Factors influencing the intriguing persistence of a *Wolbachia* symbiont in spotted wing *Drosophila*

by

Audrey E. McPherson
B.Sc., University of Victoria, 2018

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Supervisory Committee

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Abstract

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Wolbachia is a maternally inherited, endosymbiotic bacterium that infects at least 40% of terrestrial arthropods. As a facultative symbiont in the majority of its hosts, *Wolbachia* commonly act as a reproductive parasite; however, there are a number of *Wolbachia* strains that do not cause reproductive manipulations in their hosts and have no apparent fitness enhancement, yet are stably maintained in populations at low to intermediate frequencies. How these strains of *Wolbachia* persist in nature has been a long-standing question and is still unresolved. One explanation for the persistence of such strains is that they provide a context-dependent fitness advantage to their hosts. In this thesis, I investigate one such strain of *Wolbachia*, *w*Suz, which infects the agricultural pest, *Drosophila suzukii,* also known as spotted wing *Drosophila*. To explore the possibility that *w*Suz may be involved in pathogen protection, I screened wild flies for *Wolbachia* and two naturally occurring RNA viruses, Teise Virus and a recently discovered virus related to Motts Mill Virus. I did not find an association between *Wolbachia* and virus infection. Additionally, I designed an experiment to test whether *Wolbachia* increases host fitness at high larval densities. Intriguingly, although there was no effect of density, the frequency of *Wolbachia* infection changed dramatically in just one generation, but in opposite directions in replicate experiments that were performed a month apart. These results support the hypothesis that *Wolbachia* frequencies can change quickly across generations and provide some type of condition-dependent benefit. The maintenance of *Wolbachia* remains a mystery, but my study provides some exciting clues about what conditions may be playing a role.

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Chapter 1 – *Wolbachia* infection in *Drosophila suzukii*

Symbiotic relationships are intimate interactions between organisms from different species and they are incredibly diverse. Over the years, it has become increasingly clear that most organisms share some form of a symbiotic relationship with another, and these relationships shape many of the biological interactions we observe in nature. Organisms that participate in symbiotic relationships and live within the body of another are called endosymbionts and they establish long-term associations with their host (Wernegreen, 2012). Microbes are the most diverse group of organisms on the planet and account for most endosymbiotic relationships. Microbes include bacteria, fungi, viruses, and protists and can perform a number of functions in their hosts such as aiding ruminant animals in digesting cellulose (Mackie, 2002), assisting in bioluminescence in deep sea squid species (Nishiguchi *et al.*, 2004), and fixing nitrogen in root nodules of some plants (Kuypers *et al.*, 2018). Insects are no exception; they too harbor an incredible diversity of microbial symbionts (Douglas, 2011; Ferrari & Vavre, 2011; Weinert *et al.*, 2015).

Bacterial symbionts are the most common type of microbial endosymbionts in insects, with more than half of insect species carrying at least one symbiont (Ferrari & Vavre, 2011; Weinert *et al.*, 2015). Bacterial symbionts of insects have been a driving force behind insect diversification (Moran, 2007) and some share long co-evolutionary histories. For example, the bacteria *Sulcia muelleri* became an obligate symbiont of the common ancestor of the suborder Auchenorrhyncha 270 million years ago and have stably infected descendants ever since (Moran *et al.*, 2005). The effects of symbionts on their insect hosts vary tremendously and include the provision of essential nutrients (Douglas, 1998), protection from natural enemies (Oliver *et al.*, 2005; Moreira *et al.*, 2009), to alteration of their host's reproduction (Werren *et al.*, 1994). Sometimes symbionts can impose fitness costs such as reducing longevity (Min & Benzer, 1997) and offspring viability (Niebylski *et al.*, 1999).

Bacterial symbionts in insects are commonly vertically transmitted from mothers to their offspring, often in the egg cytoplasm (Bright & Bulgheresi, 2010). Maternally inherited bacteria have to strike a balance with their hosts and not be too costly to maintain since their transmission relies on the fitness of the female hosts, but also ensure they are able to spread throughout a population (Hoffmann & Turelli, 1997). If a symbiont has strong negative consequences for the host female, uninfected individuals will outperform and replace infected ones resulting in the gradual loss of the symbiont (Hoffmann & Turelli, 1997; Poinsot & Merçot, 1997; Charlat *et al.*, 2004). Therefore, it is in the best interest of the symbiont to contribute to host fitness, or at the very least not reduce fitness (Hurst & Frost, 2015). Regardless of the effects on host fitness, symbionts that are purely maternally inherited will spread if more infected female offspring are produced than uninfected (Engelstädter & Hurst, 2009; Correa & Ballard, 2016). One mechanism that is employed by some symbionts that ensures their spread throughout a population is by producing more female offspring than male offspring (Hurst & Frost, 2015). By producing more female offspring, the symbiont is increasing its own transmission at the expense of the fitness of the host (Engelstädter & Hurst 2009).

Traditionally, maternally inherited bacterial symbionts have been divided into two groups, usually described as obligate or facultative symbionts (Correa & Ballard 2016). Obligate symbionts are essential for their hosts' survival and / or reproduction. These bacteria often supplement their hosts' diet with essential amino acids or vitamins that are rare in the insects' food source (Douglas 1998). For example, the bacterium *Buchnera aphidicola* synthesizes essential amino acids that are missing from the phloem that its aphid host feeds on (Douglas, 1998; Gündüz & Douglas, 2012). Removing *Buchnera aphidicola* by antibiotic treatment leads to aphid sterility and death (Gündüz & Douglas, 2012). Even more common are facultative inherited symbionts of insects. In contrast to obligate symbionts, facultative symbionts are not required for host survival or reproduction. Facultative symbionts have evolved a number of different strategies which allows them to persist in a population, even if they do not provide a fitness enhancement to their host. These strategies can be divided into two main categories: reproductive parasites and conditional mutualists.

Reproductive parasitism is a strategy employed by many symbionts that increases the transmission of the symbiont, regardless of the consequences on the host (Werren *et al.*, 2008; Bright & Bulgheresi, 2010; Shropshire *et al.*, 2020). There are two main methods of reproductive parasitism: increasing the number of female offspring or creating incompatibilities between individuals that do not carry the symbiont and those that do. In the former, because most symbionts are vertically transmitted, symbionts finding themselves in male hosts normally would have no way of leaving descendants (Hurst & Frost 2015). Some endosymbionts have evolved ways of increasing the number of female progeny produced through feminization of genetic males (Weeks *et al.,* 2001), killing male embryos (Werren *et al.,* 1994), and development of females from unfertilized eggs (Giorgini *et al.,* 2009). These methods all create heavily - and sometimes exclusively - female-biased progeny which allows the symbiont to spread throughout a population. The other and most common method of reproductive manipulation symbionts can impose on their hosts is cytoplasmic incompatibility (CI) (Werren *et al.,* 2008). Cytoplasmic incompatibility entails a toxin-antitoxin mechanism whereby developing sperm are modified by a factor secreted from an endosymbiont which prevents the sperm from producing viable offspring (Beckmann *et al.,* 2019). When eggs bearing the same strain of symbiont are fertilized by the modified sperm, the modification is reversed and healthy embryos are produced (Beckmann *et al.,* 2019). This results in an advantage for infected females because the infected ova can be fertilized by any sperm and allows the symbiont to spread more rapidly (Kriesner & Hoffmann 2018).

Another common and important strategy employed by facultative inherited symbionts to spread through a population is to provide their host with some kind of fitness benefit. These benefits can be conditional in that in certain situations the symbiont provides a fitness enhancement, but it does not necessarily enhance fitness all of the time (Pontes & Dale, 2006). Some documented situations of conditional mutualism include: protection against natural enemies (Jaenike & Brekke, 2011; Hamilton & Perlman, 2013), increased thermal resistance (Russell & Moran, 2006), and nutritional supplementation during times of food scarcity (Ferrari & Vavre, 2011). While these symbionts can enhance fitness, they can also impose subtle and deleterious effects on hosts (Zug & Hammerstein, 2012). The most famous and best-studied facultative endosymbiont that is both a reproductive parasite and conditional mutualist of insects is the bacterium *Wolbachia*.

Wolbachia is an endosymbiotic, maternally inherited, bacterium belonging to the order Rickettsiales in the Alphaproteobacteria. *Wolbachia* is an extremely prolific endosymbiont infecting more than 40% of all insect species (Zug & Hammerstein, 2012). Given the large number of hosts, it is unsurprising that *Wolbachia* induces a wide range of phenotypes. *Wolbachia* is the only endosymbiont that displays all four known types of reproductive parasitism (CI, male-killing, parthenogenesis, and feminization), earning it the title of being the "master manipulator" of arthropod reproduction (Werren *et al.,* 2008). On the other hand, as a conditional mutualist, *Wolbachia* has been found to provide protection from pathogens (Teixeira *et al.,* 2008) and aid in iron metabolism (Gill *et al.,* 2014).

Although many strains of *Wolbachia* have been shown to affect their hosts in striking ways, from sex ratio distortion (Werren *et al.,* 2008) to virus protection (Teixeira *et al.,* 2008), how most *Wolbachia* persist in their hosts is not known. Many *Wolbachia* strains exhibit very weak or no reproductive parasitism, are not transmitted perfectly from mother to offspring (yet are not horizontally transmitted), and do not provide an obvious fitness enhancement effect. That these strains are found at intermediate to high frequencies in host populations is puzzling. In theory, infections should gradually be lost in populations where *Wolbachia* is not perfectly transmitted from mothers to their offspring or enhances fitness, and assuming there is no horizontal transmission (Hurst, 1991; Hoffmann & Turelli, 1997). Yet many such strains persist, and these have been most studied in various *Drosophila* species, including *D. melanogaster* and *D. simulans* (Hoffmann *et al.,* 1998, 1996, 1990; Kriesner *et al.,* 2013, 2016).

The strain of *Wolbachia* infecting *D. melanogaster, w*Mel, is one such strain that is mysteriously maintained in both laboratory and wild populations. Since its initial discovery in the 1930's, *w*Mel has been replacing the ancestral *w*MelCS strain in *D. melanogaster* (Riegler *et al.,* 2005) despite inducing fairly weak CI, although modifying male development time (Yamada *et al.,* 2007) or paternal grandmother age (Layton *et al.,* 2019) can cause increased CI. Additionally, *w*Mel displays imperfect maternal transmission (Hoffmann 1990, 1998). The weak CI and imperfect transmission explains why *w*Mel is not at 100% prevalence, but it does not explain the widespread distribution or persistence of *w*Mel. These observations have led to the suggestion that *Wolbachia* must confer some fitness advantage to *D. melanogaster* females (Hoffmann *et al.,* 1994, 1998). To investigate this intriguing phenomenon, a number of studies have examined a multitude of fitness traits ranging from fecundity to sperm competition to mate choice but have found no detectable fitness advantage attributable to *Wolbachia* infection (Hoffmann *et al.,* 1998; Harcombe & Hoffmann, 2004; Montenegro *et al.,* 2006; Kriesner *et al.,* 2016). *w*Mel has also been shown to protect *D. melanogaster* against pathogenic RNA viruses under laboratory conditions (Teixeira *et al.,* 2008), but whether this benefit occurs and is important in nature is not known.

My thesis focuses on another *Wolbachia* strain whose persistence remains a mystery. This strain of *Wolbachia* infects *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), a polyphagous fruit pest from Asia that has recently invaded Europe and the Americas within the last decade (Cini *et al.,* 2014; Asplen *et al.,* 2015). After it was first detected in California in 2008 (Walsh *et al.,* 2011), *D. suzukii* spread to fruit-growing regions of British Columbia in 2009 and is now an established and persistent pest (Thistlewood *et al.,* 2019). Unlike most other Drosophilidae, *D. suzukii* females have a serrated ovipositor that pierces the skin of ripening fruit where the eggs are deposited (Kanzawa 1939; Walsh *et al.,* 2011). The developing larvae then feed on the flesh of fruits causing it to rot and rendering it unmarketable, which has cost the agricultural industry hundreds of millions of dollars in losses (Walsh *et al.,* 2011).

Drosophila suzukii is infected with one strain of *Wolbachia,* designated *w*Suz (Hamm *et al.,* 2014; Cattel *et al.,* 2016; Wilches *et al.,* 2021). Most of what is known about *w*Suz comes from a seminal study by Hamm and colleagues (2014). Their study revealed that similar to *w*Mel, *w*Suz is found at intermediate frequencies and does not induce CI or other reproductive manipulations in *D. suzukii*. Interestingly, some wild-caught females infected with *w*Suz experienced a reduction in fecundity and did not transmit the symbiont perfectly. The lack of reproductive manipulation paired with leaky maternal transmission (yet no horizontal transmission), and reduced fecundity, should eventually lead to the elimination of *w*Suz, yet it has been associated with *D. suzukii* for many thousands of years and is a stable polymorphism throughout its range (Hamm *et al.* 2014; Cordaux *et al.* 2008). This raises the question: what maintains *w*Suz in *D. suzukii*? The current leading theory is that *w*Suz provides a context-dependent fitness enhancement to *D. suzukii* (Hamm *et al.,* 2014). One likely candidate for such host fitness benefits is protection against RNA viruses. *w*Suz has been found to protect its host against RNA viruses, like *w*Mel and many other strains of *Wolbachia* that infect *Drosophila,* although the protection conferred by *w*Suz may be weak compared to other strains (Martinez *et al.,* 2017).

In this thesis, I examine a number of possible factors that might explain the persistence and fitness effects of *w*Suz in *D. suzukii*. In chapter 2, I used field-collected *D. suzukii* to screen for two common RNA viruses, Teise Virus and a Motts Mill-like Virus, to determine if there is an association between *Wolbachia* and those two viruses. I present evidence that there does not appear to be an association between *w*Suz and the two RNA viruses, but there does appear to be an association between Teise Virus and Motts Mill-like Virus. In chapter 3, I examined the effect of high-density rearing conditions on the development time of *D. suzukii* individuals infected with *w*Suz. I did not find evidence that larvae infected with *w*Suz developed more quickly at high larval densities, in fact, infected individuals developed more slowly compared to uninfected larvae. Additionally, I also investigated whether there was a higher proportion of adult offspring infected with *w*Suz in high density environments. While I did find there were significant differences in *Wolbachia* frequencies, they were not driven by density or associated levels of developmental mortality.

Chapter 1 - References

Asplen, M. K., Anfora, G., Biondi, A., Choi, D. S., Chu, D., Daane, K. M., Gibert, P., Gutierrez, A. P., Hoelmer, K. A., Hutchison, W. D., Isaacs, R., Jiang, Z. L., Kárpáti, Z., Kimura, M. T., Pascual, M., Philips, C. R., Plantamp, C., Ponti, L., Vétek, G., … Desneux, N. (2015). Invasion biology of spotted wing *Drosophila* (*Drosophila suzukii*): a global perspective and future priorities. *Journal of Pest Science,* 88, 469–494. https://doi.org/10.1007/s10340-015-0681-z

Beckmann, J. F., Bonneau, M., Chen, H., Hochstrasser, M., Poinsot, D., Merçot, H., Weill, M., Sicard, M., & Charlat, S. (2019). The toxin–antidote model of cytoplasmic incompatibility: Genetics and evolutionary implications. *Trends in Genetics*, *35*(3), 175–185. https://doi.org/10.1016/j.tig.2018.12.004

Beckmann, J. F., Sharma, G. D., Mendez, L., Chen, H., & Hochstrasser, M. (2019). The *Wolbachia* cytoplasmic incompatibility enzyme CIDB targets nuclear import and protamine-histone exchange factors. *ELife*, *8*, e50026. https://doi.org/10.7554/eLife.50026

Bright, M., & Bulgheresi, S. (2010). A complex journey: Transmission of microbial symbionts. *Nature Reviews Microbiology*, *8*), 218–230. https://doi.org/10.1038/nrmicro2262

Charlat, S., Ballard, J. W. O., & Merçot, H. (2004). What maintains noncytoplasmic incompatibility inducing *Wolbachia* in their hosts: A case study from a natural *Drosophila yakuba* population. *Journal of Evolutionary Biology*, *17*(2), 322–330. https://doi.org/10.1046/j.1420-9101.2003.00676.x

Cini, A., Anfora, G., Escudero-Colomar, L. A., Grassi, A., Santosuosso, U., Seljak, G., & Papini, A. (2014). Tracking the invasion of the alien fruit pest *Drosophila suzukii* in Europe. *Journal of Pest Science*, *87*, 559–566. https://doi.org/10.1007/s10340-014-0617-z

Cordaux, R., Pichon, S., Ling, A., Pérez, P., Delaunay, C., Vavre, F., Bouchon, D., & Grève, P. (2008). Intense transpositional activity of insertion sequences in an ancient obligate endosymbiont. *Molecular Biology and Evolution*, *25*(9), 1889–1896. https://doi.org/10.1093/molbev/msn134

Correa, C. C., & Ballard, J. W. O. (2016). *Wolbachia* associations with insects: Winning or losing against a master manipulator. *Frontiers in Ecology and Evolution*, *3*, 153. https://doi.org/10.3389/fevo.2015.00153

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

Douglas, A. E. (2011). Lessons from studying insect symbioses. *Cell Host and Microbe*, *10*(4), 359–367. https://doi.org/10.1016/j.chom.2011.09.001

Engelstädter, J., & Hurst, G. D. D. (2009). The ecology and evolution of microbes that manipulate host reproduction. *Annual Review of Ecology, Evolution, and Systematics*, *40*, 127–149. https://doi.org/10.1146/annurev.ecolsys.110308.120206

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

Gill, A. C., Darby, A. C., & Makepeace, B. L. (2014). Iron Necessity: The secret of *Wolbachia*'s success? *PLoS Neglected Tropical Diseases*, *8*(10), e3224. https://doi.org/10.1371/journal.pntd.0003224

Giorgini, M., Monti, M. M., Caprio, E., Stouthamer, R., & Hunter, M. S. (2009). Feminization and the collapse of haplodiploidy in an asexual parasitoid wasp harboring the bacterial symbiont *Cardinium*. *Heredity*, *102*, 365–371. https://doi.org/10.1038/hdy.2008.135

Gündüz, A. E., & Douglas, A. E. (2012). Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1658), 987–991. https://doi.org/10.1098/rspb.2008.1476

Hamilton, P. T., & Perlman, S. J. (2013). Host defense via symbiosis in *Drosophila*. *PLoS Pathogens*, *9*(12), e1003808. https://doi.org/10.1371/journal.ppat.1003808

Hamm, C. A., Begun, D. J., Vo, A., Smith, C. C. R., Saelao, P., Shaver, A. O., Jaenike, J., & Turelli, M. (2014). *Wolbachia* do not live by reproductive manipulation alone: Infection polymorphism in *Drosophila suzukii* and *D. subpulchrella*. *Molecular Ecology*, *23*(19), 4871–4885. https://doi.org/10.1111/mec.12901

Harcombe, W., & Hoffmann, A. A. (2004). *Wolbachia* effects in *Drosophila melanogaster*: In search of fitness benefits. *Journal of Invertebrate Pathology*, *87*(1), 45–50. https://doi.org/10.1016/j.jip.2004.07.003

Hoffmann, A. A., Turelli, M., & Harshman, L. G. (1990). Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. *Genetics*, *126*(4), 933–948.

Hoffmann, A.A., & Turelli, M. (1997). Cytoplasmic incompatibility in insects, pp. 41–80 in *Influential Passengers: Inherited Microorganisms and Invertebrate Reproduction*, edited by S. L. O'Neill, A. A. Hoffmann and J. H. Werren. Oxford University Press, Oxford University Press: New York.

Hoffmann, A. A., Clancy, D., & Duncan, J. (1996). Naturally-occurring *Wolbachia* infection in *Drosophila simulan*s that does not cause cytoplasmic incompatibility. *Heredity*, *76*, 1–8. https://doi.org/10.1038/hdy.1996.1

Hoffmann, A. A., Hercus, M., & Dagher, H. (1998). Population dynamics of the *Wolbachia* infection causing cytoplasmic incompatibility in *Drosophila melanogaster*. *Genetics*, *148*(1), 221–231. https://doi.org/10.1093/genetics/148.1.221

Hurst, G. D. D., & Frost, C. L. (2015). Reproductive parasitism: Maternally inherited symbionts in a biparental world. *Cold Spring Harbor Perspectives in Biology*, *7*, a017699. https://doi.org/10.1101/cshperspect.a017699

Hurst, L. D. (1991). The incidences and evolution of cytoplasmic male killers. In *Proceedings of the Royal Society B: Biological Sciences*, *244*(1310), 91–99. https://doi.org/10.1098/rspb.1991.0056

Jaenike, J., & Brekke, T. D. (2011). Defensive endosymbionts: a cryptic trophic level in community ecology. *Ecology Letters*, *14*(2), 150–155. https://doi.org/10.1111/j.1461-0248.2010.01564.x

Kanzawa, T. (1939). Studies on *Drosophila suzukii* Mats. Kofu, Yamanashi Agricultural Experiment Station 49. *Rev. Appl. Entomol.* 29: 622.

Kriesner, P., Conner, W. R., Weeks, A. R., Turelli, M., & Hoffmann, A.A. (2016). Persistence of a *Wolbachia* infection frequency cline in *Drosophila melanogaster* and the possible role of reproductive dormancy. *Evolution, 70*(5), 979–997. https://doi.org/10.1111/evo.12923.

Kriesner, P., Hoffmann, A. A., Lee, S. F., Turelli, M., & Weeks, A. R. (2013). Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans*. *PLoS Pathogens, 9*(9), e1003607. https://doi.org/10.1371/journal.ppat.1003607

Kuypers, M. M. M., Marchant, H. K., & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nature Reviews Microbiology*, *16*, 263–276. https://doi.org/10.1038/nrmicro.2018.9

Layton, E. M., Jungmin, O., Perlmutter, J. I., Bordenstein, S. R., Shropshire, J. D. (2019). Paternal grandmother age affects the strength of *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila melanogaster*. *mBio*, 10(6), e01879-19. https:// 10.1128/mBio.01879-19

Mackie, R. I (2002). Mutualistic fermentative digestion in the gastrointestinal tract : Diversity and evolution. *Integrative and Comparative Biology*, *42*(2), 319–326. https://doi.org/10.1093/icb/42.2.319

Martinez, J., Tolosana, I., Ok, S., Smith, S., Snoeck, K., Day, J. P., & Jiggins, F. M. (2017). Symbiont strain is the main determinant of variation in *Wolbachia*-mediated protection against viruses across *Drosophila* species. *Molecular Ecology*, *26*(15), 4072–4084. https://doi.org/10.1111/mec.14164

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 of the United States of America*, *94*(20), 10792–10796. https://doi.org/10.1073/pnas.94.20.10792

Montenegro, H., Petherwick, A. S., Hurst, G. D. D., & Klaczko, L. B. (2006). Fitness effects of *Wolbachia* and *Spiroplasma* in *Drosophila melanogaster*. *Genetica*, *127*(1–3), 207–215. https://doi.org/10.1007/s10709-005-3766-4

Moran, N. A. (2007). Symbiosis as an adaptive process and source of phenotypic complexity. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 8627– 8633. https://doi.org/10.17226/11790

Moran, N. A., Tran, P., & Gerardo, N. M. (2005). Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum *Bacteroidetes*. *Applied and Environmental Microbiology, 71*(12), 8802–8810. https://doi.org/10.1128/AEM.71.12.8802

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

Niebylski, M. L., Peacock, M. G., & Schwan, T. G. (1999). Lethal effect of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). *Applied and Environmental Microbiology*, *65*(2), 773–778. https://doi.org/10.1128/aem.65.2.773-778.1999

Nishiguchi, M. K., Lopez, J. E., & Boletzky, S. v. (2004). Enlightenment of old ideas from new investigations: More questions regarding the evolution of bacteriogenic light organs in squids. *Evolution and Development*, *6*(1), 41–49. https://doi.org/10.1111/j.1525-142X.2004.04009.x

Oliver, K. M., Moran, N. A., & Hunter, M. S. (2005). Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(36), 12795–12800. https://doi.org/10.1073/pnas.0506131102

Poinsot, D., & Merçot, H. (1997). *Wolbachia* infection in *Drosophila simulans*: Does the female host bear a physiological cost? *Evolution*, *51*(1), 180–186. https://doi.org/10.1111/j.1558-5646.1997.tb02399.x

Pontes, M. H., & Dale, C. (2006). Culture and manipulation of insect facultative symbionts. *Trends in Microbiology*, *14*(9), 406–412. https://doi.org/10.1016/j.tim.2006.07.004

Riegler, M., Sidhu, M., Miller, W. J., & O'Neill, S. L. (2005). Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. *Current Biology*, *15*(15), 1428–1433. https://doi.org/10.1016/j.cub.2005.06.069

Russell, J. A., & Moran, N. A. (2006). Costs and benefits of symbiont infection in aphids: Variation among symbionts and across temperatures. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1586), 603–610. https://doi.org/10.1098/rspb.2005.3348

Shropshire, J. D., Leigh, B., & Bordenstein, S. R. (2020). Symbiont-mediated cytoplasmic incompatibility: What have we learned in 50 years? *eLife*, *9*, 1–36. https://doi.org/10.7554/ELIFE.61989

Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, *6*(12), e1000002. https://doi.org/10.1371/journal.pbio.1000002

Thistlewood, H. M. A., Rozema, B., & Acheampong, S. (2019). Infestation and timing of use of non-crop plants by *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in the Okanagan basin, Canada. *Canadian Entomologist*, *151*(1), 34–48. https://doi.org/10.4039/tce.2018.47

Walsh, D. B., Bolda, M. P., Goodhue, R. E., Dreves, A. J., Lee, J., Bruck, D. J., Walton, V. M., O’Neal, S. D., & Zalom, F. G. (2011). *Drosophila suzukii* (Diptera: Drosophilidae): Invasive pest of ripening soft fruit expanding its geographic range and damage potential. *Journal of Integrated Pest Management*, *2*(1), G1–G7. https://doi.org/10.1603/IPM10010

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

Wernegreen, J. J. (2012). Endosymbiosis. *Current Biology*, 22(14), 555–561. https://doi.org/10.1016/j.cub.2012.06.010

Werren, J. H., Hurst, G. D. D., Zhang, W., Breeuwer, J. A. J., Stouthamer, R., & Majerus, M. E. N. (1994). Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). *Journal of Bacteriology*, *176*(2), 388–394. https://doi.org/10.1128/jb.176.2.388-394.1994

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

Wilches, D., Coghlin, P., Floate, K. (2021). Next generation sequencing, insect microbiomes, and the confounding effect of *Wolbachia*: A case study using *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). *Canadian Journal of Zoology*. https://doi.org/10.1139/cjz-2020-0260.

Yamada, R., Floate, K.D., Riegler, M., O'Neill, S.L. (2007). Male development time influences the strength of *Wolbachia*-induced cytoplasmic incompatibility expression in *Drosophila melanogaster*. *Genetics*, *177*(2), 801–808. https://doi.org/10.1534/genetics.106.068486

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 2 - Association of *Wolbachia* and two RNA viruses in wild-caught *Drosophila suzukii*

Introduction

Bacteria in the genus *Wolbachia* are maternally inherited endosymbionts that produce a very diverse array of phenotypes in their arthropod hosts (Werren *et al.,* 2008). In most cases, strains of *Wolbachia* manipulate their hosts reproduction by distorting sex ratios or inducing cytoplasmic incompatibility (CI) (Werren *et al.,* 2008). In the past two decades, it has been found that some strains of this bacterium are able to block RNA virus infections in their hosts (Hedges *et al.,* 2008; Teixeira *et al.,* 2008). This has been best studied in strains of *Wolbachia* that infect *Drosophila melanogaster*. Two of these strains, *w*MelPop and *w*Mel, have been transfected from flies into *Aedes aegypti* mosquitoes, where they have been shown to block the replication of both Dengue and Zika viruses (Moreira *et al.,* 2009; van den Hurk *et al.,* 2012)

Protection from RNA viruses has been observed in a number of *Wolbachia* strains infecting *Drosophila* including *w*Mel from *D. melanogaster, w*Au and *w*Ri strains found in *D. simulans* (Osborne *et al.,* 2009), as well as *w*Tei from *D. teissieri* (Martinez *et al.,* 2017) and *w*Suz from *Drosophila suzukii* (Cattel *et al.,* 2016). In these systems, *Wolbachia* provides protection primarily through reducing viral titres as opposed to complete viral clearance (Martinez *et al.,* 2017; Osborne *et al.,* 2009). Lab work has been restricted to studying protection conferred by *Wolbachia* against only a few well-characterized RNA viruses. Drosophila C Virus (DCV) has been studied the most extensively (Teixeira *et al.,* 2008; Osborne *et al.,* 2009; Faria *et al.,* 2016; Gupta *et al.,* 2017) but although it commonly infects *Drosophila* species in the lab, it does not appear to be prevalent in wild fly populations (Webster *et al.,* 2015). Other viruses that have been examined in the lab, such as Cricket paralysis virus (CrPV) and Flockhouse virus (FHV), do not naturally infect drosophilid flies yet are routinely used in experiments (Hedges *et al.,* 2008; Faria *et al.,* 2016; Cattel *et al.,* 2016). A critical and yet unresolved question is: how does *Wolbachia* impact RNA viruses that are naturally present in their insect hosts?

To date, as far as I am aware, only two papers have explored natural virus – *Wolbachia* associations in drosophilids. Webster et al. (2015) explored the virome of *D. melanogaster* and found that not only was there no association between viruses and *Wolbachia*, but there was also a difference in the community of viruses in flies from laboratories compared to those in the wild. These results were corroborated by Shi et al. (2018) who also examined the relationship between *w*Mel and the natural virome of *D. melanogaster* and found no obvious interaction between *w*Mel and viruses. The fact that these findings under natural settings contradict laboratory studies using viruses that do not occur naturally suggests that the relationship between viruses and *Wolbachia* is complex and possibly condition-dependent.

Spotted-wing Drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) is a polyphagous fruit pest originating from Asia, that has invaded Europe and the Americas within the last decade (Cini *et al.*, 2014; Asplen *et al.*, 2015). Unlike other drosophilids, *D. suzukii* females have a serrated ovipositor that pierces the skin of ripening fruit where the eggs are deposited (Kanzawa, 1939; Walsh *et al.*, 2011;). The developing larvae feed on the flesh of fruit causing it to rot and rendering it unmarketable. This pest has caused hundreds of millions of dollars of damage in the fruit industry in North America and has proven to be very difficult to manage (Lee *et al.*, 2011).

Cordaux et al. (2008) and colleagues discovered that *D. suzukii* are infected with a strain of *Wolbachia*. This strain, designated *w*Suz, does not cause reproductive manipulations in *D. suzukii* nor is it transmitted perfectly from mother to offspring (Hamm *et al.*, 2014; Cattel *et al.*, 2016). This should limit the spread of *w*Suz if there are no positive fitness effects to offset the reduced level of transmission (Hoffmann, 1988; Hoffmann & Turelli, 1997). However, this strain of *Wolbachia* has also been found to reduce the fecundity of females in nature, but see also Mazzetto et al. (2015), but is nonetheless found at intermediate frequencies in *D. suzukii* populations (Hamm *et al.*, 2014). *Drosophila suzukii* have been infected with *w*Suz for thousands of years and there is no evidence of horizontal transmission amongst individuals (Hamm *et al.*, 2014), and yet no obvious phenotype has been found to justify this long-standing infection. This raises the question as to how this strain is maintained at intermediate frequencies in *D. suzukii* populations. A current hypothesis is that *w*Suz does provide some condition-dependent positive phenotype (Hamm *et al.*, 2014; Medd *et al.*, 2018) and recently, laboratory studies have suggested that *w*Suz provides protection to *D. suzukii* from RNA viruses (Cattel *et al.*, 2016).

Currently, it is not well understood whether *w*Suz provides protection to *D. suzukii* from naturally occurring viruses, as most studies on virus protection have been done in the laboratory (Cattel *et al.,* 2016; Martinez *et al.,* 2017) and the naturally occurring virome of *D. suzukii* has only begun to be characterized (Medd *et al.,* 2018). Cattel et al. (2016) explored the effect of DCV and FHV infection in *w*Suz-infected *D. suzukii* collected in France and Japan. They found that *Wolbachia*-infected individuals survived longer due to a reduction in viral titres in flies with a French genetic background, but not in Japanese *D. suzukii*. Another study assessed the protection of *w*Suz from FHV infection in *D. suzukii* and *D. simulans* (Martinez *et al.,* 2017). Interestingly, the authors documented that *w*Suz did not provide protection to its host from FHV and flies experienced a < 5% reduction in viral titre. The virome of *D. suzukii* was recently characterized and 18 RNA viruses were discovered (Medd *et al.,* 2018). Similar to what was reported by Webster et al. (2015), DCV and FHV were not found in *D. suzukii* (Medd *et al.,* 2018), highlighting the need to explore the relationship between *w*Suz and viruses that are naturally occurring in wild fly populations.

While the pathogen protection effect of *w*Suz has been demonstrated in an experimental setting, there is evidence that *Wolbachia*-mediated protection may not be a general trend that persists in nature (Webster *et al.,* 2015; Shi *et al.,* 2018). The aim of this study was to determine whether there is an association between *w*Suz and two naturally occurring RNA viruses, Teise Virus and a Motts Mill-like Virus, in *D. suzukii* in British Columbia, Canada. I predicted that if *w*Suz does provide *D. suzukii* protection from RNA viruses I would see flies infected with *w*Suz less frequently infected with a virus. Additionally, I wanted to measure the frequency of *w*Suz and the two RNA viruses in *D. suzukii* populations. To do this, I used PCR to screen for the presence of *Wolbachia* and the two viruses in flies collected in the Fraser Valley, British Columbia, Canada in 2019.

Materials and Methods

Screening *Drosophila suzukii* from British Columbia for two RNA viruses

I screened field-collected samples of *D. suzukii* for two RNA viruses that had been previously identified by sequencing the transcriptome of pooled samples collected from multiple sites in the Fraser Valley in British Columbia, Canada. Sequencing was performed by Genome Quebec, using Illumina HiSeq technology. Dr. Jan Dudzic, a postdoc in the Perlman lab, prepared the samples for RNA sequencing and also analyzed the resulting data set; these results will be reported elsewhere.

I chose to focus on two RNA viruses that this analysis detected, Teise Virus and a Motts Mill-like Virus. Teise Virus (TV) is a positive sense single-stranded RNA virus belonging to the *Luteoviridae* family and was discovered by Medd et al. (2018), who found that is it one of the most abundant viruses in *D. suzukii* in both Japan and Europe. This virus is closely related to Prestney virus and Motts Mill virus of *Drosophila subobscura* and *D. melanogaster*, respectively (Medd *et al.,* 2018). No phenotype has been described and it has thus far only been detected in *D. suzukii*. The TV that was detected in our samples contains two RNA fragments with three open reading frames (ORFs), two of which are identical at the amino acid level (ORF1 & 2) to the Teise Virus described by Medd et al. (2018) and the third (ORF3) is 99.8% similar. ORF1 is an RNA polymerase, and ORFs 2 and 3 encode hypothetical proteins. Overall, the TV in our samples is 99.9% similar to the TV described by Medd et al. (2018).

The Motts Mill-like Virus (MMLV) that was discovered in our samples has not been previously described. MMLV is most closely related to Motts Mill Virus, a positive-sense single-stranded RNA virus discovered by Webster et al. (2015) that has been found to infect 7% of *D. melanogaster*. MMLV consists of two ORFs, with 87% and 51% sequence similarity at the amino acid level to the Motts Mill Virus ORFs, respectively. Overall, the MMLV we detected is 65% similar to the Motts Mill Virus. ORF1 encodes an RNA polymerase and ORF2 encodes a viral coat protein. Motts Mill Virus has not been officially classified but appears to belong to a novel clade of viruses related to plant Sobemoviruses and Poleroviruses. Like TV, Motts Mill Virus also has not been characterized and no phenotype has been associated with this virus.

I chose to focus on these viruses for a few reasons. In addition to being present in our initial transcriptome survey, their presence was also confirmed in a small pilot screening study using flies captured in the early summer of 2019. I also chose to focus on single stranded positive-sense RNA viruses because *Wolbachia* has been previously shown to protect against this group of viruses (Teixeira *et al.,* 2009; Cattel *et al.,* 2016).

Site selection and insect sampling

Adult *D. suzukii* were collected at sites in the Fraser Valley, British Columbia, Canada in September 2019. At this late time in the season, adults are highly abundant. I sampled from a variety of habitats including urban, forested, and agricultural areas that all contained Himalayan blackberry, *Rubus armeniacus* (Focke) (Rosaceae), a common and widespread host for *D. suzukii*. I aspirated approximately 15 adults directly off host plants from 7 sites from along three transect lines (a northern, southern, and eastern transect line); sites on the same transect line were separated by a minimum of 10 km (Figure 2-1). Live flies were placed in vials containing Drosophila Instant Food Medium (Carolina Biological Supply Company). Upon returning to the laboratory, they were anesthetized using CO_2 and the species and sex were confirmed. *D. suzukii* males were identified based on the presence of male genitalia, a single dark spot on the leading edge of the wing near the tip, and sex combs on the first and second segments of the front legs running parallel to the length of the leg, and unbroken dark bands at the end of the abdomen (Government of B.C., 2020). Females were identified based on the presence of female genitalia, a large sclerotized ovipositor with saw-like serrations, with complete and unbroken dark bands at the end of the abdomen (Government of B.C., 2020). Flies were then individually placed in 1.5 mL Eppendorf tubes and stored in -80°C. A total of 99 flies were collected and were screened for Teise virus, Motts Mill-like virus, and *Wolbachia* (Table 2-1).

Table 2-1: Sampling information of *D. suzukii* in September 2019 in the Fraser Valley, British Columbia.

Transect	Site	GPS coordinates	Date	No. flies	Site description
South	S6	49° 1'13.72"N, 122°47'4.97"W	September 11, 2019	13	Agricultural
North	N1	49°14'30.82"N, 121°56'24.48"W	September 12, 2019	14	Agricultural
North	N3	49° 8'32.87"N, 122°18'22.88"W	September 12, 2019	15	Urban
North	N8	49°17'26.22"N, 122°53'31.98"W	September 12, 2019	14	Forested
East	E3	49°22'33.76"N, 121°30'19.35"W	September 13, 2019	15	Forested
East	E1	49°20'31.06"N, 121°44'48.88"W	September 13, 2019	13	Forested
South	S3	49° 0'33.55"N, 122°20'15.16"W	September 18, 2019	15	Agricultural

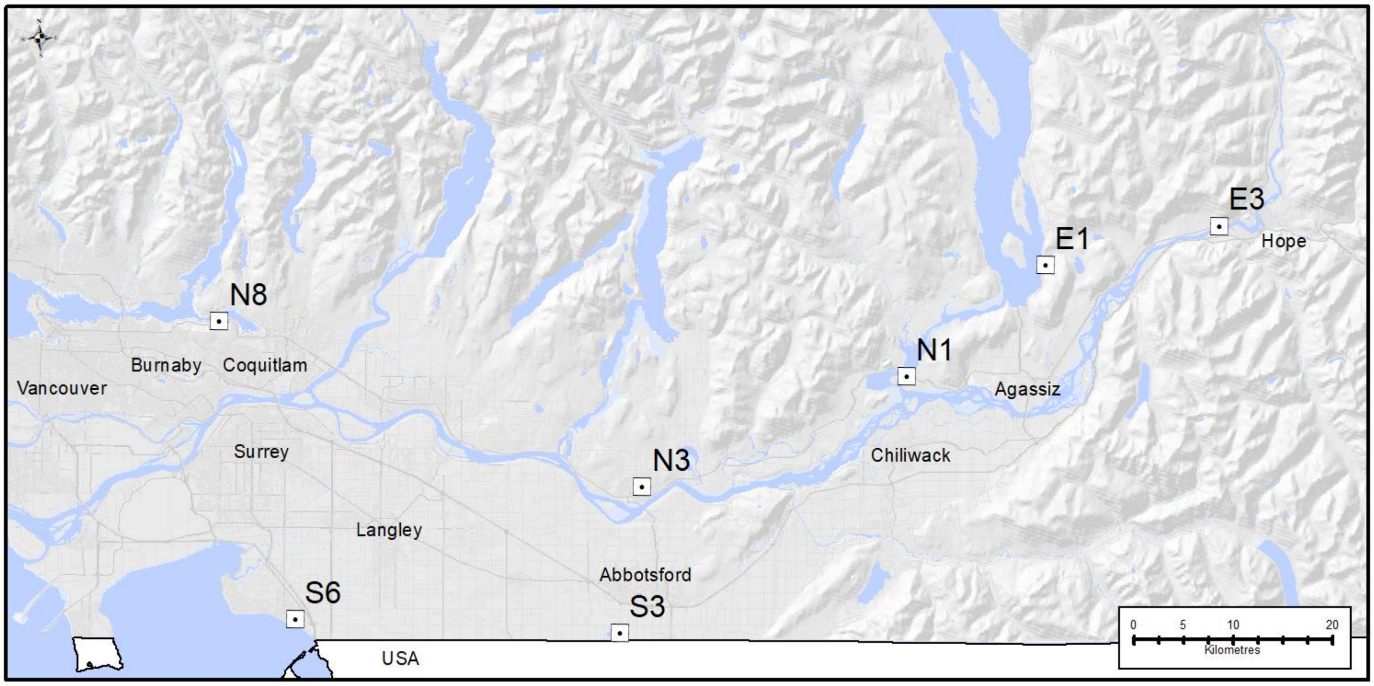

Figure 2-1: Map of sampling sites of *D. suzukii* in September 2019 in the Fraser Valley, British Columbia.

RNA extraction

RNA was extracted from individual flies by bead beating samples in 500 µL TRIzol (Invitrogen) and 1/3 volume chloroform. Samples were left at room temperature for five minutes then spun in a centrifuge at 14k RPM for 15 minutes at 4°C (Hettich Instruments, Mikro 120). The aqueous layer was removed and an equal volume of isopropanol was added to each sample as well as 0.01 mg RNA grade glycogen (Thermo Scientific) to aid with RNA precipitation and pellet visualization. Samples were centrifuged again at 14k RPM for 15 minutes at 4°C and the pellet was washed twice with 90% ethanol. The final ethanol wash was removed, the pellet was dried via evaporation and the RNA was re-dissolved in 11 µL UltraPure Distilled Water (Invitrogen). The RNA concentration and purity for each sample was assessed using Nanodrop absorbance readings.

cDNA synthesis

RNA was diluted to 500 ng/µL prior to cDNA synthesis. cDNA was obtained using the PrimeScript RT Reagent kit (TaKaRa Bio) according to the manufacturer's protocol.

Additionally, "no reverse transcription" controls were included for each sample in order to determine the presence of genomic DNA contamination.

Detection of *Wolbachia* and RNA viruses in *D. suzukii*

PCR was used to determine the presence of *Wolbachia,* Teise, and Motts Mill-like virus. *Wsp* primers (Zhou *et al.*, 1998) (wsp_81F: 5'-TGGTCCAATAAGTGATGAAGAAAC-3', wsp_691R: 5'-AAAAATTAAACGCTACTCCA-3') were used for *Wolbachia* detection with the following thermocycling conditions: 95°C × 3min, (94°C × 30 sec, 55°C × 30 sec, 72°C × 45 sec) x 30, 72°C x 10 min. I designed primers for TV and MMLV using Geneious v. 2020 1.2. I used the following primers for TV detection (TV_ 491 F: 5'-GCCACGTGGAGACTCATACC-3', TV_ 997 R: 5'-GGAACTCAACATCCCCTGGG-3') and MMLV detection (MMLV_648 F: 5'-GTCCAACAAGGCCCGTACC-3', MMLV_1169 R: 5'-ATTGGACCAACACGGAGAGG-3'). The following thermocycling conditions were used for Teise virus: 95°C × 3min, (94°C × 30 sec, 59°C × 30 sec, 72°C × 45 sec) x 30, 72°C x 10 min. The following thermocycling conditions were used for Motts Mill virus: 95°C × 3min, (94°C × 30 sec, 57°C × 30 sec, 72°C × 45 sec) x 30, 72°C x 10 min. A positive Cytochrome c oxidase (COI) control from the fly was used to ensure the extraction was successful and negative controls were included to determine that there was no contamination. COI primers (Folmer *et al.*, 1994) (LCO1490: 5'-GGTCAACAAATCATAAAGATATTG-3', HCO298R: 5'-TAAACTTCAGGGTGACCAAAAATCA-3') were used with the following thermocycling conditions: 95°C × 3min, (95°C × 1 min, 54°C × 30 sec, 72°C × 1 min) x 30, 72°C x 10 min. The PCR products were visualized on a 1% agarose gel (FroggaBio) following gel electrophoresis with the use of a 1 Kb plus DNA ladder (Invitrogen). *Wolbachia* and virus infection status was determined based on the presence/absence of a band. Positive amplicons for TV and MMLV were sequenced (Sequetech, California) to confirm the identity of the PCR product.

Data analysis

To determine if *D. suzukii* flies infected and uninfected with *Wolbachia* were more or less likely to be infected with Teise Virus and / or Motts Mill-like Virus, a Chi-square test of independence (since sample sizes were above five individuals) (Whitlock & Schluter, 2009) was conducted using the website vassarstats.net (http://vassarstats.net/tab2x2.html, 2021). I also used

Chi-square tests of independence to determine whether there was a significant association between the two viruses, as well as whether fly sex was associated with virus or *Wolbachia* infection.

Results

I found *Wolbachia*, Motts Mill-like virus, and Teise Virus infections at every site I surveyed in the Fraser Valley region of British Columbia, Canada. Overall, MMLV was more prevalent in the total population compared to *Wolbachia* and TV which infected equal number of flies (Table 2-2). At the site level, infection frequencies of *Wolbachia* and the two viruses were consistent, with every site having seven or fewer infected individuals (Table 2-2). Additionally, 18 out of 99 flies were infected with both TV and MMLV whereas 11 of 99 flies were only infected with MMLV, and 7 of 99 flies were infected with only TV.

There was no association between *Wolbachia* and virus infection, nor did sex affect infection frequency (Table 2-3). Interestingly, however, individuals were more likely to be coinfected with both RNA viruses as opposed to just one (Table 2-4).

Table 2-2: Percentage of *D. suzukii* infected with *Wolbachia*, MMLV, and TV at each site and the overall number of flies infected (n = 99).

Site	no. flies	*Wolbachia*	MMLV	TV
E1	13	23.1	46.2	23.1
E3	15	26.7	20.0	20.0
N1	14	35.7	14.3	14.3
N3	15	33.3	20.0	20.0
N8	14	21.4	50.0	42.9
S3	15	6.67	26.7	26.7
S6	13	30.8	38.5	30.8
Total no. of infected individuals	80	25	30	25

Table 2-3: Contingency table of associations between Teise Virus, Motts Mill-like Virus, and *Wolbachia* in *D. suzukii* using a Chi-square test of independence (n = 99).

Association	Chi-square test of independence
Wolbachia & MMLV	$\chi^2 = 0.69$, P = 0.41
Wolbachia & TV	$\chi^2 = 0.09$, P = 0.76
Wolbachia & Sex	$\chi^2 = 0.39$, P = 0.53
MMLV & Sex	$\chi^2 = 0$, P = 1.00
TV & Sex	$\chi^2 = 0.66$, P = 0.42

Table 2-4: Contingency table of the association between Teise Virus and Motts Mill-like Virus in *D. suzukii* using a Chi-square test of independence (n = 99).

	TV absent	TV present	Chi-square test of independence
MMLV present	12	18	$\chi^2 = 27.6$ $P \leq 0.0001$
MMLV absent	68	7	

Discussion

A number of *Wolbachia* strains, including *w*Suz in *D. suzukii,* are found at intermediate frequencies, yet do not appear to cause strong cytoplasmic incompatibility and are imperfectly maternally transmitted. How these strains persist in nature and what are the factors driving this maintenance is not well understood. One possibility is that protection from RNA viruses plays an important role in shaping these dynamics; however, protection has yet to be demonstrated to be important in nature, nor do we know what the most important pathogenic viruses in the field are. I predicted that if *Wolbachia* does provide resistance to RNA viruses that I would see flies infected with *Wolbachia* would be less frequently infected with TV or MMLV. However, I found that there does not appear to be an association between *Wolbachia* and the two viruses, meaning a fly is just as likely to be infected with either TV or MMLV if it is also infected with *Wolbachia* or not. Although I cannot directly say that *Wolbachia* does or does not provide viral blocking, the lack of association between *w*Suz and TV and MMLV could be attributed to at least two factors.

Firstly, the viruses I chose to screen for may not be very pathogenic and therefore may not be cleared by *Wolbachia* from the population as quickly, if at all, compared to viruses that have a strong negative impact on hosts. Although MMLV and TV have not been characterized, since they are fairly common in the flies I analyzed and in other studies (Webster *et al.,* 2015; Medd *et al.,* 2018) there is a trend that widespread and/or common viruses tend to not be as pathogenic (Gupta *et al.,* 2017). For example, La Jolla virus in *D. melanogaster* is widespread yet it is not protected against as much by *Wolbachia* in the same way as other more pathogenic viruses such as DCV, despite both La Jolla and DCV being picorna viruses (Webster *et al.,* 2015).

Secondly, there is the potential that TV and MMLV are resistant to the viral-protection properties of *Wolbachia*. The strongest blocking phenotypes observed in experimental settings are often the result of microinjection and using non-native virus species which can elicit a stronger immune response than what would naturally occur (Zug & Hammerstein, 2015). Anti-viral effects appear to be strongest in hosts that are transfected with a novel strain of *Wolbachia* (Zug & Hammerstein 2015; Martinez *et al.,* 2017). It has been suggested that viruses which naturally occur within a host can over time evolve a resistance to *Wolbachia* and be less susceptible to its effects (Shi *et al.*, 2018). There is the possibility that TV and MMLV have been associated with *D. suzukii* and *w*Suz for a long enough time that they are now less susceptible to the effects of *w*Suz.

Thirdly, the fact that the presence of *Wolbachia* was not associated with TV or MMLV in my study does not necessarily mean that it does not provide a protective effect; it may reduce viral titres without clearing infections completely (Osborne *et al.*, 2009; Webster *et al.,* 2015). A reduction in virus titres may allow hosts to tolerate negative fitness effects of viral infections (Zug & Hammerstein, 2015); alternatively, it is possible that *Wolbachia* might even increase tolerance to viruses, allowing for higher viral loads. Therefore, it would be important to measure titres of TV and MMLV in *Wolbachia*-infected and uninfected individuals. However, Martinez et al. (2017) found that *w*Suz reduced viral titres by a very modest amount compared to other *Wolbachia* strains indicating that *w*Suz may not have the strongest viral-blocking phenotype. To determine if *w*Suz does offer protection to flies, the titres of native RNA viruses would have to be compared to flies infected and uninfected with *Wolbachia*.

The prevalence level of *w*Suz of 25% that I found fits with what was reported by Hamm and colleagues (2014). In my study, there was also no difference in infection prevalence between males and females. This is unsurprising given that w*Suz* is not a male killer and there is no evidence of skewed sex ratios in *D. suzukii* (Hamm *et al.,* 2014; Cattel *et al.,* 2016;). *w*Mel, which is also not a strain of *Wolbachia* that manipulates reproduction, is also found with equal frequency in males and females of *D. melanogaster* (Harcombe & Hoffmann, 2004).

I found that TV and MMLV occur naturally in *D. suzukii* and commonly across all sites in the Fraser Valley. This is the first time that a common and natural viral infection has been documented in *D. suzukii* in North America. TV frequencies in my samples are comparable to those found by Medd et al. (2018) in European and Asian populations of *D. suzukii*. MMLV frequencies were also similar to those in *D. melanogaster* reported by Webster et al. (2015), despite MMLV being different from Motts Mill virus. Regarding the differences between the infection status of males and females in terms of TV and MMLV, both sexes were just as likely to be infected or uninfected. Sexual dimorphism in immune response to pathogens has been observed in many species, including drosophilids (Belmonte *et al.*, 2020). It is common for female drosophilids to have a more robust immune response to viruses compared to males (Duneau *et al.*, 2017; Palmer *et al.*, 2018) which makes our results a bit unexpected. This trend may not extend to all viruses since both male and female *D. melanogaster* are equally infected with Kallithea virus (Palmer *et al.,* 2018). The lack of difference between the sexes may be a result of both TV and MMLV not being very pathogenic and not eliciting a strong immune response, in this case, both males and females would be equally able to tolerate infection.

Although I did not find an association between *Wolbachia* and TV or MMLV, I did find that *D. suzukii* infected with one virus made them more likely to be infected with the other virus. That is, there were more flies infected with TV and MMLV than flies infected with only one of those viruses. A similar pattern has been observed in honey bees (Chen *et al.,* 2004; Remnant *et al.,* 2017) and drosophilids (Webster *et al.,* 2015; Shi *et al.,* 2016, 2018) where there were more individuals that were infected with more than one virus as opposed to being singly infected. Flies could be more susceptible to being infected with more than one virus because their immune system is already compromised from the infection of one virus or some other stressor (Palmer *et al.,* 2018). My results suggest that MMLV and TV may be mildly pathogenic and increase susceptibility of multiple infections in *D. suzukii*. This is observed in *Manduca sexta* larvae where infection with a polydnavirus increases susceptibility to being infected with M Nucleopolyhedrovirus (Washburn *et al.,* 2000). Being infected with more than one virus suggests that flies may be susceptible to a diversity of viruses simultaneously (Palmer *et al.*, 2018).

Another possible explanation for the flies in my study being more likely to be infected with more than one virus is that environmental conditions could induce stress responses that increase overall susceptibility to viral infections. Exogenous factors such as changes in temperature can increase the stress response in insects making them more susceptible to viral infections and increased transmission rates (Lambrechts *et al.*, 2011; Carrington *et al.*, 2013). Additionally, *Wolbachia* is temperature-sensitive, where both high and low temperatures have been shown to reduce *Wolbachia* titres (Wiwatanaratanabutr & Kittayapong, 2009; Kriesner *et al.*, 2016; Ross *et al.*, 2017) and *w*Suz-mediated protection from viruses has been linked to *Wolbachia* density in flies (Osborne *et al.*, 2008; Martinez *et al.*, 2017). It is possible that temperature fluctuations decrease *w*Suz densities and that could impact the protective ability of *w*Suz against viruses. The flies in this study were collected in mid-September where temperatures can fluctuate by 15°C or more daily, which could lead to flies being susceptible to multiple viral infections. All studies investigating the protective effects of *Wolbachia* have been conducted at constant temperatures, and it would be interesting to investigate how *Wolbachia*-mediated protection is impacted by temperature fluctuations.

In my study, I was able to establish that *Wolbachia* infection in *D. suzukii* was not associated with the presence of two naturally occurring RNA viruses, TV and MMLV. Interestingly, I found that flies infected with one virus were more likely to be infected with the other virus. My findings emphasize the need for further characterization of natural viruses of *D. suzukii* and understanding how *Wolbachia* may impact the immune response of *D. suzukii* to such viruses. If *Wolbachia* does provide resistance to natural RNA viruses in the field, this could explain why *Wolbachia* is maintained at intermediate frequencies in *D. suzukii* despite a lack of CI and imperfect maternal transmission. Because there is still so much still left to discover, characterizing cryptic viral infections in insects and understanding how they affect their host and interact with each other is a major frontier in insect ecology.

Chapter 2 - References

Asplen, M. K., Anfora, G., Biondi, A., Choi, D. S., Chu, D., Daane, K. M., Gibert, P., Gutierrez, A. P., Hoelmer, K. A., Hutchison, W. D., Isaacs, R., Jiang, Z. L., Kárpáti, Z., Kimura, M. T., Pascual, M., Philips, C. R., Plantamp, C., Ponti, L., Vétek, G., … Desneux, N. (2015). Invasion biology of spotted wing *Drosophila* (*Drosophila suzukii*): a global perspective and future priorities. *Journal of Pest Science*, *88*(3), 469–494. https://doi.org/10.1007/s10340-015-0681-z

Belmonte, R. L., Corbally, M. K., Duneau, D. F., & Regan, J. C. (2020). Sexual dimorphisms in innate immunity and responses to infection in *Drosophila melanogaster*. *Frontiers in Immunology*, *10*(3075), 1–18. https://doi.org/10.3389/fimmu.2019.03075

Carrington, L. B., Seifert, S. N., Armijos, M. V., Lambrechts, L., & Scott, T. W. (2013). Reduction of *Aedes aegypti* vector competence for dengue virus under large temperature fluctuations. *American Journal of Tropical Medicine and Hygiene*, *88*(4), 689–697. https://doi.org/10.4269/ajtmh.12-0488

Cattel, J., Martinez, J., Jiggins, F., Mouton, L., & Gibert, P. (2016). *Wolbachia*-mediated protection against viruses in the invasive pest *Drosophila suzukii*. *Insect Molecular Biology*, *25*(5), 595–603. https://doi.org/10.1111/imb.12245

Cattel, J., Kaur, R., Gibert, P., & Martinez, J. (2016). *Wolbachia* in European populations of the invasive pest *Drosophila suzukii* : regional variation in infection frequencies. *PLoS ONE*, *11*(1), e0147766. https://doi.org/10.1371/journal.pone.0147766

Chen, Y., Zhao, Y., Hammond, J., Hsu, H. T., Evans, J., & Feldlaufer, M. (2004). Multiple virus infections in the honey bee and genome divergence of honey bee viruses. *Journal of Invertebrate Pathology*, *87*(2–3), 84–93. https://doi.org/10.1016/j.jip.2004.07.005

Cini, A., Anfora, G., Escudero-Colomar, L. A., Grassi, A., Santosuosso, U., Seljak, G., & Papini, A. (2014). Tracking the invasion of the alien fruit pest *Drosophila suzukii* in Europe. *Journal of Pest Science*, *87*, 559–566. https://doi.org/10.1007/s10340-014-0617-z

Cordaux, R., Pichon, S., Ling, A., Pérez, P., Delaunay, C., Vavre, F., Bouchon, D., & Grève, P. (2008). Intense transpositional activity of insertion sequences in an ancient obligate endosymbiont. *Molecular Biology and Evolution*, *25*(9), 1889–1896. https://doi.org/10.1093/molbev/msn134

Duneau, D. F., Kondolf, H. C., Im, J. H., Ortiz, G. A., Chow, C., Fox, M. A., Eugénio, A. T., Revah, J., Buchon, N., & Lazzaro, B. P. (2017). The Toll pathway underlies host sexual dimorphism in resistance to both Gram-negative and Gram-positive bacteria in mated *Drosophila*. *BMC Biology*, *15*, 124. https://doi.org/10.1186/s12915-017-0466-3

Faria, V. G., Martins, N. E., Magalhães, S., Paulo, T. F., Nolte, V., Schlötterer, C., Sucena, É., & Teixeira, L. (2016). *Drosophila* adaptation to viral infection through defensive symbiont evolution. *PLoS Genetics, 12*(9), e1006297. https://doi.org/10.1371/journal.pgen.1006297

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5), 294 - 299.

Government of British Columbia. (2020). *Spotted wing Drosophila (fruit fly) pest alert.* https://www2.gov.bc.ca/gov/content/industry/agriculture-seafood/animals-and-crops/plant-health/insects-and-plant-diseases/tree-fruits/spotted-wing-drosophila

Gupta, V., Stewart, C. O., Rund, S. S. C., Monteith, K., & Vale, P. F. (2017). Costs and benefits of sublethal Drosophila C virus infection. *Journal of Evolutionary Biology*, *30*(7), 1325–1335. https://doi.org/10.1111/jeb.13096

Hamm, C. A., Begun, D. J., Vo, A., Smith, C. C. R., Saelao, P., Shaver, A. O., Jaenike, J., & Turelli, M. (2014). *Wolbachia* do not live by reproductive manipulation alone: Infection polymorphism in *Drosophila suzukii* and *D. subpulchrella*. *Molecular Ecology*, *23*(19), 4871–4885. https://doi.org/10.1111/mec.12901

Harcombe, W., & Hoffmann, A. A. (2004). *Wolbachia* effects in *Drosophila melanogaster*: In search of fitness benefits. *Journal of Invertebrate Pathology*, *87*(1), 45–50. https://doi.org/10.1016/j.jip.2004.07.003

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

Hoffmann, A. A. (1988). Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster*. *Entomologia Experimentalis et Applicata*, *48*(1), 61–67. https://doi.org/10.1111/j.1570-7458.1988.tb02299.x

Hoffmann, A.A., & Turelli, M. (1997). Cytoplasmic incompatibility in insects, pp. 41–80 in *Influential Passengers: Inherited Microorganisms and Invertebrate Reproduction*, edited by S. L. O'Neill, A. A. Hoffmann and J. H. Werren. Oxford University Press, Oxford University Press: New York

Kanzawa, T. (1939). Studies on *Drosophila suzukii* Mats. Kofu, Yamanashi Agricultural Experiment Station 49. *Rev. Appl. Entomol.* 29: 622

Kriesner, P., Conner, W. R., Weeks, A. R., Turelli, M., & Ary, A. (2016). Persistence of a *Wolbachia* infection frequency cline in *Drosophila melanogaster* and the possible role of reproductive dormancy. *Evolution*, *70*(5), 979–997. https://doi.org/10.1111/evo.12923.

Lambrechts, L., Paaijmans, K. P., Fansiri, T., Carrington, L. B., Kramer, L. D., Thomas, M. B., & Scott, T. W. (2011). Impact of daily temperature fluctuations on dengue virus transmission by *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(18), 7460–7465. https://doi.org/10.1073/pnas.1101377108

Lee, J. C., Bruck, D. J., Curry, H., Edwards, D., Haviland, D. R., Van Steenwyk, R. A., & Yorgey, B. M. (2011). The susceptibility of small fruits and cherries to the spotted-wing drosophila, *Drosophila suzukii*. *Pest Management Science*, *67*(11), 1358–1367. https://doi.org/10.1002/ps.2225

Martinez, J., Tolosana, I., Ok, S., Smith, S., Snoeck, K., Day, J. P., & Jiggins, F. M. (2017). Symbiont strain is the main determinant of variation in *Wolbachia*-mediated protection against viruses across *Drosophila* species. *Molecular Ecology*, *26*(15), 4072–4084. https://doi.org/10.1111/mec.14164

Mazzetto, F., Gonella, E., Alma, A. (2015). *Wolbachia* infection affects female fecundity in *Drosophila suzukii*. *Bulletin of Insectology*, *68*(1), 153 - 157.

Medd, N. C., Fellous, S., Waldron, F. M., Xuéreb, A., Nakai, M., Cross, J. V., & Obbard, D. J. (2018). The virome of *Drosophila suzukii*, an invasive pest of soft fruit. *Virus Evolution*, *4*(1), vey009. https://doi.org/10.1093/ve/vey009

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

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), e1000656. https://doi.org/10.1371/journal.ppat.1000656

Palmer, W. H., Varghese, F. S., & Van Rij, R. P. (2018). Natural variation in resistance to virus infection in dipteran insects. *Viruses*, *10*(3), 118. https://doi.org/10.3390/v10030118

Remnant, E. J., Shi, M., Buchmann, G., Blacquière, T., Holmes, E. C., Beekman, M., & Ashe, A. (2017). A diverse range of novel RNA viruses in geographically distinct honey bee populations. *Journal of Virology*, *91*(16), e00158-17. https://doi.org/10.1128/jvi.00158-17

Ross, P. A., Wiwatanaratanabutr, I., Axford, J. K., White, V. L., Endersby-Harshman, N. M., & Hoffmann, A. A. (2017). *Wolbachia* infections in *Aedes aegypti* differ markedly in their response to cyclical heat stress. *PLoS Pathogens*, *13*(1), e1006006 . https://doi.org/10.1371/journal.ppat.1006006

Shi, M., Lin, X. D., Tian, J. H., Chen, L. J., Chen, X., Li, C. X., Qin, X. C., Li, J., Cao, J. P., Eden, J. S., Buchmann, J., Wang, W., Xu, J., Holmes, E. C., & Zhang, Y. Z. (2016). Redefining the invertebrate RNA virosphere. *Nature*, *540*, 539–543. https://doi.org/10.1038/nature20167

Shi, M., White, V. L., Schlub, T., Eden, J. S., Hoffmann, A. A., & Holmes, E. C. (2018). No detectable effect of *Wolbachia w*Mel on the prevalence and abundance of the RNA virome of *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1883), 20181165. https://doi.org/10.1098/rspb.2018.1165

Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, *6*(12), e2 . https://doi.org/10.1371/journal.pbio.1000002 van den Hurk, A. F., 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

Walsh, D. B., Bolda, M. P., Goodhue, R. E., Dreves, A. J., Lee, J., Bruck, D. J., Walton, V. M., O'Neal, S. D., & Zalom, F. G. (2011). *Drosophila suzukii* (Diptera: Drosophilidae): Invasive pest of ripening soft fruit expanding its geographic range and damage potential. *Journal of Integrated Pest Management*, *2*(1), G1 - G7. https://doi.org/10.1603/IPM10010

Washburn, J. O., Haas-Stapleton, E. J., Tan, F. F., Beckage, N. E., & Volkman, L. E. (2000). Co-infection of *Manduca sexta* larvae with polydnavirus from *Cotesia congregata* increases susceptibility to fatal infection by *Autographa californica* M Nucleopolyhedrovirus. *Journal of Insect Physiology*, *46*(2), 179–190. https://doi.org/10.1016/S0022-1910(99)00115-8

Webster, C. L., Waldron, F. M., Robertson, S., Crowson, D., Ferrari, G., Quintana, J. F., Brouqui, J. M., Bayne, E. H., Longdon, B., Buck, A. H., Lazzaro, B. P., Akorli, J., Haddrill, P. R., & Obbard, D. J. (2015). The discovery, distribution, and evolution of viruses associated with *Drosophila melanogaster*. *PLoS Biology*, *13*(7), e1002210 . https://doi.org/10.1371/journal.pbio.1002210

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

Whitlock, M.C., & Schluter, D. (2009). Contingency analysis: Associations between categorical variables, pp. 213 - 219 in *The Analysis of Biological Data*, Roberts and Company Publishers, Roberts and Company Publishers: Greenwood Village, Colarado

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

Zhou, W., Rousset, F., and O'Neill, S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proceedings of the Royal Society B:*

Biological Sciences, *265*(1395), 509–515. doi:10.1098/rspb.1998.0324.

Zug, R., & Hammerstein, P. (2015). Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. *Biological Reviews of the Cambridge Philosophical Society*, *90*(1), 89–111. https://doi.org/10.1111/brv.12098

Chapter 3 - The role of larval density in shaping *Wolbachia* frequencies and development time in *Drosophila suzukii*

Introduction

Insects are commonly infected with facultative, inherited bacterial endosymbionts that can have diverse and important effects on their hosts (Weinert *et al.,* 2015). These symbionts are almost exclusively transmitted from mothers to their offspring, resulting in their fitness being intimately tied to that of their hosts (Hoffmann & Turelli, 1997). Because they are not required by their hosts for survival or reproduction, it is important to understand how they persist and avoid being eliminated from the host populations. This is especially intriguing as carrying a microbial infection is costly (Weinert *et al.,* 2015). One way these symbionts "stick around" is to provide conditional benefits to their hosts, like protecting them against natural enemies (Teixeira *et al.,* 2008). Finding the conditional benefit can be quite challenging and can be influenced by many different factors.

The most commonly described strategy that many facultative insect endosymbionts employ is the manipulation of host reproduction. There are four methods of manipulation: parthenogenesis-induction (Stouthamer *et al.,* 1990), male-killing (Jiggins *et al.,* 2001), feminization (Bouchon *et al.,* 1998), and cytoplasmic incompatibility (CI) (Hoffmann & Turelli, 1997). While the first three mechanisms result in female-biased offspring, CI is different in that uninfected females are at a disadvantage because they cannot produce viable offspring if they mate with infected males (Turelli & Hoffmann, 1995). The mechanism of CI entails a toxin-antitoxin mechanism where sperm is modified or "poisoned" during development and this modification is reversed or "rescued" in eggs of females that contain the same strain of symbiont (Beckmann *et al.,* 2019). This results in infected males and females having a fitness advantage over uninfected individuals (Kriesner & Hoffmann, 2018). Regardless of the mechanism of reproductive manipulation, all methods assist in the spread of the symbiont, even if they come at a cost to the host. The most well-studied facultative symbiont that exhibits all four methods of reproductive manipulation is the bacterium *Wolbachia*.

Wolbachia is a maternally inherited, endosymbiotic lineage of bacteria that has been estimated to infect at least 40% of all insects and some arthropods (Zug & Hammerstein, 2012).

This prolific symbiont has a variety of impacts on its host ranging from reproductive manipulations to mutualism (Werren *et al.*, 2008). Maintenance of these strains of *Wolbachia* that have formed a mutualistic relationship with their hosts is straight forward since they provide some fitness enhancement. For strains that act as reproductive parasites, “hijacking” the reproductive system allows *Wolbachia* to proliferate to a high degree in a population, regardless of the fitness impacts (Werren *et al.*, 2008). For strains that neither manipulate host reproduction nor confer an obvious fitness benefit, it is unknown how they are able to persist in a population (Harcombe & Hoffmann, 2004).

What can further complicate the maintenance of *Wolbachia* strains that are not reproductive parasites or mutualists is imperfect maternal transmission. For example, *w*Mel in *Drosophila melanogaster* is not perfectly maternally transmitted yet is stably maintained at intermediate frequencies in nature (Kriesner *et al.*, 2013, 2016). In order for this to occur, theory predicts that if a fraction of embryos produced are uninfected, *Wolbachia* should persist only if it enhances the relative fitness of infected females (Hoffmann & Turelli, 1997). A possible explanation for the maintenance of this mysterious *Wolbachia* strain is that it provides a context-dependent benefit to its host (Jaenike, 2012). This could include subtle effects such as conferring resistance to RNA viruses (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Unckless & Jaenike, 2012) or assisting in iron metabolism (Gill *et al.*, 2014).

Another such conditional situation where it could be beneficial to be infected with *Wolbachia* is under high-density rearing conditions. Recently, a study published by Kriesner and Hoffmann (2018) investigating the cryptic fitness benefits of *w*Au in *D. simulans* unexpectedly found that this strain came to near fixation in a short period of time. These results were very surprising given the initial *Wolbachia* frequencies were less than 30% and *w*Au does not cause reproductive manipulations. The authors found that infected females experienced a fitness advantage of 20% compared to uninfected females to explain the rapid increase in *Wolbachia* frequencies that were observed. The authors concluded that some aspect of the environment encountered by the flies enabled *Wolbachia*-infected larvae to outcompete those that were uninfected. One such environmental factor that may have led to such high *Wolbachia* frequencies was the crowded rearing conditions.

It has been established that larval density can influence adult fitness (Peters & Barbosa, 1977; Islam & Dobson, 2006; Morimoto *et al.,* 2016; Roy *et al.,* 2017). What is less well-studied are the effects of *Wolbachia* infection and larval density on host fitness, in particular *Wolbachia* strains that do not manipulate host reproduction. Most of what is known about the interactions between *Wolbachia* and density on host fitness comes from only a few papers and the results are variable. Some studies have found that *Wolbachia* negatively impacts hosts when exposed to larval crowding (Hoffmann *et al.,* 1998; Huigens *et al.,* 2004) whereas others detected no effect of *Wolbachia* on life history traits and the differences observed were attributed to the density environment (Islam & Dobson, 2006; Gavotte *et al.,* 2009). However, in drosophilids, larval crowding has been shown to impact development time and strength of CI in early emerging adults (Yamada *et al.,* 2007). These studies show that *Wolbachia* and density can have an effect on host fitness, and they also suggest that these effects are not universal and are different depending on the host species and *Wolbachia* strain.

Besides *w*Mel and *w*Au, there are other *Wolbachia* strains that are stably and mysteriously maintained in host populations. The *Wolbachia* strain *w*Suz of Spotted-wing Drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) is one such strain. *Drosophila suzukii* is an invasive polyphagous pest of soft-skinned fruits in both Europe and North America (Asplen *et al.,* 2015). The *Wolbachia* strain infecting *D. suzukii* does not induce CI, is not horizontally transmitted, and exhibits imperfect maternal transmission (Hamm *et al.,* 2014). Collectively, these factors should systematically reduce its frequency; however, despite that, *w*Suz is found at intermediate frequencies between 7 – 20% in North America (Hamm *et al.,* 2014; Wilches *et al.,* 2021). The simplest explanation for the persistence of *w*Suz is that it provides a context-dependent fitness enhancement to its host (Hamm *et al.,* 2014). Similar to *w*Mel, *w*Suz has been found to confer resistance to some RNA viruses (Cattel *et al.,* 2016; Martinez *et al.,* 2017) in a laboratory setting. However, these results have yet to be demonstrated in the field. The search for a fitness enhancement or an explanation for the continued persistence of *w*Suz in *D. suzukii* remains.

Motivated by the interesting findings of Kriesner and Hoffmann (2018), I designed an experiment to explore the possibility that *w*Suz increases the competitive ability of *D. suzukii* larvae compared to their uninfected counterparts when reared at high larval densities. I measured the development time of infected and uninfected *D. suzukii* larvae reared at three different densities and the overall infection frequencies of flies. If *w*Suz infection leads to a decrease in development time at high densities, then it would be expected that infected individuals would emerge sooner than uninfected individuals. If *w*Suz provided some other fitness enhancement at high densities then I would expect to see a higher number of infected individuals.

Materials & Methods

Establishment of D. suzukii colony

I used a pair of matched *D. suzukii* lines that only differed in terms of whether they harboured *Wolbachia*. The *Wolbachia*-positive line descended from a single *D. suzukii* female collected from Promontory, Chilliwack, British Columbia, Canada (49°05'52.9"N 121°55'28.0"W) in August of 2018 on the host plant species *Rubus armeniacus* (Focke) (Rosaceae). *Wolbachia* infection status is continually monitored via diagnostic PCR, confirming that the females in this isofemale line maintain the infection and stably transmit *Wolbachia*. To establish a genetically identical *Wolbachia*-free population, a subpopulation was treated with tetracycline (Sigma-Aldrich) mixed with prepared Instant Drosophila Medium (Carolina Biological Supply Company) at a concentration of 0.05% for three consecutive generations. *Wolbachia*-infected flies received identical food and environmental conditions, except that tetracycline was not added. After treatment, infection status was confirmed and checked every 3 – 4 generations. *Wolbachia*-positive and negative fly lines were subsequently maintained in the Perlman laboratory (University of Victoria, Canada) in a 24°C incubator (12:12 light:dark cycle), in vials with Instant Drosophila Medium (Carolina Biological Supply Company).

All flies used in the following experiments were more than 75 generations post antibiotic treatment. This is expected to provide more than sufficient time to reacquire gut bacteria and recover from adverse effects of treatment (Ballard *et al.,* 2007; Li *et al.,* 2014). Additionally, studies have also demonstrated that the gut microbiota of *D. suzukii* is heavily influenced by diet and transient in nature (Vacchini *et al.,* 2017; Jiménez-Padilla *et al.,* 2020). Flies were reared indoors in a non-climate controlled room at an average temperature of 21°C with natural light conditions of approximately 13 hours of daylight and 11 hours of darkness. A HOBO data logger was used to measure the temperature at 5-minute intervals. *Wolbachia*-infected and uninfected populations of flies were reared separately in 125mL jars (Bernardin) with 3 tbsp of Instant Drosophila Medium plus 30 mL of tap water for a total volume of 60mL of prepared food. Three frozen blueberries were added to each jar. The frozen blueberries were collected from the Agriculture and Agri-Food Canada Agassiz research station in 2019 and had been frozen for approximately a year prior to use. The tops of the jars were covered with fine insect mesh and secured with an elastic band. Each rearing jar contained 10 males and 10 females and flies were transferred to a new jar every three days. To ensure that the food media did not become too dry, jars were misted twice daily with approximately 5 mL of water.

Adult flies used in the larval density experiment were collected on the day they emerged and placed in a vial (Diamed) secured with a flug (Diamed). Each of these vials contained 30 flies consisting of 15 males and 15 females of the same age and same *Wolbachia* status. Each vial contained 1 tsp of dry Instant Drosophila Medium (Carolina Biological Supply Company) plus 8mL of tap water for a total volume of 15 mL of prepared food. One frozen blueberry from the source stated above was added to each vial. Flies were transferred to a new vial daily until they were used in the larval density experiment, which was 7 – 9 days. Vials that had > 50% mortality were not used.

Larval density experiment

This experiment was designed to examine the effect of larval competition (larval density) on *Wolbachia* infection frequency and determine if there was a difference in development time for infected versus uninfected individuals in *D. suzukii*. In this experiment, three density treatments were utilized that consisted of a constant number of flies but varying the amount of oviposition substrate available. Blueberries were used as the oviposition substrate because they are a known fruit host for *D. suzukii* (Thistlewood *et al.*, 2019) and they are readily available throughout the summer months in British Columbia. In this experiment, the “Low”, “Medium”, and “High” densities each contained 120, 60, and 20 blueberries, respectively, each with 60 adult *D. suzukii*. Of those 60 flies, 30 were from the *Wolbachia* infected colony and the other 30 were from the uninfected colony. Of the 30 flies from each colony, 15 were males and 15 females. The *Wolbachia* infection status of each fly was confirmed later (see below). Six separate temporal blocks of density experiments were conducted, with two replicates of each density per block. The experiment was executed from August 2020 to the end of September 2020, which corresponds with the natural lifecycle of *D. suzukii* as they are actively reproducing during this time in British Columbia (Thistlewood *et al.,* 2019). Due to Covid-19, this experiment was conducted outdoors on a covered porch in Agassiz, British Columbia, Canada which provided an opportunity to expose developing larvae to a realistic range of abiotic conditions such as light, temperature, and humidity. Due to time constraints, DNA extraction and *Wolbachia* screening were only performed for two of the six blocks. These two blocks, hereafter Block A and Block B, were randomly selected. Blocks A and B were set up on August 7th and September 4th, respectively. Flies from the other blocks will be processed and analyzed at a later date.

The containers used for the experiment were small square containers (Ziploc) for the high and medium density treatments and large rectangle containers (Ziploc) for the low-density treatment. A larger sized container was used for the low-density treatments to ensure all blueberries were in a single layer on the bottom of each container. The bottom of each container was lined with two paper towels that were spritzed with 15 mL of water to maintain humidity. The lids were modified by cutting out a 10cm x 10cm hole in the lid and gluing fine insect mesh over the opening. The blueberries used in this experiment came from the same organic blueberry farm (Westberry Farm, Abbotsford, British Columbia, Canada). The berries were stored for 48 hours at 4°C prior to use to ensure that any *D. suzukii* or other *Drosophila* larvae were killed. The berries were washed twice, dried, and individually inspected to ensure only undamaged berries were used.

Each temporal block was initiated by adding blueberries to their respective containers along with the 60 gently aspirated flies at 2pm, and after 24 hours the flies were removed and preserved in 95% ethanol for future *Wolbachia* detection. Using a dissecting microscope, all of the eggs in every berry were counted. Eggs were identified as an oviposition scar with the presence of spiracles (Government of B.C., 2020). Eggs laid on the exterior of the berry were not counted due to the high probability of desiccation and the larvae dying. Once the eggs were counted, all of the blueberries were placed into new small square containers (Ziploc) that were prepared as stated above. To ensure consistent development conditions for all of the density treatments, berries from the low and medium treatments were subdivided into containers containing 20 berries per container. Every container was checked daily at 10 am for emergence and to remove any excess condensation. As flies emerged, they were removed from their container, identified, and preserved in 95% ethanol for future *Wolbachia* detection. Each container was kept for 14 days after the last fly emerged to capture any other insects that may have contaminated samples, for example, parasitoids or other Drosophilids.

Determination of infection status of parents and offspring

DNA was extracted from parental mothers and all offspring in order to determine *Wolbachia* infection status. DNA was extracted by homogenizing individual flies in 50µL of DNA extracting buffer (9.8mL H_2O, 100µL 1M Tris pH 8.0, 20µL 0.5 M EDTA, 50µL NaCl) and 0.5µL of Proteinase K (BioLabs). Once flies were homogenized they were incubated at 37°C for 20 minutes followed by an incubation of 95°C for 2 minutes and stored at 4°C. Using PCR, flies were screened for *Wolbachia* using the *Wolbachia* surface protein (wsp) (Zhou *et al.*, 1998) specific primers (wsp_81F: 5'- TGGTCCAATAAGTGATGAAGAAAC-3', wsp_691R: 5'-AAAAATTAAACGCTACTCCA-3') using the following thermocycling conditions: 95°C × 3min, (94°C × 30 sec, 55°C × 30 sec, 72°C × 45 sec) x 30, 72°C x 10min . PCR products were visualized on a 1% agarose gel (FroggaBio) following gel electrophoresis with the use of a 1 Kb plus DNA ladder (Invitrogen). *Wolbachia* status was determined based on the presence/absence of a band. A *Wolbachia*-positive control was included along with a COI control for *Wolbachia*-negative samples to ensure the extraction was successful. The COI primers described by Folmer et al., (1994) (LCO1490: 5'-GGTCAACAAATCATAAAGATATTG-3', HCO298R: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') were used with the following thermocycling conditions: 95°C × 3min, (95°C × 1 min, 54°C × 30 sec, 72°C × 1 min) x 30, 72°C x 10 min .

Data analysis

All data analysis was conducted in R Studio v1.2.5019 (R Core Team, 2019).

I first compared the average number of eggs and the proportion of emerging adults among the three density treatments (High, Medium, and Low). A Generalized Linear Model with a quasipoisson error distribution was performed to determine if there was a difference in the number of eggs laid per berry between the three density treatments. There were two categorical main effects in this model: density (H, M, L) and block (A and B). An F-test with the Anova ( ) function in the "car" package (Fox & Weisberg, 2019) was used to determine statistical significance (*p* < 0.05). A Tukey multiple comparison test was performed to determine which density treatments were significantly different in the number of eggs laid using the "emmeans" package (v 1.5.2, Length, 2020). To determine if there was a difference between the density treatments in the proportion of eggs that emerged as adults, a Generalized Linear Model was performed using a quasibinomial error distribution to correct for overdispersion. The main effects in the model were proportion emergence and block. Significance was determined using an F-test.

To test the effect of density-dependent mortality (larval competition) on *Wolbachia* frequencies, a linear model was used with a normal distribution with the main effects of "Block" and "Proportion emergence". I used the proportion of emergent offspring as a main effect as opposed to "Density" because there was a large amount of variation in emergence within each density treatment and that variation was best accounted for by using "Proportion emergence" instead. To support this, I conducted a Pearson's correlation between "Proportion emergence" and "number of eggs per berry" and there is a positive correlation between mortality and number of eggs per berry (t = -1.9, df = 10, *p* = 0.03) which justifies the use of "Proportion emergence" as an estimate of density-dependent mortality. I also used a linear model with a normal distribution with the main effects of "Block" and "number of eggs per berry" to test the effect of density-dependent mortality on *Wolbachia* frequencies. An F-test with the Anova ( ) function in the "car" package (Fox & Weisberg, 2019) was used to determine statistical significance of the main effects (*p* < 0.05).

To determine whether *Wolbachia* infection status, proportion of emergence, sex of *D. suzukii* offspring, or block had an effect on emergence time of *D. suzukii* offspring, a linear mixed model using the package "lme4" (Bates *et al.*, 2015) was used. The full model with the main effects of *Wolbachia* status, proportion of emergence, sex, and block were used. The identity of each individual container was used as a random effect to account for non-independence of the *Wolbachia*-infected and *Wolbachia*-uninfected individuals emerging from the same container. None of the interaction effect factors in the model were significant, and the model was reduced to the significant main effects (block and *Wolbachia* infection status). Statistical significance of each model factor was determines using F-tests from the Anova ( ) function in the "car" package (Fox & Weisberg, 2019).

Results

Effect of density treatments

The number of eggs laid per berry varied significantly among the density treatments ($\chi^2 = 26$, df = 2, $p < 0.0001$). *Drosophila suzukii* females laid more eggs per berry with increasing fly density (Figure 3-1). The differences in the proportion of *D. suzukii* eggs emerging as adults for the three density treatments were only marginally significant (F = 3.62, df = 2, $p = 0.08$, Figure 3-2); however, more flies emerged in the Low density, followed by the Medium and High densities (Figure 3-2).

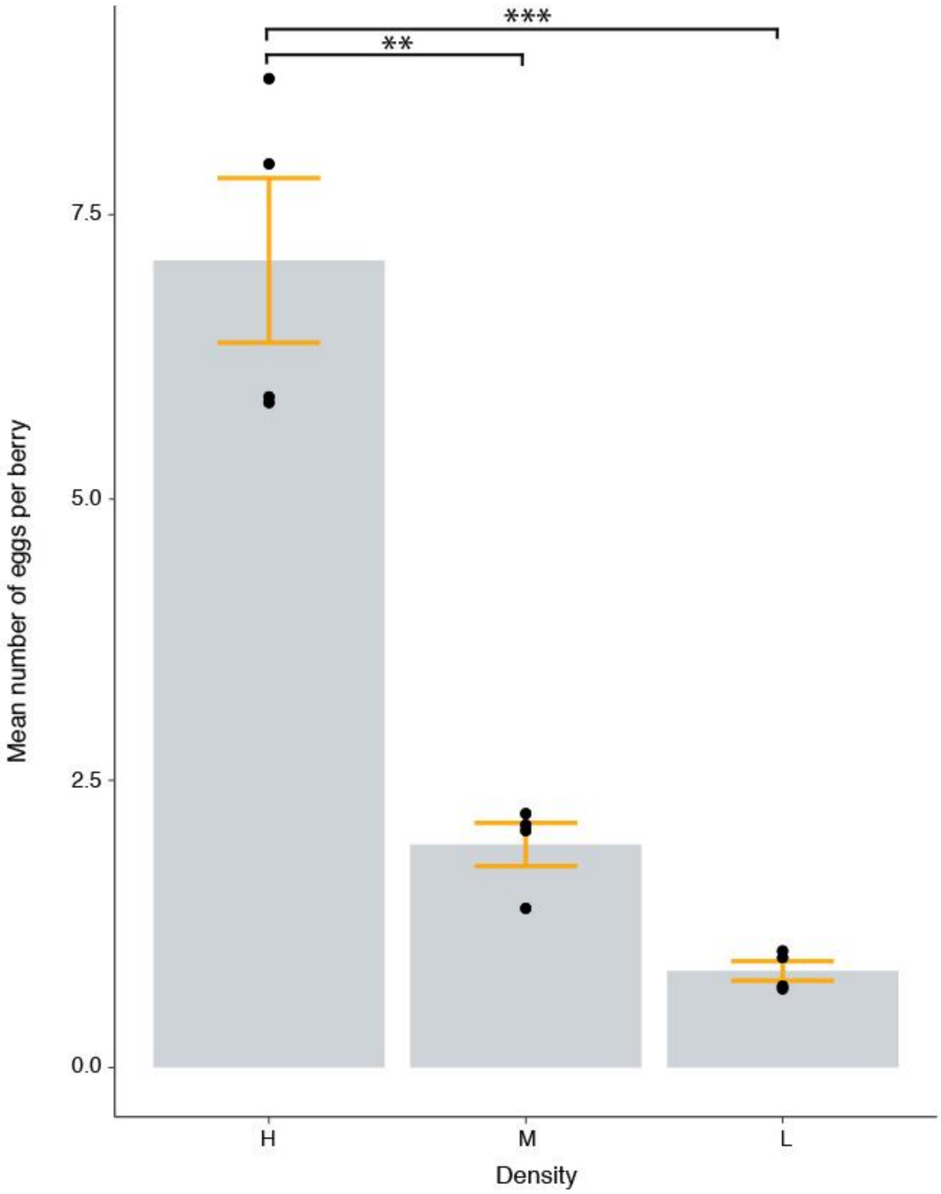

Figure 3-1: Mean number of *D. suzukii* eggs laid per berry for High, Medium, and Low density treatments in Blocks A and B. Number of stars above bars indicate significant differences (Tukey multiple comparison tests; $p < 0.0001$ = ***, $p < 0.001$ = **). Each point represents the mean number of eggs laid per berry for all females pooled together per replicate (n = 4). Error bars represent the standard error around the mean for the four replicates.

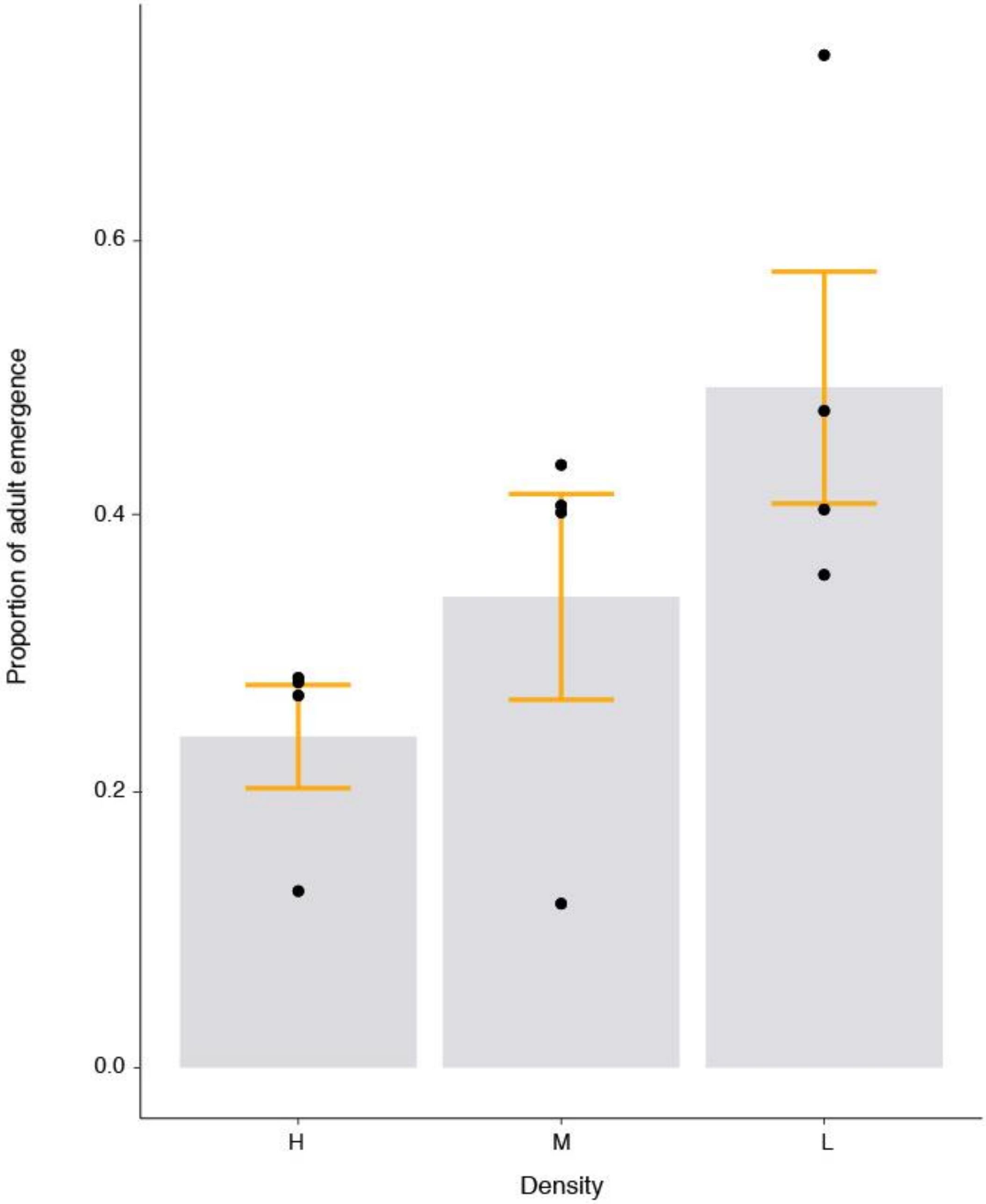

Figure 3-2: Mean proportion of *D. suzukii* emergence from High, Medium, and Low densities for Blocks A and B. Each point represents the mean proportion of emergence for all offspring pooled together in each replicate (n = 4). Error bars represent the standard error around the mean for the four replicates.

Change in Wolbachia frequencies is not driven by variation in competition intensity

In Blocks A and B, 247 and 257 adult flies emerged and were screened for *Wolbachia*, respectively. The proportion of emerging *D. suzukii* offspring ($F_{1,9}$ = 0.19, *p* = 0.67), and the egg density ($F_{1,9}$ = 0.002, *p* = 0.97) was not associated with a change in *Wolbachia* frequencies; however, the Block was associated with changes in *Wolbachia* frequencies ($F_{1,9}$ = 27.85, *p* = 0.00051; Figure 3). Block A offspring had an 18.2% (SE = 5.52%) increase in *Wolbachia* infection rates compared to the initial infection frequency of their parents and was not associated with the proportion of offspring that emerged (Figure 3-3). Block B offspring had a uniform decrease of -20.8% (SE = 3.34%) in *Wolbachia* infection frequency; again, regardless of the proportion of offspring that emerged (Figure 3-3).

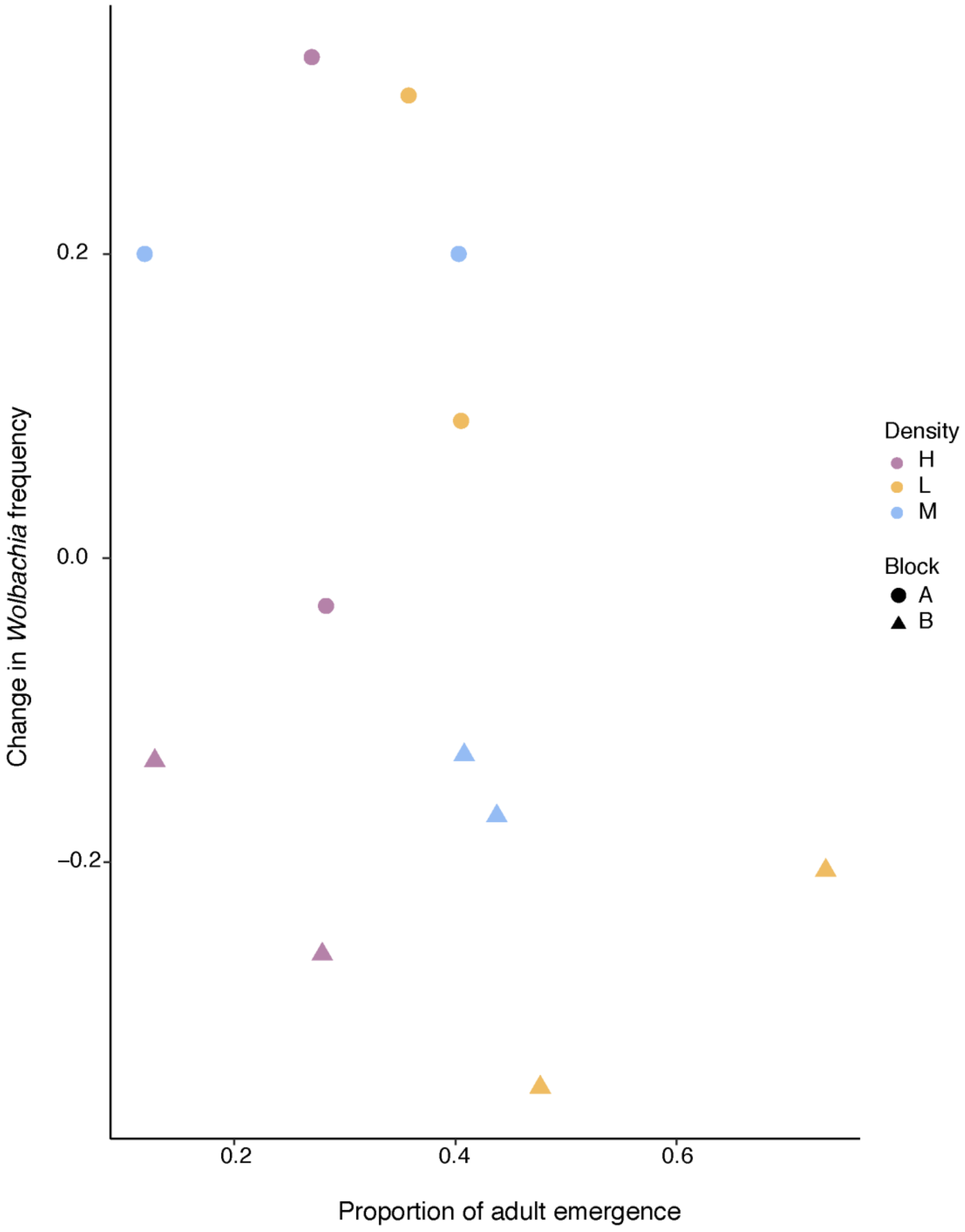

Figure 3-3: Change in *Wolbachia* infection frequency in *D. suzukii* offspring with increasing emergence in Blocks A and B for High, Medium, and Low densities.

Infection status and block impact emergence time

The emergence time of *D. suzukii* offspring was not affected by the proportion of emergent offspring, sex or any interactions between the variables. The main significant effects were reduced to block ($p = 0.00021$), and infection status ($p = 0.031$). The average development time was 18 days with Block B individuals taking 1.17 days longer to develop. Flies infected with *Wolbachia* took 0.65 days longer to develop than uninfected flies (Figure 3-4).

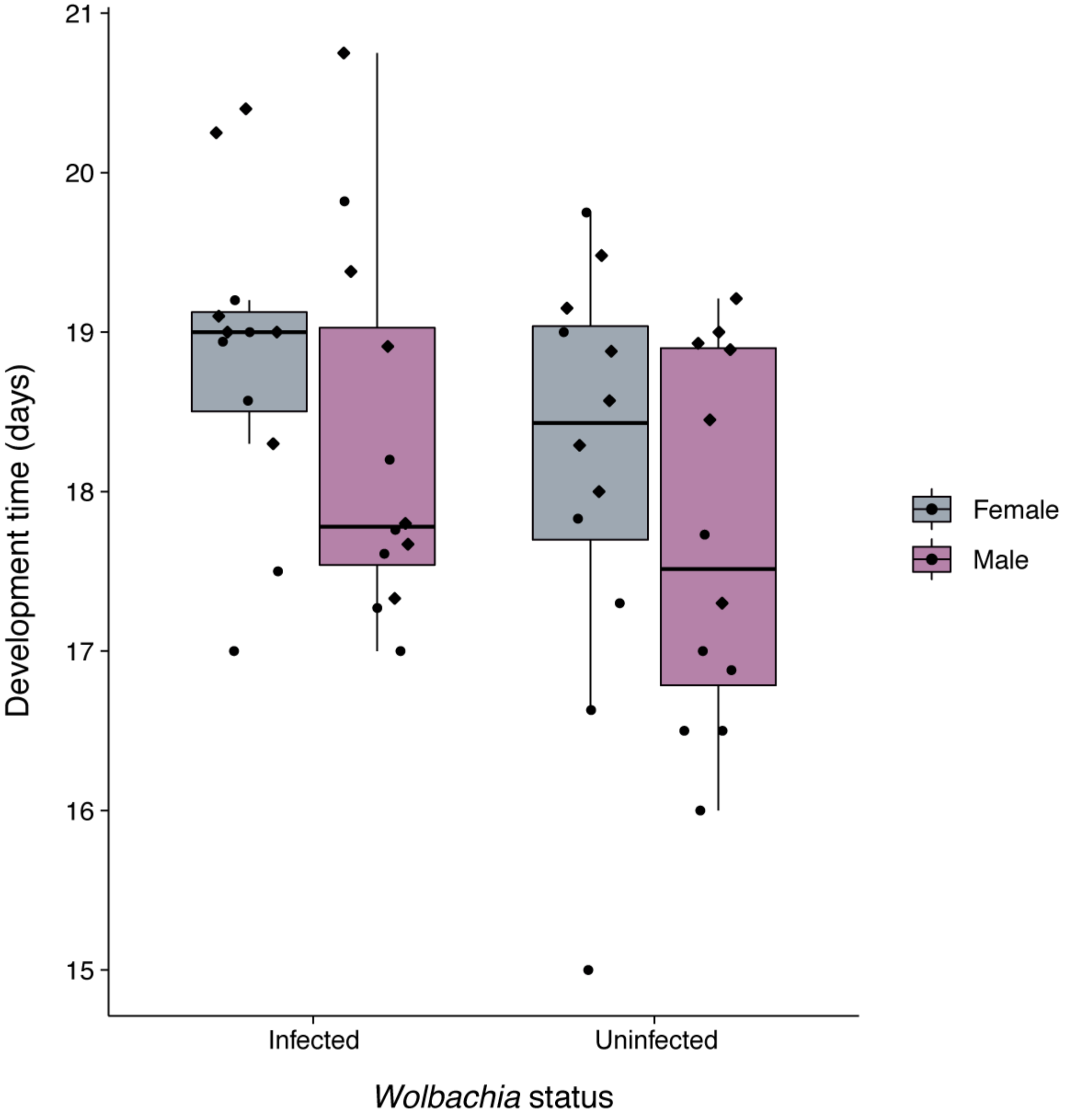

Figure 3-4: Boxplots with overlaid datapoints showing the development time of *D. suzukii* infected and uninfected with *Wolbachia*. Each point represent male and female flies infected and uninfected with *Wolbachia* pooled from Blocks A and B and all density treatments n = 48 (12* 4).

Discussion

I explored the influence of *Wolbachia* on infection frequencies and development time in *D. suzukii* larvae subjected to different crowding conditions. I found that increasing density did not lead to a higher rate of *Wolbachia* infection nor did infected larvae develop faster compared to uninfected individuals. I did find temporal differences between the infection frequency and development time for Blocks A and B, and overall, *Wolbachia* infected larvae developed more slowly compared to uninfected individuals.

I observed significant changes in *Wolbachia* frequency in both blocks in just one generation, but surprisingly, *Wolbachia* frequencies changed in oppose directions for each block. While initial frequencies were similar, *Wolbachia* infection increased by 20% in Block A and decreased by 18% in Block B. These results have some interesting implications. Firstly, *Wolbachia* frequencies are very dynamic and can change substantially in as little as one generation. This has been observed experimentally in *D. melanogaster* and *D. simulans* (Versace *et al.,* 2014; Kriesner *et al.,* 2016), both of which harbour *Wolbachia* strains that do not act as reproductive parasites and are found at moderate frequencies in nature (Hoffmann *et al.,* 1998; Harcombe *et al.,* 2004). Versace et al. (2014) tested the effects of extreme temperatures on *w*Mel frequencies in *D. melanogaster* and found that *Wolbachia* came to complete fixation in as little as 15 generations. Similarly, Kriesner and Hoffmann (2018) found both strains of *Wolbachia* infecting *D. simulans*, *w*Au and *w*Ri, came to near fixation within 10 generations. Fluctuating *Wolbachia* frequencies have also been reported in nature for other non-CI strains of *Wolbachia* (Kriesner *et al.,* 2013; Hamm *et al.,* 2014; Cooper *et al.,* 2017) indicating that external environmental factors can influence changes in infection frequencies.

Secondly, my results also suggest that some conditions encountered by flies in Block A led to an increase in infection frequencies and a decrease in Block B. Some conditions could have led infected females to lay more eggs in Block A and less in Block B compared to uninfected females. There is also the possibility that infected larvae were more successful in making it to adulthood in Block A and less in Block B compared to uninfected individuals. My results indicate that *Wolbachia* infection may confer both a fitness enhancement and cost in some conditions. I do not believe that antibiotic treatment significantly impacted the fitness of flies because if that were the case we would not see increases in the frequency of uninfected flies in one of the blocks. Exactly what situation or set of conditions that can cause both a fitness enhancement and come at a cost is yet to be determined; however, some leading candidates are temperature fluctuations (Versace *et al.,* 2014) and resistance to pathogens (Teixeira *et al.,* 2008; Cattel *et al.,* 2016; Martinez *et al.,* 2017).

My experiment was designed to test the hypothesis that *Wolbachia*-infected *D. suzukii* larvae have higher fitness than their uninfected counterparts, when developing in crowded conditions. Contrary to my initial predictions, I did not see an effect of density on the frequency of *Wolbachia* infections nor on the development time of larvae. Although I did not find an effect of density on *Wolbachia* infection, I am confident that my experimental manipulation resulted in increased density and significantly affected fitness, as only ~20% of the eggs laid in the high-density treatments survived to adulthood, compared to the ~54% in the low-density treatments.

As far as I am aware, there have been very few studies directly testing whether *Wolbachia* is affected by larval density conditions or whether infection affects host competitive ability. What few studies there are did not measure the same life history traits as I did so it is difficult to directly compare my findings to other studies. Despite that, past experimental work has found that density and *Wolbachia* impact host fitness and not in positive ways (Hoffmann *et al.,* 1998; Huigens *et al.,* 2004). These studies found that at high densities, infected individuals were less competitive in acquiring resources (Huigens *et al.,* 2004), and females were smaller (Hoffmann *et al.,* 1998). In comparison, I did not find an effect of density in my experiment in terms of *Wolbachia* frequency or development time, but infected individuals took longer to develop. Interestingly, not all hosts that harbour *Wolbachia* experience negative fitness effects when reared at high densities. Gavotte et al. (2009) and Islam and Dobson (2006) did not find that *Wolbachia* infection impacted life history traits in *Ae. albopictus* at any density but mosquitoes reared at high densities were less viable and took longer to develop. Additionally, the rapid increase in *w*Au infections in *D. simulans* population cages (Kriesner and Hoffmann, 2018) suggests that *Wolbachia*-infected flies may outperform uninfected ones at high densities. These results suggest that condition-dependent effects of *Wolbachia* on host fitness vary between species.

What difference between the two blocks could have driven the strong but opposite differences in *Wolbachia* prevalence? One possibility is temperature, as the outside average mean temperature when Block B was performed was ~3°C cooler than Block A. Temperature has been shown to influence *Wolbachia* titres in its host (Jia *et al.,* 2009; Wiwatanaratanabutr & Kittayapong, 2009; Corbin *et al.,* 2017) and can lead to fitness costs in some species. Some studies have found that at both high (Van Opijnen & Breeuwer, 1999; Ross *et al.,* 2017) and low (Kriesner *et al.,* 2016; Hague *et al.,* 2020) temperatures *Wolbachia* titres are reduced, and that can impact maternal transmission. For example, cool temperatures reduced *w*Yak titres and rate of maternal transmission in *Drosophila yakuba* (Hague *et al.*, 2020). The authors attributed the low infection frequencies observed in wild *D. yakuba* populations to the reduced level of maternal transmission caused by low temperatures in some environments. Similarly, in *D. melanogaster*, *w*Mel infected females experienced a significant reduction in fecundity and the offspring were less viable when the mothers were exposed to cooler temperatures providing a clear fitness advantage to uninfected females (Kriesner *et al.,* 2016). In my experiment, the external temperature in Block B was ~17°C which is below the reported optimal temperature of 22°C for *D. suzukii* (Calabria *et al.,* 2010; Walsh *et al.,* 2011; Tochen *et al.,* 2014). There is the possibility that the low temperature in Block B, paired with the increased development time of *Wolbachia*-infected individuals, was enough for *Wolbachia*-infected individuals to experience increased mortality in Block B. The females in Block B may also have experienced a reduction in *Wolbachia* titres and did not transmit *w*Suz to as many eggs, thus lowering the overall infection frequency. Since *D. suzukii* overwinter as adults and *Wolbachia* can negatively impact host fitness at cooler temperatures (Kriesner *et al.,* 2016), it would be interesting to explore how *Wolbachia* may impact fitness at cool temperatures in *D. suzukii*.

Another potential difference between the two blocks that could explain the changes in *Wolbachia* frequency is the presence or absence of viruses and other microbes. *Wolbachia* in *D. suzukii* has been found to provide protection from some RNA viruses in a laboratory setting (Cattel *et al.,* 2016; Martinez *et al.,* 2017). The presence or absence of viruses could have impacted my results in a couple of ways. Firstly, there could be different viruses at different times. Viruses can be episodic where they are present for some time during a certain part of the year. This has been observed in some viruses infecting honeybees (Bailey *et al.,* 1981; Runckel *et al.,* 2011; Tentcheva *et al.,* 2004) and mosquito-vectored viruses (Angel & Joshi, 2008; Novakova *et al.,* 2017). In my experiment, Block A and Block B were separated by nearly one month and it is possible that in Block A there were some viruses present that perhaps were not in

Block B and those viruses were more pathogenic but susceptible to *Wolbachia* resulting in infected individuals having an advantage. Although not much is known about viral transmission in drosophilids, there is the possibility that there were different viruses present on the berries since the blueberries were picked at different times. Alternatively, there also could be an interaction between viruses and temperature, or some other stressor. Warm temperatures can lead to the proliferation of pathogens, including some viruses (Ciota & Kramer, 2013; Reisen, 2013), and if *w*Suz does provide protection to viruses, larvae in Block A may have been exposed to increased levels of viruses and been able to outcompete uninfected larvae. To the best of my knowledge, no studies have examined *Wolbachia*-mediated protection from viruses at different temperatures and it would be interesting to examine if the strength or ability of *w*Suz to protect its host form viruses was influenced by temperature change.

Through my experiment, I was able to rule out density as a factor that may influence the fitness of *D. suzukii* harbouring *w*Suz. My results did however show that there are certain conditions that can lead to an increase in *Wolbachia* frequency. This complements the findings by Kriesner and Hoffmann (2018) and the theory that wSuz provides a context-dependent fitness benefit to *D. suzukii* (Hamm *et al.*, 2014). Other studies have reported theoretical fitness increases in hosts harbouring *w*Suz (Hamm *et al.*, 2014) and other strains of non-CI *Wolbachia* (Kriesner *et al.*, 2016; Hague *et al.*, 2020) which would explain their persistence in nature, but what conditions cause the fitness enhancement remain elusive. Given the complex nature of *Wolbachia*-host interactions, it is likely that there are a multitude of factors influencing the cost and benefit of harbouring *Wolbachia*, including abiotic conditions. Understanding what influences the determinants of *Wolbachia* spread – imperfect maternal transmission, CI, and fitness effects – is necessary to explain the prevalence and spread of *Wolbachia* in nature.

Chapter 3 - References

Angel, B., & Joshi, V. (2008). Distribution and seasonality of vertically transmitted dengue viruses in *Aedes* mosquitoes in arid and semi-arid areas of Rajasthan, India. *Journal of Vector Borne Diseases, 45*(1), 56–59.

Asplen, M. K., Anfora, G., Biondi, A., Choi, D. S., Chu, D., Daane, K. M., Gibert, P., Gutierrez, A. P., Hoelmer, K. A., Hutchison, W. D., Isaacs, R., Jiang, Z. L., Kárpáti, Z., Kimura, M. T., Pascual, M., Philips, C. R., Plantamp, C., Ponti, L., Vétek, G., … Desneux, N. (2015). Invasion biology of spotted wing *Drosophila* (*Drosophila suzukii*): a global perspective and future priorities. *Journal of Pest Science, 88,* 469–494. https://doi.org/10.1007/s10340-015-0681-z

Ballard, J. W. O., & Melvin, R. G. (2007). Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in *Drosophila*. *Insect Molecular Biology, 16*(6), 799-802.

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*(1), 1 – 48. https://doi.org/10.18637/jss.v067.i01.

Beckmann, J. F., Sharma, G. D., Mendez, L., Chen, H., & Hochstrasser, M. (2019). The *Wolbachia* cytoplasmic incompatibility enzyme CIDB targets nuclear import and protamine-histone exchange factors. *eLife, 8,* e50026. https://doi.org/10.7554/eLife.50026

Bouchon, D., Rigaud, T., & Juchault, P. (1998). Evidence for widespread *Wolbachia* infection in isopod crustaceans: Molecular identification and host feminization. *Proceedings of the Royal Society B: Biological Sciences, 265*(1401), 1081–1090. https://doi.org/10.1098/rspb.1998.0402

Calabria, G., Máca, J., Bächli, G., Serra, L., & Pascual, M. (2010). First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. *Journal of Applied Entomology, 136*(1-2), 139–147. https://doi.org/10.1111/j.1439-0418.2010.01583.x

Cattel, J., Martinez, J., Jiggins, F., Mouton, L., & Gibert, P. (2016). *Wolbachia*-mediated protection against viruses in the invasive pest *Drosophila suzukii*. *Insect Molecular Biology,* *25*(5), 595–603. https://doi.org/10.1111/imb.12245

Cattel, J., Kaur, R., Gibert, P., & Martinez, J. (2016). *Wolbachia* in European populations of the invasive pest *Drosophila suzukii*: regional variation in infection frequencies. *PLoS ONE*, *11*(1), e0147766. https://doi.org/10.1371/journal.pone.0147766

Ciota, A. T., & Kramer, L. D. (2013). Vector-virus interactions and transmission dynamics of West Nile virus. *Viruses*, *5*(12), 3021–3047. https://doi.org/10.3390/v5123021

Cooper, B. S., Ginsberg, P. S., Turelli, M., & Matute, D. R. (2017). *Wolbachia* in the *Drosophila yakuba* complex: Pervasive frequency variation and weak cytoplasmic incompatibility, but no apparent effect on reproductive isolation. *Genetics*, *205*(1), 333–351. https://doi.org/10.1534/genetics.116.196238

Corbin, C., Heyworth, E. R., Ferrari, J., & Hurst, G. D. D. (2017). Heritable symbionts in a world of varying temperature. *Heredity*, *118*, 10–20. https://doi.org/10.1038/hdy.2016.71

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5), 294 - 299.

Fox, J., & Weisberg, S. (2019). An {R} Companion to Applied Regression. Third Edition. Thousand Oaks CA: Sage. URL:https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Gavotte, L., Mercer, D. R., Vandyke, R., Mains, J. W., & Dobson, S. L. (2009). *Wolbachia* infection and resource competition effects on immature *Aedes albopictus* (Diptera: Culicidae). *Journal of Medical Entomology*, *46*(3), 451–459. https://doi.org/10.1603/033.046.0306

Gill, A. C., Darby, A. C., & Makepeace, B. L. (2014). Iron necessity: The secret of *Wolbachia*'s success? *PLoS Neglected Tropical Diseases*, *8*(10), e3224. https://doi.org/10.1371/journal.pntd.0003224

Government of British Columbia. (2020). *Spotted wing Drosophila (fruit fly) pest alert.* https://www2.gov.bc.ca/gov/content/industry/agriculture-seafood/animals-and-crops/plant-health/insects-and-plant-diseases/tree-fruits/spotted-wing-drosophila

Hague, M. T. J., Caldwell, C. N., & Cooper, B. S. (2020). Pervasive effects of *Wolbachia* on host temperature preference. *MBio*, *11*(5), e01768-20. https://doi.org/10.1128/mBio.01768-20

Hague, M. T. J., Mavengere, H., Matute, D. R., & Cooper, B. S. (2020). Environmental and genetic contributions to imperfect *w*Mel-like *Wolbachia* transmission and frequency variation. *Genetics, 215*(4), 1117–1132. https://doi.org/10.1534/genetics.120.303330

Hamm, C. A., Begun, D. J., Vo, A., Smith, C. C. R., Saelao, P., Shaver, A. O., Jaenike, J., & Turelli, M. (2014). *Wolbachia* do not live by reproductive manipulation alone: infection polymorphism in *Drosophila suzukii* and *D. subpulchrella*. *Molecular Ecology, 23*(19), 4871–4885. https://doi.org/10.1111/mec.12901

Harcombe, W., & Hoffmann, A. A. (2004). *Wolbachia* effects in *Drosophila melanogaster*: In search of fitness benefits. *Journal of Invertebrate Pathology*, *87*(1), 45–50. https://doi.org/10.1016/j.jip.2004.07.003

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

Hoffmann, A. A., Hercus, M., & Dagher, H. (1998). Population dynamics of the *Wolbachia* infection causing cytoplasmic incompatibility in *Drosophila melanogaster*. *Genetics*, *148*(1), 221–231. https://doi.org/10.1093/genetics/148.1.221

Hoffmann, A. A., & Turelli, M. (1997). Cytoplasmic incompatibility in insects, pp. 41–80 in *Influential Passengers: Inherited Microorganisms and Invertebrate Reproduction*, edited by S. L. O'Neill, A. A. Hoffmann and J. H. Werren. Oxford University Press, Oxford University Press: New York.

Huigens, M. E., Hohmann, C. L., Luck, R. F., Gort, G., & Stouthamer, R. (2004). Reduced competitive ability due to *Wolbachia* infection in the parasitoid wasp *Trichogramma kaykai*. *Entomologia Experimentalis et Applicata*, *110*(2), 115–123. https://doi.org/10.1111/j.0013-8703.2004.00126.x

Islam, M. S., & Dobson, S. L. (2006). *Wolbachia* effects on *Aedes albopictus* (Diptera: Culicidae) immature survivorship and development. *Journal of Medical Entomology*, *43*(4), 689–695. https://doi.org/10.1603/0022-2585(2006)43[689:weoaad]2.0.co;2

Jaenike, J. (2012). Population genetics of beneficial heritable symbionts. *Trends in Ecology and Evolution,* 27(4), 226–232. https://doi.org/10.1016/j.tree.2011.10.005

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-Padilla, Y., Esan, E. O., Floate, K. D., Sinclair, B. J. (2020) Persistence of diet effects on the microbiota of *Drosophila suzukii* (Diptera: Drosophilidae). *The Canadian Entomologist, 152,* 516 – 531. https://doi.org/10.4039/tce.2020.37

Jiggins, F. M., Hurst, G. D. D., Schulenburg, J. H. G. V. D., & Majerus, M. E. N. (2001). Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon. Heredity, 86,* 161–166. https://doi.org/10.1046/j.1365-2540.2001.00804.x

Kriesner, P., Conner, W. R., Weeks, A. R., Turelli, M., & Hoffmann, A.A. (2016). Persistence of a *Wolbachia* infection frequency cline in *Drosophila melanogaster* and the possible role of reproductive dormancy. *Evolution,70*(5), 979–997. https://doi.org/10.1111/evo.12923.Persistence

Kriesner, P., & Hoffmann, A. A. (2018). Rapid spread of a *Wolbachia* infection that does not affect host reproduction in *Drosophila simulans* cage populations. *Evolution, 72*(7), 1475–1487. https://doi.org/10.1111/evo.13506

Kriesner, P., Hoffmann, A. A., Lee, S. F., Turelli, M., & Weeks, A. R. (2013). Rapid sequential spread of two *Wolbachia* variants in *Drosophila simulans*. *PLoS Pathogens, 9*(9), e1003607. https://doi.org/10.1371/journal.ppat.1003607

Lenth, R. V. (2020). emmeans: Estimated marginal means, aka least-squares means. https://CRAN.R-project.org/package=emmeans.

Li, Y. Y., Floate, K. D., Fields, P. G., Pang, B. P. (2014). Review of treatment methods to remove *Wolbachia* bacteria from arthropods. *Symbiosis, 62,* 1–5. https://doi.org/10.1007/s13199-014-0267-1

Martinez, J., Tolosana, I., Ok, S., Smith, S., Snoeck, K., Day, J. P., & Jiggins, F. M. (2017).

Symbiont strain is the main determinant of variation in *Wolbachia*-mediated protection against viruses across *Drosophila* species. *Molecular Ecology*, *26*(15), 4072–4084. https://doi.org/10.1111/mec.14164

Morimoto, J., Pizzari, T., & Wigby, S. (2016). Developmental environment effects on sexual selection in male and female *Drosophila melanogaster*. *PLoS ONE*, *11*(5), e0154468. https://doi.org/10.1371/journal.pone.0154468

Novakova, E., Woodhams, D. C., Rodríguez-Ruano, S. M., Brucker, R. M., Leff, J. W., Maharaj, A., Amir, A., Knight, R., & Scott, J. (2017). Mosquito microbiome dynamics, a background for prevalence and seasonality of West Nile virus. *Frontiers in Microbiology*, *8*, 526. https://doi.org/10.3389/fmicb.2017.00526

Peters, T. M., & Barbosa, P. (1977). Influence of population density on size, fecundity, and developmental rate of insects in Culture. *Annual Review of Entomology*, *22*, 431–450.

Reisen, W. K. (2013). Ecology of West Nile virus in North America. *Viruses*, *5*(9), 2079–2105. https://doi.org/10.3390/v5092079

Ross, P. A., Wiwatanaratanabutr, I., Axford, J. K., White, V. L., Endersby-Harshman, N. M., & Hoffmann, A. A. (2017). *Wolbachia* infections in *Aedes aegypti* differ markedly in their response to cyclical heat stress. *PLoS Pathogens*, *13*(1), e1006006. https://doi.org/10.1371/journal.ppat.1006006

Roy, S. S., Aditya, G., & Ghosh, S. (2017). Impact of density and sex-dependent larval competition on selected life history traits of *Drosophila melanogaster* (Diptera: Drosophilidae). *Canadian Entomologist*, *150*(1), 87 – 99. . https://doi.org/10.4039/tce.2017.56

Runckel, C., Flenniken, M. L., Engel, J. C., Ruby, J. G., Ganem, D., Andino, R., & DeRisi, J. L. (2011). Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. *PLoS ONE*, *6*(6), e20656. https://doi.org/10.1371/journal.pone.0020656

Stouthamer, R., Luck, R. F., & Hamilton, W. D. (1990). Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera: Trichogrammatidae) to revert to sex. *Proceedings of the*

National Academy of Sciences of the United States of America, *87*(7), 2424–2427. https://doi.org/10.1073/pnas.87.7.2424

Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, *6*(12), e1000002. https://doi.org/10.1371/journal.pbio.1000002

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M. E., & Bergoin, M. (2004). Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and Varroa destructor mite populations in France. *Applied and Environmental Microbiology*, *70*(12), 7185–7191. https://doi.org/10.1128/AEM.70.12.7185-7191.2004

Tochen, S., Dalton, D. T., Wiman, N., Hamm, C., Shearer, P. W., & Walton, V. M. (2014). Temperature-related development and population parameters for *Drosophila suzukii* (Diptera: Drosophilidae) on cherry and blueberry. *Environmental Entomology*, *43*(2), 501–510. https://doi.org/10.1603/EN13200

Turelli, M., Hoffmann, A. A. (1995). Cytoplasmic incompatibility in *Drosophila simulans*: dynamics and parameter estimates from natural populations. *Genetics*, 1*40*(4),1319 - 1338.

Unckless, R. L., & Jaenike, J. (2012). Maintenance of a male-killing *Wolbachia* in *Drosophila innubila* by male-killing dependent and male-killing independent mechanisms. *Evolution*, *66*(3), 678–689. https://doi.org/10.1111/j.1558-5646.2011.01485.x

Vacchini, V., Gonella, E., Crotti, E., Prosdocimi, E. M., Mazzetto, F., Chouaia, B., Callegari, M., Mapelli, F., Mandrioli, M., Alma, A, Daffonchio, D. (2017). Bacterial diversity shift determined by different diets in the gut of spotted wing *Drosophila suzukii* is primarily reflected on acetic acid bacteria. *Environmental Microbiology Reports*, *9*(2), 91 – 103. https://doi.org/10.1111/1758-2229.12505

Van Opijnen, T., & Breeuwer, J. A. J. (1999). High temperatures eliminate *Wolbachia*, a cytoplasmic incompatibility inducing endosymbiont, from the two-spotted spider mite. *Experimental and Applied Acarology*, *23*(11), 871–881. https://doi.org/10.1023/A:1006363604916

Versace, E., Nolte, V., Pandey, R. V., Tobler, R., & Schlötterer, C. (2014). Experimental evolution reveals habitat-specific fitness dynamics among *Wolbachia* clades in *Drosophila melanogaster*. *Molecular Ecology*, *23*(4), 802–814. https://doi.org/10.1111/mec.12643

Walsh, D. B., Bolda, M. P., Goodhue, R. E., Dreves, A. J., Lee, J., Bruck, D. J., Walton, V. M., O'Neal, S. D., & Zalom, F. G. (2011). *Drosophila suzukii* (Diptera: Drosophilidae): Invasive pest of ripening soft fruit expanding its geographic range and damage potential. *Journal of Integrated Pest Management*, *2*(1), G1 – G7. https://doi.org/10.1603/IPM10010

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*, 741–751. https://doi.org/10.1038/nrmicro1969

Wilches, D., Coghlin, P., Floate, K. (2021). Next generation sequencing, insect microbiomes, and the confounding effect of *Wolbachia*: a case study using *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). *Canadian Journal of Zoology.* https://doi.org/10.1139/cjz-2020-0260.

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

Yamada, R., Floate, K. D., Riegler, M., O'Neill, S. L. (2007). Male development time influences the strength of *Wolbachia*-induced cytoplasmic incompatibility expression in *Drosophila melanogaster*. *Genetics*, *177*(2), 801–808. https://doi.org/10.1534/genetics.106.068486

Zhou, W., Rousset, F., and O'Neill, S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proceedings of the Royal Society B: Biological Sciences, 265*(1395), 509–515. doi:10.1098/rspb.1998.0324.

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