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Wolbachia-Host Interactions and the Implications to Insect Conservation and Management

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Wolbachia- Host Interactions and the Implications to Insect

Conservation and Management

by

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A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Environmental Sciences and Resources

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ABSTRACT

Parasitic reproductive endosymbionts are emerging as formidable threats to insect biodiversity. *Wolbachia* are prevalent maternally inherited intra-cellular bacteria found in >50% of arthropod species. These symbiotic bacteria interact with their hosts in diverse ways, most often they alter host reproduction causing four conditions that all selectively favor infected females: feminization, male killing, parthenogenesis, and cytoplasmic incompatibility (CI). Furthermore, depending on strain-type and host genetic background, *Wolbachia* are known to affect insect behavior, expand or shift host thermal tolerance ranges, and confer anti-viral protection to their hosts. Because *Wolbachia* both reside in and are transmitted with host cell cytoplasm, mitochondria and other cytoplasmically inherited genetic elements become linked with the bacteria. Thus, by enhancing their own transmission, *Wolbachia*-induced phenotypes can lead to mitochondrial selective sweeps, which may have profound impacts on vulnerable and small insect populations. Elucidating the extent to which endosymbionts influence biological and ecological functions is pivotal to making management decisions regarding imperiled insect species.

My dissertation investigates biological and ecological impacts of host-endosymbiont interactions by examining *Wolbachia* infections in three different host systems. First, I used the federally threatened butterfly species *Speyeria zerene hippolyta* to determine whether the general reproductive success of local populations was affected by the introduction of CI-inducing *Wolbachia*-infected butterflies through implemented species recovery programs. Next, by characterizing the *Wolbachia* infections of parasitoids associated with the *Eurema* butterfly clade, I analyzed whether host-parasitoid interactions provide a path for interspecies horizontal transmission. Finally, I conducted a

laboratory experiment using an isogenic *Drosophila melanogaster* line to determine whether *Wolbachia* influence host temperature preference. Together, my research examines how the individual level effects of host-endosymbiont interactions can expand into populations, have broader impacts on insect communities, and potentially impede the conservation and management of insects in nature.

In chapter one, I screened *S. z. hippolyta* samples from three extant populations for *Wolbachia* infection. To examine the impacts of *Wolbachia* on small populations, I analyzed and compared infected and uninfected *S. z. hippolyta* reproductive data and showed that, in a population composed of infected and uninfected *S. z. hippolyta,* uninfected butterflies had reduced reproductive success (GLMM $z = -8.067$, $P < 0.0001$). I then developed a single-population demographic theoretical model using these same reproductive data to simulate and analyze different potential dynamics of small populations resulting from population supplementation with uninfected, CI-*Wolbachia* infected, or combined uninfected and infected butterflies. Analysis of model simulations revealed that supplementation with CI-inducing butterflies significantly suppressed host-population size (ANOVA $F_{5,593} = 3349$, $P<0.0001$). A population with 300 uninfected individuals can be reduced by 88.7% from a single introduction of 50 *Wolbachia*-infected individuals (Tukey's post-hoc test $P < 0.0001$). In addition, supplementation by multiple releases using a combination of 50 infected and 300 uninfected butterflies has a less severe suppression effect, reducing the population by 75.8%, but the reduction occurs 42.6% faster than with the single release of 50 *Wolbachia*-infected butterflies (Tukey's post-doc test $P < 0.0001$).

Parasitoid-host interactions have emerged as probable ecological relationships to

facilitate horizontal transmission of *Wolbachia.* In chapter two, I addressed horizontal transmission using *Eurema* butterflies and their associated parasitoids. From four locations in Northern Queensland, Australia, I collected a total of 404 *Eurema hecabe* butterfly larvae. Twenty-three parasitoids emerged from the larvae of which 21 were Diptera and two were Hymenoptera. I amplified *COI* loci fragments from each parasitoid for BLAST query searches and found that 20 individual Diptera parasitoids matched to the genus *Exorista* and one to the genus *Senometopia.* One of the Hymenoptera parasitoids matched to the genus *Microoplitis* and the other to the genus *Cotesia.* To characterize *Wolbachia* infections, I used *Wolbachia* Multi Locus Sequencing Technique (MLST) and discovered that all 20 *Exorista* parasitoids were infected with an identical *Wolbachia* strain (ST-41), which is the same strain infecting their *Eurema hecabe* butterfly hosts. Although, further experiments are necessary to definitively determine that ST-41 *Wolbachia* are incorporated into germline cells of the parasitoids, this is the first study to provide ecological evidence for inter-ordinal *Wolbachia* transmission between Lepidoptera and Diptera. Furthermore, this discovery exposes the risk of population augmentation programs that move insects, potentially facilitating the spread of *Wolbachia* between species within a community through the accidental introduction of new *Wolbachia*-infected parasitoids.

Finally, both *Wolbachia* and their insect hosts are temperature sensitive organisms. *Wolbachia*'s replication behavior in their hosts is positively-temperature dependent, while environmental variation can have profound effects on insect's immune function, fitness, and fecundity. In chapter three, I conducted a laboratory experiment using a thermal gradient choice assay and an isogenic *Drosophila melanogaster* line with

four different *Wolbachia* infection statuses – uninfected, *w*Mel, *w*MelCS, and *w*MelPop - to assess whether a relationship existed between *Wolbachia* infection and host temperature preference. Results from my laboratory experiment revealed that *Wolbachia*-infected flies preferred cooler temperatures compared to uninfected flies. Moreover, *D. melanogaster* temperature preferences varied depending on the *Wolbachia* strain variant with which they were infected; flies infected with the *w*Mel strain had temperature preferences 2°C cooler compared to uninfected flies; flies infected with either *w*MelCS or *w*MelPop strains had preferred temperatures 8°C cooler compared to uninfected flies. *Wolbachia*-associated temperature preference variation within a species can lead to conspecifics occupying different microclimates, genetically adapting to different sets of specific environmental conditions, and may eventually result in ecological and reproductive isolation. While, reproduction isolation is recognized as one of the first stages in speciation, in small populations of endangered and threatened species, the inability to reproduce between conspecifics can drive species to extirpation or extinction.

Collectively, the three chapters of my dissertation set precedent for future integration of host-endosymbiont research prior to implementing population supplementation or translocation programs for the conservation of imperiled insects.

Dedicated to my mom and dad, my wife, and

to the loving memories of

Mike Barline and Victor Andres Vargas.

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INTRODUCTION

Ecosystems are composed of complex ecological relationships among and between species, populations, and communities (Levin 1998; Christian et al. 2015). The nature of these relationships is primarily determined by how organisms adapt on evolutionary timescales to environmental pressures (Levin 1998; Margulis 2004; Moran 2006; Moran et al. 2008). Symbiosis is arguably the most important inter-organismal interaction from which all major life forms have emerged (Margulis 2004). In its simplest form, a symbiotic relationship is defined as different species living together in long-term close associations (de Bary 1879). The constituents of symbiotic relationships have varying effects depending on the association they have formed: mutualism, parasitism, or commensalism (Moran 2006). These associations can be obligate, where the survival of one or both organisms depends on the survival of the other, or facultative, when neither organism relies on the other for their own survival (Moran et al. 2008; Oliver et al. 2010).

In the past several decades, and with the advent of relatively affordable sophisticated molecular biology techniques, ecologists and evolutionary biologists have increasingly appreciated the incredible bacterial diversity on our planet and the critical roles that microorganisms play in biological and ecological processes (Hurst & Werren 2001; Bordenstein 2003; Telschow et al. 2007; Siozios et al. 2008; Brucker & Bordenstein 2012; Chrostek et al. 2013; Kern et al. 2015; van Nouhuys et al. 2016). Some of the most interesting and complex symbiotic relationships discovered are between endosymbiotic bacteria and insects (Moran 2006; Mouton et al. 2007; Werren et al. 2008). Endosymbiotic bacteria live inside the cells of insect hosts and are passed to offspring in the cytoplasm of maternal germ line cells (Werren 1997; Margulis 2004).

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Wolbachia, one of many insect endosymbionts, are the most prevalent and well-studied group of endosymbiotic bacteria. Endosymbionts belonging to the genus *Wolbachia* receive significant attention because they are ubiquitous - estimated to infect 53% of terrestrial arthropods (Zug & Hammerstein 2012; Weinert et al. 2015) and 52% of aquatic insects (Sazama et al. 2017) - and have a wide range of phenotypic effects on their hosts including altering their hosts' reproduction and conferring anti-viral protection against insect-vector borne diseases (Werren 1997; Dobson et al. 2002; Teixeira et al. 2008; Hedges et al. 2008; Werren et al. 2008; Werren 2011; Chrostek et al. 2013; Zug & Hammerstein 2014; Kern et al. 2015). These bacteria are estimated to have invaded arthropods approximately 200 million y.a. (Gerth & Bleidorn 2016). Differing distinctly in their host distribution and biology, the primary evolutionary *Wolbachia* lineages are designated into 16 "supergroups" labeled A-H, except for G (Comandatore et al. 2015).

Parasitic strains of *Wolbachia* have developed four ways to manipulate their host's reproduction that selectively favor infected females, ensuring their intergenerational transmission and persistence in populations (Werren et al. 2008). Three of these reproductive manipulations, male killing, feminization, and parthenogenesis, selectively favor infected females over males and result in female-biased sex ratio distortions in host populations (Werren et al. 2008). The fourth phenotype, cytoplasmic incompatibility (CI), induces cytoplasmic factors in host gamete cells that cause embryonic death when an egg from an uninfected female is fertilized by a sperm from an infected male (Werren et al. 2008). Unidirectional CI occurs when a host population is infected with a single *Wolbachia* CI-inducing strain. Several insect taxa including parasitic wasps (Breeuwer et al. 1992), fruit flies (Rousset & Solignac 1995), leafcutter

ants (Van Borm et al. 2001), and bruchid beetles (Kondo et al. 2002) are known to be infected with two CI-inducing *Wolbachia* strains. Bidirectional CI occurs when a doubly infected egg is fertilized by a sperm from a male infected with a different and incompatible *Wolbachia* strain (Werren et al. 2008). Whether unidirectional or bidirectional, CI results in decreased fitness of uninfected females compared to infected females (Werren et al. 2008). Furthermore, because *Wolbachia* are transmitted in the cytoplasm of their host's cells, reproductive phenotypes that selectively favor *Wolbachia*-infected females have the consequence of indirectly selecting for other genetic elements sharing host cytoplasm, most notably mitochondria. Hence, mitochondria "hitch-hike" and spread with *Wolbachia*, resulting in mtDNA linkage disequilibrium with the *Wolbachia* strain (Turelli et al. 1992). Indirect selection and linkage disequilibrium can result in a selective sweep of the *Wolbachia* associated mtDNA haplotypes and reduced mtDNA diversity in host populations (Turelli et al. 1992; Hurst & Jiggins 2000; Jiggins 2003; Hurst & Jiggins 2005). Like other heritable selfish genetic elements, *Wolbachia* can impact genetic diversity of their host populations in ways that may lead to extinction or trigger the beginning stages of speciation (Orgel & Crick 1980; Hatcher 2000; Hurst & Werren 2001; Werren 2011).

Genetic diversity, including mtDNA haplotype diversity, is important in small populations that experience geographic isolation from source populations and restricted gene flow, particularly populations of imperiled insect species (Moritz 1994; Crandall et al. 2000; DeSalle & Amato 2004). Recent studies that examined *Wolbachia* prevalence in butterflies and moths estimated that up to 80% of all butterflies and moths (Ahmed et al. 2015) and 19 of 22 (86%) butterfly species of conservation concern are infected with

Wolbachia (Hamm et al. 2014). Emerging diseases are universally acknowledged as a serious threat to biodiversity, exceptionally so for vulnerable species (Daszak et al. 2000). Concerns were first raised about *Wolbachia* in endangered and threatened butterflies in regard to the endangered *Lycaeides melissa samuelis* and the possibility that future population supplementation efforts could introduce a novel CI-inducing *Wolbachia* strain from an infected donor population into an uninfected recipient population (Nice et al. 2009).

Imperiled butterfly conservation efforts that include population supplementation are frequently employed as a method to stimulate population growth while addressing genetic diversity (Crone et al. 2007). However, inter-population transfer of conspecifics can remarkably increase risk of novel disease emergence, which can devastate small populations (Brower 1995; Daszak, 2001; Crone et al. 2007; Altizer & de Roode 2010). Certainly, these programs employ disease-screening protocols prior to releasing individuals into new areas, but *Wolbachia* could easily evade detection as they do not produce visible morphological aberrations to their hosts. Moreover, it may take several generations for the effects of a newly introduced *Wolbachia* infection to become apparent in a population. Male-eliminating phenotypes are recognizable by distorted female to male sex ratios in populations (Hurst & Jiggins 2000; Jiggins 2003; Duplouy et al. 2010). In contrast, cytoplasmic incompatibility is more difficult to detect and, in large populations where only a few infected individuals are introduced, *Wolbachia* could potentially remain unnoticed. However, in small populations CI can cause population suppression due to mating incompatibilities between conspecifics (Zabalou et al. 2004; Perlman et al. 2008; Hancock et al. 2011). This suppression effect of *Wolbachia* strains

that induce CI was first identified as potential biological control agents by Laven (1967) to suppress pest insect populations. Subsequent research showed that when male hosts were artificially infected with CI-inducing *Wolbachia* they became effectively "sterile" because they did not produce viable offspring when they mated with uninfected females (Laven 1967; Dobson et al. 2002; Zabalou et al. 2004; Bourtzis 2008). Theoretical models predict that, depending on the size of a population and the number of *Wolbachia*-infected individuals released, population suppression can be successful after only a single exposure to a novel CI-inducing *Wolbachia* strain (Dobson et al. 2002; Gebiola et al. 2016).

Although *Wolbachia* are primarily vertically transmitted, their prevalence among insect species suggests they may occasionally transfer horizontally between individuals and diverged taxa (Riegler et al. 2005; Schuler et al. 2013; Morrow et al. 2014; Ahmed et al. 2016; Schuler et al. 2016). The exact mechanisms responsible for horizontal transmission have not yet been discovered; however, research indicates that close ecological interactions appear to be fundamental to interspecies transfer, including host-parasitoid interactions and shared resources (Heath et al. 1999; Huigens et al. 2004; Morrow et al. 2014; Raychoudhury et al. 2014; Ahmed et al. 2015a; Li et al. 2016). The opportunity for interspecies *Wolbachia* transmission is presented when insect parasitoid larvae develop on or within the body of their arthropod hosts (Maure et al. 2014). The most likely direction of *Wolbachia* transmission is from parasitoid-host to the parasitoid because parasitoids most often kill their hosts. However, some hosts do survive and even reproduce following the emergence of a parasitoid (DeVries 1984; Shaw & Huddleston 1991). *Wolbachia* could possibly be transferred from parasitoid to their host if the host

survives emergence of a parasitoid and reproduces. Population supplementation and translocation programs likely present more opportunities for species to species *Wolbachia* transfer than would otherwise be possible, including transfer to vulnerable insect species (Snyder et al. 1996; Daszak et al. 2001) as introduced individuals may carry *Wolbachia* that will later transfer into and affect a new host's population dynamics.

The effects of *Wolbachia* on insect populations expand beyond theoretical bottlenecking via reproductive manipulations and may also include strong effects due to host-endosymbiont conflicts. *Wolbachia,* like their insect hosts, are also extremely sensitive to temperature and have positive temperature-dependent replication behavior (Mouton et al. 2006; Mouton et al. 2007; Chrostek et al. 2013; Strunov et al. 2013a). Overlapping host and symbiont thermal tolerance ranges may be narrow and independent organismal physiological requirements may disagree (Mouton et al. 2007). Several scenarios could emerge from host-symbiont temperature preference conflicts: 1) uninfected and infected conspecifics might experience genetic differentiation as they adapt to different sets of specific environmental conditions (Martin & Huey 2008; Hoffmann & Sgrò 2011; Huey et al. 2012; Rajpurohit & Schmidt 2016), 2) over- or under-replication of *Wolbachia* under different temperatures could induce various costs or benefits to the host (Chrostek et al. 2013), or 3) *Wolbachia* could be selected against and lost from the host population as observed by Chrostek et al. (2013). Theory predicts that with global climate change, temperature will be the principal factor affecting insects, forcing them to either perish or migrate to suitable habitats (Bale et al. 2002). Studies addressing the importance of *Wolbachia* on host temperature preference and host response to climate change, which could have significant impacts on endangered and

threatened species, remain unrepresented in the literature.

To date, despite the growing list of endangered and threatened species, few studies have addressed the biological and ecological impacts of extremely common reproductive endosymbionts like *Wolbachia* on their respective hosts. My overall research goals were to elucidate ways in which *Wolbachia* impact their host's biology and ecology. I was particularly interested in investigating the impact of spillovers into populations and communities and the implications to the conservation and management of endangered and threatened insects. In this context, in chapter 1, I provide the first empirical investigation of *Wolbachia* infection in a federally listed insect species, the butterfly *Speyeria zerene hippolyta*. Importantly, this species has a population supplementation program as part of its species recovery plan. I developed a single-population demographic model to demonstrate the *Wolbachia*-driven dynamics of different supplementation scenarios by using multiple years of empirical reproductive data.

In chapter two, I address the lack of ecological studies to empirically support phylogenetic analyses indicating horizontal transmission of *Wolbachia* by examining parasitoids that afflict butterfly species from the genus *Eurema* in Australia. Previous studies have revealed an identical CI-inducing *Wolbachia* strain in two Japanese *Eurema* species, *E. hecabe* and *E. mandarin* (Hiroki et al. 2004; Narita et al. 2007a) and five Australian *Eurema* species (Kern et al. unpublished). I hypothesized that if parasitoid interactions are a route for interspecies horizontal transmission of *Wolbachia, Eurema* butterfly species and their associated parasitoids would be infected with identical *Wolbachia* strains. Using multi-locus sequencing technique (MLST) (Baldo et al. 2006), I

characterized the *Wolbachia* strains of Australian *Eurema* butterflies and their parasitoids.

Finally, in chapter 3, I explore endosymbiont-host conflict that may arise from divergent physiological needs of each organism by testing whether a relationship exists between *Wolbachia* and host temperature preference. I developed a three-dimensional thermal gradient chamber apparatus and conducted a laboratory experiment comparing temperature preferences of uninfected and *Wolbachia*-infected fruit flies (*Drosophila melanogaster*). This study is the first to employ thermal choice assays to address *Wolbachia* associated temperature-dependent behavioral phenotypes. Results from my experiment begin to fill the knowledge gap regarding *Wolbachia*'s influence on host physiology and ways in which temperature preference disparities of conspecifics may affect a species ecology.

This dissertation introduces a new approach, rooted in empirical data, to better understand how *Wolbachia*-host interactions behave in natural and laboratory conditions, and discusses ways in which this endosymbiont could profoundly affect population trajectories. In addition, these field- and experiment-informed studies fills crucial gaps of knowledge in the *Wolbachia* field and provides platforms from which future research can build. My research indicates that understanding the population trajectories of species given their endosymbionts is imperative prior to implementation of population supplementation or translocation programs.

Chapter 1: *Wolbachia*'s butterfly effect: An emerging adversary to the conservation of threatened insect species

INTRODUCTION

Ecologists and natural resource managers have developed various population management programs, including population supplementation, that are aimed at mitigating some of the risks associated with small populations of endangered and threatened species (Snyder et al. 1996; Crone et al. 2007). Population supplementation aims to enhance populations that are at risk of extinction while maintaining or increasing genetic diversity. Small unstable populations are supplemented with individuals of the same species from the same population or from a larger, more stable population (Crone et al. 2007). The latter type of supplementation brings populations together that otherwise may have lacked gene flow. In the United States, at least 10 programs breed and release butterflies as a conservation method for threatened and endangered species, some of which are considered important pollinators (Schultz et al. 2008). While supplementation programs are diligent about not releasing visibly infected butterflies into recipient populations, movement between populations has the potential to spread less obvious hitchhiking microbes such as endosymbiotic bacteria like *Wolbachia.*

Widespread in terrestrial insects, *Wolbachia* comprise a diverse group of heritable endosymbiotic bacteria, some of which are characterized as parasitic as they manipulate their host's reproductive system in one of four ways: feminization, male killing, parthenogenesis, or cytoplasmic incompatibility (CI) (Werren et al. 2008; Zug & Hammerstein 2012; Kern et al. 2015; Weinert et al. 2015). Each of these phenotypes favors selection for infected females, ensuring *Wolbachia*'s persistence through

intergenerational transmission. Cytoplasmic incompatibility is the most extensively found reproductive manipulation in insects. Cytoplasmic incompatibility does not cause a female biased sex ratio distortion in their host's population, but rather embryo mortality, which ranges from barely detectable to complete, when an egg from an uninfected female is fertilized by a sperm from an infected male, or when an infected egg is fertilized by a sperm from a male infected with a different and incompatible *Wolbachia* strain (Yen & Barr 1973; Werren 1997; Zabalou et al. 2004; Werren et al. 2008). The consequences of CI are reduced fitness of uninfected females and a reproductive advantage to infected females (Werren et al. 2008). Such reproductive manipulation can cause reproductive isolation and lead to speciation (Brucker & Bordenstein 2012).

Cytoplasmic incompatibility-inducing *Wolbachia* were first identified as potential biological control agents by Laven (1967) to suppress pest insect populations. Subsequent research showed that when male hosts were artificially infected with CI-inducing *Wolbachia* they became effectively "sterile" because they did not produce viable offspring when they mated with uninfected females (Laven 1967; Dobson et al. 2002; Zabalou et al. 2004; Bourtzis 2008). Theoretical models predict that population suppression can be successful after only a single exposure to a novel CI-inducing *Wolbachia* strain (Dobson et al. 2002).

Wolbachia was recently detected in 19 Lepidoptera species of conservation concern, including one species spotlighted for captive rearing efforts (*Neonympha mitchelli mitchelli*) (Tolson 2008; Hamm et al. 2014). Accidental exposure of CI-inducing *Wolbachia* to threatened or endangered insect populations through a population supplementation program may unintentionally have similar suppression effects on

recipient populations (Nice et al. 2009). Thus, the nature of population supplementation programs and the ubiquity of *Wolbachia* in butterflies of conservation concern prompted me to investigate the prevalence of *Wolbachia* in populations of the threatened butterfly species *Speyeria zerene hippolyta,* (common name: Oregon silverspot butterfly). Here I examined the potential consequences that endosymbiotic bacteria *Wolbachia* might have had on several isolated populations of *S. z. hippolyta,* which carry *Wolbachia* and are managed by a population supplementation program. The goal of this study was to assess whether accidental introduction of *Wolbachia* could have negative impacts on small, at risk populations of insects.

I hypothesized that if *Wolbachia* had been accidently introduced and spread through *S. z. hippolyta* populations, the proportion of infected butterflies would increase over time. If the *Wolbachia* strain type induced CI, it would decrease reproductive output of uninfected females and may confer a positive frequency dependent fitness advantage to *Wolbachia*-infected female butterflies. I further hypothesized that CI-inducing *Wolbachia* could cause a temporary or permanent reduction in *S. z. hippolyta* population sizes due to offspring mortality**.** To address these hypotheses, I sampled female *S. z. hippolyta* butterflies that had been used in a population supplementation program, screened them for *Wolbachia*, and collected and analyzed their reproductive output data. Furthermore, I developed a single-population demographic model to simulate different potential dynamics of small populations resulting from population supplementation with uninfected, CI-*Wolbachia* infected, or combined uninfected and infected butterflies.

The infection dynamics of CI-inducing *Wolbachia* invasion into naïve host populations have previously been described for single populations, parapatric

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populations, and populations with a mainland-island structure (Caspari & Watson 1959; Fine 1978; Hoffmann et al. 1990; Hurst 1991; Turelli 1994; Telschow et al. 2007; Flor et al. 2007; Turelli 2010). Extensions of these models have been used to describe the release of CI-inducing *Wolbachia*-infected hosts into large populations with the goal of either successful population suppression of pest insects or identifying population replacement strategies for mosquitoes that carry insect-vector borne diseases that affect humans (Dobson et al. 2002; Zabalou et al. 2004; Jansen et al. 2008; Hancock et al. 2011). In contrast, my model aims to build on previous models by describing the population dynamics when CI-inducing *Wolbachia* is released into small populations of endangered insect species.

This study is the first, to my knowledge, to use reproductive data from threatened or endangered insects to show that the spread of *Wolbachia* in a natural population is associated with reduced fertility of uninfected hosts in the population and that this fitness reduction is positively frequency dependent. My results have important implications not only for *S. z. hippolyta* conservation, but for the conservation of all imperiled insect species, including some key pollinators.

MATERIALS AND METHODS

Butterfly populations

Reduced to five geographically isolated populations, the federally threatened *S. z. hippolyta* (Family: Nymphalidae) historically inhabited coastal prairies and grasslands from southern Washington to northern California. Three of the five extant populations, Bray Point, Cascade Head, and Rock Creek, all of which are located in Oregon, are

supplemented by the captive rearing and release program as dictated by the *S. z. hippolyta*

Recovery Plan (US Fish & Wildlife Service 2001). Cascade Head (45°03'24"N

124°00'17"W; elevation 371m), Rock Creek (44°10'49"N 124°03'21"W; elevation 56

m), and Mount Hebo (45°12'52"N 123°45'23"W; elevation 962 m), the fourth Oregon

population, have all been used as source populations at some time during the program,

though the most stable population, Mount Hebo, has been the primary source population.

Measurement, tissue collection, *Wolbachia* screen, and reproductive data

Individual leg tissue samples were taken from 234 *S. z. hippolyta* female

specimens used in the population supplementation program from 1999 and 2001-2011(no

butterflies were collected in 2000) at the Oregon Zoo (Portland, Oregon) and Woodland

Park Zoo (Seattle, Washington). Because *S. z. hippolyta* is a federally listed threatened

species, I had limited tissue available for use in my study. Therefore, I extracted DNA

from only a portion of each tissue sample and preserved the remaining portion in case a

second DNA extraction was required. All tissue samples were stored individually at -

20˚C in 1.5ml microcentrifuge tubes that contained absolute ethanol. Three of the

sampled butterflies were males, and thus were included in the *Wolbachia* screen but

excluded from analyses on reproduction data. Individual tissues were homogenized in

1.5ml microcentrifuge tubes using liquid nitrogen and microtube pestles (Scientific

Specialties Inc., Lodi, CA). All DNA extractions were performed using the Qiagen

DNeasy Blood and Tissue kit, following the manufacturer's protocol (Qiagen, Valencia,

CA, USA). Diagnostic PCR amplification of *28S* rDNA was performed to test the quality

of DNA using universal arthropod primer sets (Werren et al. 1995a). For samples that

failed to detect *28S* rDNA or that yielded light bands, I performed a second DNA extraction.

I screened individual *S. z. hippolyta* for *Wolbachia* by performing PCR amplification using *Wolbachia* specific *16S* rDNA primer sets and thermocycling conditions as described by Werren & Windsor (2000). Polymerase chain reactions were each a total of 25µl comprised of 12.5µl 2X Phusion High-Fidelity PCR Master Mix (New England BioLabs Inc.), 2-4µl template DNA, and nuclease-free molecular grade water to 25µl. All PCR products were visualized with ethidium bromide on 1% agarose gels. For samples from which arthropod *28S* was successfully amplified, but indicated a negative result for *Wolbachia*-infection, PCR amplification of *16S* rDNA was repeated with a higher concentration of genomic DNA template. Primers and thermocycler conditions are listed in Tables 1.1 and 1.2.

Table 1.1: List of primers used for PCR amplification of loci, targeted genome, and references.

Loci	Primers	Target genomes	Sequences (5'-3')	References
16S	16SW spec F	Mitochondria	CATACCTATTCGAAGGGATAG	Werren & Windsor 2000
	16SW spec R		AGCTTCGAGTGAAACCAATTC	
28S	28SF	Nuclear	CCCTGTTGAGCTTGACTCTAGTCTGGC	Werren et al. 1995a
	28SR		AAGAGCCGACATCGAAGGATC	

Table 1.2: Thermocycler conditions for PCR of each loci.

	Mitochondrial loci *16S* and *COI*	Nuclear loci *28S*
Denaturation	98°C for 4 min	98°C for 2 min
Cycling	5 cycles: 98°C for 2 min, 60°C for 1 min, and 72°C for 1 min 35 cycles: 98°C for 30 sec, 60°C for 45 sec, and 72°C for 1 min	1 cycle: 98°C for 1 min, 56°C for 1 min, and 72°C for 2 min 35 cycles: 98°C for 15 sec, 56°C for 1min, and 72°C for 2 min 1 cycle: 94°C for 15 sec, 56°C for 1 min
Final elongation	72°C for 10 min	72°C for 7 min

Both zoos collected fertility data for every female butterfly used in the captive

rearing and release program during the years 2003-2011 (N=205). Before looking at the

infection status of the butterflies, I used these fertility data to determine three measures of

fertility: 1) whether butterflies laid eggs, 2) the number of eggs laid by females that did

lay eggs, and 3) the number of eggs that successfully developed into larvae (hatched)

compared to the number that did not hatch. I also employed these data in the

demographic model. To determine if butterfly size affected their reproductive output,

expired butterflies were placed onto Cartesian graph paper next to a centimeter ruler aligned with their abdomen, thorax, and head. I used a Panasonic Lumix DMC-FZ18 camera that was stationed above the butterflies to capture images. Images were scaled and butterflies were measured using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Data analysis

For each Year and Site from which *S. z. hippolyta* were used in the captive rearing and release program, I determined the number of *Wolbachia*-infected butterflies versus the number of butterflies that were uninfected. To determine whether body sizes were associated with reproductive output, I used linear regression and compared slopes of the regression lines describing the relationships between body sizes and number of eggs laid by uninfected and infected butterflies. I repeated the previous described statistical test for comparison using proportion of eggs that hatched instead of number of eggs laid for uninfected and infected butterflies. In addition, a t-test was used to determine whether uninfected and *Wolbachia*-infected butterflies body sizes were significantly different. A Mann-Whitney test was used to compare the number of eggs laid by uninfected and *Wolbachia*-infected female butterflies.

Using R statistical software version 3.3.2 (R Development Core Team 2016) and linear-mixed model package "lme4" (Bates 2014), I created a general linear mixed effects model (GLMM) to compare Hatch Success (the number of laid eggs that hatched compared to the number that did not hatch) between butterflies of two different Infection Statuses: uninfected female butterflies and *Wolbachia*-infected female butterflies. The

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predictor variable in my model was *Wolbachia* Infection Status and had a binomial

distribution of the number hatched given the number of eggs that did not hatch (using

column bind). A GLMM was used for this analysis because 1) it allows for the use of

binomial data and 2) it allows for the inclusion of random effects. The random effects in

my model were Year and Site, with Site (Cascade Head, Rock Creek, and Mt. Hebo)

nested within Year (2003-2011). Finally, my GLMM model used a binomial error

distribution and logit link function. The R code used and GLMM model outputs are in

Appendix A.

I created a scatterplot of the proportion of eggs that hatched (number of hatched

eggs from the total number of eggs laid) from uninfected female butterflies against the

frequency of *Wolbachia*-infected butterflies among my specimens to visually assess the

relationship between the frequency of *Wolbachia*-infection among my specimens and the

fertility of uninfected butterflies. To avoid creating a multiplicity issue, I used these plots

to visualize trends, but did not perform statistical analyses on them.

I also used the reproductive data and the systems dynamics modeling software STELLA

v. 10.0.6 (ISEE systems 2014) to develop a demographic population model (model

description below).

Population demographic model

I referenced Hoffman *et al.* (1990) and Dobson *et al.* (2002) and used an

extension of the logistic growth equation to develop a model describing the population

dynamics of a single panmictic butterfly population with discrete and non-overlapping

generations under four different scenarios (Table 1.3). My model assumed that all

butterflies mated only once. Additionally, my model assumed a 1:1 female to male sex ratio in all populations, as a sex ratio distortion in *S. z. hippolyta* has not been detected. I first modeled a control population to describe the population dynamics of a completely uninfected butterfly population without supplementation and an initial population size of 300. Let u_t denote the number of uninfected butterflies, w_t denote number of *Wolbachia*-infected butterflies, and N_t denote the total number of butterflies in the population at time t. Then I described the intergenerational changes in the number of uninfected butterflies by

$$(1) \quad u_{t+1} = u_t r \left(1 - \frac{u_t}{K}\right) p,$$

where r denotes the population growth rate and is calculated as the product of the number of eggs laid and the proportion of eggs that hatch, p denotes adult survival, and K denotes carrying capacity of the population. The number of eggs laid per female butterfly was set to 162; a value derived from previously collected *S. z. hippolyta* reproductive data. I included stochasticity in the model by randomizing larval survival rates yearly between 0.015 and 0.023, which represents seasonal variation in butterfly abundance (US Fish & Wildlife Service). Carrying capacity K was set at 500 butterflies, but can be as low as 200-500 in some of the *S. z. hippolyta* populations (McCorkel et al. 1980). Adult butterfly survival was set to 65% based on the best available data, adult survival during release events. Model symbols and definitions are listed in Table 1.4.

Table 1.3: Model conditions for simulating different population supplementation scenarios.

Model numbers and description	Number of times butterflies were released	Number of uninfected butterflies released each time	Number of *Wolbachia*-infected butterflies released each time
Model 1: Uninfected no – CRR	0	0	0
Model 2: Single release uninfected 300	1	300 at t_5	0
Model 3: Single release uninfected 1000	1	1000 at t_5	0
Model 4: Single release *Wolbachia*-infected 50	1	0	50 at t_5
Model 5: Single release *Wolbachia*-infected 300	1	300 at t_5	0
Model 6: Single release *Wolbachia*-infected 1000	8	0	1000 at t_5
Model 7: Multiple release uninfected 300	8	300 at t_5 to t_{19}	0
Model 8: Multiple release *Wolbachia*-infected 300		0	300 at t_5 to t_{19}
Model 9: Combined multiple release uninfected 300, *Wolbachia*-infected 50	8	300 at t_5 to t_{19}	50 at t_5 to t_{19}
Model 10: Combined multiple release uninfected 50, *Wolbachia*-infected 30	8	50 at t_5 to t_{19}	300 at t_5 to t_{19}

Table 1.4: Definition of parameters and symbols used in the model.

Symbols	Definitions
u_t	Number of uninfected butterflies at time t
w_t	Number of *Wolbachia*-infected butterflies at time t
N_t	Total number of butterflies at time t
s	Number of butterflies released to supplement population
r	The product of the number of eggs laid, and larvae survival
p	Adult butterfly survival
K	Carrying Capacity

I then extended equation (1) to include supplementation. I simulated the release of virgin adult butterflies into the control population either once at time point t_5 or eight times on every odd time point from t_5 to t_{19}. In all models, I assumed that virgin adult butterflies were released corresponding to the time that resident adult butterflies reached reproductive age; therefore, supplementation aimed to increase the absolute population size. I assumed that supplemented and resident adult butterflies had the same survival rate and included supplementation with the release of uninfected virgin adult butterflies by

$$(2) \quad u_{t+1} = \left[u_t r \left(1 - \frac{u_t}{K} \right) + s_u \right] p$$

where s_u denotes the number of adult uninfected butterflies released.

Next, the population dynamics when *Wolbachia* was introduced into the uninfected control population were described. Under the same assumptions as equation (2), I simulated a single release of virgin adult *Wolbachia*-infected butterflies by

$$(3) \quad w_{t+1} = \left[w_t r \left(1 - \frac{w_t}{K} \right) + s_w \right] p,$$

where s_w denotes the number of virgin adult *Wolbachia*-infected butterflies released into the population. Matings between uninfected females and *Wolbachia*-infected males were assumed to be completely reproductively inviable (i.e. they exhibited complete CI). Thus, I define CI as the probability that an uninfected female mated with an uninfected male butterfly. I accounted for this probability in the model by including the proportion of uninfected males in the population by $\frac{u}{N}$. Therefore, when *Wolbachia* was introduced into the population, equation (1) became

$$(4) \qquad u_{t+1} = \frac{u_t^2 r}{N_t}\left(1 - \frac{u_t}{K}\right)p.$$

Lastly, I described the population dynamics of supplementation into the control population by eight combined releases of uninfected and *Wolbachia*-infected butterflies from t_5 to t_{19}. Equation (4) became

$$(5) \qquad u_{t+1} = \left[\frac{u_t^2 r}{N_t}\left(1 - \frac{u_t}{K}\right) + s_u\right]p$$

to include the release of uninfected butterflies in the presence of *Wolbachia*-infected butterflies. I described the changes in the total number of butterflies in the population from one generation to the next as the sum of equations (3) and (5).

Maternal transmission of *Wolbachia* and embryonic mortality rates from CI are unknown for *S. z. hippolyta,* but are known to vary depending on host species, host genetic background, and the *Wolbachia* strain type (Zabalou et al. 2004; Duplouy et al. 2015). Based on several studies for other butterfly species (Narita et al. 2007; Hornett et al. 2008; Narita et al. 2009), my model assumed perfect maternal transmission and 100% embryonic mortality due to CI; thus, the highest impact from CI-inducing *Wolbachia* on the butterfly population dynamics was demonstrated in the models.

I simulated population dynamics from each supplementation scenario for 100 years. For each model, I ran 100 iterations then calculated the average value across the iteration for each time point. The time point from each model that had the smallest average value was defined as the year with the lowest population size. To determine whether different supplementation scenarios had different effects on population size, I compared the models with analysis of variance (ANOVA) in two ways. First, I used the population size at the lowest point in each model iteration (n=100 iterations/model). Similarly, I also compared the population size at the 50-year time point in each of four models, using the 100 iterations per model in the ANOVA. I performed a Tukey's post-hoc test for each ANOVA to determine which of each of the single release and multiple release population models differed from one another.

RESULTS

I found that 62.7% (146/233) of female *S. z. hippolyta* samples from Mount Hebo, Rock Creek, and Cascade Head were infected by *Wolbachia* (Appendix A Figure A1). Additionally, the proportion of *Wolbachia*-infected butterflies increased over time (Figure 1.1), from an average of 30% during the first three years of the data (1999-2002), to 78% between 2008-2010, and finally reaching 100% in 2011. This increase suggests *Wolbachia* spread through the populations over a 13-year period.

Uninfected female *S. z. hippolyta* laid fewer eggs than *Wolbachia*-infected butterflies from 1999-2011 (Figure 1.2; Mann-Whitney test: $U = 3785$, $P_{exact} = 0.019$). The body sizes of uninfected and *Wolbachia*-infected butterflies were not significantly different (t-test: $t = -1.041$, $N = 120$, $P = 0.301$) and I concluded that butterfly body size

did not affect the number of eggs a female butterfly laid (Regression uninfected equation

Y = -13.79*X + 372.2, *Wolbachia*-$F_{1,119}$ = 0.018, P = 0.893) or the Hatch Success

(Regression uninfected equation Y = 0.01959*X + 12.78, *Wolbachia*-infected equation Y

= -0.2163*X + 13.12, $F_{1,119}$ = 0.392, r^2 = 0.004, P = 0.533).
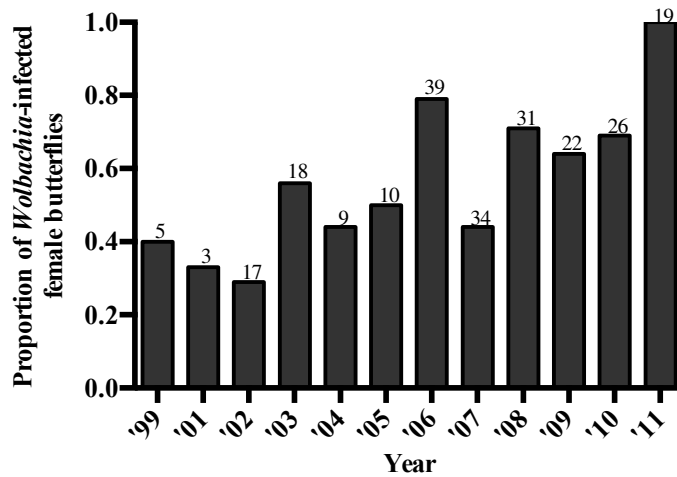
Figure 1.1: Proportion of *Wolbachia*-infected female *S. zerene hippolyta* for each year. Numbers above

bars represent the number of combined sampled females from three populations, Cascade Head, Rock

Creek, and Mt. Hebo for years 1999 and 2001-2011.
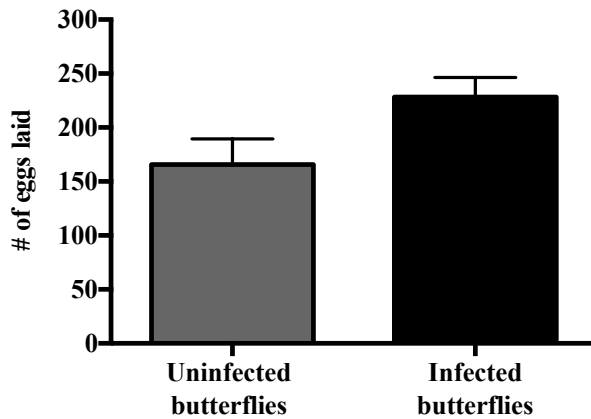
Figure 1.2: Comparison of the number of eggs laid by uninfected (N=70) versus *Wolbachia*-infected

(N=135) female *S. z. hippolyta* butterflies. Uninfected butterflies laid fewer eggs than infected butterflies.

Bars represent SE.

Scatterplots of Hatch Success against frequency of *Wolbachia*-infected butterflies suggested that as the proportion of *Wolbachia*-infected butterflies increased, the number of eggs that hatched from uninfected butterflies decreased (Figure 1.3a). Moreover, Figure 1.3b shows a negligible change in the number of eggs that hatched from *Wolbachia*-infected butterflies as the frequency of *Wolbachia*-infected butterflies increased. Figure 1.3c demonstrates that the ratio of Hatch Success from uninfected to *Wolbachia*-infected butterflies decreased as the frequency of *Wolbachia*-infected butterflies increased. These trends suggest a positive-frequency dependent relationship between *Wolbachia*-infection frequency and fertility reduction of uninfected butterflies.

Finally, statistical analysis of *S. z. hippolyta* reproductive data detected a significant relationship between *Wolbachia*-infection status and egg Hatch Success. Results from the GLMM showed that uninfected female butterflies (N = 70) had significantly lower Hatch Success, measured as the number of eggs that hatched (68.34 ± 11.73 mean ± SE) given the number that failed to hatch, compared to Hatch Success of *Wolbachia*-infected butterflies (81.20 ± 7.4, N = 135; Table 1.5; GLMM z = -8.067, P < 0.0001).

Uninfected eggs that hatched

Wolbachia-infected eggs that hatched

Uninfected:Infected eggs that hatched
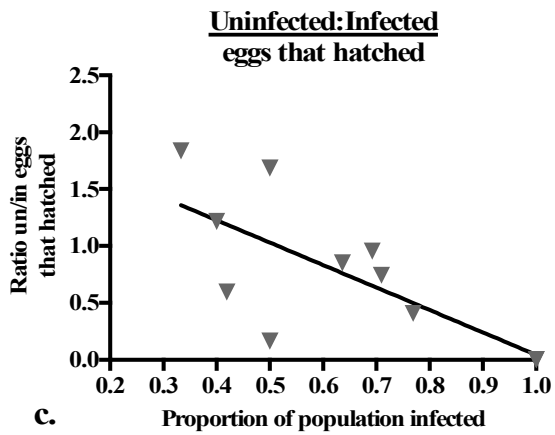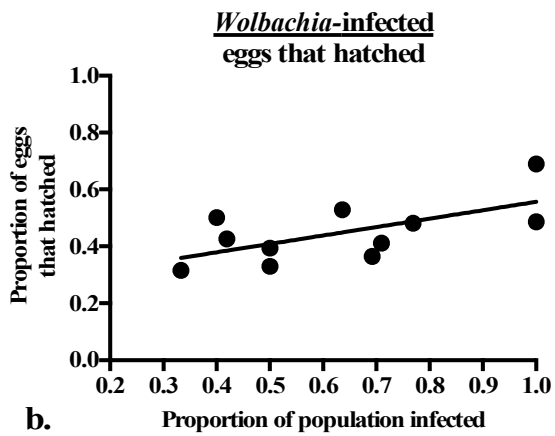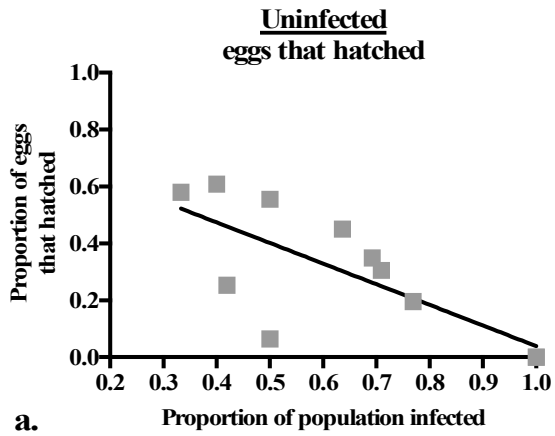
*Figure 1.3***:** Average proportion of eggs that developed into larvae a) from uninfected female butterflies, b) from infected female butterflies, and c) as a ratio of uninfected to infected butterflies, each as a function of the proportion of *Wolbachia*-infected.

Model simulations of population supplementation with single release scenarios using empirical values revealed that supplementation with CI-inducing *Wolbachia*-infected butterflies significantly depressed the host-population size compared to no supplementation, whether examining the smallest population size or population size after 50 years. This effect was even more pronounced when compared to supplementation with *Wolbachia*-free butterflies (Figures 1.4, 1.5, Appendix A Tables A1-A4; ANOVA: $F_{5,593}$ = 3349 P < 0.0001, r^2 = 0.97 and $F_{5,593}$ = 1526, P < 0.0001, r^2 = 0.93 respectively). All models differed significantly from one another regardless of whether I compared smallest population sizes or population sizes at 50 years (Figure 1.5; Tukey test: *P* < 0.0001 for all combinations; Appendix A Tables A1.2 & A1.4). Model 2 and 3, which simulated population supplementation by the addition of 300 and 1000 uninfected butterflies respectively during year five, produced a significantly larger population size compared to the other single release models, models 4, 5, and 6 (P < 0.0001). Model 4, which simulated the effects of a single release of 50 *Wolbachia*-infected butterflies into the population, had a significantly smaller population size compared to all other models (*P* < 0.0001). The models showed that cytoplasmic incompatibility reduced the absolute population size of uninfected butterflies in these initially moderate-sized simulated initial populations.

Model simulations of population supplementation with multiple release scenarios also showed that supplementation with CI-inducing *Wolbachia*-infected butterflies significantly depressed the host-population size compared to no supplementation, whether examining the smallest population size or population size after 50 years. This effect was even more pronounced when compared to supplementation with *Wolbachia*-

free butterflies (Figures 1.4, 1.5, Appendix A Tables A.5-A.8; ANOVA: $F_{4,524} = 1891$, P <

0.0001, $r^2 = 0.94$ and $F_{4, 524} = 1559$, P < 0.0001, $r^2 = 0.92$ respectively). Except for models 8 and 10

(P = 0.992), all models differed significantly from one another regardless of whether I

compared smallest population sizes or population sizes at 50 years (Figure 1.5; Tukey

test: P < 0.0001 for all combinations; Appendix A Tables A1.6 & A1.8). Model 7, which

simulated population supplementation by the addition of 300 uninfected butterflies during

each of eight different years, produced a significantly larger population size compared to

the other models (P < 0.0001). Model 9, in which I used 300 uninfected and 50

Wolbachia-infected butterflies to supplement the population produced a significantly

smaller population compared to models 7, 8, and 10 (P < 0.0001). The models showed

that cytoplasmic incompatibility reduced the absolute population size of uninfected

butterflies in these initially moderate-sized simulated initial populations.
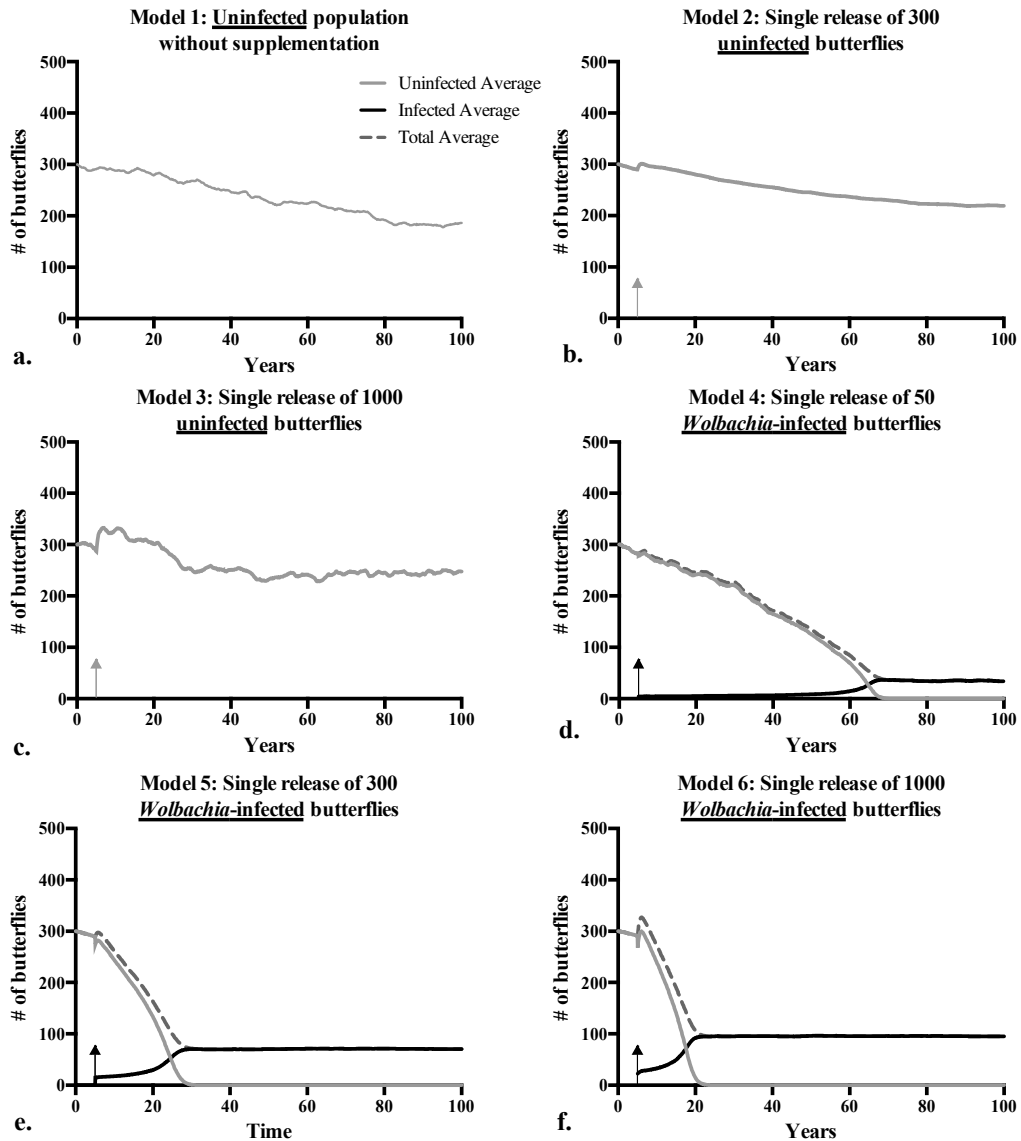
Figure 1.4: Simulation results from 100 iteration averages of single populations with non-overlapping generations under different single release at time point five supplementation scenarios. Graphs illustrate the dynamics of a butterfly population (a) without supplementation, (b) supplementation with 300 uninfected butterflies, (c) supplementation with 1000 uninfected butterflies, (4) supplementation with 50 *Wolbachia*-infected butterflies, (5) supplementation with 300 *Wolbachia*-infected butterflies, (6) supplementation with 1000 *Wolbachia*-infected butterflies.

Model 1: <u>Uninfected</u> population without supplementation

— Uninfected Average
— Infected Average
-- Total Average

a.

Model 7: Multiple releases of 300 <u>uninfected</u> butterflies

b.

Model 8: Multiple release of 300 <u>*Wolbachia*-infected</u> butterflies

c.

Model 9: Combined multiple releases of 300 <u>uninfected</u> and 50 <u>*Wolbachia*-infected</u> butterflies

d.

Model 10: Combined multiple releases of 50 <u>uninfected</u> and 300 <u>*Wolbachia*-infected</u> butterflies

e.

Figure 1.5: Simulation results from 100 iteration averages of single populations with non-overlapping generations under different multiple release at eight different time point (odd time points from t_5 to t_{19}) supplementation scenarios. Graphs illustrate the dynamics of a butterfly population (a) without supplementation, (b) supplementation with 300 uninfected butterflies, (c) supplementation with 300 *Wolbachia*-infected butterflies, (4) supplementation with 300 uninfected 300 and 50 *Wolbachia*-infected butterflies, (d) supplementation with 50 uninfected butterflies, *Wolbachia*-infected 300, and (e) supplementation with 50 uninfected and 300 *Wolbachia*-infected butterflies.

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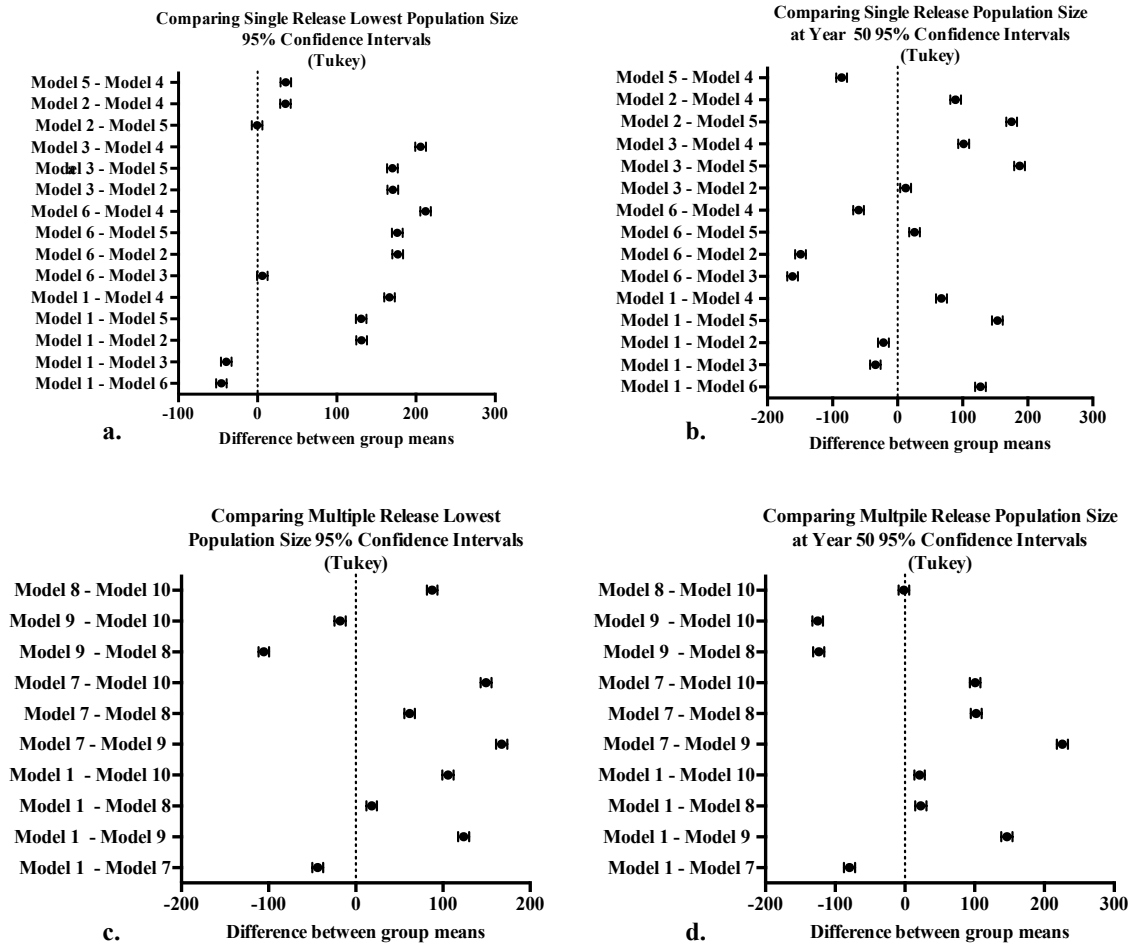
Figure 1.6: Confidence intervals for model comparisons of (a) single release lowest population sizes, (b) single release population sizes at year 50, (c) multiple release population lowest population sizes, and (d) multiple release populations sizes at year 50.

DISCUSSION

The decline of *S. z. hippolyta* has been attributed to habitat destruction and fragmentation, as well as to the introduction of invasive plant species that compete with the butterfly's host plant (Schultz & Chang 1998). Despite recent population supplementation efforts with captive rearing and release, four of the five extant

populations of *S. z. hippolyta* remain at risk of extirpation. I screened for and detected the presence of *Wolbachia* in *S. z. hippolyta* female butterflies in all sampled populations. I discovered that the frequency of infection has increased and that the reproductive output of uninfected females has decreased in the three populations tested since the beginning of the population supplementation program (from 1999-2011). I conclude that *Wolbachia* may have been and could continue to be an additional stressor to *S. z. hippolyta* populations. In addition to the reproductive effects, in some species, *Wolbachia* can reduce host dispersal distances (Goodacre et al. 2009). Such dispersal reduction to *S. z. hippolyta* could increase species mortality from vehicle collisions as they rely on their dispersal ability in at least one of their populations, Rock Creek, where habitats for larval food plant (*Viola adunca*) and nectaring plants were divided by a busy coastal highway (Zielin et al. 2016).

Wolbachia's phenotypes encompass several reproductive manipulations: CI, parthenogenesis, feminization, and male killing. Cytoplasmic incompatibility is the most commonly described *Wolbachia*-induced phenotype in insects, causing reduced fertility of uninfected females mated with infected males (Yen & Barr 1973; Werren 1997; Zabalou et al. 2004; Werren et al. 2008). As the *S. z. hippolyta* populations maintain a relatively equal sex ratio, the induction of parthenogenesis, feminization, and male killing by *Wolbachia* in this butterfly species seems unlikely. In contrast, my results are consistent with the hypothesis that the *Wolbachia* induces CI in *S. z. hippolyta* butterflies. Uninfected female *S. z. hippolyta* had reduced fertility as they laid significantly fewer eggs and showed significantly reduced hatch success compared to *Wolbachia*-infected female butterflies. Hatch Success was also highest in years and populations with lowest

infection prevalence. Additionally, I showed that the fertility of uninfected female butterflies decreases as *Wolbachia* prevalence increases through *S. z. hippolyta* populations. Together, these results suggest that uninfected females have compromised fitness and that *Wolbachia* benefits infected female *S. z. hippolyta* butterflies, as has been previously shown in other CI-inducing *Wolbachia* infected host species (Dobson et al. 2004; Weeks et al. 2007; Brownlie et al. 2009). Unfortunately, I was unable to empirically measure CI in *S. z. hippolyta* as the butterflies do not mate in captivity; however, my results suggest that uninfected females sampled for this study suffered from CI and that CI may have resulted in population-level costs.

Small isolated populations face many threats that can lead to extirpation and extinction. Through model simulations, I showed that even a single exposure event to a small number of *Wolbachia*-infected butterflies can have a long-term negative effect on overall population size (Figure 1.4c). When a population is already small and afflicted by the risks associated with small populations (inbreeding depression, genetic drift, reduced population growth, decreased disease resistance), the introduction of *Wolbachia* can potentially push a vulnerable population to extinction. Although, it is unlikely that a population will recover to pre-infection size (Figure 1.4a-d), my model demonstrates that, under certain conditions, host populations can recover after *Wolbachia* introduction (Figure 1.4d); however, this is not without the incurred population-level cost by the extinction of uninfected haplotype(s) and subsequent loss of some genetic diversity. If one or more incompatible CI-inducing *Wolbachia* strains are introduced into a population, it could put populations at risk of extinction as butterflies are moved among populations through supplementation. Cytoplasmic incompatibility causes post-mating

reproductive isolation, which reduces gene flow, and can lead to evolutionary selection of pre-mating isolation (Jaenike et al. 2006). Reproductive isolation reduces breeding opportunities and overall fitness leading to reduced population size in subsequent generations.

Using butterfly specimens that were collected before 1999, McHugh *et al.* (2013) screened six *S. zerene* subspecies, including *S. z. hippolyta,* for *Wolbachia.* While *Wolbachia* was detected in two of the subspecies, it was not detected in any of the 12 *S. z. hippolyta* individuals tested. There are several potential reasons for why McHugh *et al.* (2013) did not detect *Wolbachia* from their *S. z. hippolyta* specimens. First, *Wolbachia* may not have been present in the *S. z. hippolyta* populations at the time the samples were collected. Alternatively, *Wolbachia* could have been present but they might not have detected *Wolbachia* because their sample size was small and may not have been representative of the populations. Third, they stated in their paper that they experienced limited success with PCR amplification of nuclear DNA from *S. z. hippolyta* samples, which suggests they potentially encountered similar issues with the amplification of *Wolbachia* DNA, therefore, yielding false negatives.

Wolbachia are not the only endosymbiotic bacteria that manipulate their host's reproduction. The stage for reproductive parasitism is shared with *Spiroplasma, Rickettesia,* and most notably, *Cardinium*, which all are known to induce CI and are commonly found in arthropods (Hunter 2007; Jaenike et al. 2007; Weinert et al. 2015). However, *Wolbachia* linked selective mitochondrial sweeps have been shown in many species and in small populations the mtDNA haplotype associated with uninfected individuals may become extinct (Turelli et al. 1992; Jiggins 2003; Rasgon & Scott 2003;

Hurst & Jiggins 2005; Schuler et al. 2013). With my discovery of *Wolbachia* in the extant

populations of this *S. z. hippolyta*, the mixing of infected and uninfected butterflies

during supplementation could lead to the extinction of uninfected butterflies, further

reducing genetic diversity.

Echoing the conclusions from Nice *et al.* (2009), I suggest that conservation

programs currently using population supplementation strategies consider the risks of

Wolbachia to endangered or threatened species. As with all threatened and endangered

species recovery plans, success of these programs requires not only screening for visibly

infected pathogens but also extensive knowledge of the biology of the species (Snyder et

al. 1996), including intracellular symbiotic relationships such as those with *Wolbachia*,

which may affect the reproductive success of the species. A more thorough analysis is

needed to understand the effects that *Wolbachia* have on their specific hosts, their host's

populations, and their ecosystems. Results from this study can be used to inform all

invertebrate species conservation plans; particularly those currently supplemented by

captive propagation or that are being considered as potential candidates for

supplementation.

Chapter 2: *Eurema* butterflies are quite hospitable: characterization of their parasitoids revealed some sharing of identical *Wolbachia* strains

INTRODUCTION

Host-bacteria relationships, particularly those between endosymbionts and insects, are increasingly accepted as pivotal to biological and ecological processes (McFall-Ngai et al. 2013; Archibold 2014). Endosymbiotic bacteria are primarily vertically transmitted (from mother to offspring) and form relationships with their hosts that are either obligate of facultative. In obligate endosymbiosis, the host bacteria are crucial to host survival. Conversely, facultative endosymbiotic bacteria, such as *Wolbachia*, can also be transmitted horizontally between species, are not necessary for host survival, and can have a range of effects on host fitness (Oliver et al. 2006; Moran et al. 2008). Whether an endosymbiont is facultative or obligate, their vertical transfer is coupled with other maternally inherited genetic elements present in host cytoplasm, most notably, mitochondria (Cosmides & Tooby 1981).

Wolbachia are a group of facultative endosymbiotic bacteria that are estimated to be present in approximately 53% of terrestrial arthropod species and 52% of aquatic insects (Zug & Hammerstein 2012; Weinert et al. 2015; Sazama et al. 2017). They have varying effects on their hosts; however, they have garnered interest because of the reproductive phenotypes they induce in their hosts: feminization, male killing, parthenogenesis, or cytoplasmic incompatibility (CI) (Werren et al. 2008; Werren 2011). Reproductive manipulating *Wolbachia* strains resemble selfish genetic elements by altering their host's reproductive phenotypes to selectively favor infected females and enhance their own transmission (Werren 1997; Hatcher 2000; Werren et al. 2008; Werren

2011). *Wolbachia* induced feminization, male killing, and parthenogenesis are phenotypes that favor infected females by reducing males in the population (Hurst & Werren 2001; Werren et al. 2008). Cytoplasmic incompatibility is the most prevalent phenotype, characterized by increased embryo mortality when an egg from an uninfected female is fertilized by a sperm from an infected male; thereby, *Wolbachia*-infected females are selectively favored over uninfected females (Hurst & Werren 2001; Werren et al. 2008).

It is well established that *Wolbachia* are able to perpetuate in a host population because of their nearly complete to complete intergenerational vertical transmission (Bandi et al. 2001). Despite being widespread and present in both terrestrial and aquatic insects, the routes by *Wolbachia* for interspecies transmission are less understood. Moreover, explanations for *Wolbachia* in phylogenetically distantly diverged taxa cannot be explained by vertical transmission. Molecular phylogenetic analyses of endosymbiont and host mitochondria have revealed incongruence between *Wolbachia* and host species, providing strong evidence that supports frequent horizontal movement of *Wolbachia* between host species (O'Neill et al. 1992; Vavre et al. 1999; Haine et al. 2005; Baldo et al. 2006; Miller & Riegler 2006). Though, the mechanisms of horizontal transmission in nature are not fully understand, close ecological interactions appear fundamental to *Wolbachia*'s ability to cross species boundaries (Raychoudhury et al. 2009; Stahlhut et al. 2010; Schuler et al. 2013; Ahmed et al. 2015b; Li et al. 2016).

Previous studies have shown that horizontal transmission events can be mediated during larval development by sharing a food source (Sintupachee et al. 2006), through cannibalistic consumption (Le Clec'h et al. 2013), or through blood to blood to contact

that can result from an altercation (Rigaud & Juchault 1995). Interspecific horizontal transmission can also be mediated by vector species such as parasitoids (Werren et al. 1995b) as was accomplished in a laboratory experiment that demonstrated a parasitoid successfully transferring *Wolbachia* from *Drosophila simulans* to *Leptopilina boulardi* (Heath et al. 1999). Since emerging as potential routes of interspecies transmission, host-parasitoid interactions have dominated *Wolbachia* horizontal transmission research. These interspecies interactions are both ecologically important and provide an opportunity for a parasitoid to acquire *Wolbachia* through a trophic interaction: parasitoid development requires consuming their tissues of their larval host thought to transmit *Wolbachia* (Schilthuizen & Stouthamer 1997; West et al. 1998; Heath et al. 1999; Huigens et al. 2004; Morrow et al. 2014; Ahmed et al. 2015b).

Eurema (Lepidoptera, Pieridae) comprises a diverse group of butterfly species commonly known as grass yellows, with extensive distribution throughout Africa, Asia and Australia (Kemp 2002; Kemp 2008). Two different strains of *Wolbachia* with very different effects on host reproduction, the CI-inducing strain *w*CI and the feminization strain *w*Fem, were discovered in *Eurema* populations from Japan (Hiroki et al. 2004; Narita et al. 2007a; Narita et al. 2007b). The *w*Fem *Wolbachia* strain has been further detected in *Eurema* species from China, Indonesia, Vietnam (Narita et al. 2006), and India (Salunke et al. 2012).

In Australia, the six *Eurema* butterfly species – *E. smilax, E. hecabe, E. alitha, E. laeta, E. herla,* and *E. brigitta* – inhabit various geographic and climatic regions. Each species uses a specific life history strategy (diapause vs. migration) to cope with extended periods of drought and seasonal precipitation changes in tropical and subtropical

Australia (Jones & Rienks 1987; Jones 1992). Except for *E. smilax,* all Australian *Eurema* species are infected with *Wolbachia* (Kern et al. unpublished). Four of these species share a *Wolbachia* strain that is identical to *w*CI and designated to sequence type (ST) ST-41 (Figure 2.1) (Kern et al. unpublished), which is a frequently identified strain type in various butterfly species around the world (Ilinsky & Kosterin 2017). *Eurema herla* and *E. brigitta* are infected with another strain designated to ST-125 (Figure 2.1). Recent phylogenetic analysis of mtDNA from *Eurema* butterfly species revealed that mitochondrial lineages of each species lack geographical structure and that *Wolbachia*-infected species have far less mtDNA diversity compared to the uninfected species, *E. smilax* (Kern et al. unpublished). Additionally, results from the phylogenetic analysis showed that each Australian *Eurema* species had a unique mtDNA sequence and that mtDNA was not shared between the species (Kern et al. unpublished). This result indicates that none of the species are interspecies hybrids, the species have diverged, and there is no evidence of the mitochondrial hitchhiking that is generally associated with CI-inducing *Wolbachia* strains, which if present, would have suggested that the strain originated with *E. smilax* (Kern et al. unpublished). Given these findings that the same *Wolbachia* strain exists in multiple fully diverged *Eurema* species with overlapping geographic distributions, a compelling case can be made that horizontal transmission has been paramount to *Wolbachia* invasion into this group of butterflies.

I postulated that horizontal transmission of *Wolbachia* in *Eurema* species was most likely mediated by parasitoid vectors. Although parasitoids have been characterized for a vast number of butterfly species (Smith et al. 2007; Smith et al. 2008; Janzen et al. 2009), parasitoids afflicting this group of butterflies have not yet been extensively

investigated. In this study, my aims were to 1) characterize parasitoids from *Eurema* butterfly species, 2) screen parasitoids for *Wolbachia*, and 3) determine whether parasitoids are infected with the same *Wolbachia* strain as their hosts. I addressed these aims by surveying *Eurema* larvae collected at four different locations in Queensland, Australia for parasitoids and by conducting a *Wolbachia* screen using a genetic approach based on Multi Locus Sequencing Technique (MLST) of *Wolbachia* house-keeping genes (Baldo et al. 2006) and the *Wolbachia* surface protein gene, *wsp* (Zhou et al. 1998).
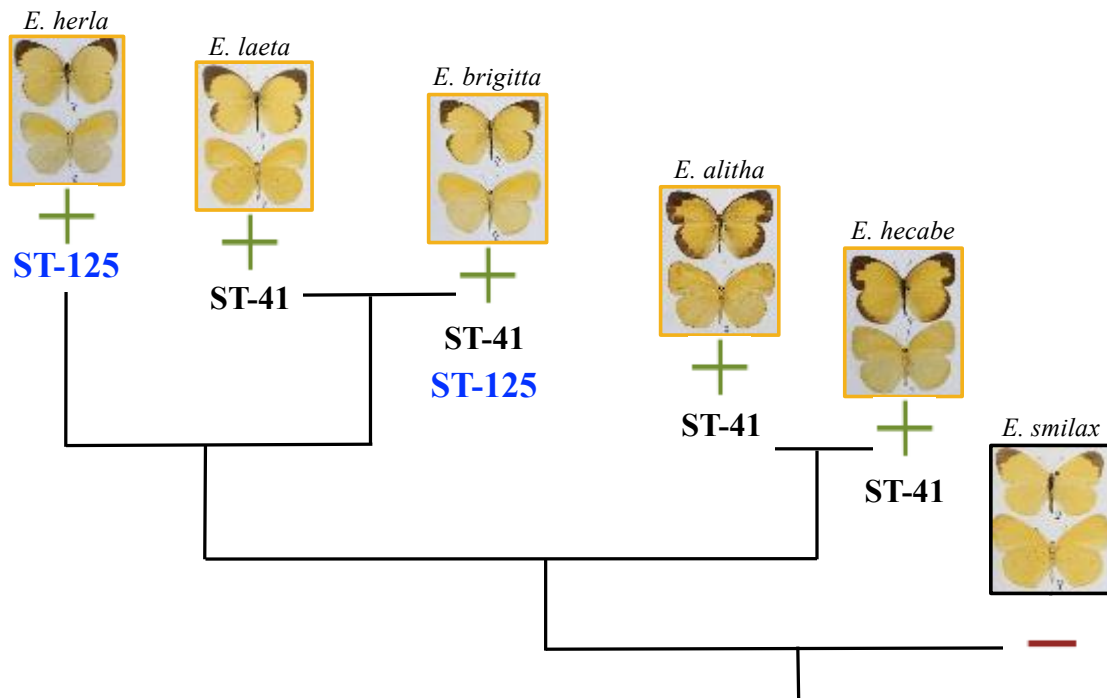
Figure 2.1: Schematic of a phylogeny tree that depicts the relationship between Australian *Eurema* butterfly species and their associated *Wolbachia* strain types (STs) as listed below each species. Green plus signs below a species indicates the species is infected with *Wolbachia* and the red minus sign indicates the species does not carry *Wolbachia*.

Building on molecular phylogenetic analysis suggesting horizontal transmission between divergent *Eurema* species (Kern et al. unpublished), I predicted that parasitoids

and butterfly hosts would share *Wolbachia* strains, which would bolster support for the hypothesis that host-parasitoid interactions are probable routes for interspecies *Wolbachia* transmission. Results from this research provide the first characterization of the parasitoids associated with *Eurema* butterflies, estimates of parasitization rates in *E. hecabe*, and the presence of shared *Wolbachia* strains between host and parasitoids. Additionally, I provide the first empirical field evidence demonstrating inter-ordinal sharing of identical *Wolbachia* strains in species that engage in close ecological interactions.

MATERIALS AND METHODS

Eurema and parasitoid collection and identification

Eurema butterfly larvae were collected from host plants in four locations in northern Queensland, Australia, between March and April 2015 (Table 2.1). Larvae and host plant were transported to the laboratory where they were reared separately in individual translucent 60ml plastic containers (Polar Ice PIJS040200). I examined all collected larvae for signs of parasitization, indicated by the presence of melanization, resulting from the encapsulation of the parasitoid egg by the host's immune response. As parasitoid larvae exited hosts, they were isolated in fresh 60ml containers where they were allowed to pupate. Dead hosts, either larvae or pupae, and newly emerged parasitoids were stored in absolute ethanol. Ethanol preserved samples were transferred to the laboratory and stored at -20°C. Larvae that were not affected by parasitoids and developed in adult butterflies were preserved in specimen envelopes. Parasitoids were then assigned to morphotypes.

Table 2.1: Australia field collection site locations, coordinates, and altitudes.

Site	Location	Latitude	Longitude	Altitude (m)
Cairns	Machan Beach Rd., Machans Beach QLD 4878	-16.86282	145.732963	2
Mt. Sophia	69727 Bruce HWY, Aloomba QLD 4861	-17.161521	145.874346	14
Babinda	Clyde Rd., Babinda QLD 4861	-17.335391	145.93606	8
Mareeba Wetlands	Pickford Rd., Biboohra QLD 4880	-16.91913	145.404252	387

DNA extraction

To eliminate foreign DNA and microbes from external surfaces of parasitoids and larvae or pupae hosts, prior to DNA extraction, individual specimens were rinsed five times with molecular grade water and five times with absolute ethanol before being transferred to sterile microcentrifuge tubes that contained absolute ethanol. DNA was extracted using a small tissue sample (2-5mm^3) from individual *Eurema* larvae and their adult parasitoids. Three *Eurema* larvae from which parasitoids emerged survived and developed into adult butterflies. I extracted DNA from the three parasitoid surviving adults and from a subsample of parasitoid-free adult butterflies. To avoid false negatives in the *Wolbachia* screen that can sometimes occur when using DNA extracted from legs (Duron et al. 2010), I used abdomen tissue samples from adult specimens. Tissues were homogenized in 1.5ml microcentrifuge tubes using microtube pestles (Scientific Specialties Inc., Lodi, CA). All DNA extractions were performed using Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. I tested DNA quality by PCR amplification of arthropod 28S rDNA (Werren et

al. 1995a; Table 2.2). For samples that yielded weak or no amplification of the 28S rDNA fragment (N = 8 *Eurema* larvae and N = 2 parasitoids), DNA was extracted for a second time. The second round of DNA extractions were all successful and no subsequent extraction were needed.

Table 2.2: List of primers used for PCR amplification of loci, targeted genome, and references.

Loci	Primers	Target genomes	Sequences (5'-3')	References
16S rDNA	16SW spec F	Mitochondria	CATACCTATTCGAAGGGATAG	Werren & Windsor 2000
	16SW spec R		AGCTTCGAGTGAAACCAATTC	
28S rDNA	28SF	Nuclear	CCCTGTTGAGCTTGACTCTAGTCTGGC	Werren et al. 1995a
	28SR		AAGAGCCGACATCGAAGGATC	
COI	LCO1490	Mitochondria	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
	HCO2198		TAAACTTTCAGGGTGACAAAAAATCA	
wsp	81F	*Wolbachia*	TGGTCCAATAAGTGATGAAGAAAC	Zhou et al. 1998
	691R		AAAAATTAAACGCTACTCCA	
gatB	gatB F1	*Wolbachia*	GAKTTAAAYCGYGCAGGBGTT	Baldo et al. 2006
	gatB R1		TGGYAAYTCRGGYAAAGATGA	
coxA	coxA F1	*Wolbachia*	TTGGRGCRATYAACTTTATAG	Baldo et al. 2006
	coxA R1		CTAAAGACTTTKACRCCAGT	
hcpA	hcpA F1	*Wolbachia*	GAAATARCAGTTGCTGCAAA	Baldo et al. 2006
	hcpA R1		GAAAGTYRAGCAAGYTCTG	
ftsZ	ftsZ F1	*Wolbachia*	ATYATGGARCATATAAARGATAG	Baldo et al. 2006
	ftsZ R1		TCRAGYAATGGATTRGATAT	
fbpA	fbpA F1	*Wolbachia*	GCTGCTCCRCTTGGYWTGAT	Baldo et al. 2006
	fbpA R1		CCRCCAGARAAAAYYACTATTC	

Detection and identification *Wolbachia*

  Wolbachia experience high rates of recombination; thus, to avoid the

misidentification of *Wolbachia* strains I used a multi-gene protocol rather than a single

gene approach (Baldo et al. 2006). An initial diagnostic *Wolbachia* screen of individual

samples was conducted by PCR amplification using *Wolbachia* specific 16S rDNA

(Werren & Windsor 2000) and *wsp* primers (Zhou et al. 1998). For characterization of *Wolbachia* strains, I performed PCR amplification of five *Wolbachia* MLST loci - *gatB, coxA, ftsZ, hcpA,* and *fbpA*. All polymerase chain reactions were performed using a Bio-Rad T100™ thermocycler (Hercules, CA. USA). Primers are listed in Table 2.2.

To determine whether *Wolbachia* was present on the external surfaces of parasitoids, I tested for the presence of *Wolbachia* DNA in the ethanol in which the specimens were stored using direct PCR amplification of arthropod 28S rDNA and *Wolbachia* specific 16S rDNA primer sets (Table 2.2).

I used PCR to amplify the mitochondrial cytochrome oxidase I subunit (*COI*) loci from all individuals with the primer set LCO1490 and HCO2198 (Folmer et al. 1994) to estimate haplotype diversity of both parasitoids and *Eurema* butterflies. *Eurema* species identity was then established with the help of existing DNA barcodes for Australian *Eurema* species.

Polymerase chain reactions were each a total of 25µl comprised of 1.25µl of each 10µM forward and reverse primer, 12.5µl of 2X Phusion High-Fidelity PCR Master Mix (New England BioLabs Inc.), 2-4µl template DNA, and nuclease-free water to 25µl. To confirm that PCR products were the same size as target genes and to determine whether multiple bands were present, I visualized 5µl of each on a 1% agarose gel. The remaining 20µl of crude PCR products were sent to Functional Biosciences (Madison, WI) for sequencing. Specific annealing temperatures and thermocycler conditions for the amplification of each gene are listed in Tables 2.3 and 2.4.

Table 2.3: Annealing temperatures specific to primers sets.

Loci and primers	Primer specific annealing temperatures (T)
16S rDNA - 16SWspecF/16SWspecR	T = 60°C
28S rDNA - 28SF/28SR	T = 56°C
COI - LCO1490/HCO2198	T1 = 45°C T2 = 51°C
wsp - 81F/691R	59°C
gatB - gatB F1/gatB R1	56°C
coxA - coxA F1/coxA R1	55°C
hcpA - hcpA F1/hcpA R1	53°C
ftsZ - ftsZ F1/ ftsZ R1	54°C
fbpA - fbpA F1/fbpA R1	56.5°C

Table 2.4: Thermocycler conditions for PCR of each loci.

	Wolbachia MLST loci *wsp, gatB, coxA, hcpA, ftsZ,* and *fbpA*	Mitochondrial loci *16S* rDNA and *COI*	Nuclear loci *28S* rDNA
Denaturation	98°C for 2 min	98°C for 4 min	98°C for 2 min
Cycling	35 cycles: 98°C for 30 sec, **loci T** for 30 sec, and 72°C for 2 min	5 cycles: 98°C for 2 min, *16S* **T** or *COI* **T1** for 1 min, and 72°C for 1 min 35 cycles: 95°C for 30 sec, *16S* **T** or *COI* **T2** for 45 sec, and 72°C for 1 min	1 cycle: 98°C for 1 min, *28S* **T** for 1 min, and 72°C for 2 min 35 cycles: 98°C for 15 sec, *28S* **T** for 1min, and 72°C for 2 min 1 cycle: 94°C for 15 sec, *28S* **T** for 1 min
Final elongation	72°C for 2 min	72°C for 10 min	72°C for 7 min

Phylogenetic analysis

I carefully screened sequence chromatograms of the *coxA*, *fbpA, ftsZ, gatB,* and *hcpA* genes for ambiguities to exclude the presence of multiple infections. Sequences were then edited manually and trimmed using Geneious 3 8.1.8 software (Kearse et al. 2012). I determined DNA sequences by BLAST analysis and compared *Wolbachia* sequences to the *Wolbachia* MLST database (http://pubmlst.org/wolbachia). Reference species MLST profiles were retrieved from the database and aligned with sequences obtained in this study using the MUSCLE algorithm within the Geneious 3 8.1.8 software (Kearse et al. 2012). I determined pairwise genetic distances between different *Wolbachia* strains using the Tamura 3-parameter model in Mega 7.0.21 (Kumar et al. 2016).

Sequence chromatograms of the *COI* gene were edited manually and trimmed using Geneious 3 8.1.8 software (Kumar et al. 2016). I then used BLAST query analysis to compare obtained sequences against the NCBI database. Reference sequences were retrieved from GenBank and aligned with my sequences using the MUSCLE algorithm within Geneious 3 8.1.8 software (Kearse et al. 2012). The pairwise genetic distances between different *COI* sequences were calculated using Kimura 2 parameter model in Mega 7.0.21 (Kumar et al. 2016).

The substitution models with the lowest BIC values were selected from Find Best DNA Model using Mega 7.0.21 (Kumar et al. 2016) for phylogenetic analysis of MLST and *COI*. Maximum Likelihood phylogenetic analyses were performed and tree topology robustness was determined by non-parametric bootstrapping with 1000 iterations. The MLST profile ST-35 from *Bruglia malayi* was used as an outgroup to root the *Wolbachia*

MLST tree. The parasitoid *COI* gene tree was rooted with *Nasonia vistripennis* and *Eurema COI* gene tree was rooted with *Colias erate.*

RESULTS

In total, I collected 404 larvae from *Eurema* host plants across four sites. Collectively, these larvae yielded a combined 24 fly and wasp parasitoids, which I characterized and screened for *Wolbachia* along with the *Eurema* hosts from which they emerged (Table 2.5). Results from *COI* gene analysis revealed all larvae with parasitoids and a subsample of adult butterflies that did not yield parasitoids were *E. hecabe* (Figure 2.2). *Eurema hecabe* had a 5.9% rate of parasitization. Apart from three larvae that survived, all larvae from which parasitoids emerged died. Of the larvae that survived, wasps emerged from two and a fly emerged from one. In 16 other *E. hecabe* larvae, I detected evidence of the host's immune response as indicated by melanization. Since no parasitoids emerged from these 16 larvae and they all developed into adult butterflies, I assumed they survived parasitization by killing the parasitoid. An additional surprising result was the discovery of a parasitoid moth species that developed inside a butterfly pupa. The parasitoid moth was not included in the analyses.

Table 2.5: Lists the number of larvae and parasitoids collected at each site.

Site	Number of *Eurema* larvae collected	Number of parasitoids	% larvae with parasitoids
Cairns	90	7	7.8
Mt. Sophia	78	4	5.1
Babinda	109	2	1.8
Mareeba Wetlands	127	11	8.7

E 549 MW *
E. hecabe 4
62
E 551 MW *
E. hecabe 2
E. alitha 4
E 495 MtS *
E 31 MtS
E 46 BC *
E 73 BC *
50
E 202 CM *
E 452 MW *
E 526 MW *
E 527 MW *
E 498 MtS
65
E 538 MW *
E 505 MtS
E. hecabe 3
E. hecabe 5
99
E. mandarina
KF881051.1 Lethe albolineata
27
E 1 CM *
97
E. hecabe
E. alitha 3
E. alitha
100
84
94
E. alitha 2
E. smiulatrix
28
E. laeta
99
E. laeta 2
65
E. brigitta 3
E. brigitta
100
57
E. brigitta 2
37
E. herla
E. herla 3
99
E. herla 2
49
E. herla 4
E. smilax 3
E. smilax
99
47
E. smilax 2
JQ922063.1 Colias erate

0.02

Figure 2.2: Inferred phylogenetic tree based on COI sequence fragments (544bp) of *Eurema* constructed by maximum likelihood method (Model T92+G). *Eurema hecabe* samples from this study are shaded in grey boxes, with dark grey boxes highlight *E. hecabe* samples collected at Mt. Sophia. Outgroups from other *Eurema* species were retrieved from the NCBI database (accession numbers not listed here are listed in Appendix C). Numbers at nodes represent bootstrap values (1000 replicates) > 50 % and scale bar represents number of nucleotide substitutions per site.

Parasitoids were morphologically grouped into fly or wasp morphotypes. Matches based on *COI* sequence BLAST searches indicated that the parasitoid wasp species were *Miroplitis demolitor* (N=1) and *Cotesia congregate* (N=1)*, and two species of flies, *Exorista cantans* (N=18) and *Senometopia cinerea* (N=1)*. According to phylogenetic analysis, the remaining three flies belonged to the *Exorista* genus (Figure 2.3). The two wasps, *M. demolitor, C. congregata,* and unique fly species, *S. cinerea,* were collected from the same location, Mt. Sophia (Figure 2.3).
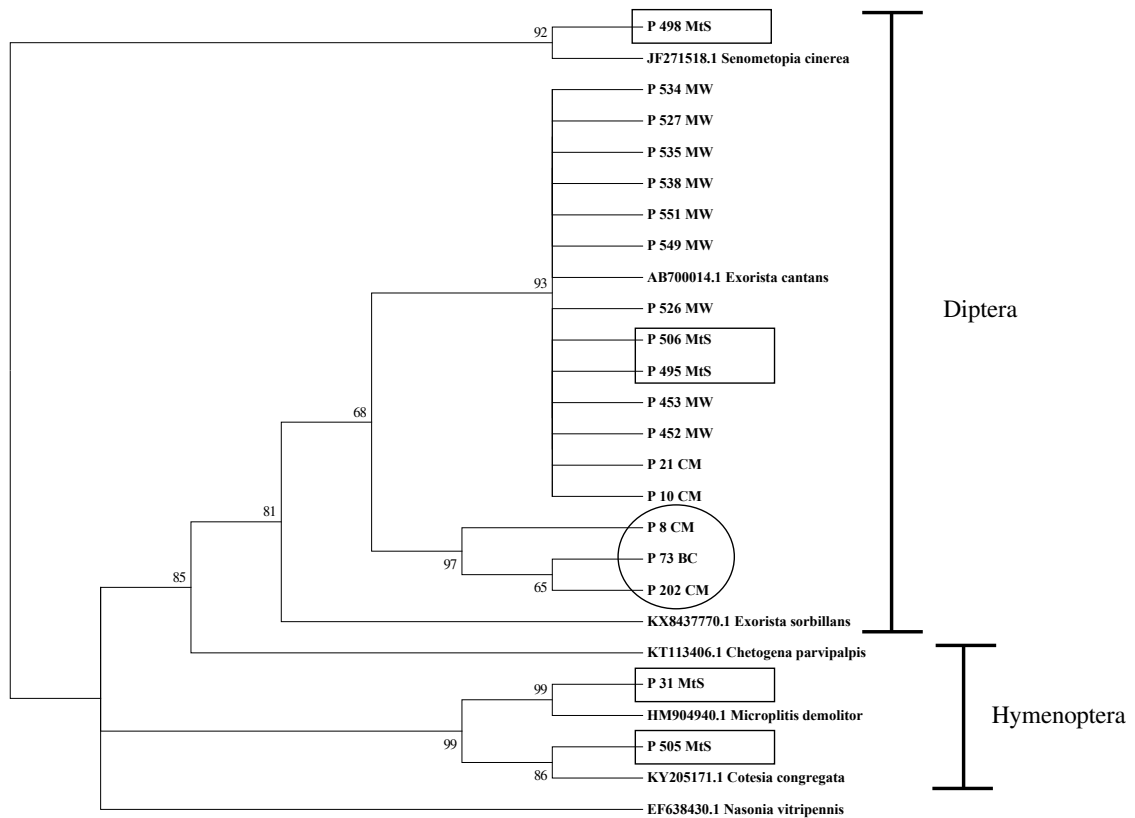
Figure 2.3: Phylogenetic tree based on COI sequence fragments (523bp) constructed by maximum likelihood method (Model T93+G+I). Parasitoid samples from this study are shaded in grey boxes, with dark grey boxes highlighting parasitoids collected from Mt. Sophia. Species identified from *COI* sequence BLAST queries were retrieved from the NCBI database and are listed by name and accession number Numbers at nodes represent bootstrap values (1000 replicates) > 50 %.
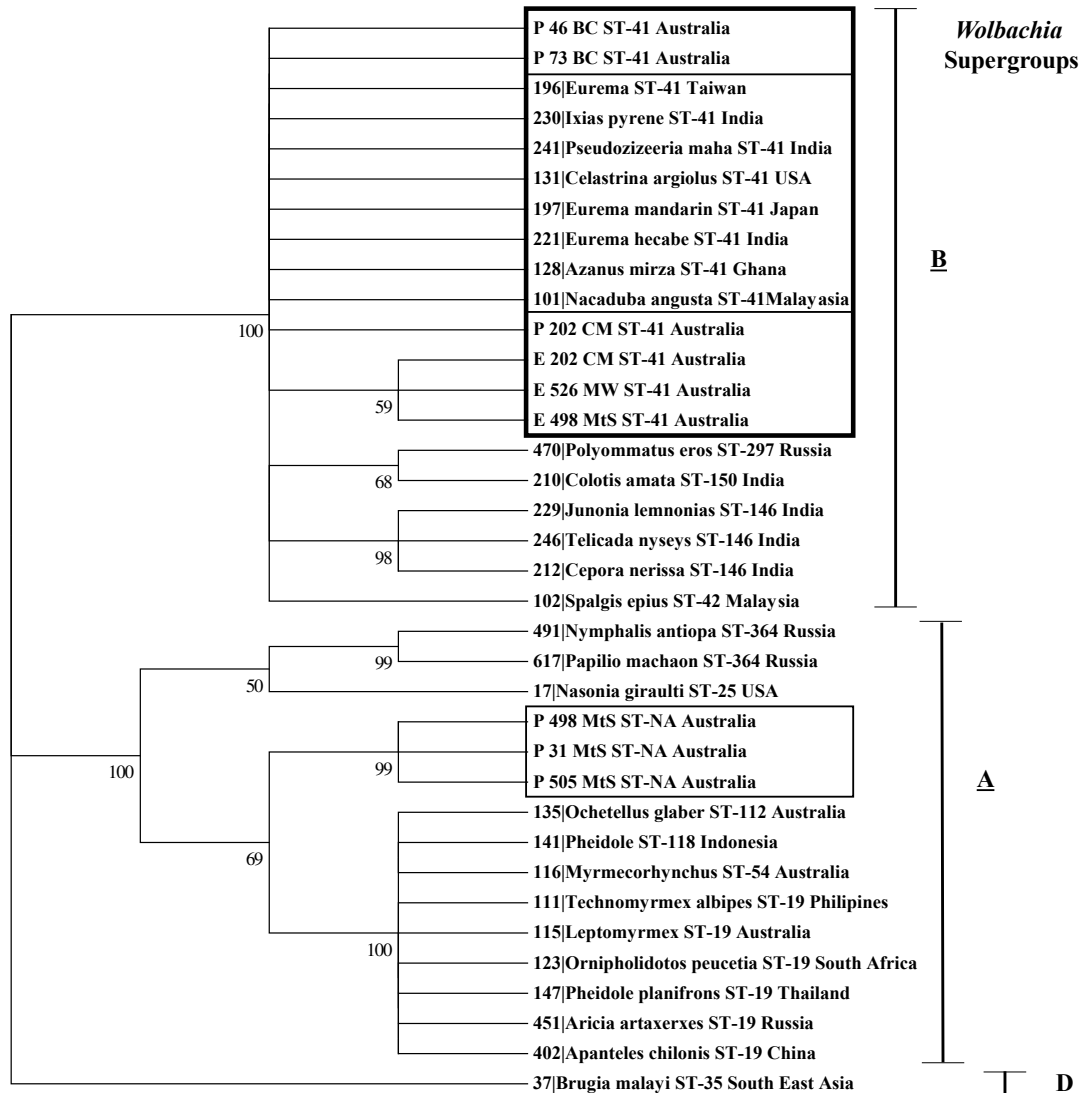
Figure 2.4: Phylogenetic tree based on concatenated MLST genes (2079bp) constructed by maximum likelihood method (Model T93+G). Outgroups from other known *Wolbachia* ST and supergroups, listed by ID, host name, and sequence type, were retrieved from the *Wolbachia* MLST database. Host names of sequences isolate from this study are delineated by grey boxes. Numbers at nodes represent bootstrap values (1000 replicates) > 50 % and letters indicate *Wolbachia* supergroups.

All 24 parasitoids and 24 hosts were positive for *Wolbachia* infection. Direct sequencing of marker genes from the *Wolbachia* MLST complex produced clear chromatograms and complete profiles for each *Eurema* specimen and for all but one parasitoid specimen, which was missing sequence data for the *fbpA* locus. Overall, two MLST profiles were found, one complete and one that was incomplete. The MLST profiles revealed that all 24 *E. hecabe* host larvae and 20 of the 24 emerged parasitoids were infected with a *Wolbachia* strain designated to the sequence type (ST) ST-41. This *Wolbachia* strain, *w*CI, has been previously identified in both Australian and Japanese *Eurema* species (Table 2.6 and 2.7) (Narita et al. 2006; Kern et al. unpublished). Interestingly, alleles from the incomplete MLST profile match to STs from both supergroup A and B, which could indicate presence of a double infection (Table 2.8, Figure 2.4).

Table 2.6: Results showing alleles from PCR and sequencing analysis of the five *Wolbachia* MLST loci from each parasitoid collected. Parasitoids morphotypes are listed as: D stands for Diptera and H for Hymenoptera, D1 = Tachinid fly (N = 17), D2 = Tachinid unique from the others (N = 3), H1 = small Braconids, larvae yielded six small wasps, but they were pooled for DNA extraction (N = 1), and H2 = large Braconid (N = 1). Parasitoids from Mt. Sophia are highlighted in grey. Samples that had unique strains are listed in bold text.

Parasitoid	Morphotype	Site	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*	ST	Supergroup
1	D1	CM	39	14	40	36	4	41	B
C9	D1	BC	39	14	40	36	4	41	B
8	D1	CN	39	14	40	36	4	41	B
10	D1	CM	39	14	40	36	4	41	B
21	D1	CM	39	14	40	36	4	41	B
31	**H1**	**MtS**	**89**	**1**	**1**	**3**	**1**	**New**	**A**
46	D1	BC	39	14	40	36	4	41	B
73	D1	BC	39	14	40	36	4	41	B
202	D1	CM	39	14	40	36	4	41	B
452	D1	MW	39	14	40	36	4	41	B
453	D1	MW	39	14	40	36	4	41	B
458	D1	MW	39	14	40	36	4	41	B
495	D1	MtS	87	9	6	70	n/a	n/a	n/a
498	**D2**	**MtS**	**89**	**1**	**1**	**3**	**1**	**New**	**A**
505	**H2**	**MtS**	**89**	**1**	**1**	**3**	**1**	**New**	**A**
506	D1	MtS	39	14	40	36	4	41	B
526	D1	MW	39	14	40	36	4	41	B
527	D1	MW	39	14	40	36	4	41	B
538	D1	MW	39	14	40	36	4	41	B
549	D1	MW	39	14	40	36	4	41	B
551	D1	MW	39	14	40	36	4	41	B
552	D1	MW	39	14	40	36	4	41	B

Table 2.7: Allelic profiles at the five *Wolbachia* MLST loci from each *Eurema* butterfly tested. Butterflies collected at Mt. Sophia are highlighted in grey. The life stage listed for each *Eurema* is the stage from which we sampled tissues for DNA extractions. *Eurema* that survived adult the stage are listed in bold text.

Eurema	Life stage died	Site	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*	ST	Supergroup
1	Pupal	CM	39	14	40	36	4	41	B
C9	**Adult**	**BC**	**39**	**14**	**40**	**36**	**4**	**41**	**B**
8	Pupal	CM	39	14	40	36	4	41	B
10	Pupal	CM	39	14	40	36	4	41	B
21	Pupal	CM	39	14	40	36	4	41	B
31	**Adult**	**MtS**	**39**	**14**	**40**	**36**	**4**	**41**	**B**
46	Pupal	BC	39	14	40	36	4	41	B
73	Pupal	BC	39	14	40	36	4	41	B
202	Pupal	CM	39	14	40	36	4	41	B
452	Pupal	MW	39	14	40	36	4	41	B
453	Pupal	MW	39	14	40	36	4	41	B
458	Pupal	MW	39	14	40	36	4	41	B
495	Pupal	MtS	39	14	40	36	4	41	B
498	Pupal	MtS	39	14	40	36	4	41	B
505	**Adult**	**MtS**	**39**	**14**	**40**	**36**	**4**	**41**	**B**
506	Pupal	MtS	39	14	40	36	4	41	B
526	Pupal	MW	39	14	40	36	4	41	B
527	Pupal	MW	39	14	40	36	4	41	B
538	Pupal	MW	39	14	40	36	4	41	B
549	Pupal	MW	39	14	40	36	4	41	B
551	Pupal	MW	39	14	40	36	4	41	B
552	Pupal	MW	39	14	40	36	4	41	B

*Table 2.8***:** Results from BLAST search using COI sequences of parasitoids. Species listed were the closest

match in the NCBI database where N indicates the number of samples.

COI BLAST search closest species match	Site	Strain	N	Query coverage %	e-value	Indentity %
Microplitis demolitor	Mt. Sophia	New	1	100	0.0	99
Senometopia cinerea	Mt. Sophia	New	1	99	0.0	96
Cotesia congregata	Mt. Sophia	New	1	99	0.0	98
Exorista cantans	Mareeba Wetlands	ST-41	17	100	0.0	98
	Cairns					
	Babinda					
Exorista sp.	Mareeba Wetlands	ST-41	3	100	0.0	95
	Cairns					
	Babinda					

DISCUSSION

Horizontal transmission of *Wolbachia* has been largely inferred from

phylogenetic analyses that provide indirect evidence consistent with such transmission

(Haine et al. 2005; Sintupachee et al. 2006; Ahmed et al. 2016). Recent ecological studies

have revealed identical *Wolbachia* strains in multiple species among two different genera

belonging to the same order (Morrow et al. 2014; Schuler et al. 2016). Results presented

here are the first, to my knowledge, to identify a shared identical *Wolbachia* strain (*w*CI)

among a Lepidoptera host, *E. hecabe*, and Diptera parasitoids from the genus *Exorista*.

This finding indicates that the *Wolbachia* strain is shared across species within the genus.

Frequent occurrence of shared *Wolbachia* strains among host and parasitoid strongly

suggests that horizontal transmission was paramount to the successful invasion of

Wolbachia in this group of butterflies.

The sequence type I identified in both parasitoids and *E. hecabe*, ST-41, is especially common in Lepidoptera with 34 of the 38 ST-41 isolates in the MLST database assigned to Lepidoptera species from all continents apart from Europe. A previous study suggests evidence for inter-ordinal transfer of ST-41, ST-19, and ST-37 *Wolbachia* (Ahmed et al. 2016). However, the evidence is based on identification of STs among species belonging to different orders from different parts of the world. Although an important finding, they did not find species of multiple orders at the same location sharing STs.

Because I tested parasitoids that emerged from infected *E. hecabe* for *Wolbachia*, in theory there are perhaps three reasons why they all tested positive: 1) they all carry heritable infections, meaning that *Wolbachia* reside in the parasitoids' germline cells, 2) they could all carry infections that are not heritable and *Wolbachia* is localized in somatic tissues, and 3) they are not infected at all and I detected *Wolbachia* DNA carryover or contamination. To determine which of these infection scenarios is true for parasitoids of *E. hecabe* requires laboratory experiments and testing several generations of offspring from parasitoids for *Wolbachia* infection. It is feasible that *Wolbachia* infections in these parasitoids are non-heritable and reside in somatic tissues. A laboratory embryo micro-injection study detected positive adult parasitoids emerging from the embryos, but their offspring were uninfected (Zabalou et al. 2004). I postulate that if the parasitoids have somatic infections, such infections could be a starting point for *Wolbachia*-spillover and, consequently, potentially establish inheritable infection in new lineages.

Although non-specific DNA was not detected in DNA extractions from either host or parasitoid, I am not able to eliminate the possibility that parasitoids were exposed

to *Wolbachia* during development. I took several precautions to avoid contamination between parasitoids and *Eurema* during every stage of this investigation. Evidence that my results are not an artifact from contamination include 1) *COI* gene amplifications were specific and there was no detection of non-target DNA and 2) I detected unique strains from four parasitoid DNA extractions that were not detected in host *E. hecabe* DNA extractions.

Larvae and pupae have developed immune responses to defend themselves against different parasites and parasitoids (Altizer & de Roode 2010). Clusters of immune cells called hemocytes adhere to parasitoid eggs, encapsulating them with the deposition of melanin pigment, in an attempt to kill them by asphyxiation (Altizer et al. 2010). Melanization of parasitoids in *Eurema* butterflies is easily visible and indicates the invasion and presence of one or more endoparasitoids. Thus, to estimate *Eurema* survival from parasitoids, I screened *Eurema* larvae for melanization and recorded whether they survived to the adult stage. In total, I found that 19 *E. hecabe* in this study survived parasitization. I postulate that when a larva launches an immune response that kills the parasitoid and emerges as an adult butterfly, there may be an opportunity for horizontal transmission of *Wolbachia* from an infected parasitoid to an uninfected larva host. Acquisition of *Wolbachia* by a larva could occur by *Wolbachia* escaping the dead parasitoid cells and migrating to the larva's germ line cells, which are responsible for *Wolbachia*'s vertical transmission (Van Meer & Stouthamer 1999; Huigens et al. 2004; Frydman et al. 2006). Conversely, a parasitoid could potentially acquire *Wolbachia* during development inside a larva. This is a probable route of passage for *Wolbachia* from a larva to a parasitoid because parasitoid development depends exclusively on

consumption of host tissue. Once inside of the parasitoid digestive tract, *Wolbachia* has the potential to migrate into parasitoid germ lines and be passed to the next generation (Frydman et al. 2006).

Approximately 10,000 described parasitoid species worldwide comprise the Tachinidae family, the most speciose family of Diptera. Though morphologically diverse, many tachinids are parasitoids of caterpillars (Irwin et al. 2003). While seemingly immense, the Tachnidae are all but eclipsed by the Bracondidae family (Hymenoptera) consisting of up to 45,000 species of parasitoid wasps (Jones et al. 2009). The substantial biodiversity of parasitoids poses many challenges to species classification and identification, as only approximately 1% of the species can be identified using *COI* barcoding methods (Godfray 1994). Consequently, the extent to which parasitoids vector *Wolbachia* between closely or distantly related taxa is difficult to assess or even predict.

Furthermore, I discovered that three of the five *E. hecabe* collected from Mt. Sophia had unique parasitoids that shared an identical *Wolbachia* strain, which is unique from the *w*CI strain I found in *E. hecabe* and parasitoid *Exorista* species. Parasitoids, especially tachinid flies, have been widely accepted as generalists, parasitizing multiple host species (Stireman et al. 2005; Stireman et al. 2006). However, host-specificity among parasitoids is widely debated, may vary between geographic regions, and may depend on the presence or absence of available host species at a given time (Stireman et al. 2005; Smith et al. 2006; Stireman et al. 2006). Mount Sophia may be void of other hosts of the three parasitoids species and *E. hecabe* larvae were available. The variables affecting host-specificity might interfere with predictive accuracy on host-parasitoid *Wolbachia* horizontal transmission events as generalist parasitoids have more

opportunities to both expose novel hosts to and be exposed to the endosymbiont.

Climate change may lead to increased parasitization as temperate regions warm, subsequently spreading *Wolbachia* to novel hosts (Ahmed et al. 2016). Some strains of *Wolbachia* induce CI and can lead to decreased host genetic diversity (Hurst & Jiggins 2005). Hence, understanding the mechanisms of horizontal transmission will give researchers better predictive power and ability to inform management decisions for vulnerable insect species.

Finally, while the original aim of this study was to collect multiple *Eurema* species to investigate whether parasitoids among multiple species share identical *Wolbachia* strains, I was successful only in collecting *E. hecabe.* It is possible that I was unsuccessful in collecting multiple *Eurema* species because *E. hecabe* is extremely prevalent in the region where I collected larvae and have less restricted geographic distribution compared to some of the other *Eurema* species. In addition, morphological identification of different *Eurema* species is impossible at the larval stage and I was not able to discern species at time of collection. This study of a host-parasitoid system presents a platform for further investigations into the mechanisms of *Wolbachia* horizontal transmission. Future studies should include a more exhaustive and long-term survey of *Eurema* larvae and their parasitoid communities, followed with multi-generational laboratory experiments to show interspecies transmission.

Chapter 3: Chill out: *Wolbachia* associated cooling off of *Drosophila melanogaster* in thermal gradient temperature preference choice assays

INTRODUCTION

Endosymbiotic bacteria interact with insects in multifarious ways, including altering the reproductive phenotype of their hosts to enhance their own transmission (Werren et al. 2008). One such group endosymbionts are extremely widespread, infecting approximately 53% of arthropod species and belong to the genus *Wolbachia* (Zug & Hammerstein 2012; Weinert et al. 2015; Sazama et al. 2017).

Wolbachia have garnered extensive interest due to the reproductive manipulations they inflict on their hosts: parthenogenesis, male killing, feminization, and cytoplasmic incompatibility (reviewed by Werren et al. 2008). The relationships they form with their hosts are diverse and not all *Wolbachia* are master manipulators. For example, some strains are obligate mutualists while others form facultative relationships (Miller 2013; Zug & Hammerstein 2014). There are *Wolbachia* strains that increase fecundity to female hosts (Miller et al. 2010) and strains that provide their hosts with protection against viruses (Hedges et al. 2008; Teixeira et al. 2008; Martinez et al. 2014). Additionally, *Wolbachia*-induced cytoplasmic incompatibility (CI) can promote mate discrimination (Miller et al. 2010; Buellesbach et al. 2014), which can lead to reproductive isolation - one of the key driving forces of speciation (Coyne & Orr 2004).

All terrestrial insects infected with *Wolbachia* are ectotherms that seek external sources of heat, cold, or shelter to maintain their body temperature within a thermoregulatory range (Angilletta 2009). In addition, *Wolbachia'*s replication behavior is positively temperature-dependent (Hoffmann et al. 1990; Reynolds et al. 2003; Mouton

et al. 2006; Mouton et al. 2007; Bordenstein & Bordenstein 2011; Correa & Ballard 2012; Chrostek et al. 2013; Strunov et al. 2013a; Strunov et al. 2013b; Murdock et al. 2014; Versace et al. 2014). Host-symbiont conflict could arise due to the disparities between the physiological requirements of *Wolbachia* and those of their hosts. For example, some insects induce behavioral fever (Louis et al. 1986) or behavioral chill (Fedorka et al. 2016) as an immune strategy to fight pathogen infection. Conversely, some bacterial symbionts are known to alter their host's thermal tolerance range (Russell & Moran 2006; Dunbar et al. 2007; reviewed by Wernegreen 2012).

Whether host or symbiont, an organism's ability to remain within its upper and lower thermal tolerance limits is paramount to its survival (Huey & Berrigan 2001; Angilletta et al. 2004; Hoffmann 2010; Huey et al. 2012). Temperature preference can vary between species within a genus, or among populations of a species that have been exposed to different biological factors, including pathogens (Matute et al. 2009). Research has shown that geography, elevation, and genetic background can influence *D. melanogaster* environmental temperature preferences (Martin & Huey 2008; Hoffmann & Sgrò 2011; Huey et al. 2012; Rajpurohit & Schmidt 2016). In addition, research examining temperature effects on *Wolbachia* have found that replication of some strains increases at warmer temperatures (Mouton et al. 2006; Chrostek et al. 2013; Strunov et al. 2013). However, despite widespread and diverse *Wolbachia* infections in *Drosophila* species on every continent, no studies have explicitly addressed *Wolbachia*-dependent temperature preference in *Drosophila.* Thus, I conducted a laboratory experiment to determine whether *Wolbachia* affects the environmental temperature preference of *D. melanogaster.*

Drosophila melanogaster is infected with several closely related genetic variants of the *Wolbachia* strain *w*Mel: *w*Mel, *w*MelCS, and *w*MelPop (Riegler et al. 2005). All three variants cause weak CI and provide virus protection to varying degrees (Osborne et al. 2009; Chrostek et al. 2013). The *w*Mel-strain variants *w*Mel and *w*MelCS infect natural populations of *D. melanogaster,* whereas the *w*MelPop was isolated from a laboratory *Drosophila* stock during a survey for genetic mutations and is not found in natural populations (Min & Benzer 1997; Riegler et al. 2005; Richardson et al. 2012). The *w*MelPop variant is considered a unique example of a pathogenic symbiont (Min & Benzer 1997). The life-span of *D. melanogaster* infected with *w*MelPop is reduced two-fold as a result of the bacteria over-replicating in host tissues, such as the brain, retina, and muscles (Min & Benzer 1997; Strunov et al. 2013b). Additionally, at 25°C, *w*MelPop reaches densities in its hosts that are 20 times higher than *w*Mel and at least twice as high as *w*MelCS (Chrostek et al. 2013).

I performed this study to determine the effects of *Wolbachia* on *D. melanogaster* temperature preferences using a laboratory reared population. My goal was to determine whether *Wolbachia*-infected flies have different preferred temperatures compared to uninfected flies. In *D. melanogaster,* different genetic variants of *Wolbachia* are known to provide varying extents of anti-viral protection (Chrostek et al. 2013). In addition, temperature preference divergence could negatively affect insects of conservation concern if *Wolbachia*-infected and uninfected conspecifics adapt to different sets of environmental conditions. The resulting diverged adaptation could lead to reproductive isolation, ultimately reducing the overall fecundity of the population. Therefore, I wanted to know whether flies of the same genetic background infected with different genetic

variants of *Wolbachia*-strains had different temperature preferences. I hypothesized that *Wolbachia*-infected flies would prefer lower temperatures compared to uninfected flies as a response aimed at regulating or inhibiting *Wolbachia* replication. Additionally, I hypothesized that *w*MelPop infected flies would prefer a lower temperature compared to the other three *D. melanogaster*-*Wolbachia* combinations because *w*MelPop actively proliferates and is pathogenic at 25°C and warmer (Strunov et al. 2013b).

Here, I compare temperature preferences of the isogenic strain *D. melanogaster* infected by different *w*Mel strain variants – *w*Mel, *w*MelCS, and *w*MelPop – to temperature preferences of uninfected (w-) flies. I define temperature preference as the temperature along the gradient where the highest number of flies aggregate (Appendix B Figure B.1). I demonstrate that the temperature preference of *D. melanogaster* correlates with the presence or absence of *Wolbachia.* In addition, I show that a significant relationship exists between temperature preference of *D. melanogaster* and the *w*Mel-strain variant with which the flies are infected. This research elucidates fundamental ecological conflicts between host and symbiont that may arise in natural insect populations.

MATERIALS AND METHODS

To determine whether *D. melanogaster'*s environmental temperature preference varied with *Wolbachia* infection status and strain-type variant, I conducted a behavioral laboratory experiment using a thermal gradient apparatus (Appendix B Figure B.1). I used flies from each of four different infection scenarios: uninfected (w-) and flies infected with *w*Mel, *w*MelCS, or *w*MelPop. In each assay, I introduced 75-100 flies to the

thermal gradient chamber, allowed them to settle, and then recorded their preferred temperature distribution within the chamber. I performed seven replicates in a randomly selected order for each of these infection scenarios. I thoroughly cleaned the equipment before and after each run. In this section and in the appendices, I describe the fly lines used, a detailed description of the thermal gradient apparatus, and the method used for confirming *Wolbachia* infection and *w*Mel- strain variant.

Fly Lines

Wolbachia infected and uninfected *Drosophila melanogaster* ^{w1118} (mutant white-eyed) fly lines were kindly provided by Luis Teixeira and previously described by Chrostek *et al.* (2013). For all assays, I used *D. melanogaster* without *Wolbachia* (w-) as well as three genetic variants of the *Wolbachia w*Mel-strain, *w*Mel, wMelCS, and *w*MelPop all set in the w^{1118} isogenic background.

Flies were incubated at 25°C, in a 12:12 light - dark cycle with constant 45% humidity, and raised on *Drosophila* Formula 4-24® Instant Medium (Carolina®, NC) that was supplemented with fresh yeast. Vials contained approximately 30 flies each. Approximately equal numbers of male and female flies were used in each assay except for assays that explicitly addressed whether behavior was different between male and female flies.

Confirmation of fly *Wolbachia* strains

Genome sections that contain hypervariable regions or hypervariable regions and tandem repeats are used as genetic markers to differentiate *Wolbachia* strains and strain

variants (O'Neill et al. 1992; Werren et al. 1995; Zhou et al. 1998; Riegler et al. 2012).

To confirm *Wolbachia*-infection status, I performed diagnostic PCR amplification using primers for a gene that encodes the *Wolbachia* surface protein, *wsp* (Jeyaprakash & Hoy 2000), and for an intergenic region with 141bp tandem repeats, VNTR-141 loci (Riegler et al. 2005). The PCR reactions for *wsp* amplification were each a total of 10µl containing 2µl Promega 5x Green GoTaq buffer, 4mM Promega $MgCl_2$, 0.8µM of forward and reverse primers, 35µM of each dNTP, 0.04 U Promega GoTaq DNA Polymerase, and 1µl of genomic DNA template. Diagnostic VNTR-141 PCR reactions were each a total of 10µl comprised of the following: 2µl Promega 5x Green GoTaq buffer, 1.5mM Promega $MgCl_2$, 0.3µM of forward and reverse primers, 35µM of each dNTP, 0.04 U Promega GoTaq DNA Polymerase, and 1µl of genomic DNA template. Sequences and thermocycler conditions are listed in Tables 3.1 and 3.2. PCR products were visualized on a 1% agarose gel (Figure 3.1).
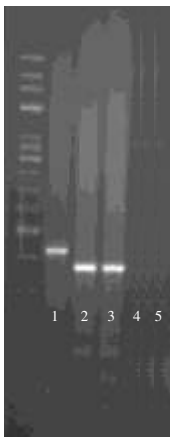
Figure 3.1: 1% agarose gel with 1kb DNA ladder on the far left and PCR amplification products of VNTR-141 loci from: *w*Mel in lane 1, *w*MelCS in lane 2, *w*MelPop in lane 3, w- in lane 4, and blank quality control in lane 5.

Table 3.1: List of primers used for PCR amplification of loci, targeted genome, and references.

Loci	Primers	Sequences (5'-3')	References
wsp	wsp-F	TGGTCCAATAAGTGATGAAGAAACTAGCTA	Jeyaprakash & Hoy 2000
	wsp-R	AAAAATTAAACGCTACTCCAGCTTCTGCAC	
VNTR-141	VNTR-F	GGAGTATTATTGATATGCG	Riegler et al. 2005
	VNTR-R	GACTAAAGGTTAGTTGCAT	

Table 3.2: Thermocycler conditions for PCR of each loci.

	wsp	**VNTR-141**
Denaturation	94°C for 2 min	94°C for 2 min
Cycling	10 cycles: 94°C for 10 sec, 65°C for 30 sec, and 68°C for 1 min 25 cycles: 94°C for 10 sec, 65°C for 30 sec, and 68°C for 1 min, plus an additional 20 sec added for every consecutive cycle	35 cycles: 94°C for 30 sec, 55°C for 1min, and 72°C for 1 min
Final elongation	68°C for 10 min	72°C for 10 min

Thermal gradient apparatus

Temperature preference assays were performed using a three-dimensional thermal gradient apparatus (adapted from Rajpurohit & Schmidt 2016; Appendix B Figure B.4). A temperature gradient was created along an aluminum rod (length 74.93cm, diameter 3.02cm; Part #R31-316 Metals Depot, Winchester, KY) that was encased within a 58.76cm long and 6.35cm inside diameter polycarbonate tube, creating an enclosed chamber allowing for three-dimensional movement. Constant voltage was applied to Peltier devices on each end of the aluminum rod to create a temperature gradient inside the thermal preference chamber. Temperatures along the gradient were measured at seven evenly spaced (8.39cm) points using K-type thermocouples and two four-channel thermocouple recorders. I recorded temperatures on the aluminum rod and inside polycarbonate tube surfaces (bottom, top, and mid-point between the top and bottom surfaces; Appendix B Figure B.5). The average temperatures from each thermocouple point on all surfaces from 18 different assays are depicted in Figure 3.2. Mean temperatures increased linearly and ranged from 12°C at the coldest point to 40°C at the hottest point of the aluminum rod, 58.76 cm distance (Figure 3.2). Along the aluminum rod, for every 4.2cm from cold to hot, the temperature increased 2°. Temperatures along each of the measured polycarbonate tube surfaces (bottom, mid-point, and top) increased 1°C every 4.2cm from cold to hot. The gradient reached thermal stability after approximately 20 minutes and remained stable for at least 3 hours. Assays were only conducted when the device had attained thermal stability.
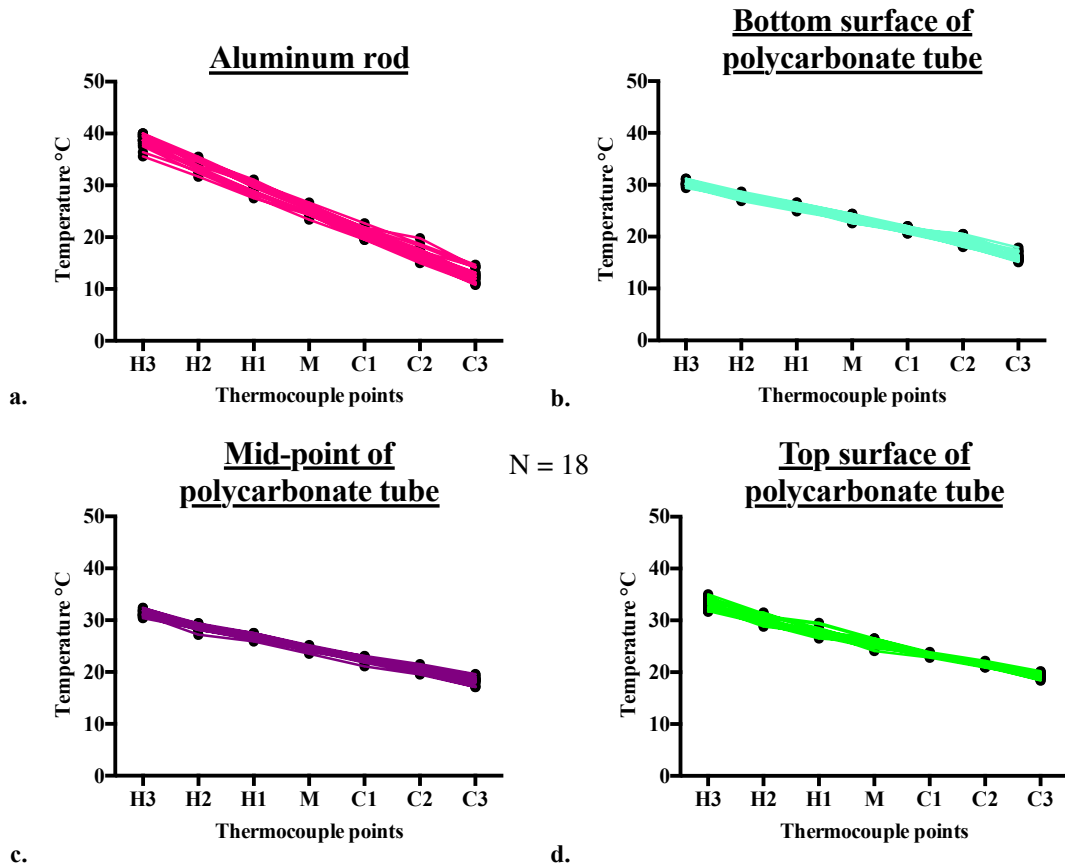
Figure 3.2: Plots showing linearity of temperature change for the different surfaces (a. aluminum rod, b. top, c. bottom, and mid-point of the polycarbonate tube) as measured with K-type thermocouples at regular intervals along the length of apparatus from the hottest end (H3) to the coldest (C3).

Thermal preference assays

All experiments were conducted in a room with a constant temperature of 24°C and constant 40% humidity. I determined the number of flies that represented a meaningful distribution along the thermal gradient without over-crowding in preferred temperature ranges to be 75-100 flies for each assay. Therefore, I used 75-100 flies that were aged 3-7 days old for each assay. Flies were introduced by aspiration into the temperature gradient chamber through a small hole located halfway along the top of the

polycarbonate tube, where the temperature averaged 25°C. Flies used for thermal

preference assays were never anesthetized because of the strong effects that CO_2 has on

Drosophila behavior. The assays ran for thirty minutes. Between assays, the temperature

gradient chamber was taken apart and thoroughly cleaned to avoid contamination from

any pheromone particles. All aluminum parts were cleaned using 95% ethanol. Because

ethanol and polycarbonate are chemically incompatible, the polycarbonate tube and end

caps were cleaned using hot water and soap, followed by a four-minute rinse with hot

water to ensure that surfaces were free of soap residue.

Data collection and analyses

Using three GoPro HERO3+ cameras, I collected data for each assay in the format

of digital images. To capture images of the entire thermal gradient and the flies within it,

I mounted the cameras above, lateral to, and below the apparatus, capturing images every

30 seconds for the duration of each treatment (30 minutes). Images were analyzed using

Adobe Photoshop CS6. All 60 images from each assay were reviewed, from which I

determined that A) the flies were highly active, retaining the ability to relocate as

necessary, for the entire assay, and B) after being introduced to the thermal gradient,

actively flew around for up to 15 mins before they settled on either the aluminum rod or

polycarbonate tube surfaces. Therefore, I selected images for analysis of fly distribution

at the 20-minute time point as representative of the 30-minute experiment. For each

assay, I manually counted flies and marked the location of flies on a custom grid that

delineated gradient surfaces and surface temperatures.

I confirmed that the data approximated a normal distribution using the

Kolmogrov-Smirnov test and homoscedastic variance using the Brown-Forsythe test. I performed a two-way ANOVA to examine the effects of *Wolbachia* infection status (w-, *w*Mel, *w*MelCS, and, *w*MelPop), temperature, and *Wolbachia* infection X temperature interaction on the preferred temperature of *D. melanogaster*. To identify significant pair-wise comparisons, I performed Tukey's multiple comparison post-hoc tests.

RESULTS

The effect of *Wolbachia* on temperature preference

Results from a two-way analysis of variance (ANOVA) elucidated that uninfected and *Wolbachia*-infected *D. melanogaster* had significantly different environmental temperature preferences (*Wolbachia*-infection status * temperature interaction effect $F_{21, 128}$ = 49.54, P < 0.0001; Table 3.3). Using Tukey's post-hoc pairwise comparison, I determined that for each *Wolbachia*-infection status the temperature at which the most flies landed had significantly more flies compared to the number of flies that landed at each of the other temperatures on the gradient. I concluded that the temperature at which the highest number flies settled, hereon called 'temperature preferences', for each *Wolbachia*-infection status was 26°C for w-, 24°C for *w*Mel-infected flies, and 18°C for both *w*MelCS- or *w*MelPop-infected *D. melanogaster* (P always <0.0001; Table 3.4, Appendix B Table B6, and Figure 3.3).

*Table 3.3***:** Results from two-way ANOVA examining the effects of *Wolbachia*-infection, strain-variant, and temperature on *D. melanogaster* temperature preference.

Two-way ANOVA table	SS	DF	MS	F(DFn, DFd)	P value
Wolbachia-strain variation	2.12E-05	3	7.05E-06	F(3,128) = 0.0051	P = 0.9995
Temperature	1.57	7	0.2243	F(7,128 = 163.3	P < 0.0001
Interaction	1.429	21	0.6803	F(21,128) = 49.54	P < 0.0001
Residual	0.1758	128	0.00137		

*Table 3.4***:** Results from Tukey's post hoc multiple comparison test comparing the temperature preference values (the temperature at which the *w*Mel- variant had the highest proportion of flies) to the proportion of flies at all the other available temperatures.

Temperature °C	w- 26°C	*w*Mel 24°C	*w*MelCS <18°C	*w*MelPop <18°C
18	P < 0.0001	P < 0.0001	x	x
20	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
22	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
24	P < 0.0001	x	P < 0.0001	P < 0.0001
26	x	P < 0.0001	P < 0.0001	P < 0.0001
28	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
30	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
32	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001

In addition, preferred temperatures for w- flies and *w*Mel-infected flies significantly differed from one another and from both *w*MelCS and *w*MelPop-infected

flies (Tukey's post hoc pairwise comparison P always <0.0001; Table 3.5). Finally,

temperature preferences from *w*MelCS and *w*MelPop-infected flies were not significantly

different (P >0.9999; Table 3.4. Figure 3.3).

Age (Appendix B Figure B.2) or sex of flies (Appendix B Figure B.3), time of

day, and orientation of the apparatus had no apparent influence on the flies' behavior or

choice of temperature preference.

***Table 3.5*:** Results from Tukey's post hoc multiple comparison test comparing the temperature preference
values of each *Wolbachia* infection treatment (w-(uninfected) *D. melanogaster* and *w*Mel, *w*MelCS, and
*w*MelPop-infected *D. melanogaster*). The temperature where most of the flies landed varied based on their
infection: the temperature preference of w- flies significantly differed from that of *w*Mel infected flies and
both were significantly different from those of *w*MelCS and *w*MelPop

	w- 26°C	***w*Mel 24°C**	***w*MelCS**
w- 26°C			
***w*Mel 24°C**	P < 0.0001		
***w*MelCS 18°C**	P < 0.0001	P < 0.0001	
***w*MelPop 18°C**	P < 0.0001	P < 0.0001	P > 0.9999
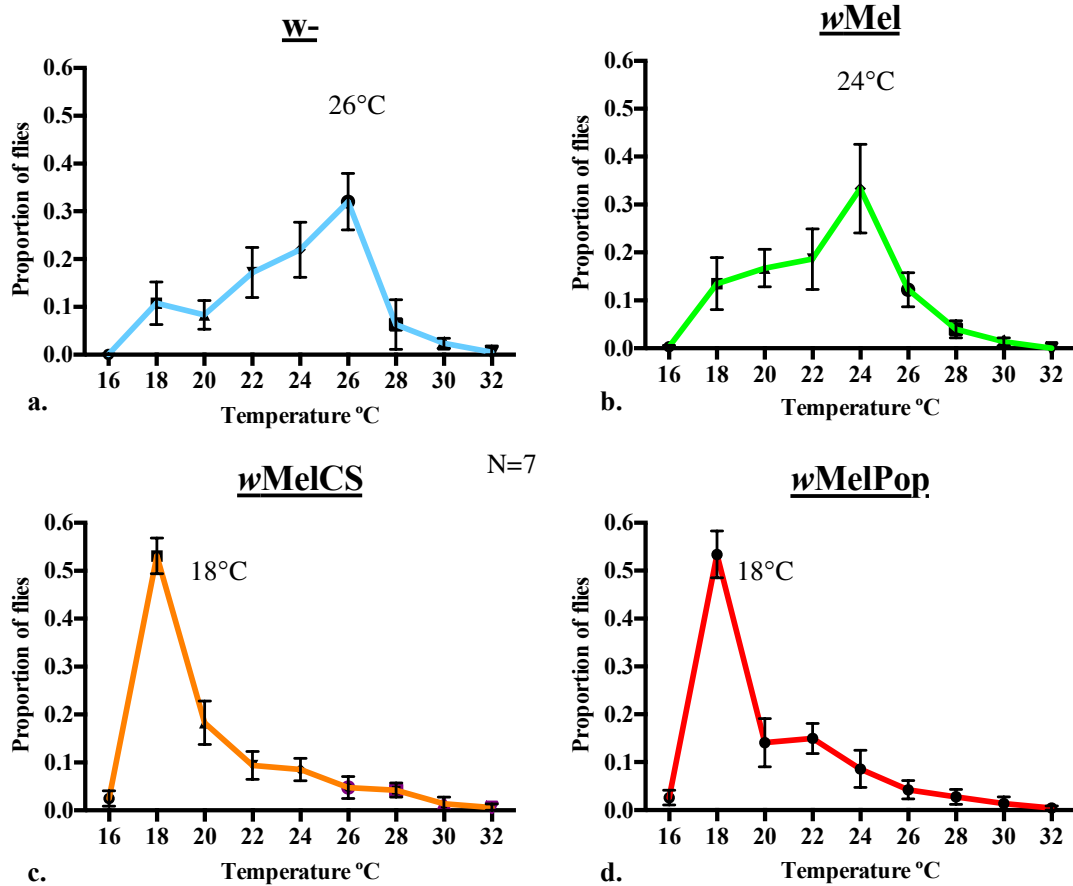
Figure 3.3: Results from temperature preference assays with standard error bars; Y-Axis in each graph is the proportion of flies and the X-Axis represents range of the temperature where flies congregated. Two-way ANOVA results revealed a significant relationship between *Wolbachia*-infection and the temperature preference of *D. melanogaster* (*Wolbachia*- infection X temperature interaction $F_{21,128} = 49.54$, $P < 0.0001$. Tukey's multiple comparison post-hoc tests showed that: a) w- flies had a significantly higher proportion at 26°C ($P < 0.0001$); b) *w*Mel-variant infected flies had a significantly higher proportion at 24°C ($P < 0.0001$); c) *w*MelCS-variant infected flies had a significantly higher proportion at 18°C ($P < 0.0001$); and d) *w*MelPop-variant infected flies had a significantly higher proportion at 18°C ($P < 0.0001$).

DISCUSSION

This study elucidates, for the first time, a significant relationship between temperature preference of *D. melanogaster* and the presence of *Wolbachia* infection. Using behavior choice thermal gradient assays, I showed that *Wolbachia* infected *D. melanogaster* prefer 2°C to 8°C cooler temperatures, depending on with which *w*Mel-strain variant they are infected, compared to uninfected conspecifics. Uninfected *D. melanogaster* flies preferred 26°C, *w*Mel-variant infected flies preferred 24°C, and both *w*MelCS and *w*MelPop-variant infected flies preferred 18°C. I postulate that, in my study, *D. melanogaster* responded to *Wolbachia* by inducing a self-medicating behavior or behavioral chill (Fedorka et al. 2016), which may be a mechanism they use to attenuate the fitness costs associated with *Wolbachia* infection.

Temperature preference can vary significantly between populations of the same species (Matute et al. 2009; Rajpurohit & Schmidt 2016) and can have profound effects on immune function, fitness, and fecundity (Huey & Berrigan 2001; Martin & Huey 2008; Hoffmann 2010). The first stages of speciation result from geographical and/or ecological isolation (Keller & Seehausen 2012). If ecologically diverging populations do not come into contact, reproductive isolation follows, causing individuals to lose the ability to produce viable offspring (Keller & Seehausen 2012). Interestingly, Matute *et al.* (2009) discovered that differences in thermal tolerance and temperature preference produced reproductive isolation between two tropical *Drosophila* sister species with sympatric populations, *D. santomea* and *D. yakuba.* This is a unique example in *Drosophila* demonstrating different temperature preferences corresponding to ecological and reproductive isolation (Matute et al. 2009). Nuclear genes and organelles have

largely dominated the speciation paradigm as the sole mechanisms paramount to driving

speciation (reviewed by Brucker & Bordenstein 2012). Yet, recent studies have

discovered microbes as additional genetic elements that catalyze speciation and they have

been incorporated into the speciation paradigm (reviewed by Brucker & Bordenstein

2012; Bordenstein & Theis 2015). *Wolbachia*-associated temperature preference

variation within a species could lead to conspecifics occupying different microclimates

and habitats. Conspecifics experience genetic adaptation to different sets of specific

environmental conditions, which can eventually result in reproductive isolation. For

endangered and threatened insects, such reproductive isolation of conspecifics can further

reduce the number of individuals in a population. Species recovery programs that employ

population supplementation should be careful to not release *Wolbachia*-infected

individuals into an uninfected population until the effect of the endosymbiont on their

host physiology has been determined.

Small fluctuations in temperature can cause considerable modifications to host-

symbiont interactions (Blanford & Thomas 1999). Pathogenicity of *w*MelPop is

attributed to its active proliferation in host tissues at temperatures $\geq$ 19°C. The increase of

*w*MelPop density confers strong anti-viral protection but leads to a two-fold reduction in

host lifespan. However, at temperatures < 19°C, anti-viral protection is weakened and

pathogenicity of *w*MelPop is eliminated (Reynolds et al. 2003). *Wolbachia'*s ability to

provide anti-viral protection to their hosts has emerged as the most promising approach to

combatting insect-vector borne pathogens that pose serious health risks to humans, such

as Dengue Fever and Zika (Hedges et al. 2008; Teixeira et al. 2008; Iturbe-Ormaetxe et

al. 2011). However, this anti-viral protection is a temperature sensitive trait that is absent

under certain thermal conditions. My findings suggest that thermal preference of *Wolbachia*'s hosts could have implications for the field application of arbovirus vector-release control programs. Most applied biology research regarding *Wolbachia* use fixed temperatures, ignoring the innate thermoregulatory behavior of insects, which might impact the efficacy and success of the applications. Thus, prior to field applications using *Wolbachia* as a biocontrol measure, it is important to understand specific host-insect temperature preferences in the presence and absence of *Wolbachia.*

Finally, recent analyses of *w*Mel-variants and mitochondria from *D. melanogaster* have provided evidence that in the past few thousand years, *w*MelCS variants have been largely replaced in the field by *w*Mel variants (Riegler et al. 2005; Richardson et al. 2012; Chrostek et al. 2013; reviewed by Miller 2013). Although the reason for the sweep remains elusive, it has been proposed that *w*Mel has adapted better to the genetic background of *D. melanogaster* and less cost is incurred to the host compared to cost associate with *w*MelCS, which ensures *Wolbachia*'s persistence in the population (Chrostek et al. 2013; reviewed by Miller 2013). I postulate that results from the current study provide evidence that the *w*Mel-variant was selected for after invading *w*MelCS-infected *D. melanogaster* populations because flies infected with *w*Mel have temperature preferences within a natural ecological range (Petavy et al. 2001).

In conclusion, this study is the first to correlate the presence of *Wolbachia* to the environmental temperature preference of their insect host. I present an example of an ecological conflict between host and symbiont that has profound effects on host physiology. Further understanding of the ecological intersection between *Wolbachia* and their hosts is needed.

GENERAL CONCLUSIONS

The goal of my research was to investigate the impact of spillovers into populations and communities and the implications to the conservation and management of endangered and threatened insects. I accomplished this goal by examining the biological and ecological impacts of *Wolbachia* infections in three different host systems: a federally threatened butterfly species, *Speyeria zerene hippolyta*; a commonly used laboratory biological model organism, *Drosophila melanogaster*; and *Eurema hecabe* and its parasitoids. I analyzed reproductive data from uninfected and *Wolbachia*-infected *S. z. hippolyta* to determine whether *Wolbachia* had an impact on host fertility. I expanded on this analysis by developing a single-population demographic model to examine population dynamics from different supplementation scenarios. Next, to investigate horizontal of *Wolbachia,* I collected and surveyed *E. hecabe* larvae from four locations in Queensland, Australia for parasitoids. I screened both parasitoids and larvae for *Wolbachia,* and characterized their *Wolbachia* strains. Finally, I designed a three-dimensional thermal gradient choice assay and conducted a laboratory experiment to show temperature preferences of *D. melanogaster* with four different *Wolbachia*-infection statuses.

Uninfected *S. z. hippolyta* butterflies suffered a reduction to their fertility when *Wolbachia*-infected butterflies were present in a population. The reduction to the number of offspring produced by uninfected butterflies, consequently decreased the population's net fecundity. *Wolbachia'*s impact on *S. z. hippolyta* fecundity was reflected in results from model simulations that showed even a single exposure event to a small number of *Wolbachia*-infected butterflies can have a long-term negative effect on overall population

size. In some supplementation scenarios, populations can recover after *Wolbachia* introduction; however, the population is unlikely to return to pre-infection size and not without the incurred population-level cost by the loss of genetic diversity. While a previous study presented hypothetical research regarding *Wolbachia* and translocation of endangered butterfly species, this study is the first to use empirical reproductive data from a federally listed species with populations supplemented from a captive rearing and release program. Furthermore, these results suggest that *Wolbachia* is an emerging threat to insect biodiversity and, if infected individuals evade detection, *Wolbachia* could hinder species recovery efforts. The phenotypes *Wolbachia* express in a host differ depending on a host's genetic background, with some strains causing reduced fitness and some strains producing no reproductive phenotype. Therefore, prior to implementing population supplementation or translocation programs and beyond screening a species for reproductive manipulating endosymbionts, I recommend that experiments be conducted to determine the phenotype induced in a specific host by *Wolbachia*. If screening for *Wolbachia* or conducting experiments prior to supplementation is not possible, I recommend that managers release offspring into the same population from which their parents were taken.

I provide the first empirical ecological evidence demonstrating inter-ordinal *Wolbachia* transmission. I identified an identical *Wolbachia* strain in a Lepidopteran butterfly species, *E. hecabe*, and their Dipteran parasitoid species, *Exorista cantans*. Twenty of 24 parasitoids and all 24 host larvae were infected with an identical *Wolbachia* strain type. A recent review on *Wolbachia* in Lepidoptera identified identical *Wolbachia* strains in species belonging to different arthropod orders (Ahmed et al. 2016); however,

the strains were identified in separate studies and from species on different continents. Therefore, this study is the first to identify identical strains in Lepidoptera and Diptera species in a single study. I was not able to accomplish my initial goal of characterizing parasitoids and their *Wolbachia* strains from all six Australia *Eurema* species. To do so would require more extensive sampling and would need to be conducted over multiple seasons. Additionally, I suggest that a laboratory experiment be performed to test for direct interspecies and inter-generational transfer of *Wolbachia* between *E. hecabe* and *E. cantans.* My results contribute significantly to advancing the understanding of horizontal transmission by *Wolbachia* and reaffirm that conservation managers be careful to not move or release *Wolbachia* infected individuals among or between populations until the effect of the bacteria on the host species is known.

Finally, my research elucidates, for the first time, that a significant relationship exists between temperature preference of *D. melanogaster* and the presence of *Wolbachia* infection. I showed that *Wolbachia* infected *D. melanogaster* prefer 2°C to 8°C cooler temperatures, depending on with which *w*Mel-strain variant they are infected, compared to uninfected conspecifics. I postulate that *Wolbachia*-infected *D. melanogaster* prefer cooler temperatures as a behavioral mechanism to attenuate the fitness costs associated with over-replication of *Wolbachia* at warmer temperatures (Chrostek et al. 2013). I recommend that this research be expanded to test temperature preferences of *Wolbachia* hosts additionally infected with viruses. My discovery of *Wolbachia*-influenced temperature preferences has implications for research associated with *Wolbachia* use as biocontrol agents against insect vector-borne human diseases. I further recommend these results be integrated into climate change predictions for insects and used to inform

species conservation decisions, particularly when programs include population augmentation. Within species temperature preference disparities could lead to reproductive and ecological isolation of conspecifics, which reduces mating opportunities and fecundity of an insect population.

The threat diseases pose to insect biodiversity is widely accepted (Daszak 2000; Dobson & Foufopoulos 2001; Harvell et al. 2002; Altizer & de Roode 2010; Graystock et al. 2013). However, there has yet to be a foundational framework developed for natural resource managers and conservation biologists to use for identifying and preventing anthropogenic disease spread through population augmentation programs. Managing for endosymbiont infections presents new challenges that will require integrating new technology into current protocols.

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APPENDIX A: Chapter 1 supplemental tables and figures

Table A.1: Using values across 100 iterations, this table shows ANOVA results comparing the number of individuals at the time point representing the lowest population size for each of the single release models – (1) no supplementation, (2) uninfected 300 butterflies, (3) uninfected 1000 butterflies, (4) *Wolbachia*-infected 50, (5) *Wolbachia*-infected 300, (6) *Wolbachia*-infected 1000.

ANOVA table	SS	DF	MS	F(DFn,DFd)	P values
Treatment	4.595e + 006	5	918912	F(5, 593) = 3349	P < 0.0001
Residual	162726	593	274.4		
Total	4.757e+006	598			

ANOVA results: F (5, 593) = 3349 P < 0.0001, r^2 = 0.9658

Table A.2: Using values across 100 iterations, this table shows Tukey's multiple comparisons results comparing the number of individuals at the time point representing the lowest population size for each of the single release models to each other – (1) no supplementation, (2) uninfected 300 butterflies, (3) uninfected 1000 butterflies, (4) *Wolbachia*-infected 50, (5) *Wolbachia*-infected 300, (6) *Wolbachia*-infected 1000.

Tukey's Post-hoc	Single CRR no-W 300	Single CRR no-W 1000	Single CRR *W* 50	Single CRR *W* 300	Single CRR *W* 1000
No CRR	MD = 131.3 P < 0.0001	MD = -39.45 P < 0.0001	MD = 166.5 P < 0.0001	MD = 25.70 P < 0.0001	MD = -45.61 P < 0.0001
Single CRR no-W 300		MD = 170.8 P < 0.0001	MD = 35.24 P < 0.0001	MD = -0.4786 P < 0.0001	MD = 176.9 P < 0.0001
Single CRR no-W 1000			MD = 206.0 P < 0.0001	MD = 170.3 P < 0.0001	MD = 6.16 P = 0.0826
Single CRR *W* 50				MD = 35.72 P < 0.0001	MD = 212.2 P < 0.0001
Single CRR *W* 300					MD = 176.4 P < 0.0001

Table A.3: Using values across 100 iterations, this table shows ANOVA results comparing the number of individuals at the 50-year time point for each of the single release models – (1) no supplementation, (2) uninfected 300 butterflies, (3) uninfected 1000 butterflies, (4) *Wolbachia*-infected 50, (5) *Wolbachia*-infected 300, (6) *Wolbachia*-infected 1000.

ANOVA table	SS	DF	MS	F(DFn,DFd)	P values
Treatment	3.162e + 006	5	632474	F(5, 593) = 1526	P < 0.0001
Residual	245717	593	414.4		
Total	3.408e+006	598			

ANOVA results: F (5, 593) = 1526, P < 0.0001, r^2 = 0.9279

*Table A.4***:** Using values across 100 iterations, this table shows Tukey's multiple comparisons results comparing the number of individuals at the 50-year time point for each of the single release models to each other – (1) no supplementation, (2) uninfected 300 butterflies, (3) uninfected 1000 butterflies, (4) *Wolbachia*-infected 50, (5) *Wolbachia*-infected 300, (6) *Wolbachia*-infected 1000.

Tukey's Post-hoc	Single CRR no-W 300	Single CRR no-W 1000	Single CRR W 50	Single CRR W 300	Single CRR W 1000
No CRR	MD = -21.77 P < 0.0001	MD = -34.10 P < 0.0001	MD = 67.37 P < 0.0001	MD = 153.4 P < 0.0001	MD = 127.3 P < 0.0001
Single CRR no-W 300		MD = 12.33 P = 0.0003	MD = 89.15 P < 0.0001	MD = 175.2 P < 0.0001	MD = -149.1 P < 0.0001
Single CRR no-W 1000			MD = 101.5 P < 0.0001	MD = 187.5 P = 0.0001	MD = -161.4 P < 0.0001
Single CRR W 50				MD = -86.04 P < 0.0001	MD = -59.97 P < 0.0001
Single CRR W 300					MD = 26.07 P < 0.0001

Table A.5: Using values across 100 iterations, this table shows ANOVA results comparing the number of individuals at the lowest population size time point for each of the multiple release models – (1) no supplementation, (2) uninfected 300, (3) *Wolbachia*-infected 300, (4) uninfected 300, *Wolbachia*-infected 50, (5) uninfected 50, *Wolbachia*-infected 300.

ANOVA table	SS	DF	MS	F(DFn,DFd)	P values
Treatment	2.057e + 006	4	514202	F(4, 524) = 1891	P < 0.0001
Residual	142469	524	271.9		
Total	2.199e+006	528			

ANOVA results: F (4, 524) = 1891, P < 0.0001, r^2 = 0.94

Table A.6: Using values across 100 iterations, this table shows Tukey's multiple comparisons results comparing the number of individuals at the lowest population size time point for each of the multiple release models – (1) no supplementation, (2) uninfected 300, (3) *Wolbachia*-infected 300, (4) uninfected 300, *Wolbachia*-infected 50, (5) uninfected 50, *Wolbachia*-infected 300.

Tukey's Post-hoc	Multiple CRR no-W 300	Mulltpile CRR *W* 300	Combined CRR 300 no-W, 50 *W*	Combined CRR 50 no-W, 300 *W*
No CRR	MD = -43.60 P < 0.0001	MD = 18.29 P < 0.0001	MD = 123.8 P < 0.0001	MD = 105.9 P < 0.0001
Multiple CRR no-W 300		MD = 61.88 P < 0.0001	MD = 167.4 P < 0.0001	MD = 149.5 P < 0.0001
Multiple CRR *W* 300			MD = -105.5 P < 0.0001	MD = 87.64 P < 0.0001
Combined CRR 300 no-W, 50 *W*				MD = -17.90 P < 0.0001

Table A.7: Using values across 100 iterations, this table shows ANOVA results comparing the number of individuals at the 50-year time point for each of the multiple release models – (1) no supplementation, (2) uninfected 300, (3) *Wolbachia*-infected 300, (4) uninfected 300, *Wolbachia*-infected 50, (5) uninfected 50, *Wolbachia*-infected 300.

ANOVA table	SS	DF	MS	F(DFn,DFd)	P values
Treatment	2.623e + 006	4	655629	F(5, 623) = 1559	P < 0.0001
Residual	220414	524	420.6.3		
Total	2.843e+006	528			

ANOVA results: F (4, 524) = 1559, P < 0.0001, r^2 = 0.92

Table A.8: Using values across 100 iterations, this table shows Tukey's multiple comparisons results comparing the number of individuals at the 50-year time point for each of the multiple release models – (1) no supplementation, (2) uninfected 300, (3) *Wolbachia*-infected 300, (4) uninfected 300, *Wolbachia*-infected 50, (5) uninfected 50, *Wolbachia*-infected 300.

Tukey's Post-hoc	Multiple CRR no-W 300	Multiple CRR *W* 300	Combined CRR 300 no-W, 50 *W*	Combined CRR 50 no-W, 300 *W*
No CRR	MD = -79.52 P < 0.0001	MD = 22.64 P < 0.0001	MD = 146.2 P < 0.0001	MD = 21.07 P < 0.0001
Multiple CRR no-W 300		MD = 102.2 P < 0.0001	MD = 225.8 P < 0.0001	MD = 100.6 P < 0.0001
Multiple CRR *W* 300			MD = -222.1 P < 0.0001	MD = -1.565 P = 0.9927
Combined CRR 300 no-W, 50 *W*				MD = -125.2 P < 0.0001

R script A.1: R script for the Generalized linear mixed effects R script model, which was produced using "lme4" and fit by maximum likelihood

```
glmer (cbind (Number.Eggs.Hatch, Number.Eggs.Unhatched) ~ Wolbachia.treatment +

(1|site/Year), data = data, family = binomial, + control = glmerControl

(optimizer = "bobyqa", optCtrl = list (maxfun=100000)))
```

Table A.9: Scaled residuals from general linear mixed-effects model.

Min	1Q	Median	3Q	Max
-28.350	-6.788	0	4.596	17.937
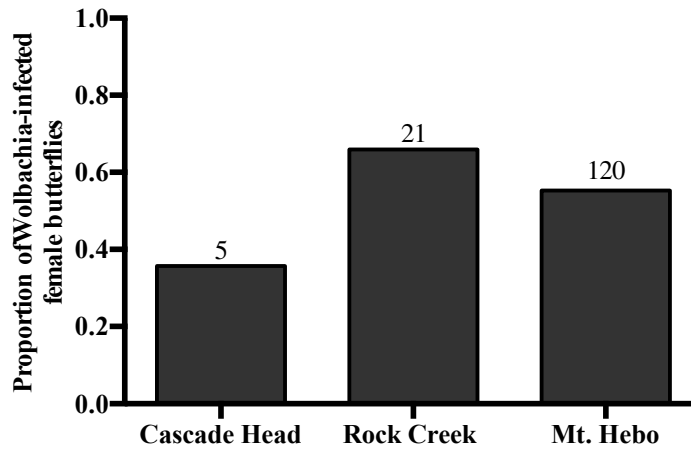
Figure A.1: Proportion of female *Wolbachia*-infected butterflies from each site sampled. Numbers above bars represents sample size for each site.

Figure A.2: Comparison of the number of *Wolbachia*-infected and uninfected female butterflies that did not lay eggs.

APPENDIX B: Chapter 3 supplemental tables and figures

w- 26°C	Mean Difference	95% CI of difference		P values
w-:26 vs. w-:18	-0.2224	-0.3165 to -0.1283	****	< 0.0001
w-:26 vs. w-:20	-0.2517	-0.3458 to -0.1576	****	< 0.0001
w-:26 vs w-:22	-0.163	-0.2571 to -0.06889	****	< 0.0001
w-:26 vs. w-:24	-0.1333	-0.2274 to -0.03917	****	< 0.0001
w-:26 vs. w-:28	0.2584	0.1643 to 0.3524	****	< 0.0001
w-:26 vs. w-:30	0.3056	0.2115 to 0.3997	****	< 0.0001
w-:26 vs. w-:32	0.3254	0.2313 to 0.4195	****	< 0.0001

a.

wMel 24°C	Mean Difference	95% CI of difference		P values
wMel:24 vs. wMel:18	-0.1983	-0.2924 to -0.1042	****	< 0.0001
wMel:24 vs. wMel:20	-0.1658	-0.2599 to -0.07169	****	< 0.0001
wMel:24 vs. wMel:22	-0.1472	-0.2413 to -0.05307	****	< 0.0001
wMel:24 vs. wMel:26	0.2111	0.1170 to 0.3052	****	< 0.0001
wMel:24 vs. wMel:28	0.2935	0.1994 to 0.3876	****	< 0.0001
wMel:24 vs. wMel:30	0.3197	0.2256 to 0.4137	****	< 0.0001
wMel:24 vs. wMel:32	0.3332	0.2391 to 0.4273	****	< 0.0001

b.

wMelCS 18°C	Mean Difference	95% CI of difference		P values
wMelCS:18 vs. wMelCS:20	0.3479	0.2539 to 0.4420	****	< 0.0001
wMelCS:18 vs. wMelCS:22	0.4371	0.3430 to 0.5312	****	< 0.0001
wMelCS:18 vs. wMelCS:24	0.4456	0.3515 to 0.5397	****	< 0.0001
wMelCS:18 vs. wMelCS:26	0.483	0.3889 to 0.5770	****	< 0.0001
wMelCS:18 vs. wMelCS:28	0.4884	0.3943 to 0.5825	****	< 0.0001
wMelCS:18 vs. wMelCS:30	0.517	0.4229 to 0.6111	****	< 0.0001
wMelCS:18 vs. wMelCS:32	0.5252	0.4311 to 0.6193	****	< 0.0001

c.

| wMelPop 18°C	Mean Difference	95% CI of difference		P values
wMelPop:18 vs. wMelPop:20	0.4268	0.3327 to 0.5208	****	< 0.0001
wMelPop:18 vs. wMelPop:22	0.4092	0.3151 to 0.5033	****	< 0.0001
wMelPop:18 vs. wMelPop:24	0.4681	0.3740 to 0.5622	****	< 0.0001
wMelPop:18 vs. wMelPop:26	0.5099	0.4158 to 0.6040	****	< 0.0001
wMelPop:18 vs. wMelPop:28	0.5208	0.4267 to 0.6149	****	< 0.0001
wMelPop:18 vs. wMelPop:30	0.5354	0.4413 to 0.6295	****	< 0.0001
wMelPop:18 vs. wMelPop:32	0.5488	0.4547 to 0.6429	****	< 0.0001

d.

Table B.1: Results from Tukey's multiple comparison post-hoc tests showing significant temperature preferences for each *D. melanogaster – Wolbachia* combination and w- flies.
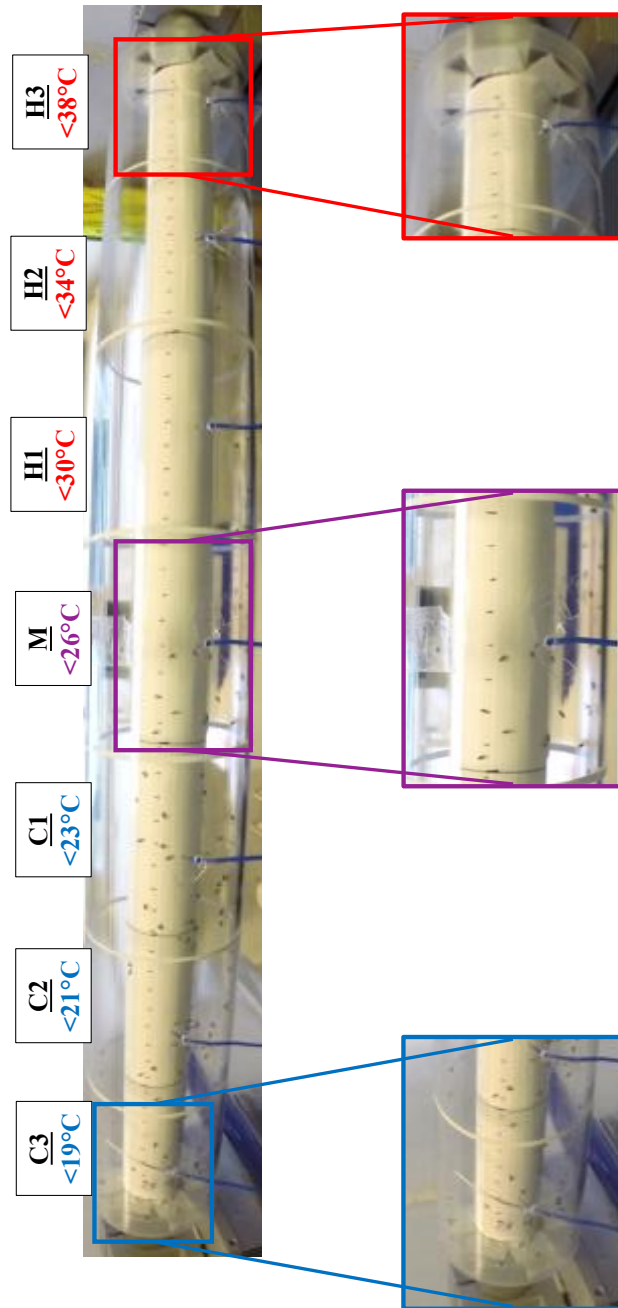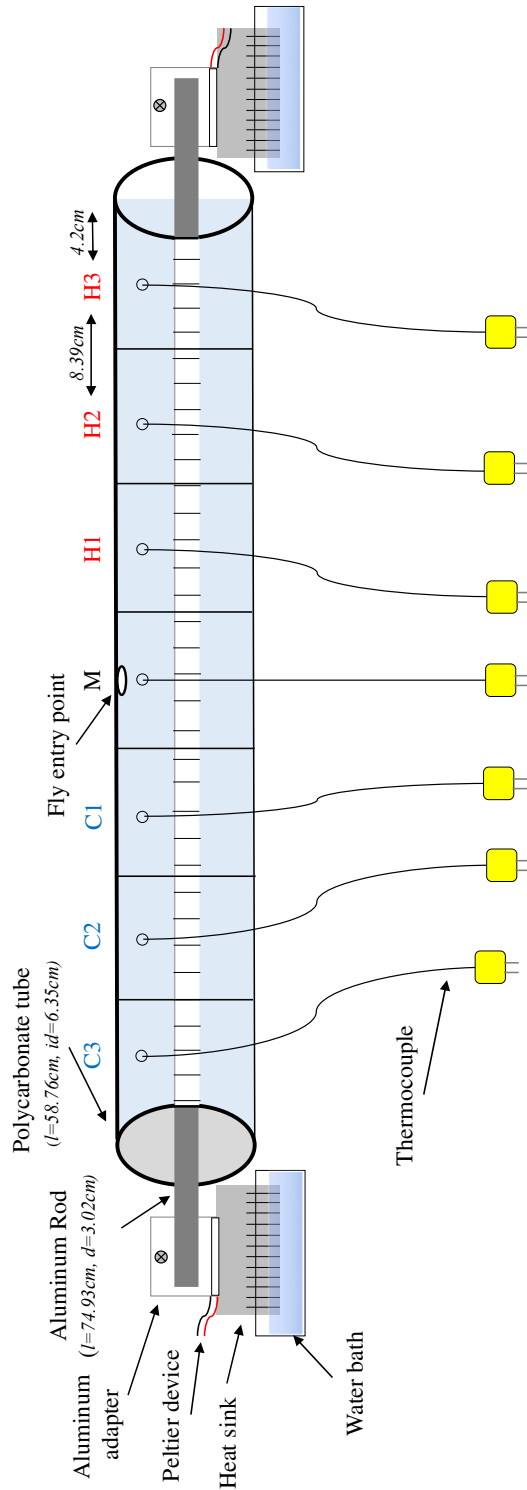
Figure B.1 Thermal gradient apparatus gradient depicting different temperature zones and fly dispersion (*w*MelPop).
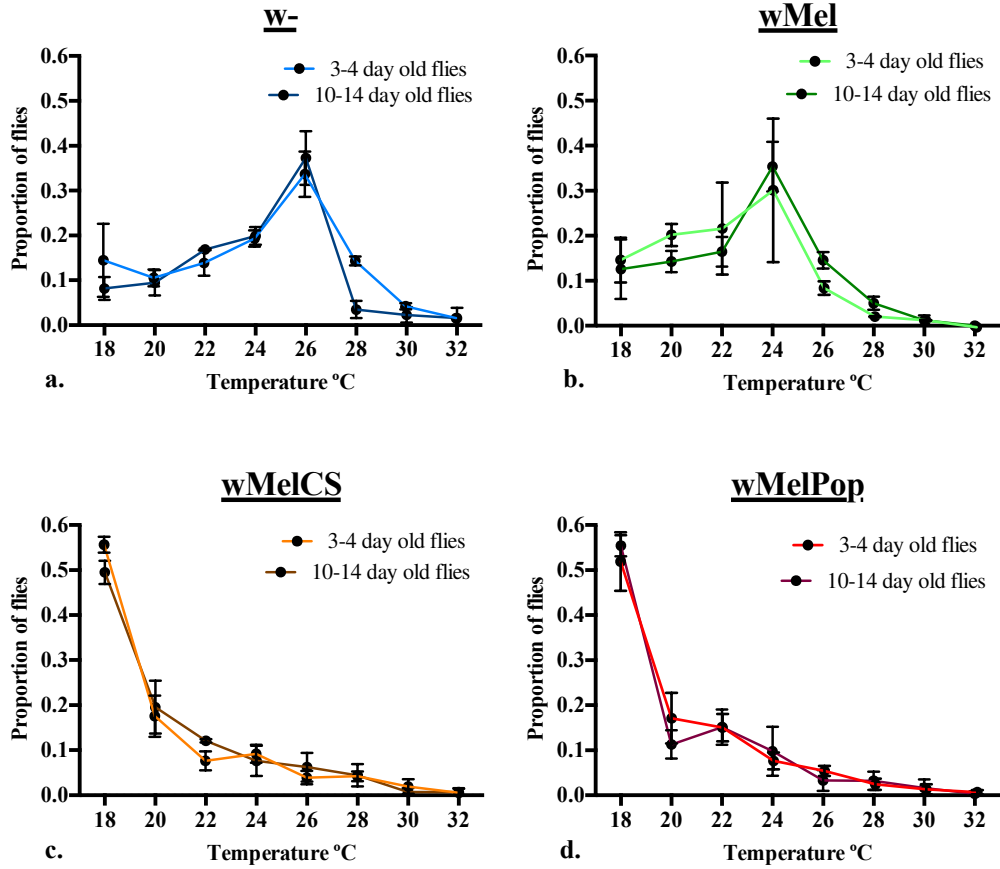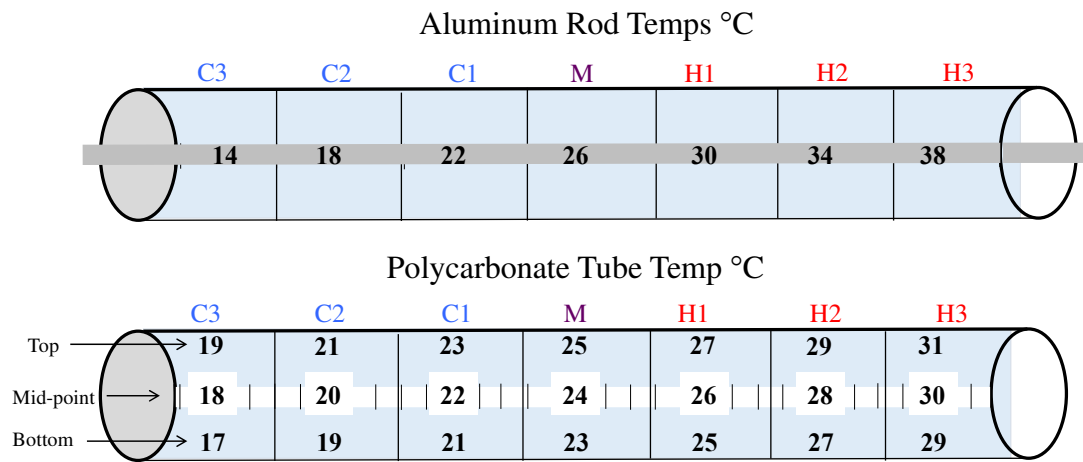
Figure B.2: Results from assays comparing temperature preferences of 3-4 day old flies to 10-14 day old flies. Graphs showing that age did not influence temperature preference of the flies.
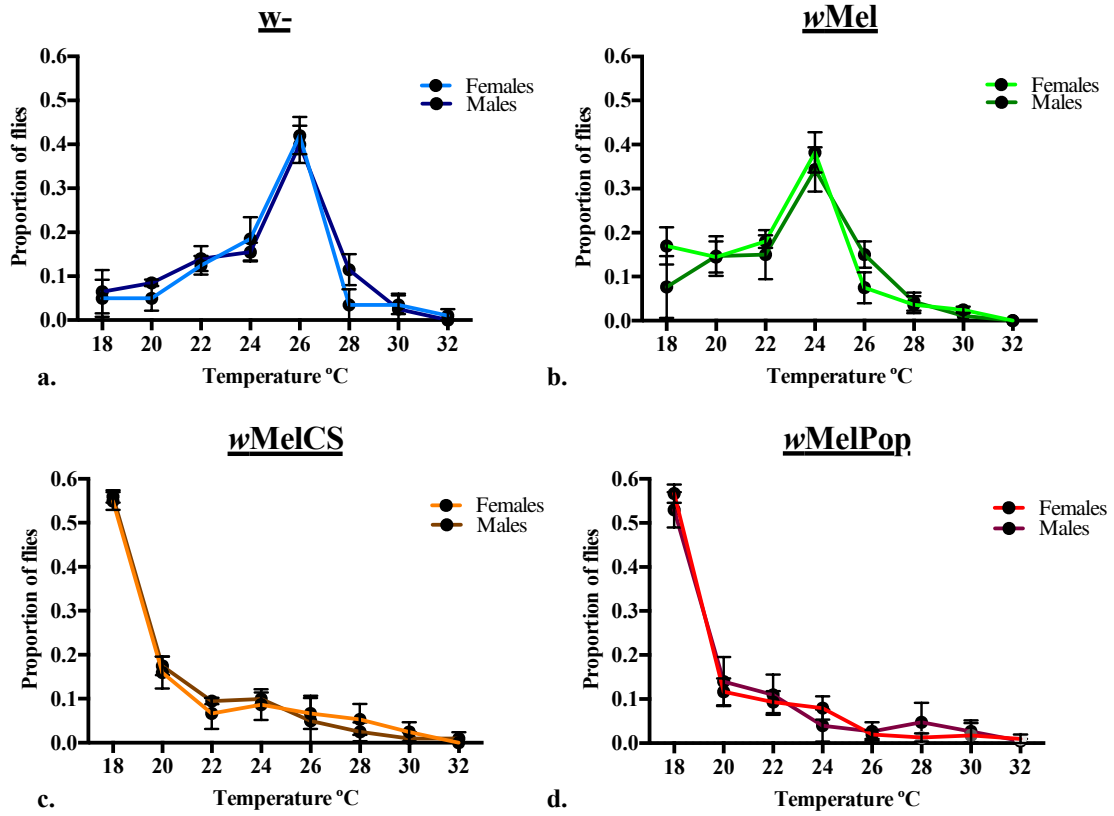
Figure B.3: Results from assays comparing temperature preferences of females and males. Graphs showing that sex did not influence temperature preference of the flies.

***Figure B.4*:** Schematic of the thermal gradient apparatus used for thermal gradient assays as adapted from

Rajpurohit & Schmidt (2016). The polycarbonate tube and length of aluminum gradient with the tube were

58.76cm and temperature was recorded

Aluminum Rod Temps °C

C3	C2	C1	M	H1	H2	H3
14	18	22	26	30	34	38

Polycarbonate Tube Temp °C

	C3	C2	C1	M	H1	H2	H3
Top	19	21	23	25	27	29	31
Mid-point	18	20	22	24	26	28	30
Bottom	17	19	21	23	25	27	29

Figure B.5: Average + 0.5°C (SD) temperatures from 18 runs that were recorded at each surface measured using k-type thermocouples. There was a linear increase in temperature from cold to hot as measured at each of seven evenly spaced (8.39cm)

APPENDIX C: Cascades to Coast GK-12 Curriculum

Exploring Variation: Diversity Within and Between Species

Fellows: Amy Truitt and Monica Mogilewsky (Environmental Science and Resources)

Teachers: Linda Wolf (Glencoe High School, Hillsboro, OR) and Jennie Richard

(Gresham

High School, Gresham, OR)

Advisers: Dr. Catherine E. de Rivera (Environmental Science) and Dr. Natalie Vasey

(Anthropology)

Learning Goal

Students will learn to recognize variation between individuals of a species and

distinguish it from variation between closely related species (species within the same

order). Students will learn that variation within species arises from both genetic and

environmental factors. They will also learn how scientists examine phylogenetic variation

within and between species. Students will be introduced to some key terms for

understanding the theory of evolution.

Students' Learning Objectives

☐ To recognize that all species exhibit individual variation

☐ To learn the causes of individual variation within species

☐ To recognize distinguish between within and between species variation

☐ To relate the causes of individual variation to variation between species

☐ To learn vocabulary pertinent to the theory of evolution Target Grade: 10th Grade biology or integrated science

Next Generation Science Standards

HS.LS3.1 *Ask questions to clarify relationships about the role of DNA and chromosomes in coding the instructions for characteristic traits passed from parents to offspring.*

HS.LS3.2 *Make and defend a claim based on evidence that inheritable genetic variations may result from: (1) new genetic combinations through meiosis, (2) viable errors occurring during replication, and/or (3) mutations caused by environmental factors.*

Exploring Variation_GK12 Curriculum

HS.LS3.3 *Apply concepts of statistics and probability to explain the variation and distribution of expressed traits in a population.*

Unit Summary

This unit will introduce students to the sources of individual variation and the vocabulary that scientists use to discuss that variation. Students will learn the two sources of variation and will practice distinguishing within species variation from between species variation. They will learn the foundations for how scientists distinguish between species.

120

Required Knowledge/Skills

Students should be familiar with the hierarchical Linnaean system of classification and how to use an identification guide. Previous work with identification is helpful but not necessary.

Activity Plan

This activity is designed to be completed in two 50-60 minute class periods.

Part I

<u>*Scaffolding – Sources of variation*</u>

Instructor will introduce the definition of variation, types of variation, and sources of variation, preferably using local examples. Materials provided with this lesson plan use at least some local examples from Level III ecoregions (Cascade Mountains, Willamette Valley, and Coast Range Mountains). The instructor will compare within species variation with between species variation, preparing students to distinguish within species variation from between species variation in the hands-on exercise.

<u>*Activity*</u>

Materials needed

☐ *Exploring Variation presentation*

☐ *Exploring Variation student worksheet*

☐ *An Inventory of a Few Human Traits student worksheet*

Methods

1. *As a class define each trait on "An Inventory of a Few Human Traits" student worksheet.*

2. *Divide the class into groups of 4-5 students.*

3. *Working in groups, have each student complete the inventory for themselves and then*

4. *compare their answers to that of their group members.*

5. *Once students have finished their inventories, have students report their results to the class. For example, the first group might report that 2 of 4 members have pierced ears. In the interest of time, the instructor may wish to limit the number of traits report.*

6. *The instructor should record each group's results on the board and calculate the total number of students displaying each trait. If time allows, instructor should make histograms for two or three of the traits.*

7. *Use Exploring Variation power point presentation to formally introduce the primary concepts of biological variation. Have students fill out the worksheet while watching the presentation; be sure to cue them so that they know which slides will help them answer worksheet questions. Connect the concepts in the power point to traits from the Inventory of Human Traits activity.*

8. *After the presentation, have students again work in groups to prepare a list of traits for butterflies.*

9. *As a class, compile a master list of potential butterfly traits.*

10. *If there is time, have students determine which of the butterfly traits are continuous and*

11. *which are discontinuous. Have students predict which traits would be most useful for distinguishing species and which traits would be most useful for distinguishing individuals.*

Part 2

<u>*Scaffolding*</u>

The instructor should remind students of the definition of variation and should make the master list of potential butterfly traits available – either on the board or on handouts.

<u>*Activity*</u>

Materials Needed

☐ *Exploring Variation student worksheet*

☐ *A collection of diverse specimen within a single order; for example, 30-50 butterflies (Order Insecta) representing 8-15 species. Collections may be created by the instructor by capturing local insects or by ordering specimen from online sources (see Credits). Alternatively, collections may be available for loan from local zoos and/or natural history museums.*

☐ *Foam core*

☐ *Mounting pins*

☐ *Identification guides (for the collection of specimen)*

☐ *Exploring Variation Student Instruction Sheet*

Method

Prepare prior to class:

1. *For each student group, prepare a tray with foam core, mounting pins, masking tape and*

2. *5-10 individual specimen. Number of specimen provided will depend on total number*

3. *of specimen available and number of student groups. Include in each group multiple representatives of 2-4 different species. Create a key for each group, so that you know the number and identity of species provided to each group.*

4. *During class:*

5. *Start by asking students for examples of variation in humans – have them first talk in*

6. *groups and then call on groups to contribute examples to the entire classroom.*

7. *Use power point presentation to introduce the following definitions: variation,*

8. *continuous variation, discontinuous variation, genetic variation, and environmental variation. Have students fill out the worksheet while watching the presentation; be sure to cue them so that they know which slides will help them answer worksheet questions.*

9. *Divide students into groups of 4-5 and give each group an identification tray.*

10. *Each student group should sort their specimen into species.*

11. *Species should be identified using the identification guide provided.*

12. *Specimen should be mounted to the foam core in the following manner:*

a. *Butterflies should be pinned to foam core using mounting pins through the wings to help preserve the butterflies for long term use.*

b. *Each species should have its own row on the foam core.*

c. *Individual specimen of the same species should be mounted in a single line.*

d. *Below each row (one row per species), students should record the species*

13. *identification on a piece of masking tape.*

14. *Students should then answer the reflection questions on the Exploring Variation student*

15. *worksheet.*

16. *If time allows, have groups share their answers to the reflection questions with each*

17. *other.*

Credits

- *Power point presentation modified from www.skinnerscience.com/Biology/variation.ppt Retrieved 6 December 2014*

- *An Inventory of a Few Human Traits student worksheet: http://science.education.nih.gov/supplements/nih1/Genetic/guide/pdfs.htm Retrieved 9 December 2014*

- **Online sources for insect collections:*

http://landing.carolina.com/insects?s_cid=ppc_gl_insects_ion&gclid=CM3bxaGNssICF

RFafgo duosACwhttp://www.insects4sale.com/

*Website addresses valid as of 6 December 2014

Attachments

☐ *Exploring Variation presentation*

☐ *Exploring Variation student worksheet*

☐ *An Inventory of a Few Human Traits student worksheet*

☐ *Exploring Variation Student Instruction Sheet*