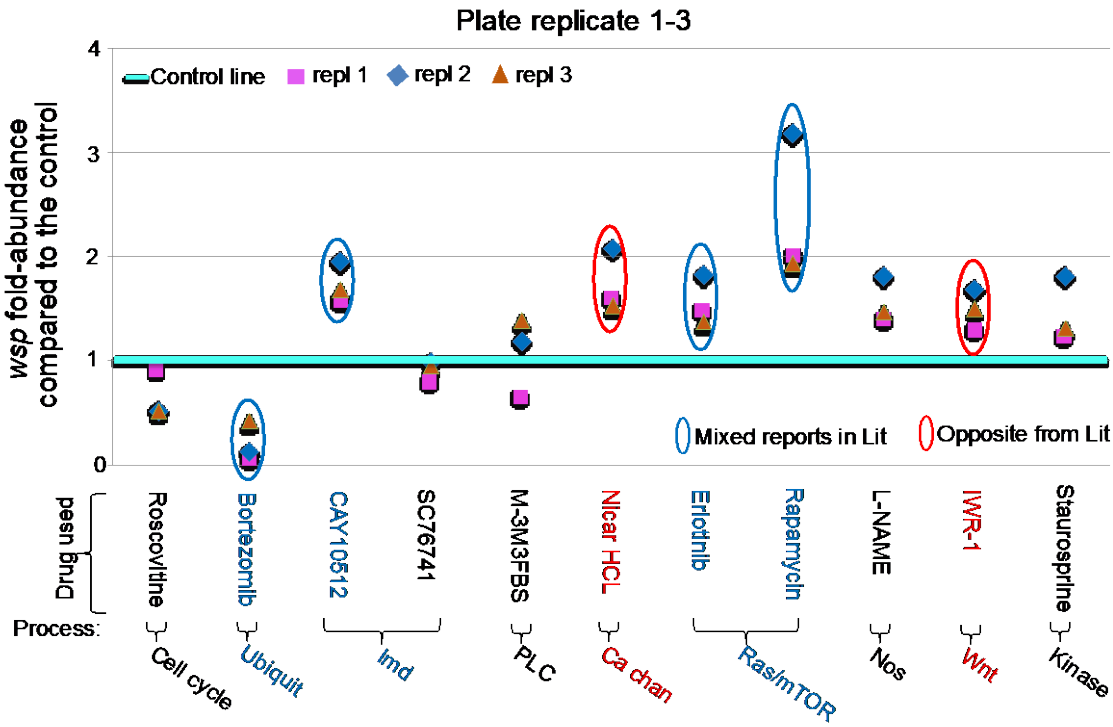

Figure 4.5 Results from whole body-based drug screening with Dsim *w*Ri. Y-axis shows average absolute *wsp* copy numbers normalized to their respective DMSO controls. X-axis shows 11 chemicals tested on Dsim *w*Ri representing 9 different biological processes. Circled: 6 compounds identified as "hits" corresponding to 5 different cellular processes. Green circles: outcome consistent with field literature. Red circles: outcomes opposite of the literature. Blue circles: mixed records in the literature. Purple squares: data from plate replicate 1. Blue diamonds: data from plate replicate 2. Orange triangles: data from plate replicate 3. Cyan line: baseline titer, DMSO control. Appropriate statistics were applied on the raw data. Significance was set at * $p < 0.05$.

Developmental RNA-seq data		
Developmental stages	Actin5C	daughterless
Embryo 0-2hr	1417	103
Embryo12-14hr	1285	48
Embryo 22-24hr	1825	20
Larva L1	1325	22
Larva L2	1744	13
Larva L3 12hr	1063	12
Pupa 1d	1978	41
Pupa 3d	382	27
Pupa 4d	1458	24
Adult female 1d	1133	43
Adult female 5d	1126	54

Color	Expression
	extremely high
	very high
	moderately high
	moderate

Figure 4.6: Expression profiles of Actin5C and daughterless genes at different developmental stages. The data is obtained from modENCODE project (Graveley et al., 2011), as communicated via the community resource website *Flybase.net*

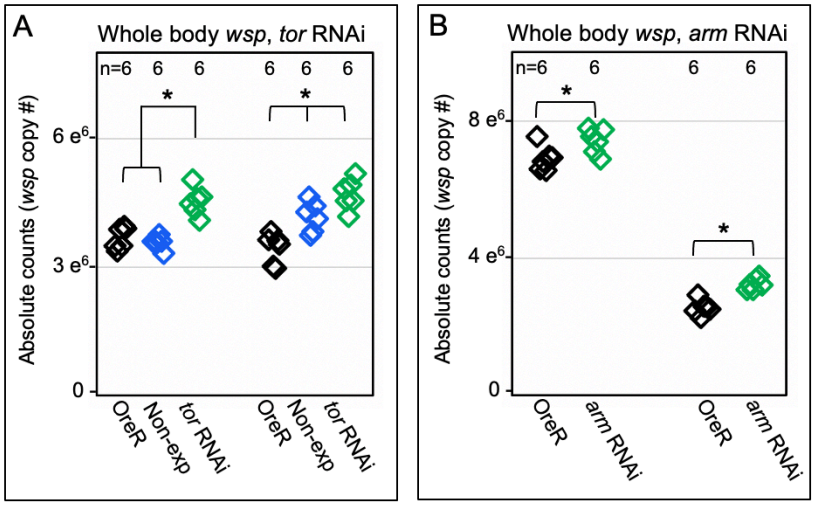

Figure 4.7 Absolute *wsp* abundance of whole-body samples from da-GAL4:UAS-RNAi knockdown flies versus control flies. A) *tor* RNAi as compared to non-expressing sibling controls (non-exp) and an OreR outcrossed control (OreR). B) *armadillo* RNAi (*arm*) vs only an OreR outcrossed control (OreR), as non-expressing siblings were not available. Panels show data from 2 independent plate replicates. “n” represents 6 technical replicates from 3 sample tubes. Black diamonds: OreR control. Blue diamonds: non-expressing sibling control. Green diamonds: RNAi expressing lines. Appropriate statistics were applied on the raw data. Significance was set at * $p < 0.05$.

Process	Drug name	Catalouge #	Vendor
Cell cycle	Flavopiridol	S1230	Selleckchem
	Roscovitine	A1723	ApexBio
	Sodium butyrate	B5887	Sigma
Cytoskeleton	Colchicine	CO115	Sigma
	Cytochalasin D	B6645	ApexBio
	Taxol	T7402	Sigma
Ubiquitin-proteasome system	Bortezomib	S1013	Selleckchem
	Epoxomicin	S7038	Selleckchem
Ros/ Mitochondria	Butylated hydroxytoluene	S25212A	Thermo fisher
	L-Glutathione	G4251	Sigma
	Mitoblocker	505759	Sigma
	Niclosamide	N3510	Sigma
	Resveratrol	R5010	Sigma
Apoptosis	ABT-199	6960	Tocris
	Apoptosis inhibitor	178488	Sigma
	Caspase 8 inhibitor	SC-3084	Santa cruz
GPCR	Caffeine	C0750	Sigma
	SQ22536	S8283	Selleckchem
IMD	CAY10512	sc-205237	ApexBio
	SC76741	SML2382	Sigma
PLC	M-3M3FBS	T5699	Sigma
	U73122	1268	Tocris
Calcium Channel	Nicardipine Hcl	N7510	Sigma
	Verapamil	V4629	Sigma
Ras	Erlotinib hcl	SML2156	Sigma
	Trametinib	S2673	Fisher
mTor	Rapamycin	553211	Sigma
	Wortmanin	S2758	Selleckchem
Nitric oxide synthase	L-NAME	N5751	Sigma
	Methylxanthine	69720	Sigma
	Sildenafil	SIL-984	TSZ Chem
JAK-STAT	Ruxolitinib	S1378	Selleckchem
	SH-4-54	B4789	ApexBio
Wnt	IWR-1	B2306	ApexBio
	Ly090314	SML1438	Sigma
Kinase	Pyrvinium pamoate	P0027	Sigma
	Staurosprine	S1421	Selleckchem

Table 4.1 List of drugs tested in the screen

Process	Stock #	Gene name	Valium	Chromosome
Ubiq-Prot	53974	*Prosalpha6*	Val20 Trip line	2
	42631	*Ubc6*	Val20 Trip line	2
Imd	33661	*Relish*	Val20 Trip line	3
	53377	*Tak1*	Val20 Trip line	2
Ca channel	33413	*Ca-alpha1D*	Val20 Trip line	3
	77174	*Cacophony*	Val20 Trip line	2
Ras/mTOR	60012	*Egfr*	Val20 Trip line	2
	58312	*Tor*	Val20 Trip line	2
Wnt	35004	*Armadillo*	Val20 Trip line	3
	38293	*Sgg*	Val20 Trip line	2

Table 4.2 RNAi lines used for the genetic analyses

Class	Genus	# species researched	# species pursued
Alphaproteobacteria	*Brucella*	5	2
Alphaproteobacteria	*Rickettsia*	5	5
Alphaproteobacteria	*Anaplasma*	3	1
Alphaproteobacteria	*Ehrlichia*	3	3
Alphaproteobacteria	*Wolbachia*	1	1
Gammaroteobacteria	*Legionella*	8	1
Gammaroteobacteria	*Francisella*	3	1
Gammaroteobacteria	*Salmonella*	2	1
Gammaroteobacteria	*Wigglesworthia*	1	1
Gammaroteobacteria	*Coxiella*	1	1
Gammaroteobacteria	*Pseudomonas aeruginosa*	3	1
Actinobacteria	*Mycobacterium*	2	2
Bacilli	*Listeria*	5	1
Chlamydiae	*Chlamydia*	3	2
Clostridia	*Candidatus Arthromitus*	1	1
Mollicutes	*Spiroplasma*	1	1
Spirochaetales	*Borrelia*	5	1

Table 4.3 Different taxa discussed in literature with respect to titer control of other microbes

Table: Testable pathways				
Candidate host processes	Number of organisms represented	Number of classes represented	Name of the Organisms	References
Cell cycle modulation	4*	4	*M. fortuitum*; *Listeria monocytogenes*; *Chlamydia caviae*; *Wolbachia*	Agaisse et al., 2005; Derre et al., 2007; Grobler et al., 2018
Cytoskeletal	6*	4	*Ehrlichia risticii; Ehrlichia canis*; *Listeria monocytogenes*; *Mycobacterium fortuitum*; *Chlamydia caviae*; *Wolbachia*	Rikihisa et al., 2004; Kuhbacher et al., 2015; Derre et al., 2007; Philips et al., 2005; Ferree et al., 2005, Serbus et al., 2011; Newton et al., 2015; Sheehan et al., 2016
Ubiquitination pathway	3*	1	*Ehrlichia chaffeensis*; *Anaplasma phagocytophilum*; *Wolbachia;*	Zhu et al., 2017; Severo et al., 2013., White et al., 2017
Mitochondrial/ Antioxidant	4*	4	*Chlamydia cavia*; *Wolbachia*; *Francisella tularensis*; *Borrelia burgdorferi*	Derre et al., 2007; White et al., 2017
Apoptotic pathway	3	3	*Brucella abortus*; *Legionella pneumophila*; *Mycobacteria*	Wei et al., 2015; Gross et al., 2000; Ashida et al., 2011
IMD pathway	5*	4	*Rickettsia typhi*; *Wigglesworthia glossinidia*; *Borrelia burgdorferi*; *Spiroplasma poulsonii*; *Pseudomonas aeruginosa*	Rennoll et al., 2018; Wang et al., 2009; Carroll et al., 2019; Herren & Lemaitre et al., 2011; Limmer et al., 2011
GPCR Signaling	5*	2	*Rickettsia conorii*; *Coxiella burnetii*; *Brucella abortus*; *Legionella pneumophila*; *Wolbachia*; *E. chaffeensis*	Czyz et al., 2014; Kim et al., 2012; White et al., 2017
Phospholipase-related	3	1	*Ehrlichia chaffeensis*; *Ehrlichia canis*; *Rickettsia rickettsii*;	Lin et al., 2002; Levenhagen et al., 2012; Walker et al., 1983

Table 4.4: Mechanisms that affect intracellular density of commonly studied microbes.

(continue on the next page)

Table: Testable pathways				
Candidate host processes	Number of organisms represented	Number of classes represented	Name of the Organisms	References
Calcium signaling	7	2	*E. risticii; Ehrlichia chaffeensis; Ehrlichia canis*; *Rickettsia conorii*; *Coxiella burnetii*; *Brucella abortus*; *Legionella pneumophila*	Rikihisa & Park., 1995; Lin et al., 2002; Levenhagen et al., 2012; Czyz et al., 2014; Kim et al., 2012;
Ras/ mTor pathway	13*	7	*Salmonella typhimorium; Chlamydia cavia; Listeria monocytogenes; Wolbachia; Franciella tularensis; Candidatus Arthromitus; Ehrlicia chaffeensis; Rickettsia australis; Mycobacterium tuberculosis*; *Pseudomonas aeruginosa; Anaplasma phagocytophilum; Coxiella burnetii ; Brucella suis*	Xiong et al., 2009; Czyz et al., 2014; Bagues et al., 2005; Luo et al., 2017; Birmingham et al., 2006; Derre et al., 2007; Steele et al., 2013; Yang et al., 2018; Niu et al., 2008; Niu et al., 2012; Lina et al., 2017; Bechelli et al., 2019; Gutierre et al., 2004; Wang et al., 2013; Yuan et al., 2012; Foldenauer et al., 2013
Nitric oxide synthase	4	2	*Ehrlicia; Rickettsia prowazekii*; *Brucella abortus*; *Chlamydia cavia; Listeria monocytogenes*	Banerjee et al., 2000; Turco et al., 1998; Czyz et al., 2016; Zhang et al., 2012; Cole et al., 2012
JAK-STAT signaling	4*	2	*Anaplasma phagocytophilum*; *Wolbachia*; *Rickettsia conorii*; *Chlamydia trachomatis*	Liu et al., 2012; Grobler et al., 2018; Colonne et al., 2011; Lad et al., 2005; Zhang et al., 2018
Wnt pathway	4*	3	*Ehrlichia chaffeensis*; *Wolbachia*; *Chlamydia trachomatis*; *Mycobacterium tuberculosis*	Luo et al., 2016; Grobler et al., 2018; Kintner et al., 2017; Kessler et al., 2012; Rogan et al., 2019; Pean et al., 2017
Kinase modulator	7*	4	*Ehrlichia risticii & chaffeensis & canis*; *Mycobacterium tuberculosis*; *S. typhimurium*; *Coxiella burnetii*; *Brucella abortus*; *Pseudomonas aeruginosa*	Zhang & Rikihisa., 1997; Lin et al., 2002; Levenhagen et al., 2012; Kuijl et al., 2007; Czyz et al 2014; Czyz et al., 2016; Alhazmi et al., 2018

Table 4.4: Mechanisms that affect intracellular density of commonly studied microbes. (continued from previous page).

Processes	Drug name	Plate replicate	Median of treatment	Median of DMSO	Comaparative analysis	Resulting p-value	Impact on *Wolbachia* titer
Cell cycle	Flavopiridol	**1**	**12305000**	**7928500**	**Mann-Whitney U**	**0.002**	**Increased**
		2	**7656000**	**6488000**	**Indep. T-test**	**0.014**	**Increased**
		3	**16915000**	**17860000**	**Indep. T-test**	**0.111**	**no**
	Roscovitine	1	6570277	5754829	Indep. T-test	0.003	Increased
		2	8249002	6720730	Welch's T-test	0.004	Increased
		3	5815500	5479257	Mann-Whitney U	0.009	Increased
Cytoskeletal	Taxol	**1**	**3474462**	**3119664**	**Indep. T-test**	**0.043**	**Increased**
		2	**4153869**	**3635670**	**Indep. T-test**	**0.005**	**Increased**
		3	**8526000**	**10750000**	**Indep. T-test**	**0.011**	**Decreased**
Ubiquitin-proteasome	Bortezomib	1	2216088	3119664	Indep. T-test	<0.001	Decreased
		2	2444533	3635670	Indep. T-test	<0.001	Decreased
		3	5168500	10750000	Indep. T-test	<0.001	Decreased
Apoptosis	ABT-199	**1**	**8963500**	**6263500**	**Indep. T-test**	**<0.001**	**Increased**
		2	**7975750**	**5958000**	**Indep. T-test**	**0.01**	**Increased**
		3	**23455000**	**17860000**	**Indep. T-test**	**0.103**	**no**
Imd	CAY10512	1	5159500	4682500	Indep. T-test	0.017	Increased
		2	10715000	7314500	Indep. T-test	0.001	Increased
		3	7285000	6374500	Indep. T-test	0.047	Increased
	SC76741	1	9276500	7690500	Indep. T-test	0.022	Increased
		2	8608500	7921000	Indep. T-test	0.02	Increased
		3	7483750	5479257	Indep. T-test	<0.001	Increased
PLC	U73122	**1**	**3612828**	**3119664**	**Indep. T-test**	**0.013**	**Increased**
		2	**4533621**	**3635670**	**Indep. T-test**	**0.012**	**Increased**
		3	**3996500**	**5047000**	**Indep. T-test**	**0.004**	**lower**
	M-3M3FBS	1	6056000	4682500	Indep. T-test	0.004	Increased
		2	9336000	7314500	Indep. T-test	0.002	Increased
		3	9619500	6760500	Mann-Whitney U	0.002	Increased
Calcium channel	Nicardipine Hcl	1	8540500	7690500	Indep. T-test	0.016	Increased
		2	8825500	7921000	Indep. T-test	0.012	Increased
		3	6945000	5479257	Indep. T-test	<0.001	Increased
Ras	Erlotinib	1	7971500	6263500	Indep. T-test	<0.001	Increased
		2	7772500	5958000	Indep. T-test	0.002	Increased
		3	8104500	5479257	Indep. T-test	<0.001	Increased
mTor	Rapamycin	1	8508228	5754829	Indep. T-test	0.001	Increased
		2	6720730	2108747	Indep. T-test	<0.001	Increased
		3	10744500	7314500	Indep. T-test	<0.001	Increased
Nos	L-NAME	1	5826000	4636000	Indep. T-test	0.002	Increased
		2	6973500	6004500	Mann-Whitney U	0.002	Increased
		3	7214500	5479257	Indep. T-test	<0.001	Increased
Wnt	IWR-1	1	7287916	5754829	Indep. T-test	0.001	Increased
		2	10361054	6720730	Indep. T-test	<0.001	Increased
		3	9802500	7314500	Indep. T-test	<0.001	Increased
Kinase	Pyrvinium pamoate	**1**	**7645500**	**4636000**	**Mann-Whitney U**	**0.002**	**Increased**
		2	**7321000**	**6004500**	**Indep. T-test**	**<0.001**	**Increased**
		3	**9185000**	**10750000**	**Indep. T-test**	**0.234**	**no**
	Staurosprine	1	11895000	7928500	Mann-Whitney U	0.002	Increased
		2	7451500	6488000	Indep. T-test	0.006	Increased
		3	11480000	7314500	Mann-Whitney U	0.002	Increased

Table 4.5: Required statistical analyses for DB *w*Mel whole body *Wolbachia* titer in response to drug treatment. Data shows median *wsp* abundance of drug treatments and associated DMSO control. Conditions that induced a consistent titer change in all plate replicates are indicated in grey-shaded areas.

Processes	Drug name	Plate replicate	Median of treatment	Median of DMSO	Comparative analysis	Resulting p-value	Impact on *Wolbachia* titer
Cell cycle	Roscovitine	1	15925526	16784969	Indep. T-test	0.283	no
		2	14174327	18582671	Indep. T-test	0.013	Decreased
		3	10290344	14001082	Indep. T-test	0.006	Decreased
Ubiquitin-proteasome	Bortezomib	1	9340971	16784969	Welch's T-test	<0.001	Decreased
		2	10405154	18582671	Indep. T-test	<0.001	Decreased
		3	9592492	14001082	Indep. T-test	<0.001	Decreased
Imd	CAY10512	1	21345402	16784969	Indep. T-test	0.001	Increased
		2	27645268	18582671	Indep. T-test	<0.001	Increased
		3	19419795	14001082	Indep. T-test	<0.001	Increased
	SC76741	1	15095678	16784969	Indep. T-test	0.171	no
		2	18490031	18582671	Indep. T-test	0.561	no
		3	13655230	14001082	Indep. T-test	0.936	no
PLC	M-3M3FBS	1	14130000	16784969	Indep. T-test w/bootstrap	0.044	Increased
		2	20361562	18582671	Indep. T-test	0.84	no
		3	17098326	14001082	Indep. T-test	0.009	Increased
Calcium channel	Nicardipine Hcl	1	15460000	12956000	Indep. T-test	0.006	Increased
		2	22236522	17503958	Mann-Whitney U test	0.002	Increased
		3	24983535	18640999	Indep. T-test	<0.001	Increased
Ras	Erlotinib	1	14905000	12956000	Indep. T-test	0.024	Increased
		2	21166624	17503958	Welch's T-test	0.014	Increased
		3	23166453	18640999	Indep. T-test	<0.001	Increased
mTor	Rapamycin	1	18613380	12315759	Indep. T-test	<0.001	Increased
		2	24661569	12413239	Indep. T-test	<0.001	Increased
		3	17113581	11267502	Indep. T-test	<0.001	Increased
Nos	L-NAME	1	14580000	12956000	Indep. T-test	0.056	no
		2	21041754	17503958	Indep. T-test	0.001	Increased
		3	24220632	18640999	Indep. T-test	0.004	Increased
Wnt	IWR-1	1	14130000	12956000	Mann-Whitney U test	0.041	Increased
		2	20553900	17503958	Mann-Whitney U test	0.002	Increased
		3	24607540	18640999	Welch's T-test	<0.001	Increased
Kinase	Staurosprine	1	13776775	12315759	Indep. T-test	0.05	Increased
		2	17000535	12413239	Indep. T-test	0.003	Increased
		3	13228434	11267502	Indep. T-test	0.061	no

Table 4.6: Required statistical analyses for Dsim *w*Ri whole body *Wolbachia* titer in response to drug treatment. Data shows median *wsp* abundance of drug treatments and associated DMSO control. Conditions that induced a consistent titer change in all plate replicates are indicated in grey-shaded areas.

Processes	RNAi	Plate replicate	RNAi median	Control type	Control median	Comparative analysis	Resulting p-value	Significant difference?
Imd	Relish	1	15400000	non-exp	14100000	Indep. T-test	0.005	higher
		2	12600000	non-exp	11300000	Indep. T-test	0.128	no
	Tak1	1	16500000	non-exp	14100000	Mann-Whitney U	0.132	no
		2	9430000	non-exp	6610000	Mann-Whitney U	0.002	higher
Calcium channel	Ca1alphaD	1	6940000	non-exp	6570000	Indep. T-test	0.135	no
		2	Did not pursue					
Wnt	Armadilo	1	13400000	non-exp	10000000	Indep. T-test	0.001	higher
		2	12700000	non-exp	10700000	Indep. T-test	0.004	higher

Table 4.7 Required statistical analyses of whole body *Wolbachia* titer in response to Actin5C-GAL4:UAS-RNAi mediated gene knockdown. Data shows median *wsp* abundance of RNAi knocked down flies, and non-expressed control flies.

Processes	RNAi	Plate replicate	RNAi median	Control type	Control median	Comparative analysis	Resulting p-value	Significant difference?
Ubiquitin-proteasome	Pros alpha	1	5530000	Ore-R	5240000	Indep. T-test	0.11	no
				non-exp	4630000	Indep. T-test	0.004	higher
		2	7410000	Ore-R	6670000	Indep. T-test	0.065	no
				non-exp	6760000	Indep. T-test	0.071	no
	Ubc6	1	12700000	Ore-R	12000000	Indep. T-test	0.077	no
				non-exp	11000000	Indep. T-test	0.394	no
		2	7340000	Ore-R	5520000	Indep. T-test	<0.001	higher
				non-exp	6660000	Indep. T-test	0.077	no
Imd	Relish	1	4310000	Ore-R	4680000	Indep. T-test	0.653	no
				non-exp	n/a	n/a	n/a	n/a
		2	5050000	Ore-R	5960000	Indep. T-test	0.038	lower
				non-exp	n/a	n/a	n/a	n/a
	Tak1	1	4580000	Ore-R	5340000	Indep. T-test	0.147	no
				non-exp	n/a	n/a	n/a	n/a
		2	5210000	Ore-R	4640000	Indep. T-test	0.548	no
				non-exp	n/a	n/a	n/a	n/a
Calcium channel	Ca1alpha	1	3830000	Ore-R	3680000	Indep. T-test	0.272	no
				non-exp	n/a	n/a	n/a	n/a
		2	8220000	Ore-R	8660000	Mann-Whitney U	0.394	no
				non-exp	n/a	n/a	n/a	n/a
	Cac	1	3970000	Ore-R	4470000	Mann-Whitney U	0.589	no
				non-exp	n/a	n/a	n/a	n/a
		2	8220000	Ore-R	8660000	Indep. T-test	0.337	no
				non-exp	n/a	n/a	n/a	n/a
Ras/mTor	Egfr	1	9540000	Ore-R	8200000	Indep. T-test	<0.001	higher
				non-exp	6640000	Indep. T-test	<0.001	higher
		2	5670000	Ore-R	6200000	Indep. T-test	0.258	no
				non-exp	4190000	Welch's T-test	0.002	higher
	Tor	1	4540000	Ore-R	3680000	Indep. T-test	<0.001	higher
				non-exp	3610000	Indep. T-test	<0.001	higher
		2	4690000	Ore-R	3580000	Indep. T-test	<0.001	higher
				non-exp	4210000	Indep. T-test	0.028	higher
Wnt	Armadillo	1	7470000	Ore-R	6880000	Indep. T-test	0.034	higher
				non-exp	n/a	n/a	n/a	n/a
		2	3240000	Ore-R	2540000	Indep. T-test	<0.001	higher
				non-exp	n/a	n/a	n/a	n/a
	Sgg	1	5540000	Ore-R	4420000	Indep. T-test	0.008	higher
				non-exp	5590000	Welch's T-test	0.722	no
		2	4050000	Ore-R	3950000	Indep. T-test	0.702	no
				non-exp	3540000	Indep. T-test	0.002	higher

Table 4.8: Required statistical analyses of whole body *Wolbachia* titer in response to da-GAL4:UAS-RNAi mediated gene knockdown. Data shows median *wsp* abundance of RNAi knocked down flies, non-expressed control flies, and OreR control flies. n/a: Flies were not available for the comparison. Conditions that induced a consistent titer change in all replicates are indicated in grey-shaded areas.

Processes	RNAi	Plate replicate	RNAi median	Control type	Control median	Comparative analysis	Resulting p-value	Significant difference?
Ubiquitin-proteasome	Pros alpha	1	1990000	Ore-R	1470000	Indep. T-test	0.001	higher
				non-exp	1790000	Indep. T-test	0.329	no
		2	3400000	Ore-R	3420000	Welch's T-test	0.869	no
				non-exp	3170000	Indep. T-test	0.064	no
	Ubc6	1	5000000	Ore-R	5000000	Indep. T-test	0.001	lower
				non-exp	4470000	Indep. T-test	0.014	higher
		2	2490000	Ore-R	3080000	Indep. T-test	0.081	no
				non-exp	1790000	Indep. T-test	<0.001	higher
Imd	Relish	1	1650000	Ore-R	2030000	Indep. T-test	0.101	no
				non-exp	n/a	n/a	n/a	n/a
		2	2160000	Ore-R	2410000	Indep. T-test	0.152	no
				non-exp	n/a	n/a	n/a	n/a
	Tak1	1	1070000	Ore-R	1470000	Mann-Whitney U	0.028	lower
				non-exp	n/a	n/a	n/a	n/a
		2	1880000	Ore-R	2810000	Mann-Whitney U	<0.001	lower
				non-exp	n/a	n/a	n/a	n/a
Calcium channel	Ca1alpha	1	1420000	Ore-R	1390000	Indep. T-test	0.642	no
				non-exp	n/a	n/a	n/a	n/a
		2	3730000	Ore-R	3390000	Mann-Whitney U	0.394	no
				non-exp	n/a	n/a	n/a	n/a
	Cac	1	1410000	Ore-R	1750000	Mann-Whitney U	0.589	no
				non-exp	n/a	n/a	n/a	n/a
		2	3690000	Ore-R	3540000	Indep. T-test	0.801	no
				non-exp	n/a	n/a	n/a	n/a
Ras/mTOR	Egfr	1	3810000	Ore-R	1980000	Indep. T-test	0.625	no
				non-exp	3550000	Indep. T-test	<0.001	higher
		2	1080000	Ore-R	1100000	Indep. T-test	<0.001	lower
				non-exp	1840000	Indep. T-test	0.408	no
	Tor	1	955000	Ore-R	738000	Welch's T-test	0.003	higher
				non-exp	750000	Welch's T-test	<0.001	higher
		2	1870000	Ore-R	1830000	Welch's T-test	0.711	no
				non-exp	1530000	Indep. T-test	0.015	higher
Wnt	Armadillo	1	3580000	Ore-R	3260000	Indep. T-test	0.377	no
				non-exp	n/a	n/a	n/a	n/a
		2	1510000	Ore-R	1550000	Indep. T-test	0.92	no
				non-exp	n/a	n/a	n/a	n/a
	Sgg	1	1070000	Ore-R	1030000	Welch's T-test	0.334	no
				non-exp	1020000	Indep. T-test	0.087	no
		2	1830000	Ore-R	2450000	Indep. T-test	0.002	lower
				non-exp	1290000	Indep. T-test	<0.001	higher

Table 4.9: Required statistical analyses of ovarian *Wolbachia* titer in response to da-GAL4:UAS-RNAi mediated gene knockdown. Data shows median *wsp* abundance of ovary tissues from RNAi knocked down flies, non-expressed control flies, and OreR control flies. n/a: Flies were not available for the comparison.

Species	Disruption tool/method	Effect on density	Reference
A. phagocytophilum	Rapamycin	Increase	Niu et al., 2008
Candidatus Arthromitus	*atg5* null mutant	Increase	Yang et al., 2018
Chlamydia cavia	*tor* RNAi	Decrease	Derre et al., 2007
Ehrlicia chaffeensis	*rheb* RNAi	Decrease	Lina et al., 2017
Listeria monocytogenes	*tor* RNAi	Decrease	Derre et al., 2007
Mycobacterium tuberculosis	Rapamycin	Decrease	Gutierrez et al., 2004
Pseudomonas aeruginosa	Rapamycin	Decrease	Yuan et al., 2012
Rickettsia australis	Rapamycin	Increase	Bechelli et al., 2019
Salmonella typhimurium	*atg5* null mutant	Increase	Birmingham et al., 2006
Wolbachia spp.	Rapamycin (*B. malayi*)	Decrease	Voronin et al., 2012
	Rapamycin (*D. mel*)	Decrease	Voronin et al., 2012
	atg1 RNAi (*D. mel* germline)	Decrease	Deehan et al., 2021
	atg8 RNAi (*D. mel* germline)	Decrease	Deehan et al., 2021
	Rapamycin (*D. mel* germline)	Increase	Serbus et al., 2015
	atg6 RNAi (*D. mel* cell culture)	Increase	Grobler et al., 2018
	atg18b RNAi (*D. mel* cell culture)	Increase	Grobler et al., 2018

Table 4.10: Impact of host autophagy pathway on the intracellular load of various intracellular bacteria, according to field literature.

References

Allocca, M., Zola, S., & Bellosta, P. (2018). The fruit fly, *Drosophila melanogaster:* The making of a model (Part I). *Drosophila Melanogaster - Model for Recent Advances in Genetics and Therapeutics*. https://doi.org/10.5772/intechopen.72832

Azoulay-Alfaguter, I., Elya, R., Avrahami, L., Katz, A., & Eldar-Finkelman, H. (2015). Combined regulation of mTORC1 and lysosomal acidification by GSK-3 suppresses autophagy and contributes to cancer cell growth. *Oncogene*, *34*(35), 4613–4623. https://doi.org/10.1038/onc.2014.390

Bechelli, J., Vergara, L., Smalley, C., Buzhdygan, T. P., Bender, S., Zhang, W., Liu, Y., Popov, V. L., Wang, J., Garg, N., Hwang, S., Walker, D. H., & Fang, R. (2018). Atg5 supports *Rickettsia australis* infection in macrophages in vitro and in vivo. *Infection and Immunity*, *87*(1). https://doi.org/10.1128/IAI.00651-18

Birmingham, C. L., Smith, A. C., Bakowski, M. A., Yoshimori, T., & Brumell, J. H. (2006). Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole. *The Journal of Biological Chemistry*, *281*(16), 11374–11383. https://doi.org/10.1074/jbc.M509157200

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development (Cambridge, England)*, *118*(2), 401–415.

Camacho, M., Oliva, M., & Serbus, L. R. (2017). Dietary saccharides and sweet tastants have differential effects on colonization of *Drosophila* oocytes by *Wolbachia* endosymbionts. *Biology Open*, *6*(7), 1074–1083. https://doi.org/10.1242/bio.023895

Caspi, M., Zilberberg, A., Eldar-Finkelman, H., & Rosin-Arbesfeld, R. (2008). Nuclear GSK-3β inhibits the canonical Wnt signalling pathway in a β-catenin phosphorylation-independent manner. *Oncogene*, *27*(25), 3546–3555. https://doi.org/10.1038/sj.onc.1211026

Christensen, S., Camacho, M., Sharmin, Z., Momtaz, A. J. M. Z., Perez, L., Navarro, G., Triana, J., Samarah, H., Turelli, M., & Serbus, L. R. (2019). Quantitative methods for assessing local and bodywide contributions to *Wolbachia* titer in maternal germline cells of *Drosophila*. *BMC Microbiology*, *19*. https://doi.org/10.1186/s12866-019-1579-3

Christensen, S., Pérez Dulzaides, R., Hedrick, V. E., Momtaz, A. J. M. Z., Nakayasu, E. S., Paul, L. N., & Serbus, L. R. (2016). *Wolbachia* endosymbionts modify *Drosophila* ovary protein levels in a context-dependent manner. *Applied and Environmental Microbiology*, *82*(17), 5354–5363. https://doi.org/10.1128/AEM.01255-16

Czyż, D. M., Potluri, L.-P., Jain-Gupta, N., Riley, S. P., Martinez, J. J., Steck, T. L., Crosson, S., Shuman, H. A., & Gabay, J. E. (2014). Host-directed antimicrobial drugs with broad-spectrum efficacy against intracellular bacterial pathogens. *MBio*, *5*(4). https://doi.org/10.1128/mBio.01534-14

da Rocha Fernandes, M., Martins, R., Pessoa Costa, E., Casagrande Pacidônio, E., Araujo de Abreu, L., da Silva Vaz, I., Moreira, L. A., da Fonseca, R. N., & Logullo, C. (2014). The modulation of the symbiont/host interaction between *Wolbachia pipientis* and *Aedes fluviatilis* embryos by glycogen metabolism. *PLoS ONE*, *9*(6). https://doi.org/10.1371/journal.pone.0098966

de Cuevas, M., & Spradling, A. C. (1998). Morphogenesis of the *Drosophila* fusome and its implications for oocyte specification. *Development (Cambridge, England)*, *125*(15), 2781–2789.

Deehan, M., Lin, W., Blum, B., Emili, A., & Frydman, H. (2021). Intracellular Density of *Wolbachia* Is mediated by host autophagy and the bacterial cytoplasmic incompatibility gene cifb in a cell type-dependent manner in *Drosophila melanogaster*. *MBio*, *12*(1). https://doi.org/10.1128/mBio.02205-20

Derré, I., Pypaert, M., Dautry-Varsat, A., & Agaisse, H. (2007). RNAi screen in *Drosophila* cells reveals the involvement of the tom complex in *Chlamydia* Infection. *PLoS Pathogens*, *3*(10). https://doi.org/10.1371/journal.ppat.0030155

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J., & Hurst, G. D. (2008). The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology*, *6*, 27. https://doi.org/10.1186/1741-7007-6-27

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., & Stackebrandt, E. (Eds.). (2006). The Prokaryotes: Vol. 5: proteobacteria: Alpha and beta subclasses (3rd ed.). Springer-Verlag. https://www.springer.com/us/book/9780387307459

Ehrman, L. (1998). Influential Passengers: Inherited microorganisms and arthropod reproduction. Scott L. O'Neill , Ary A. Hoffmann , John H. Werren. *The Quarterly Review of Biology*, *73*(4), 514–515. https://doi.org/10.1086/420470

Ferree, P. M., Frydman, H. M., Li, J. M., Cao, J., Wieschaus, E., & Sullivan, W. (2005). *Wolbachia* utilizes host microtubules and dynein for anterior localization in the *Drosophila* oocyte. *PLoS Pathogens*, *1*(2). https://doi.org/10.1371/journal.ppat.0010014

Ford, D., Hoe, N., Landis, G. N., Tozer, K., Luu, A., Bhole, D., Badrinath, A., & Tower, J. (2007). Alteration of *Drosophila* life span using conditional, tissue-specific expression of transgenes triggered by doxycyline or RU486/Mifepristone. *Experimental Gerontology*, *42*(6), 483–497. https://doi.org/10.1016/j.exger.2007.01.004

Goto, S., Anbutsu, H., & Fukatsu, T. (2006). Asymmetrical Interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host.

Applied and Environmental Microbiology, *72*(7), 4805–4810. https://doi.org/10.1128/AEM.00416-06

Grobler, Y., Yun, C. Y., Kahler, D. J., Bergman, C. M., Lee, H., Oliver, B., & Lehmann, R. (2018). Whole genome screen reveals a novel relationship between *Wolbachia* levels and *Drosophila* host translation. *PLoS Pathogens*, *14*(11), e1007445. https://doi.org/10.1371/journal.ppat.1007445

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I., & Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell*, *119*(6), 753–766. https://doi.org/10.1016/j.cell.2004.11.038

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., & Werren, J. H. (2008). How many species are infected with *Wolbachia*? – A statistical analysis of current data. *Fems Microbiology Letters*, *281*(2), 215–220. https://doi.org/10.1111/j.1574-6968.2008.01110.x

Hoffmann, A. A., Turelli, M., & Simmons, G. M. (1986). Unidirectional incompatibility between populations of *Drosophila Simulans*. *Evolution*, *40*(4), 692–701. https://doi.org/10.1111/j.1558-5646.1986.tb00531.x

Hussain, M., Frentiu, F. D., Moreira, L. A., O'Neill, S. L., & Asgari, S. (2011). *Wolbachia* uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector *Aedes aegypti*. *Proceedings of the National Academy of Sciences*, *108*(22), 9250–9255. https://doi.org/10.1073/pnas.1105469108

Jaenike, J. (2009). Coupled population dynamics of endosymbionts within and between hosts. *Oikos*, *118*(3), 353–362. https://doi.org/10.1111/j.1600-0706.2008.17110.x

Jennings, B. H. (2011). Drosophila – a versatile model in biology & medicine. *Materials Today*, *14*(5), 190–195. https://doi.org/10.1016/S1369-7021(11)70113-4

Kessler, M., Zielecki, J., Thieck, O., Mollenkopf, H.-J., Fotopoulou, C., & Meyer, T. F. (2012). Chlamydia trachomatis disturbs epithelial tissue homeostasis in fallopian tubes via paracrine Wnt signaling. *The American Journal of Pathology*, *180*(1), 186–198. https://doi.org/10.1016/j.ajpath.2011.09.015

Kühbacher, A., Emmenlauer, M., Rämo, P., Kafai, N., Dehio, C., Cossart, P., & Pizarro-Cerdá, J. (2015). Genome-wide siRNA screen identifies complementary signaling pathways involved in *Listeria* infection and reveals different actin nucleation mechanisms during *listeria* cell invasion and actin comet tail formation. *MBio*, *6*(3), e00598-00515. https://doi.org/10.1128/mBio.00598-15

Lim, T.-S., & Loh, W.-Y. (1996). A comparison of tests of equality of variances. *Computational Statistics & Data Analysis*, *22*(3), 287–301. https://doi.org/10.1016/0167-9473(95)00054-2

Lina, T. T., Luo, T., Velayutham, T.-S., Das, S., & McBride, J. W. (2017). Ehrlichia Activation of Wnt-PI3K-mTOR signaling inhibits autolysosome generation and autophagic destruction by the mononuclear phagocyte. *Infection and Immunity*, *85*(12). https://doi.org/10.1128/IAI.00690-17

Luo, T., Dunphy, P. S., Lina, T. T., & McBride, J. W. (2016). *Ehrlichia chaffeensis* exploits canonical and noncanonical host wnt signaling pathways to stimulate phagocytosis and promote intracellular survival. *Infection and Immunity*, *84*(3), 686–700. https://doi.org/10.1128/IAI.01289-15

Luo, T., Dunphy, P. S., & McBride, J. W. (2017). *Ehrlichia chaffeensis* tandem repeat effector targets differentially influence infection. *Frontiers in Cellular and Infection Microbiology*, *7*. https://doi.org/10.3389/fcimb.2017.00178

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). Identifying off-target effects and hidden phenotypes of drugs in human cells. *Nature Chemical Biology*, *2*(6), 329–337. https://doi.org/10.1038/nchembio790

McGraw, E. A., Merritt, D. J., Droller, J. N., & O'Neill, S. L. (2002). *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proceedings of the National Academy of Sciences*, *99*(5), 2918–2923. https://doi.org/10.1073/pnas.052466499

Min, K.-T., & Benzer, S. (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proceedings of the National Academy of Sciences*, *94*(20), 10792–10796. https://doi.org/10.1073/pnas.94.20.10792

Monod, J. (1949). The Growth of Bacterial Cultures. *Annual Review of Microbiology*, *3*(1), 371–394. https://doi.org/10.1146/annurev.mi.03.100149.002103

Moran, N. A., McCutcheon, J. P., & Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, *42*, 165–190. https://doi.org/10.1146/annurev.genet.41.110306.130119

Mouton, L., Dedeine, F., Henri, H., Boulétreau, M., Profizi, N., & Vavre, F. (2004). Virulence, multiple infections and regulation of symbiotic population in the *Wolbachia-Asobara tabida* Symbiosis. *Genetics*, *168*(1), 181–189. https://doi.org/10.1534/genetics.104.026716

Newton, I. L. G., Savytskyy, O., & Sheehan, K. B. (2015). *Wolbachia* utilize host actin for efficient maternal transmission in *Drosophila melanogaster*. *PLoS Pathogens*, *11*(4). https://doi.org/10.1371/journal.ppat.1004798

Niu, H., Yamaguchi, M., & Rikihisa, Y. (2008). Subversion of cellular autophagy by *Anaplasma phagocytophilum*. *Cellular Microbiology*, *10*(3), 593–605. https://doi.org/10.1111/j.1462-5822.2007.01068.x

Pan, X., Pike, A., Joshi, D., Bian, G., McFadden, M. J., Lu, P., Liang, X., Zhang, F., Raikhel, A. S., & Xi, Z. (2018). The bacterium *Wolbachia* exploits host innate immunity to establish a symbiotic relationship with the dengue vector mosquito *Aedes aegypti*. *The ISME Journal*, *12*(1), 277–288. https://doi.org/10.1038/ismej.2017.174

Perrimon, N., Ni, J.-Q., & Perkins, L. (2010). In vivo RNAi: Today and tomorrow. *Cold Spring Harbor Perspectives in Biology*, *2*(8), a003640. https://doi.org/10.1101/cshperspect.a003640

Petherick, K. J., Williams, A. C., Lane, J. D., Ordóñez-Morán, P., Huelsken, J., Collard, T. J., Smartt, H. J., Batson, J., Malik, K., Paraskeva, C., & Greenhough, A. (2013). Autolysosomal β-catenin degradation regulates Wnt-autophagy-p62 crosstalk. *The EMBO Journal*, *32*(13), 1903–1916. https://doi.org/10.1038/emboj.2013.123

Philips, J. A., Rubin, E. J., & Perrimon, N. (2005). *Drosophila* RNAi screen reveals CD36 family member required for mycobacterial infection. *Science (New York, N.Y.)*, *309*(5738), 1251–1253. https://doi.org/10.1126/science.1116006

Rietveld, T., & van Hout, R. (2015). The t test and beyond: Recommendations for testing the central tendencies of two independent samples in research on speech, language and hearing pathology. *Journal of Communication Disorders*, *58*, 158–168. https://doi.org/10.1016/j.jcomdis.2015.08.002

Rikihisa, Y., Zhang, Y., & Park, J. (1994). Inhibition of infection of macrophages with *Ehrlichia risticii* by cytochalasins, monodansylcadaverine, and taxol. *Infection and Immunity*, *62*(11), 5126–5132.

Rikihisa, Y., Zhang, Y., & Park, J. (1995). Role of Ca2+ and calmodulin in ehrlichial infection in macrophages. *Infection and Immunity*, *63*(6), 2310–2316.

Sazama, E. J., Bosch, M. J., Shouldis, C. S., Ouellette, S. P., & Wesner, J. S. (2017). Incidence of *Wolbachia* in aquatic insects. *Ecology and Evolution*, *7*(4), 1165–1169. https://doi.org/10.1002/ece3.2742

Scialo, F., Sriram, A., Stefanatos, R., & Sanz, A. (2016). Practical recommendations for the Use of the GeneSwitch Gal4 system to knock-down genes in *Drosophila melanogaster*. *PLoS ONE*, *11*(8). https://doi.org/10.1371/journal.pone.0161817

Serbus, L. R., Casper-Lindley, C., Landmann, F., & Sullivan, W. (2008). The genetics and cell biology of *Wolbachia*-host interactions. *Annual Review of Genetics*, *42*, 683–707.

Serbus, L. R., Ferreccio, A., Zhukova, M., McMorris, C. L., Kiseleva, E., & Sullivan, W. (2011). A feedback loop between *Wolbachia* and the *Drosophila* gurken mRNP complex influences *Wolbachia* titer. *Journal of Cell Science*, *124*(Pt 24), 4299–4308. https://doi.org/10.1242/jcs.092510

Serbus, L. R., Landmann, F., Bray, W. M., White, P. M., Ruybal, J., Lokey, R. S., Debec, A., & Sullivan, W. (2012). A cell-based screen reveals that the albendazole metabolite, albendazole sulfone, targets Wolbachia. *PLoS Pathogens*, *8*(9), e1002922. https://doi.org/10.1371/journal.ppat.1002922

Serbus, L. R., White, P. M., Silva, J. P., Rabe, A., Teixeira, L., Albertson, R., & Sullivan, W. (2015). The impact of host diet on *Wolbachia* titer in *Drosophila*. *PLoS Pathogens*, *11*(3). https://doi.org/10.1371/journal.ppat.1004777

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (Complete Samples). *Biometrika*, *52*(3/4), 591–611. https://doi.org/10.2307/2333709

Turelli, M. (1994). Evolution of incompatibility-inducing microbes and their Hosts. *Evolution*, *48*(5), 1500–1513. https://doi.org/10.1111/j.1558-5646.1994.tb02192.x

Voronin, D., Cook, D. A. N., Steven, A., & Taylor, M. J. (2012). Autophagy regulates *Wolbachia* populations across diverse symbiotic associations. *Proceedings of the National Academy of Sciences*, *109*(25), E1638–E1646. https://doi.org/10.1073/pnas.1203519109

Wasserman, S. (1994). Review of a handbook for data analysis in the behavioral sciences; (Vol. 1) Statistical Issues; (Vol. 2) Methodological Issues. [Review of *Review of A Handbook for Data Analysis in the Behavioral Sciences; (Vol. 1) Statistical Issues; (Vol. 2) Methodological Issues.*, by G. Keren & C. Lewis]. *Journal of the American Statistical Association*, *89*(426), 715–717. https://doi.org/10.2307/2290884

Weeks, A. R., Turelli, M., Harcombe, W. R., Reynolds, K. T., & Hoffmann, A. A. (2007). From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLOS Biology*, *5*(5), e114. https://doi.org/10.1371/journal.pbio.0050114

Weinert, L. A., Araujo-Jnr, E. V., Ahmed, M. Z., & Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1807), 20150249. https://doi.org/10.1098/rspb.2015.0249

Werren, J. H., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*(10), 741–751.

White, P. M., Pietri, J. E., Debec, A., Russell, S., Patel, B., & Sullivan, W. (2017)b. Mechanisms of horizontal cell-to-cell transfer of *Wolbachia spp*. in *Drosophila melanogaster*. *Applied and Environmental Microbiology*, *83*(7). https://doi.org/10.1128/AEM.03425-16

White, P. M., Serbus, L. R., Debec, A., Codina, A., Bray, W., Guichet, A., Lokey, R. S., & Sullivan, W. (2017a). Reliance of *Wolbachia* on high rates of host proteolysis revealed by a genome-wide rnai screen of *Drosophila* cells. *Genetics*, *205*(4), 1473–1488. https://doi.org/10.1534/genetics.116.198903

Wiwatanaratanabutr, I., & Kittayapong, P. (2009). Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of *Aedes albopictus*. *Journal of Invertebrate Pathology*, *102*(3), 220–224. https://doi.org/10.1016/j.jip.2009.08.009

Wu, D., & Pan, W. (2010). GSK3: A multifaceted kinase in Wnt signaling. *Trends in Biochemical Sciences*, *35*(3), 161–168. https://doi.org/10.1016/j.tibs.2009.10.002

Yamaguchi, M., & Yoshida, H. (2018). *Drosophila* as a Model Organism. In M. Yamaguchi (Ed.), *Drosophila Models for Human Diseases* (pp. 1–10). Springer Singapore. https://doi.org/10.1007/978-981-13-0529-0_1

Yang, L., Liu, C., Zhao, W., He, C., Ding, J., Dai, R., Xu, K., Xiao, L., Luo, L., Liu, S., Li, W., & Meng, H. (2018). Impaired autophagy in intestinal epithelial cells alters gut microbiota and host immune responses. *Applied and Environmental Microbiology*, *84*(18). https://doi.org/10.1128/AEM.00880-18

Yuan, K., Huang, C., Fox, J., Laturnus, D., Carlson, E., Zhang, B., Yin, Q., Gao, H., & Wu, M. (2012). Autophagy plays an essential role in the clearance of *Pseudomonas aeruginosa* by alveolar macrophages. *Journal of Cell Science*, *125*(Pt 2), 507–515. https://doi.org/10.1242/jcs.094573

Zhang, G., Hussain, M., O'Neill, S. L., & Asgari, S. (2013). Wolbachia uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(25), 10276–10281. https://doi.org/10.1073/pnas.1303603110

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*(6), e38544. https://doi.org/10.1371/journal.pone.0038544

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., & van 't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, *56*(6), 1875–1881.

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

Endosymbiont density dynamics is an emerging topic in host-microbe interactions, with *Wolbachia*-host systems serving as informative models for exploring that. The density of *Wolbachia* has shown to be correlated with the strength of *Wolbachia*-induced reproductive manipulations (Bourtzis et al., 1996; Boyle et al., 1993; Poinsot et al., 1998) and the suppression of disease transmission (Hedges et al., 2008; Teixeira et al., 2008). There is a broad interest in intentional/strategic manipulation of *Wolbachia* titer. *Wolbachia* mediated viral suppression is often density dependent (Lu et al., 2012). Therefore, increasing titer within the host would elevate the viral suppression effect. By contrast, filarial nematodes depend on *Wolbachia* for their survival and reproduction (Makepeace et al., 2006; Townson et al., 2000). Decreasing titer, thereby, would potentiate the suppression of filarial disease. In either case, host mechanisms that fundmanentally affect how *Wolbachia* loads are specified is not clear. This same knowledge gap broadly applies to most endosymbionts. Therefore, my overall goal is to identify and pursue mechanisms that affect titer in vivo. We are using the *Wolbachia* example to investigate how this is achieved.

Measurements of bacterial titer carried by a whole organism are critical to inform the mechanisms involved in host colonization. There is currently no agreed-upon standard for how such experiments should be done. Lack of comparability across studies has created disconnects in the literature. The work of this thesis helps to fill that void by providing a standardized assay to carry out titer related experiments. Chapter II demonstrated the stepwise, empirical optimization of an absolute count, real-time PCR assay to quantify *Wolbachia* within whole *Drosophila* hosts. Employing the optimized assay, we showed the maximum detection of *Wolbachia* titer within a whole organism. Statistical certainty, tailored to the format of the data, was also incorporated.

Consistency of this data was further supported by our use of a 24-well plate format, to facilitate simultaneous drug testing or other dietary treatments against *Wolbachia*-infected *Drosophila*. Overall, the methodology presented can be further applied to test body-wide colonization in response to chemical treatments, genetic conditions, new host-endosymbiont combinations. It is also possible to adapt these procedures to follow-up analyses of dissected organs and tissue as well.

Systematic optimization of the body-wide *Wolbachia* quantification protocol in Chapter II opened the possibility of asking titer-related mechanistic questions. In Chapter III, we examined whether mechanisms that affect germline *Wolbachia* titer also influence body-wide *Wolbachia* abundance. Our results show that whole body *Wolbachia* titers are unresponsive to yeast-enriched host diets. Host ovary tissues showed titer reduction in response to dietary yeast, however, which is consistent with previous germline-based outcomes (Christensen et al., 2019; Serbus et al., 2015). Taken together, these data suggest that germline and somatic titers are differentially responsive to host nutrition, specifically yeast-induced insulin signaling. Perhaps diet-driven insulin signaling changes the location of *Wolbachia* replication within the body, and/or changes patterns of organ invasion in vivo. We hypothesize that the invasion scenario would be marked by higher levels of *Wolbachia* in the hemolymph. There were technical difficulties with our initial attempts to quantify hemolymph titer directly. In the future, we will calculate hemolymph titer subtractively, by measuring average titer values from whole females, from ovarectomized females, and from dissected ovarian tissues, all from shared populations of flies. Running control and yeast-rich conditions in parallel will show if a *Wolbachia* shift to the hemolymph has occurred or not.

Chapter III also addressed the relevance of absolute qPCR over relative qPCR while measuring bacterial titer responses to varying treatments. Relative counts don’t

exactly show “wrong” answers, but the method does constrain interpretation. A shift in the ratio of *Wolbachia/*host DNA abundance markers could indicate a host copy number shift just as easily as a *Wolbachia* titer shift. In the dietary yeast example, host gene copy number increased 1.5-1.8 fold in the yeast-enriched dietary condition, which reduces the *Wolbachia/* host ratio, though *Wolbachia* titer was unchanged. Therefore, to answer titer-related questions in the context of biology, absolute quantification is required for accurate qPCR data interpretation.

Chapter IV investigated how candidate host mechanisms affect *Wolbachia* titer in vivo. Comprehensive RNAi screens were carried out previously in tissue culture cells to identify host factors that regulate *Wolbachia* titer (Grobler et al., 2018; White et al., 2017a). However, there are concerns regarding tissue culture cells. In cell culture studies, it is not clear why titer changes occurred, as invasion and replication dynamics are indistinguishable in that context. We carried out a systematic pilot screen using an intact fly model. A major strength of this physiologically appropriate system to study colonization is that we can separately analyze replication dynamics vs. invasion dynamics. In this case, we focused on analysis of *Wolbachia* titer changes, to start creating a foundation for addressing replication dynamics.

Our screen strategy was to first identify candidate titer-related host pathways by screening small molecule inhibitors, then retest the function of those pathways using targeted genetic disruptions. The pilot drug screen revealed 6 “hit” drugs that altered *Wolbachia* titer in 6 total replicate tests across 2 host/strain combinations. These hits corresponded to several different host pathways, and further suggest that most of the implicated host pathways generally suppress body-wide titer. Perhaps this endosymbiotic relationship is commensal due to the restraint of *Wolbachia* by cellular pathways of the *Drosophila* host. In future experiments, we would like to see the extent

to which the drugs exert tissue-specific effects in vivo. This can be achieved by comparing *Wolbachia* abundance from whole body, and specific organs, such as: ovary, brain, crop, or fat body samples. It will also be important to test the candidate hit drugs on other, more disparate *Wolbachia/*host systems, for instance, mosquitoes, wasps and nematodes. As part of such analyses, the dose-dependency of the drug impact can and should be measured in each case.

The pathway functions from the drug screening were retested using the somatic da-GAl4: UAS-RNAi genotype for genetic validation. However, not all of the processes implicated by drug hits were confirmed by this genetic knockdown method. Only mTOR and Wnt pathways were reconfirmed as hits. One possibility is that the non-corroborated pathways are the result of off-target effect from the drugs. Another possibility is that the targets specifically disrupted by da-GAl4: UAS-RNAi expression were compensated for by functional redundancy of other factors. Alternatively, RNAi expression that is mild enough to allow host development, as per the daughterless-GAL4 driver, may not have been a strong enough function disruption to elicit a titer phenotype in adulthood. Our attempts to induce pathway disruption with the stronger somatic actin-GAL4 driver were lethal. Future use of an inducible GeneSwitch GAL4 driver, which can induce a strong knockdown upon induction in adults, could help to further interpret host pathway function impacts on *Wolbachia* titer in adult insects.

It has been revealed by the both drug screen and RNAi knockdowns that inhibition of mTOR and Wnt signaling increased body-wide *Wolbachia* titer. The observed effects for *tor* (mTOR pathway) and *armadillo* (Wnt pathway) RNAi knockdown on body-wide titer were less robust than seen for the drugs. The magnitude of titer increase for the drugs was 1.5-2.5 fold, whereas the RNAi experiments showed 1.09-1.31 fold increase. It should be noted that the RNAi experiments were preliminary, and

do not yet include sub-sampling analyses. After determining appropriate sample sizes, the impact of the hit pathways can be tested for their spatial versus temporal regulation. This can be achieved by the tissue specific knockdown of a gene of interest. For instance, using nanos-GAL4 driver to knock down target genes specifically in the ovary, or elav-GAL4 to knockdown gene function in the brain. This will specifically address whether host effects on titer occur in a tissue-autonomous manner. Conducting a large-scale RNAi screen is also a possibility, as per our pilot screen, which demonstrated that preliminary hits will be detected in the first and every pass of screening. Successive retests of only the “hit” lines each time will identify the reproducible ones, to pursue in sufficient follow-up replicates for statistical rigor.

Organismal quantification of *Wolbachia* titer can also be performed to understand *Wolbachia* replication dynamics throughout host development. Chrostek and colleagues previously determined that relative qPCR counts of the *w*MelPop *Wolbachia* strain increase over time in adult *Drosophila* males (Chrostek et al., 2013). However, considering the influence of host gene copy number in generating relative *Wolbachia* counts, a final interpretation cannot be made from this report. To address this, we are now examining *Wolbachia* abundance across host life cycle using absolute counts. The study involves titer measurements at 24-hour intervals from embryo to adult. Subsequently, the replication rate will be determined at timepoints that show the greatest increases in *Wolbachia* titer. *Wolbachia* replication rates will be calculated by measuring ploidy within the *Wolbachia* chromosome, as a ratio of the Origin of replication (Ori) versus the replication Terminus (Ter). Since the Ori region is copied first during chromosome duplication, it is expected that replicating bacterial populations will exhibit an Ori/Ter ratio exceeding 1, whereas stationary phase populations will show an Ori/Ter ratio close to 1. Preliminary experiments show an Ori/Ter ratio of 1.2-1.7 during *D.*

melanogaster larval stages when organismal *Wolbachia* counts show a rapid titer increase. There is now great potential in applying these methods to characterize *Wolbachia* replication dynamics within the physiological context of their insect hosts. This will provide context for what *Wolbachia* titer changes represent by helping to distinguish between changes in replication vs. clearance by the host.

This project has created a template for understanding titer control across endosymbiotic systems. Using the fascinating endosymbiont *Wolbachia* as an example, our work has effectively demonstrated the cellular basis of *Wolbachia*-host interactions, which was largely unknown until now. The tools that we have introduced to widen the mechanistic understanding of titer control, showed complete transparency of how techniques were validated. This opens up the possibility to retest mechanisms across systems informing the basis for commensalism.

References

Bourtzis, K., Nirgianaki, A., Markakis, G., & Savakis, C. (1996). *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. *Genetics*, *144*(3), 1063–1073.

Boyle, L., O'Neill, S. L., Robertson, H. M., & Karr, T. L. (1993). Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science (New York, N.Y.)*, *260*(5115), 1796–1799.

Christensen, S., Camacho, M., Sharmin, Z., Momtaz, A. J. M. Z., Perez, L., Navarro, G., Triana, J., Samarah, H., Turelli, M., & Serbus, L. R. (2019). Quantitative methods for assessing local and bodywide contributions to *Wolbachia* titer in maternal germline cells of Drosophila. *BMC Microbiology*, *19*. https://doi.org/10.1186/s12866-019-1579-3

Chrostek, E., Marialva, M. S. P., Esteves, S. S., Weinert, L. A., Martinez, J., Jiggins, F. M., & Teixeira, L. (2013). *Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: A phenotypic and phylogenomic analysis. *PLoS Genetics*, *9*(12), e1003896. https://doi.org/10.1371/journal.pgen.1003896

Grobler, Y., Yun, C. Y., Kahler, D. J., Bergman, C. M., Lee, H., Oliver, B., & Lehmann, R. (2018). Whole genome screen reveals a novel relationship between *Wolbachia*

levels and *Drosophila* host translation. *PLoS Pathogens*, *14*(11). https://doi.org/10.1371/journal.ppat.1007445

Hedges, L. M., Brownlie, J. C., O'Neill, S. L., & Johnson, K. N. (2008). *Wolbachia* and virus protection in insects. *Science (New York, N.Y.)*, *322*(5902), 702. https://doi.org/10.1126/science.1162418

Lu, P., Bian, G., Pan, X., & Xi, Z. (2012). *Wolbachia* induces density-dependent inhibition to Dengue virus in mosquito cells. *PLoS Neglected Tropical Diseases*, *6*(7). https://doi.org/10.1371/journal.pntd.0001754

Makepeace, B. L., Rodgers, L., & Trees, A. J. (2006). Rate of elimination of *Wolbachia pipientis* by Doxycycline in vitro increases following drug withdrawal. *Antimicrobial Agents and Chemotherapy*, *50*(3), 922–927. https://doi.org/10.1128/AAC.50.3.922-927.2006

Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C., & Merçot, H. (1998). *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: Host effect and cytoplasmic incompatibility relationships. *Genetics*, *150*(1), 227–237.

Serbus, L. R., White, P. M., Silva, J. P., Rabe, A., Teixeira, L., Albertson, R., & Sullivan, W. (2015). The impact of host diet on *Wolbachia* titer in *Drosophila. PLoS Pathogens*, *11*(3). https://doi.org/10.1371/journal.ppat.1004777

Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, *6*(12). https://doi.org/10.1371/journal.pbio.1000002

Townson, S., Hutton, D., Siemienska, J., Hollick, L., Scanlon, T., Tagboto, S. K., & Taylor, M. J. (2000). Antibiotics and *Wolbachia* in filarial nematodes: Antifilarial activity of rifampicin, oxytetracycline and chloramphenicol against *Onchocerca gutturosa, Onchocerca lienalis* and *Brugia pahangi*. *Annals of Tropical Medicine and Parasitology*, *94*(8), 801–816.

White, P. M., Serbus, L. R., Debec, A., Codina, A., Bray, W., Guichet, A., Lokey, R. S., & Sullivan, W. (2017a). Reliance of *Wolbachia* on high rates of host proteolysis revealed by a genome-wide RNAi screen of *Drosophila* Cells. *Genetics*, *205*(4), 1473–1488. https://doi.org/10.1534/genetics.116.198903

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Publications and presentations

Sharmin Z, Samarah H, Serbus LR. Systematic screens reveal mechanisms of *Wolbachia* colonization in the *Drosophila* host. In prep.

Christensen S, Camacho* M, Sharmin* Z, Momtaz AJMZ, Perez L, Navarro G, Triana J, Samarah H, Turelli M, Serbus LR. Quantitative methods for assessing local and bodywide contributions to *Wolbachia* titer in maternal germline cells of *Drosophila*. BMC Microbiol. 2019 Sep 3;19(1):206. doi: 10.1186/s12866-019-1579-3.

* Equal contributions

Serbus LR, Rodriguez BG, Sharmin Z, Momtaz AJMZ, Christensen S. Predictive Genomic Analyses Inform the Basis for Vitamin Metabolism and Provisioning in Bacteria-Arthropod Endosymbioses. G3 (Bethesda). 2017 Jun 7;7(6):1887-1898. doi: 10.1534/g3.117.042184.

Sumit AF, Das A, Sharmin Z, Ahsan N, Ohgami N, Kato M, Akhand AA. Cigarette smoking causes hearing impairment among Bangladeshi population. PLoS One. 2015 Mar 17;10(3):e0). doi: 10.1371/journal.pone.0118960.

Sharmin Z, Samarah H, Serbus LR. Understanding density regulation of endosymbiotic *Wolbachia* bacteria within the host. BSI annual research symposium, FIU, 2020.

Sharmin Z, Camacho M, Momtaz AJMZ, Christensen S, Serbus LR. Regulating the density of endosymbiotic *Wolbachia* bacteria in insects. South Florida *Drosophila* Research Consortium, Scripps research center, Jupiter, 2019.

Sharmin Z, Acle E, Momtaz AJMZ, Christensen S, Serbus LR. Regulating the density of endosymbiotic *Wolbachia* bacteria in insects. South Florida *Drosophila* Research Consortium, Scripps research center, Jupiter, 2018.

Sharmin Z, Acle E, Momtaz AJMZ, Christensen S, Serbus L. Regulating the density of endosymbiotic *Wolbachia* bacteria in insects. Bio symposium, FIU, 2018.