Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter know, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Rohini Swamy April 18th 2012

The butterfly *Danaus plexippus* is infected with the bacteria *Wolbachia* and *Spiroplasma*

By

Rohini Swamy

Jacobus de Roode
Adviser

Department of Biology

Jacobus de Roode

Adviser

Nicole Gerardo

Committee Member

Tracy Morkin

Committee Member

2012

The butterfly *Danaus plexippus* is infected with the bacteria *Wolbachia* and *Spiroplasma*

By

Rohini Swamy

Dr. Jacobus de Roode

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2012

Abstract

The butterfly *Danaus plexippus* is infected with the bacteria *Wolbachia* and *Spiroplasma*

By Rohini Swamy

Very little is known about the microorganisms that exist within Monarch butterflies (*Danaus plexippus)*. Several studies have been carried out looking at the microorganisms of other insect species; research shows that large amounts of insect species are infected with *Wolbachia* and *Spiroplasma*. These bacteria have varying phenotypes within their hosts, as well as different relationships with their specific hosts. This study shows that Monarch butterflies are, too, infected with *Wolbachia* and *Spiroplasma.* The prevalence rates of these bacteria were determined using bacterium-specific Polymerase Chain Reactions. *Wolbachia* had an extremely low prevalence rate ~1%, while *Spiroplasma* had a high prevalence rate ~80%. Interestingly, the prevalence rates of both bacteria, especially *Spiroplasma,* were different across the populations screened. There was no significant difference in the number of *Wolbachia* infected males vs. females, but there was a significant difference between *Spiroplasma* infected males vs. females. Sequencing the bacteria and further phylogenetic analyses will be important in determining the strains of the bacteria and the phenotypes they cause in Monarchs.

The butterfly *Danaus plexippus* is infected with the bacteria *Wolbachia* and *Spiroplasma*

By

Rohini Swamy

Dr. Jacobus de Roode

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2012

Acknowledgements

I would like to thank Dr. de Roode for his invaluable comments, guidance, support, assistance and encouragement throughout the year, and his help with statistical analyses; Dr. Gerardo for providing valuable suggestions for the experimental set up and comments about future directions for my project; members of the de Roode and Gerardo labs for assisting me with extractions, protocols and other lab technique related issues; Amanda Pierce, my graduate student mentor who worked closely with me on this project, for guiding me throughout, patiently answering my endless questions, and assisting me whenever possible.

TABLE OF CONTENTS

Introduction

Insects are the most diverse group within the animal kingdom with over a million species found in a wide range of existent habitats, except the sea (Vilmos & Kurucz 1998). As a result of their extensive distribution, the world of insects harbors a diverse array of bacterial species (Tòth et. al 2006), in addition to other forms of life. Bacteria are unicellular (prokaryotic) organisms that lack a nucleus and other cell organelles. Despite their seemingly apparent simplicity, bacteria are capable of complex relationships with animals, plants and the environment (Horner-Devine et. al 2004). The interactions between insects and bacteria can be either symbiotic or pathogenic. In 1879, Heinrich Anton de Bary coined the term "symbiosis" to mean "the living together of unlike organisms" (Sanchez-Contreras & Vlisidou 2008). Depending on the fitness (reproductive capability viz. number of offspring produced) effects on the organisms within the association, symbiosis can be further categorized into mutualism, parasitism and commensalism (Moya et. al 2008). In general terms, mutualism, often interchangeable with symbiosis, occurs when both species benefit from the association. Parasitism refers to an association between two species in which one increases its fitness while the other is harmed by the relationship. Commensalism occurs when one species benefits, but does no harm or provides no benefit to the other species.

Pathogenic relationships, on the other hand, refer to those associations in which one species, the pathogen, produces disease in the other, its host. Sometimes, the same organism can behave like a pathogen or a mutualist depending on host fitness effects and the environmental circumstances, therefore creating some ambiguity in the differentiation between mutualists and pathogens. Studies have shown that interactions that began as pathogenic have, on occasion,

evolved towards a tolerance for the pathogen, and/or even beneficial exchange – mutualism (Sanchez-Contreras & Vlisidou 2008).

Most insects contain inherited bacteria that perform any of these potential aforementioned roles within the insect. In some circumstances, these inherited bacteria do not show any long-term evolutionary relationship with the host (Baumann et. al 1995). More broadly though, bacteria can be maintained within insect populations through horizontal transmission or vertical transmission. Horizontal transmission is the process by which bacteria are passed on to other arthropod[1] hosts often through a plant or vertebrate intermediate host (Hurst & Jiggins 2000). On the other hand, vertical transmission is the process by which bacteria are passed from parent to offspring, possibly connected to the mechanisms of host reproduction (Hurst & Jiggins 2000). Of these vertically transmitted bacteria, many are maternally, but not paternally, transmitted mainly due to the fact that there is no fitness benefit associated with male hosts (Dyson et. al 2002), and these bacteria are then passed on to the next generation. These complex interactions have been studied extensively in a range of insect species, but surprisingly not in Monarchs. Research into the bacteria that inhabit the intestinal tract of Monarchs is ongoing, but very little is known about the bacteria that are naturally present in Monarchs (Kingsley 1972).

Monarch butterflies have always been an intriguing species to study; their migration and overwintering behaviors are extremely fascinating and exemplary. Every winter, Monarch butterflies undergo a massive migration to overwintering sites where large numbers of Monarchs can be found together (Davis et. al 2009). This paper will focus on the detection of two widely occurring bacteria in insects, *Wolbachia* and *Spiroplasma*, (Werren et. al 1995; Ebbert 1991) in

[1] The Arthropods is the largest animal phylum (classification rank) that includes insects, spiders, mites and crustaceans among others. They consist of invertebrate animals having jointed limbs, segmented bodies and an exoskeleton made of chitin (complex carbohydrate).
[2] Microorganisms are single celled living things (organisms) that are extremely small – invisible

the diverse populations of Monarch butterflies. To test for the presence of these bacteria, bacterium specific polymerase chain reaction (PCR) was used. PCR is a molecular diagnostic tool used to amplify specific copies of DNA, usually generating several copies of a specific DNA sequence. See Appendix B for an explanation of the process. Through PCR, data on the existence and nature of these microorganisms[2] can be applied to future research about the roles of these bacteria within the host, their relationship – symbiotic or pathogenic – to the host, and the potential protective effects, if any, against parasitism.

Wolbachia

Wolbachia are alpha proteobacteria[3] (Family: *Rickettsiaceae*) that are widespread in arthropods, now known to infect 25-70% of insect species (Werren et. al 1995; Kozek & Rao 2007). These bacteria infect the reproductive tissue, ovary and testes, of arthropods and are transmitted through the cytoplasm of the egg to the next generation i.e. vertical transmission (Werren et. al 1995). These bacteria are capable of manipulating host reproduction in a number of ways (Werren et. al 1995; Werren 1997) namely, reproductive incompatibility (also known as cytoplasmic incompatibility or CI), male killing, parthenogenesis, and feminization of genetic males (O'Neill et. al 1992; Rousset et. al 1992; Stouthamer et. al 1993; Werren et. al 1995; Werren 1997; Zhou et. al 1998). Different strains of *Wolbachia* are responsible for specific reproductive manipulations.

- Cytoplasmic incompatibility (CI) is a condition that occurs when *Wolbachia* infected males (sperm) are unable to mate with uninfected females (egg) (Zhou et. al 1998; Werren 1997). Both infected and uninfected males (sperm) are able to mate with infected

[2] Microorganisms are single celled living things (organisms) that are extremely small – invisible to the naked eye.
[3] Proteobacteria is a kingdom within bacteria, exhibiting great metabolic diversity. It is further subdivided (alpha through zeta) based on different ribosomal RNA sequences.

females. (Werren 1997). This refers to unidirectional incompatibility. Bidirectional incompatibility occurs when eggs from infected females are incompatible with sperm from infected males because they have different strains of *Wolbachia*. The particular strain of *Wolbachia* that infects the male has "encrypted" the developing sperm, rendering it unable to fertilize an infected egg. In order for fertilization to take place, the same strain of *Wolbachia* has to be present in the infected egg to "decode" the encrypted sperm (Werren 1997; Werren 2003). The mechanism by which this takes place is still unknown, but researchers are aware that different strains of *Wolbachia* have unique encryption systems. This is an interesting finding since this raises the possibility of *Wolbachia's* role in speciation, by preventing insects' gene pools from mixing (Werren 2003). Werren cites the example of jewel wasps, a genus (*Nasnia)* of small parasitic wasps that kill fly pupae, where this circumstance might have occurred. Three closely related species of wasps were infected with different strains of *Wolbachia*. Thus, the bacteria prevent inter-species mating, and reduce the reproduction among uninfected females, thereby successfully spreading the infection to the next generation (Knight 2001).

- Male killing refers to the killing of males during early larval development due to the presence of maternally inherited genetic elements (Hurst et. al 1999). This is very common in insects. *Wolbachia* has male-killing strains, which have been found in the butterfly *Acraea encedana* and in the two-spot ladybird *Adalia bipunctata*, among other insects (Hurst et. al 1999; Jiggins et. al 2000). Research suggests that male-killers invade host populations and continue to persist because the death of male embryos benefits their female siblings, who then go on to pass the male-killer to the next generation of

offspring i.e. vertical transmission of the male-killer (Jiggins et. al 2000). As a result of male killing by certain organisms, including *Wolbachia,* there has been a rise in the number of females. The consequent female-biased population sex ratios will have, or in some cases already have, evolutionary repercussions viz. sexual selection (Jiggins et. al 2000).

- Parthenogenesis refers to the ability to reproduce without males i.e. without fertilization taking place (Knight 2001). This is most commonly seen in wasp species where populations of all female wasps are able to persist in nature due to infection with *Wolbachia* (Werren 2003). In strains of *Trichogramma* wasps, antibiotic treatment allowed them to revert to sexual reproduction, and subsequent research showed that bacteria responsible for parthenogenesis were in fact *Wolbachia* (Stouthamer et. al 1990; Stouthamer & Werren 1993; Werren 1997; Werren 2003).
- Feminization is the process in which *Wolbachia* turns males into sexually functioning females (Werren 2003). This has been seen in *Wolbachia* infected woodlouse *Armadillidium vulgare*, where it suppresses an androgenic gland, producing reproductively competent females (Werren 1997).

Wolbachia can potentially cause any of the aforementioned conditions in Monarch butterflies. Studies cited above have involved species within the *Lepidoptera* order; it would not be surprising if *D. plexippus,* too, contains *Wolbachia*. Furthermore, it would be interesting to determine if there is a difference in the strain of *Wolbachia* harbored by the Monarchs and the particular phenotype[4] it causes.

[4] Phenotype – refers to the visible character traits of an organism such as behavior, structure, morphology and the like. Phenotypes result from an interaction between gene expression and the environment.

Spiroplasma

Bacteria from the genus *Spiroplasma* are motile, helical and lack a cell wall (Davis & Worley 1973). By 1975, researchers had established the interaction between arthropod hosts and *Spiroplasma*; specifically, *Spiroplasma* has an obligate association with insects – mutualists, pathogens or commensals (Gasparich 2002). They, like *Wolbachia*, are capable of horizontal and vertical transmission. They most commonly occur in the guts of insects, but can also be found in the salivary glands, reproductive tissue, and brain cells (Gasparich 2002). Studies show that within the insect community *Spiroplasma* plays a role in insect disease, distortions in sex ratios, and most recently discovered protection against parasites or pathogens (Gasparich 2002; Jiggins et. al 2000; Xie 2010).

- *Spiroplasma* has been suspected of having a negative effect on the fitness and survival of *Dalbulus* leafhoppers, and is known to cause disease in Honeybees as well (Gasparich 2002). That is, it reduces the reproductive capability of infected individuals, as well as reduces life span and lowers quality of life.
- Like *Wolbachia,* several strains of *Spiroplasma* are male-killing and in turn distort the sex ratio of the infected populations. The bacteria are passed to the next generation by the females and kill the male offspring. This has been studied in *Drosophila* species, beetle species *Harmonia axyrids* and *Adalia bipunctata,* and *Danaus chrysippus* butterflies (Gasparich 2002). These organisms were infected with different strains of *Spiroplasma,* providing evidence that there maybe more than one male-killing strain.
- Several studies carried out on *Drosophila* flies show that several of these species are infected with *Spiroplasma*. Research has shown that at least nine strains of *Spiroplasma* that have been identified in these species have no specific phenotype. That is, they do not

play a role in reproductive manipulation, male killing, or fitness and survival, but they are still highly prevalent in *Drosophila* populations (Xie et. al 2010). Researchers figured that there had to be an explanation for their persistence within the population and tested *Spiroplasma's* role in defense against enemies. Xie et. al found that a highly prevalent strain of *Spiroplasma* provides protection to its natural host *Drosophila hydei* against parasitism by the wasp *Leptopilinia heterotoma* (Xie et. al 2010).

This thesis deals with investigating these bacteria in Monarch butterflies. If Monarchs are infected with either *Wolbachia* or *Spiroplasma*, or both, it provides evidence for the existence of a specific relationship between the bacteria and its host. With the various phenotypes both *Wolbachia* and *Spiroplasma* are known to cause in other insects, they could cause similar phenotypes in Monarchs, or there is a possibility that Monarchs are infected with novel strains of these bacteria whose phenotype has yet to be identified. These bacteria serve as ideal candidates for the discovery of new interactions with insects.

Methods

Monarch butterfly Collections

The main aim was to determine if Monarchs were infected with *Wolbachia* and *Spiroplasma* and if so, to estimate the prevalence rates of these bacteria in adult Monarch butterflies. Several different populations were used to ensure a better understanding of the existence of these bacteria over a broad spectrum. The de Roode lab had already collected and/or obtained butterfly samples, which were then used in this experiment. Adult Monarch samples from migratory East and West populations in the U.S, non-migratory Monarch butterfly populations, and newly established Monarch populations were used to obtain a diverse data set. The map below, see Fig. 1, provides an overview of the Monarch populations and their locations.

The East and West Populations in the U.S. included St. Marks, FL (October 2009), Santa Barbara, CA (November 2009), and Pismo Beach, CA (February 2009). St. Marks, FL is a migration stopping point for Monarchs on their way to Mexico (Urquhart & Urquhart 1978), while Santa Barbara and Pismo Beach are the two biggest Californian overwintering sites (Frey et. al 1992). Newly established populations (~170 years ago) included Spain (January 2012), Hawaii (Hawaii 2009) and New Zealand (January 2011); these are also non-migratory populations. The Monarch sample IDs, season, their respective populations and infection status, are listed in Table 2, Appendix A.

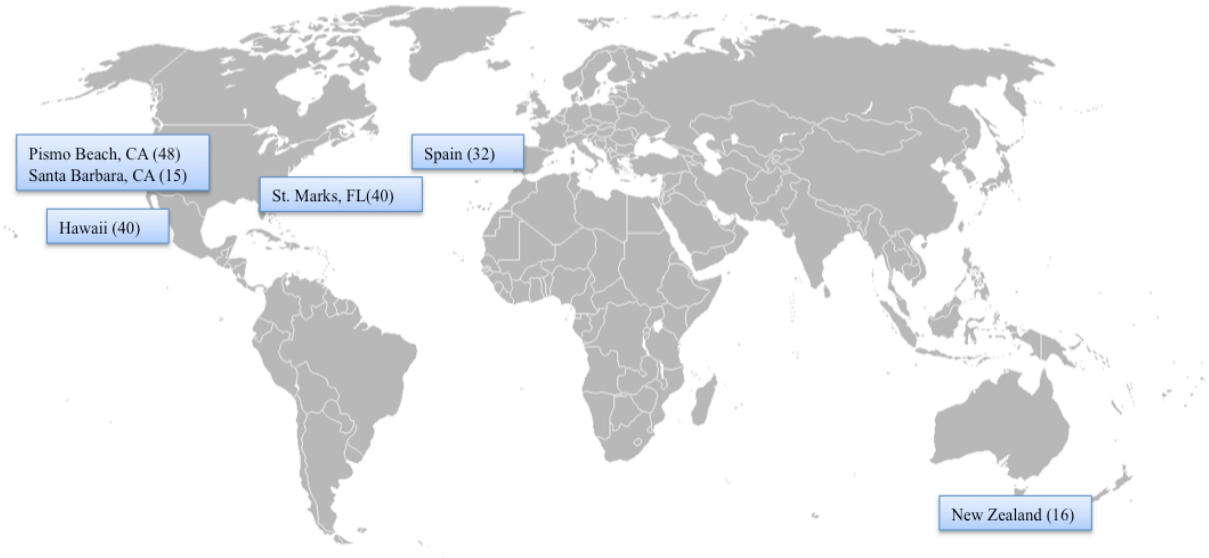


Fig 1. Map of Monarch populations used. The number in parentheses represents the sample size used in this experiment. The numbers in parentheses represent the sample sizes.

Bacterium-Specific Polymerase Chain Reactions and DNA Extractions

For PCR, genomic DNA was extracted from a 0.5 mm section of adult male and female butterfly abdomen using the UltraClean™ Tissue and Cells DNA Isolation Kit from MOBIO (Carlsbad, CA) and quantified using a Nanodrop 2000. The protocol is listed in Appendix C. We extracted DNA from male and female abdomens to procure gonadal tissue because both *Wolbachia* and *Spiroplasma* bacteria are known to infect the gonads of invertebrate hosts (Vandekerckhove et. al. 2006, Mateos et. al. 2006). PCR was carried out in 25 µl reactions using the GoTaq® Hot Start Green Master Mix (Promega), screening *Wolbachia* and *Spiroplasma* separately. Each reaction contained 0.2 µM of each primer and 30-50 ng DNA template. Thermal cycling reactions for the amplifications consisted of an initial 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at the primer-specific annealing temperature (see Table 1), and 30 s at 72°C. A final step of 30 min at 72°C was included to complete any partial polymerizations, followed by an incubation step at 15°C. At the end, 5 µl of PCR product was run on 1.8% Agarose gel at 90 Volts for 30 minutes to determine the presence of amplified DNA in relation to the specific positive and negative control.

For *Wolbachia*:

To detect the presence of *Wolbachia* in adult Monarchs *Drosophila melanogaster* was used as a positive control in the PCR reaction (Min et. al 1997), while PCR grade water was used in the negative control. The primer set W-Specf/W-Specr was used to amplify 438bp of the 16s rDNA gene to assay for the presence of *Wolbachia* (Werren & Windsor 2000). See Table 1 below for annealing temperatures and primer sequences.

For *Spiroplasma*:

To test for the presence of *Spiroplasma* in adult Monarchs, *Drosophila neotestacea* (Jaenike, 2011, Jaenike et al. 2010) was used as a positive control in the PCR reaction, while PCR grade water was used in the negative control. The primer set 23f/KSSsp was used to amplify approximately 410 base pair fragment of bacterial 16S rDNA to assay for the presence of Spiroplasma (Watts et. al 2009). See Table 1 for annealing temperatures and primer sequences.

For every sample, subsequent arthropod specific PCR was carried out to ensure the reliability of the results obtained from the *Spiroplasma* and *Wolbachia* specific reactions. That is, any negative results were due to the absence of the bacteria itself and not the absence of successfully extracted DNA. The primer set A28sF/A28sR was used to amplify highly conserved region of eukaryotic 28s rDNA (Werren et. al 1995).

Table 1
Primer sequences and annealing temperatures (T_A) for all primer sets used.

Type	Primer Set	Primer sequence	T_A
Wolbachia	W-specf	F: 5'-CATACCTATTCGAAGGGATAG-3'	55°C
	W-Specr	R: 5'-AGCTTCGAGTGAAACCAATTC-3'	
Spiroplasma	23f	F: 5′-CTCAGGATGAACGCTGGCGGCAT-3'	55°C
	TKSSsspR	R: 5'-TAGCCGTGGCTTTCTGGTAA-3'	
Arthropod	A28sF	F: 5'-TACCGTGAGGGAAAGTTGAAA-3'	55°C
	A28sR	R: 5'-AGACTCCTTGGTCCGTGTTT-3'	

Statistics

All the statistical tests were run in R programming software version 2.14.2 for Mac OS X. The data were analyzed using chi-square tests, except Fig 4.a and Fig 5, which were analyzed using a linear regression model.

Results

Identification and prevalence of Wolbachia and Spiroplasma

The prevalence of *Wolbachia* and *Spiroplasma* in the aforementioned Monarch populations is summarized in Table 2.

Table 2
Prevalence of *Wolbachia* and *Spiroplasma*

Population	Season	No. Of Spiroplasma (S)	No. Of Wolbachia (W)	Sample Size	Prevalence Rate (S)	(W)
St. Marks, FL	Oct-09	19	1	40	48%	2.50%
Pismo Beach, CA	Feb-09	48	0	48	100%	0%
Santa Barbara, CA	Nov-09	12	0	15	80%	0%
Hawaii	Nov-09	32	0	40	80%	0%
New Zealand	Jan-11	15	1	16	94%	6.25%
Spain	Jan-12	30	1	32	94%	3.13%
Total		156	3	191	81.68%	1.57%

Wolbachia was found in St. Marks, FL, New Zealand and Spain, while there was no *Wolbachia* observed in the remaining populations. The prevalence rates were 2.50%, 6.25% and 3.13% respectively. Specifically, all the infected individuals in St. Marks, New Zealand, and Spain were male. Altogether, 191 randomly selected individuals were screened for *Wolbachia* by PCR, out of which 3 male individuals were found to be positive. This low prevalence rate overall (1.57%) suggested that the observed difference between males and females was likely to be irrelevant. It was found that there was no difference in the prevalence *of Wolbachia* in males vs. females (X^2=1.21, df=1, P=0.2711) across all the populations. See Fig 2(a). To make sure there were no false negatives, i.e. testing negative for *Wolbachia* when it is actually present, the presence of successfully extracted DNA was determined using eukaryotic 28s rDNA primers. This ensured that the results were negative because *Wolbachia* was absent and not because of an unsuccessful DNA extraction.

Spiroplasma

Spiroplasma was more widespread in monarchs compared to *Wolbachia*. *Spiroplasma* was found in all populations screened in this experiment. Infected individuals were found in St. Marks, Pismo Beach, Hawaii, Santa Barbara, Spain and New Zealand. It was found that the prevalence of *Spiroplasma* was different in the different populations screened (X^2=30.78, df=5, p=<0.001). See Fig 3. In the case of the Hawaii population, double bands were observed for some positive samples on the PCR gels. For the purpose of this experiment, all individuals that displayed a band on the gel were considered infected. On the other hand, the individuals that lacked a band altogether were considered uninfected. See Appendix D for examples of gel images. There was a difference in the infection with *Spiroplasma* between male and female individuals (X^2=4.46, df=1, P=0.03) across all the populations. See Fig. 2(b). There were more infected males than females. To make sure there were no false negatives, i.e. testing negative for *Spiroplasma* when it is actually present, the presence of successfully extracted DNA was determined using eukaryotic 28s rDNA primers. This ensured that the results were negative because *Spiroplasma* was absent and not because of an unsuccessful DNA extraction.

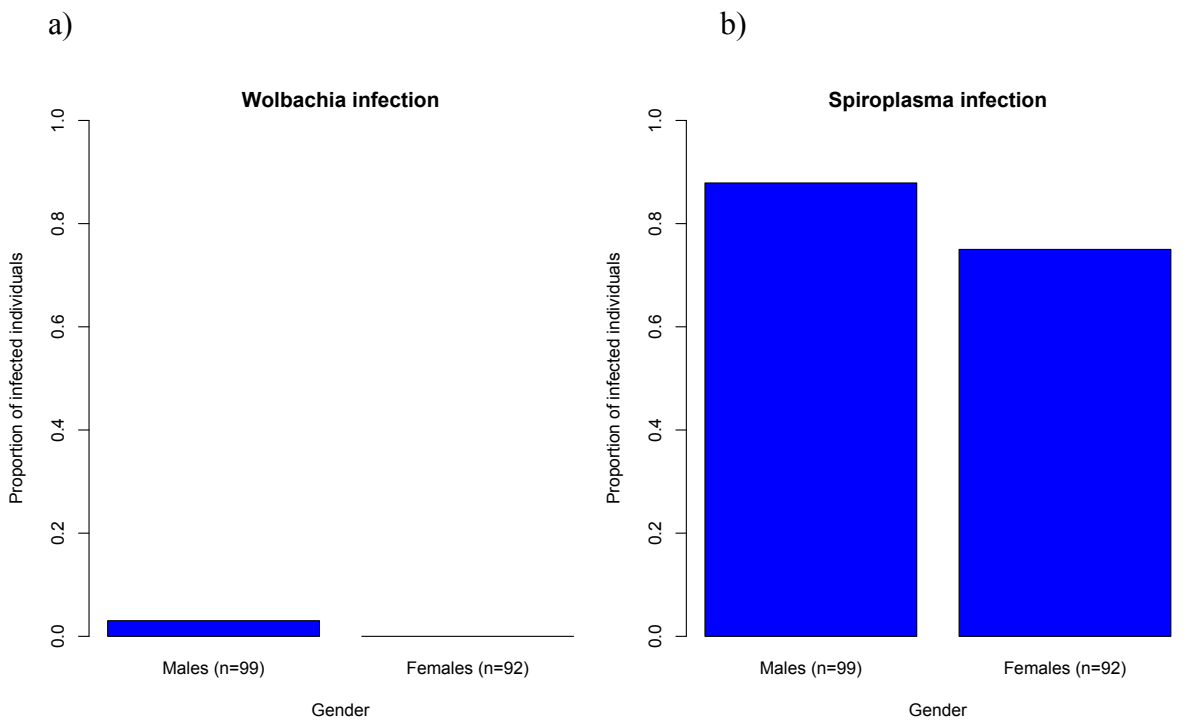


Fig 2. (a) This shows the proportion of *Wolbachia* infected male and female individuals. (b) This shows the proportion of *Spiroplasma* infected male and female individuals. The numbers in parentheses represent the sample sizes used.

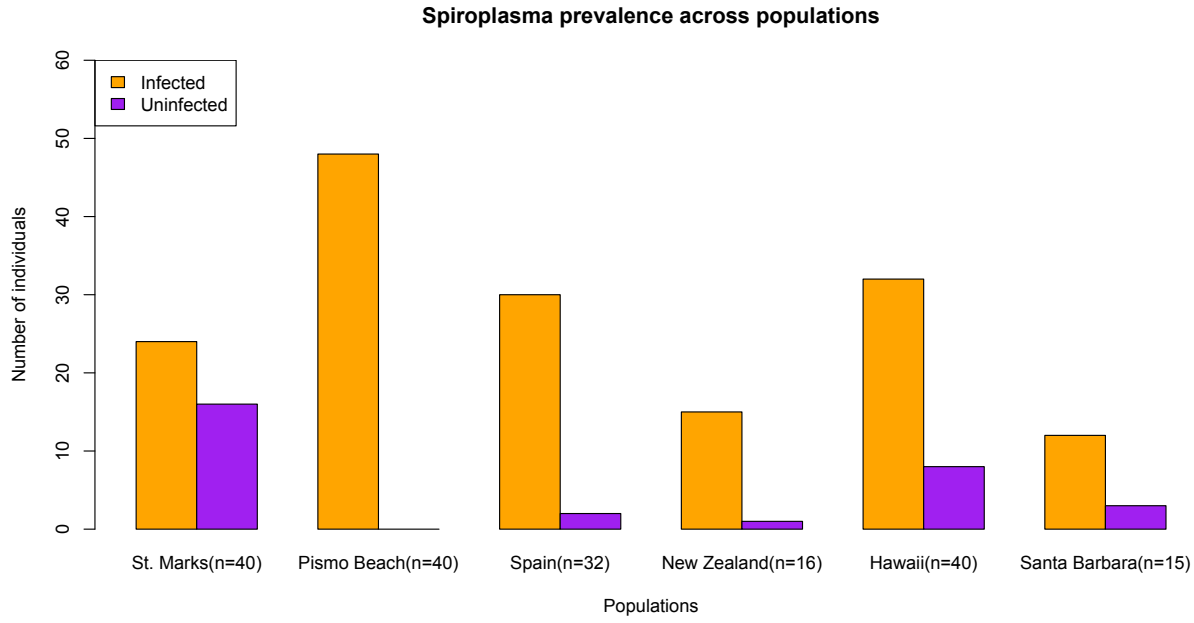


Fig 3. This graph shows the difference in the prevalence rates of *Spiroplasma* across the populations screened in this experiment. The numbers in parentheses represent the sample sizes used.

In order to test the reliability of the results obtained from this study, a quality control step was carried out. In the case of *Wolbachia*, all the individuals (3) that tested positive were screened again using PCR, and in the case of *Spiroplasma* 10 randomly selected males and 10 randomly selected females were screened again using PCR as well. The results obtained were the same as the initial results.

With such high prevalence rates in males and females, another possible relationship between *Spiroplasma* and its host was looked at – host protection. Given that 81.68% of the individuals screened tested positive for an infection with *Spiroplasma*, data comparing the prevalence rates of *Spiroplasma* and the Monarch's naturally occurring protozoan parasite[5] *Ophyryocystis elektroscirrha* (de Roode et. al 2008). *O. elektroscirrha* infects Monarch and Queen butterflies; there are no other known hosts. Infected individuals have spores on the exterior of the cuticle and can be detected in Monarchs by examining crushed abdomens under a microscope (McLaughlin & Myers 1970). The parasite is known to exhibit vertical transmission, and once infected the Monarchs cannot recover. Infected individuals have reduced survival, smaller body size and shorter life spans (Altizer & Oberhauser 1999). It was found that *Spiroplasma* does not appear to provide protection against *O. elektroscirrha* at the individual (X^2=0.093, df=1, P=0.76) or population level ($F_{1,4}$=0.362, P=0.58). See Fig. 4. We would have expected that populations with higher prevalence of *Spiroplasma* had lower parasite infection rates, while those with lower prevalence of *Spiroplasma* had higher parasite infection rates (See Fig. 3a), but this was not the case. Fig. 6 shows the *Spiroplasma* protection at the individual level for each population. No statistical analysis could be carried out due to the presence of several

[5] A protozoan parasite is a unicellular eukaryotic (true nucleus with nuclear membrane) organism that has attacked an individual by invading and living in the cells of that individual. Protozoan parasites cannot live independently like other protozoa and need another organism to provide them with food and protection.

zeros in the data set. That is, most individuals were not infected with the protozoan parasite in these populations.

Since double bands were only noticed in the Hawaii population, it is expected that there is something unusual taking place. Without sequencing, there is no way to ascertain the reason for the double bands. Given this ambiguity regarding the Hawaii population, another analysis of *Spiroplasma's* protection against *O. elektroscirrha* was done without the Hawaii population. It was found that at the population level, with increased *Spiroplasma* prevalence, the prevalence of the parasite decreased ($F_{1,3}$=19.88, P=0.02). At the individual level, however, removing Hawaii from the analysis did not affect the results. See Fig 5.

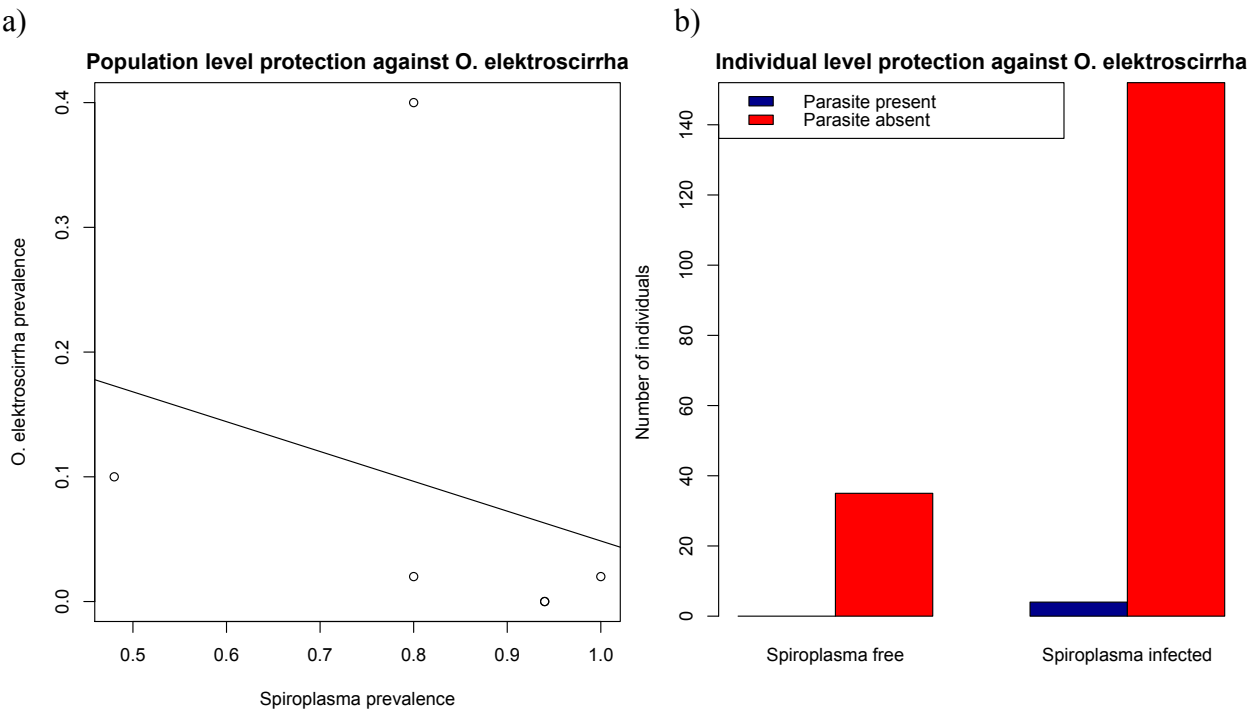


Fig 4. a) This graph shows the population infection rates against the prevalence of *Spiroplasma* to determine if there is any protection at the population level. b) This graph looks at the individual infection with the protozoan parasite and its *Spiroplasma* infection status.

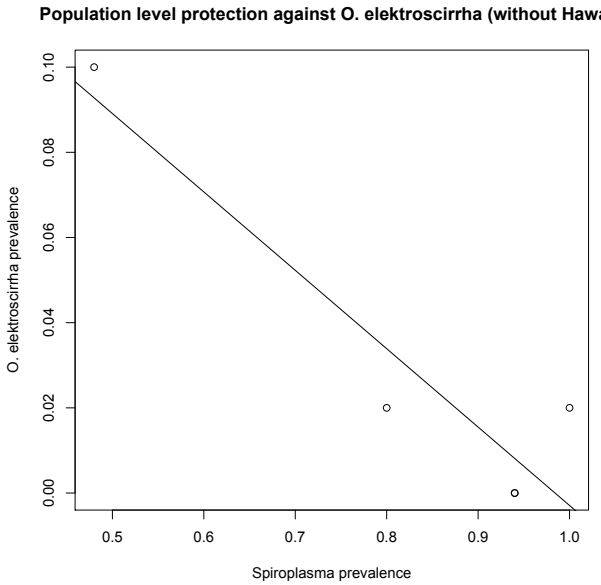


Fig. 5. **This graph shows the analysis of population level protection with the absence of the Hawaii population.**

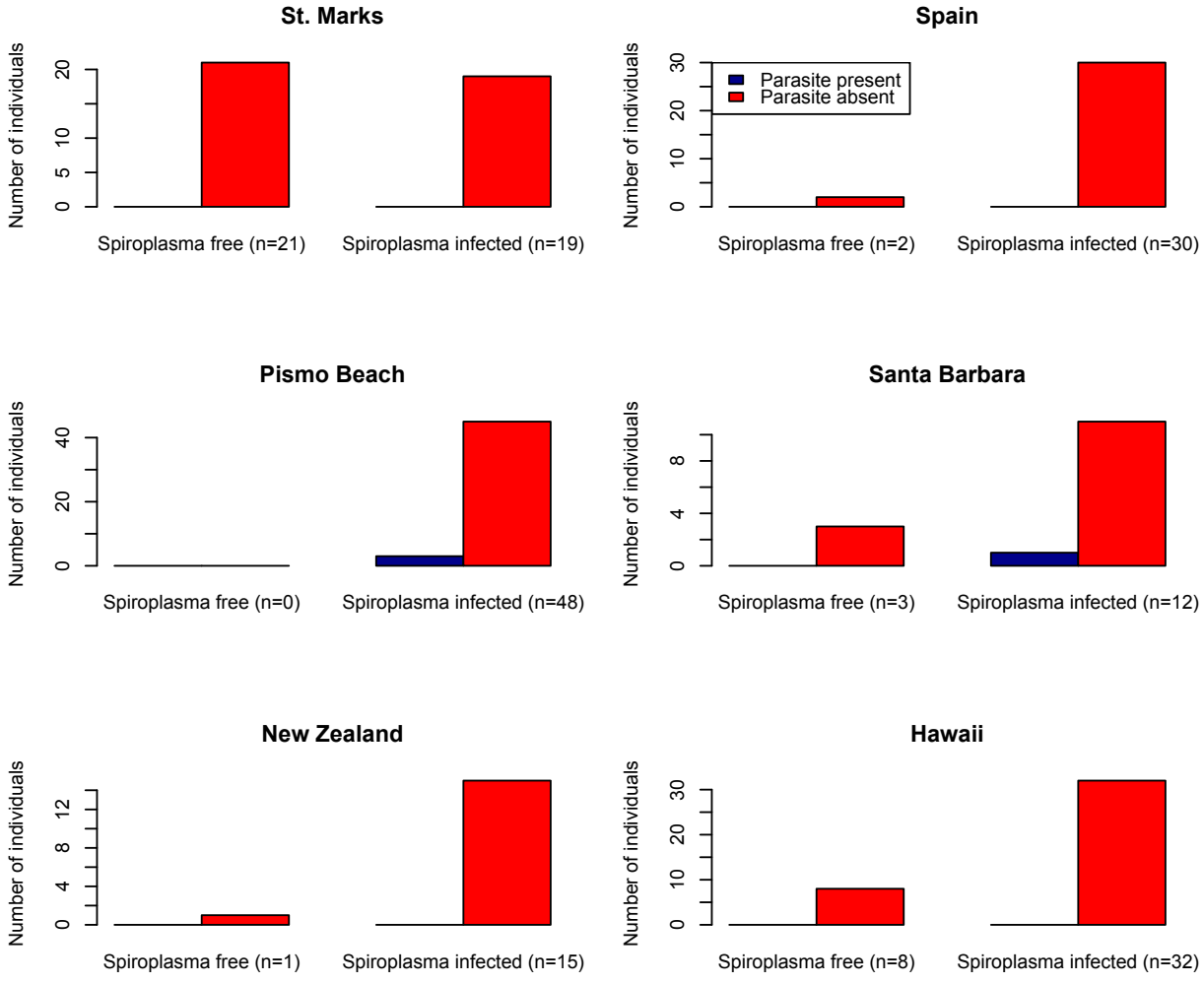


Fig 6. These graphs show the individual level *Spiroplasma* protection against *O. elektroscirrha* for each population. A chi-squared test could not be carried out due to the presence of several zeros in this sample size. The legend is applicable to all the graphs

Discussion

This study clearly shows that *Danaus plexippus* is infected with *Wolbachia* and *Spiroplasma*.

Wolbachia

Wolbachia infected individuals were found in isolated populations of St. Marks, FL, New Zealand and Spain. Based on these data, there are two hypotheses. CI seems to be the most common phenotype caused by *Wolbachia* (Zhou et al 1998). It is possible that the extremely low prevalence rate of *Wolbachia* is because Monarchs are infected with the CI inducing strain of *Wolbachia.* To recap, males infected with CI inducing strains of *Wolbachia* can only mate with females infected with the same strain, but are incompatible with uninfected females or females infected with another strain of *Wolbachia*. Consequently, we would expect that if infected males can only mate with infected females, *Wolbachia* infected males will eventually disappear from the population when no infected females are present, because this cross will lead to embryo death. However, if both infected males and females were present, it is likely that *Wolbachia* will be passed on to the next generation and persist in the population. With such a low prevalence rate, the former scenario is more likely. Furthermore, it would be expected that if *Wolbachia* induced CI in Monarchs, it would be extremely weak, since it was found that there was no difference in *Wolbachia* infection between males and females. Sequencing the *Wolbachia* found in the infected individuals can shed light on the particular strain of *Wolbachia,* and if it is in fact a CI inducing strain. To confirm this hypothesis, after determining the strain, CI can be tested in monarchs by infecting females with the same strain of *Wolbachia* and mating infected males

with both infected and uninfected males. If they are able to mate with infected females and not the uninfected females, it is highly likely that *Wolbachia* causes CI in monarchs.

It is more difficult to hypothesize if Monarchs are infected with a parthenogenetic strain of *Wolbachia,* just by looking at the prevalence rates. If it were parthenogenetic, we would expect more females to be infected than males because it provides infected females with the ability to reproduce in the absence of males. The infected individual would then pass the *Wolbachia* on to their offspring (which would, of course, be all female viz. asexual reproduction). However, the fact that there was no significant difference in the prevalence of *Wolbachia* between males and females suggests that the hypothesis that Monarchs are infected with a parthenogenetic strain of *Wolbachia* is not likely to be true.

Recent research on Monarch population demographics have shown that the number of females have been on the decline. Every winter, Monarch butterflies undergo a massive migration to overwintering sites where large numbers of Monarchs can be found together (Davis et. al 2009). In previous research studies, these overwintering sites have been studied in great detail where Monarchs have been captured and their sexes been recorded (Van Hook 1993). When recent attempts were made to compile sex ratio data from these previous records, it was found that the proportion of female Monarchs has gradually decreased over time (Davis et.al 2009). In light of this observation, it is unlikely that the strain of *Wolbachia* found in Monarchs is male killing or causes feminization of males. These phenotypes would suggest a female-biased sex ratio, or in cases of higher prevalence of *Wolbachia*, ultimately an all female population. A male-biased sex ratio is not expected if male mortality (male killing) is high. Furthermore, there was no significant difference in the prevalence of *Wolbachia* in males vs. females, suggesting that there is no gender-specific advantage or disadvantage associated with *Wolbachia* infection.

Second, this study suggests that there is horizontal transfer of *Wolbachia* to *Danaus plexippus* from other insect or arthropod species in the three infected populations. Since *Wolbachia* was not uniformly present in all populations, it suggests that there is something different in the environments of the butterflies in these populations. It is likely that Monarchs were only recently exposed to *Wolbachia*, because otherwise we would have expected *Wolbachia* to be prevalent in all populations, i.e. we would have expected *Wolbachia* to be present in Monarch populations when they spread around the world, thereby carrying the bacteria to their "new" location. Seeing as this is not the case, it is likely that *Wolbachia* transfer to Monarchs in the three infected populations happened from a closely related host or as a result of their specific environments. Furthermore, it is possible for horizontal transfer to occur within species itself. It is possible that one Monarch population received *Wolbachia* from an infected individual in another population. Seeing as they have extensive migration capabilities, horizontal transfer within Monarchs is plausible. It would be interesting to sequence the *Wolbachia* from the infected individuals and determine if the aforementioned populations are infected with the same strain of *Wolbachia* or different, and if they cause the same phenotype in all the infected populations.

Spiroplasma

It was found that proportion of males infected with *Spiroplasma* was higher than females. It is possible that *Spiroplasma* has a different effect on males vs. females, that is that males are more prone to infection than females. To understand the relationship between *Spiroplasma* and Monarchs, sequencing the bacteria will be helpful. This can help determine the strain and if there is a difference in the strain present in males vs. females. If there is a difference, then *Spiroplasma* produces different phenotypes in males and females, where it can benefit one and harm the other.

It is also possible that there is a genetic basis for the difference, i.e. males are genetically predisposed to acquire *Spiroplasma* or they were born with it. Furthermore, it is known that some *Spiroplasma* can cause disease in their host or can act as mutualists; keeping this in mind, there can be two hypotheses to explain the relationship between *Spiroplasma* and Monarchs, after sequencing. To determine the relationship, carrying out an experiment comparing the survival or fitness of infected and infected individuals will be helpful. First, if the survival or fitness of *Spiroplasma* infected individuals is less than uninfected individuals, then *Spiroplasma* is pathogenic for its host. At the same time, looking if there is a difference between the sexes can explain if *Spiroplasma* has a greater impact on one sex over the other. Second, if the reverse is true, i.e. if infected individuals have enhanced survival or fitness compared to uninfected individuals, then it is possible that *Spiroplasma* confers some kind of benefit to its host.

What is interesting to note about the infected individuals in Hawaii is that some individuals displayed two bands, while some individuals displayed either one or the other. In Appendix D, for individuals each band was labeled as *Spiroplasma* A for the heavier (top) band and *Spiroplasma* B for the lighter (bottom band). The latter band lined up with the positive control. There are three possible explanations for this. First, the individuals with two bands in the Hawaii population have a mutation at the 16s rDNA region that was being amplified. Second, individuals in the Hawaii population could be infected with multiple strains of *Spiroplasma* that have similar 16s rDNA regions. Third, mispriming could have occurred i.e. the primers were not specific enough at the chosen annealing temperature. This seems less likely since Hawaii was the only population in which double bands were seen.

It was also found that *Spiroplasma* does not provide protection to Monarchs against the protozoan parasite *O. elektroscirrha*. Although most individuals that were infected with

Spiroplasma did not have the parasite, there were a significant number of *Spiroplasma*-free individuals that did not have the parasite either. At the same time, there was a small number of infected individuals that contained the parasite, and there were no *Spiroplasma*-free individuals that contained the parasite. There was no difference in infection with the protozoan parasite in individuals infected or uninfected with *Spiroplasma.* This suggests that there is not a high level of protection from *Spiroplasma* against *O. elektroscirrha.* However, once the Hawaii population was removed from the analysis, we found a significant trend at the population level. There was a visible negative trend noticed, where with an increase in *Spiroplasma* prevalence the prevalence of *O. elektroscirrha* decreased. This shows that there might be some level of protection offered by *Spiroplasma*, but cannot be confirmed just yet. This is an interesting observation, further suggesting that there is something going in the Hawaii population that is different from the other populations screened in this experiment. It is important to note, however, that the infection with the protozoan parasite was relatively low in the individuals screened in this experiment. Protozoan infection was measured on a 0-5 scale, 0 being no infection and 5 being heavily infected. Any individual with an infection score of 4 or higher was considered infected.

These conclusions imply that there has to be another explanation for the high prevalence of *Spiroplasma* in Monarch populations. With such a high prevalence rate, there is the possibility that *Spiroplasma* has an effect on the fitness or survival of Monarchs. *Spiroplasma* might exhibit efficient vertical transmission, providing some benefit to the offspring, enhancing the survival and fitness of individuals, or might have the opposite effect on fitness. More importantly, the prevalence rates differed between the populations studied in this experiment; there could be two potential explanations for this. It might be possible that Monarchs are infected with different strains of *Spiroplasma,* which is extremely likely in Hawaii at least. If so, the bacterium may

cause different phenotypes in infected populations. Second, selection pressures favoring *Spiroplasma* may also vary within these populations, thus conferring different benefits to its host. That is, *Spiroplasma* may be in symbiosis with Monarchs, or may share a pathogenic relationship. These hypotheses have yet to be confirmed.

Future Directions

Now that it has been established that Monarchs are infected with *Spiroplasma* and *Wolbachia,* the next step would be to sequence the bacteria to determine the particular strain and phenotype the strain causes. This would provide useful information about the relationship between the Monarch and these bacteria. Once the strain is determined, it will be interesting to carry out some infection experiments to understand the effect of these bacteria on the fitness and survival of the Monarch. Furthermore, experiments that screen Monarch eggs for these bacteria would help determine if these bacteria exhibit vertical or horizontal transmission, and if they benefit the larvae in some way. Lastly, carrying out experiments that can explain the differing prevalence rates between Monarch populations can shed light on the effect of environment on the prevalence of these bacteria

References

Altizer, S.M. and Oberhauser, K.S. (1999). Effects of the protozoan parasite, *Ophyrocystis elektroscirrha,* on the fitness of monarch butterflies (*Danaus plexippus)*. *Journal of Invertebrate Pathology,* 74, 76-88.

Baumann, P., Baumann, L., Lai, C-Y, Rouhbakhsh, D., Moran, NA., Clark, MA. (1995). Genetics, physiology, and evolutionary relationships of the genus Buchnera: intracellular symbionts of aphids. *Annual Review of Microbiology,* 49, 55-94.

Davis, Andrew K., and Rendon-Salinas, Eduardo. (2009). Are Female Monarch Butterflies Declining in Eastern North America? Evidence of a 30-year Change in Sex Ratios at Mexican Overwintering Sites. *Biology Letters,* 6, 45-47.

Davis, R.E., Worley, J.F. (1973). Spiroplasma: motile, helical microorganism associated with corn stunt disease. *Phytopathology*, 63, 403–408.

De Roode, J.C., Pedersen, A.B., Hunter, M.D., Altizer, S. (2008a). Host plant species affects virulence in monarch butterfly parasites. *Journal of Animal Ecology*, 77, 120-126.

Dyson, E. A., Kamath, M. K. and Hurst, Gregory D.D. (2002). Wolbachia Infection Associated with All-female Broods in *Hypolimnas Bolina* (Lepidoptera: Nymphalidae): Evidence for Horizontal Transmission of a Butterfly Male Killer. *Heredity,* 88, 166-71.

Ebert, Mercedes A. (1991). The Interaction Phenotype in the *Drosophila Willistoni* – Spiroplasma Symbiosis. *Evolution,* 45, 971-988.

Frey, D., Leong, K. L. H., Fredericks, D., and Raskowitz, S. (1992). Clustering patterns of monarch butterflies (Lepidoptera: Danaidae) at two California central coast overwintering sites. *Entomological Society of America* 85, 148-153.

Gasparich, G. (2002). *Spiroplasmas*: evolution, adaptation and diversity *Front. Biosci,* 7, 619–640.

Hoffmann AA, Hercus M, Dagher H. (1998) Population dynamics of the Wolbachia infection causing cytoplasmic incompatibility in Drosophila melanogaster. *Genetics,* 148, 221-231.

Horner-Devine, M. Claire, Carney, Karen M. and Bohannan, Brendan J. M. (2004). An Ecological Perspective on Bacterial Biodiversity. *Proceedings of the Royal Society B: Biological Sciences* 271, 113-22.

Hurst, Gregory .D.D., Jiggins, Francis M. J. Hinrich Graf von der Schulenburg, Dominique Bertrand, Stuart A. West, Irina I. Goriacheva, Ilia A. Zakharov,

Hurst, Gregory D.D., and Jiggins, Francis M. (2000). Male-Killing Bacteria in Insects: Mechanisms, Incidence, and Implications. *Emerging Infectious Diseases* 6, 329-36.

Jaenike, J., Unckless, R., Cockburn, S.N., Boelio, L.M. and Perlman, S.J. (2010). Adaptation via Symbiosis: Recent Spread of a Drosophila Defensive Symbiont. *Science,* 329, 212-15.

Jaenike, John. (2011). Population genetics of beneficial heritable symbionts. *Trends in Ecology and Evolution,* 27, 226-32.

Jiggins, F.M., Hurst, G. D. D., Jiggins, C. D., Schulenburg, J. H. G. v. d. and Majerus, M. E. N. (2000). The butterfly Danaus chrysippus is infected by a male-killing Spiroplasma bacterium. *Parasitology,*120, 439-446.

John H. Werren, Richard Stouthamer and Michael E. N. Majerus. (1999). *Proceedings of the Royal Society B: Biological Sciences,* 266, 735-740.

Kingsley, V. V. (1972). Persistence of intestinal bacteria in the developmental stages of the Monarch butterfly (Danaus plexippus). *Journal of Invertebrate Pathology,* 20, 51-58.

Knight, Jonathan. (2001). Meet the Herod Bug. *Nature*, 412, 12-15.

Kozek, Wieslaw J., Rao, Ramakrishna U. (2007). The Discovery of Wolbachia in Arthropods and Nematodes – A Historical Perspective. *Issues in Infectious Diseases*, 5, 1–14.

Mateos, M., Castrezana, S. J., Nankivell, B. J., Estes, A. M., Markow, T. A. and Moran, N. A. (2006). Heritable Endosymbionts of Drosophila. *Genetics*, 174, 363-76.

McLaughlin, R.E. and Myers, J. (1970). *Ophryocystis elektroscirrha* sp. n. a neogregarine pathogen of the monarch butterfly *Danaus plexippus* (L.) and the Florida queen butterfly *Danaus gilippus berenice* Cramer. *Journal of Protozoology*, 17, 300-305.

Min, Kyung-Tai, and Benzer, Seymour. (1997). *Wolbachia*, Normally a Symbiont of Drosophila, Can Be Virulent, Causing Degeneration and Early Death. *PNAS,* 94, 10792-0796.

Moya, A., Pereto, J., Gil, R. & Latorre, A. (2008). Learning how to live together: genomic insights into prokaryote-animal symbioses. *Nat. Rev. Genet.*, 9, 218-29.

O'Neill, S.L., Giordano, R., Colbert, A.M.E., Karr, T.L. & Robertson, H.M. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci.* USA, 94, 2699-2702.

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P. & Solignac, M. (1992). *Wolbachia* endosymbionts responsible for various sexual alterations of sexuality in arthropods. *Proc. R. Soc. Lond. B*, 250, 91-98.

Sanchez-Contreras, Maria and Vlisidou, Isabella. (2008) The Diversity of Insect-bacteria Interactions and its Applications for Disease Control. *Biotechnology and Genetic Engineering Reviews,* 25, 203-244.

Stouthamer, R. (1990). Proceedings and abstracts, Vth International Colloquium on Invertebrate Pathology and Microbial Control, Adelaide, Australia, pp. 417-421.

Stouthamer, R. & Werren, J.H. (1993). Microbes associated with parthenogenesis in wasps of the genus *Trichogramma*. *J. Invertebr. Pathol*., 61, 6-9.

Stouthamer, R., Breeuwer, J. A. J., Luck, R. F. & Werren, J. H. (1993). Molecular identification of microorganisms associated with parthenogenesis. *Nature. Lond.,* 361, 66-68.

Stouthamer, R., R.F. Luck & W.D. Hamilton. (1990). Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Nat. Acad. Sci.* 87, 2424-2427.

Tóth E. M., Hell, É., Kovács, G., Borsodi, A. K., Márialigeti, K. (2006). Bacteria Isolated from the Different Developmental Stages and Larval Organs of the Obligate Parasitic Fly, *Wohlfahrtua magnifica* (Diptera: Sacrophagidae). *Microbial Ecology,* 51, 13-21.

Urquhart, F. A. AND Urquhart, N. R. (1978). Autumnal migration routes of the eastern population of the monarch butterfly (*Danaus p. plexippus* L.; Danaidae; Lepidoptera) in North America to the overwintering site in the Neovolcanic plateau of Mexico. *Can. J.Zool*. 56, 1759–1764.

Van Hook, T. (1993). Non-random mating in monarch butterflies overwintering in Mexico. *Biology and conservation of the monarch butterfly* (eds S. B. Malcolm & M. P.Zalucki), 49–60.

Vandekerckhove TTM, Watteyne S, Willems A, Swings JG, Mertens J, Gillis M(1999) Phylogenetic analysis of the 16SrRNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. *FEMS Microbiol Lett*, 180, 279-286.

Vilmos, P. & Kurucz, E. (1998). Insect immunity: evolutionary roots of the mammalian innate immune system. *Immunol Lett*, 62, 59-66.

Watts, Thomas, Haselkorn, Tamara S., Moran, Nancy A. and Markow, Therese A. (2009). Variable Incidence of Spiroplasma Infections in Natural Populations of Drosophila Species. *PLoS ONE,* 4, E5703.

Werren, J.H. (1997). Biology of *Wolbachia*. *Ann. Rev. Entom*., 42, 587-609

Werren, J.H. (2003) Invasion of the Gender Benders. *Natural History,* 112, 58-63.

Werren, John H., and Windsor, Donald M. (2000) Wolbachia Infection Frequencies in Insects: Evidence of a Global Equilibrium? *Proceedings: Biological Sciences,* 267, 1277-285.

Werren, John H., Windsor, Donald and Guo, Lirong. (1995) Distribution of Wolbachia among Neotropical Arthropods. *Proceedings of the Royal Society B: Biological Sciences,* 262, 197-204.

Werren, John H., Zhang, Wan and Guo, Li Rong. (1995). Evolution and Phylogeny of Wolbachia: Reproductive Parasites of Arthropods. *Proceedings of the Royal Society B: Biological Sciences*, 261, 55-63.

Yen, J.H. and Barr, A.R. (1971) New hypothesis of the cause for cytoplasmic incompatibility in *Culex pipiens*. *Nature* , 232, 657-968.

Zhou, W., Rousset, F. & O'Neill, S. (1998) Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. Lond. B*, 265, 509-515.

Appendix A

Table 3: Summary of individual Monarchs used and infection status. Individuals that were infected with the bacteria =1, infection-free=0.

Location	Season	Monarch	Gender	Spiroplasma infection	Wolbachia infection	Protozoan infection
St. Marks	Oct-09	86	F	1	0	0
St. Marks	Oct-09	82	F	1	0	0
St. Marks	Oct-09	67	F	0	0	0
St. Marks	Oct-09	71	F	1	0	0
St. Marks	Oct-09	47	F	1	0	0
St. Marks	Oct-09	93	F	0	0	0
St. Marks	Oct-09	78	F	1	0	0
St. Marks	Oct-09	6	F	0	0	0
St. Marks	Oct-09	68	F	0	0	0
St. Marks	Oct-09	84	F	0	0	0
St. Marks	Oct-09	45	F	1	0	0
St. Marks	Oct-09	48	F	1	0	0
St. Marks	Oct-09	90	F	0	0	0
St. Marks	Oct-09	160	F	0	0	0
St. Marks	Oct-09	157	F	0	0	0
St. Marks	Oct-09	186	F	0	0	0
St. Marks	Oct-09	161	F	0	0	0
St. Marks	Oct-09	170	F	0	0	0
St. Marks	Oct-09	178	F	0	0	0
St. Marks	Oct-09	175	F	0	0	0
St. Marks	Oct-09	132	M	0	0	0

St. Marks	Oct-09	120	M	1	0	0
St. Marks	Oct-09	156	M	0	0	0
St. Marks	Oct-09	173	M	0	1	0
St. Marks	Oct-09	116	M	0	0	0
St. Marks	Oct-09	118	M	1	0	0
St. Marks	Oct-09	121	M	0	0	0
St. Marks	Oct-09	122	M	1	0	0
St. Marks	Oct-09	126	M	1	0	0
St. Marks	Oct-09	137	M	1	0	0
St. Marks	Oct-09	135	M	1	0	0
St. Marks	Oct-09	134	M	1	0	0
St. Marks	Oct-09	133	M	1	0	0
St. Marks	Oct-09	129	M	1	0	0
St. Marks	Oct-09	148	M	1	0	0
St. Marks	Oct-09	155	M	0	0	0
St. Marks	Oct-09	185	M	0	0	0
St. Marks	Oct-09	181	M	1	0	0
St. Marks	Oct-09	138	M	0	0	0
St. Marks	Oct-09	158	M	1	0	0
Spain	Jan-12	ESP1	F	1	0	0
Spain	Jan-12	ESP2	M	0	1	0
Spain	Jan-12	ESP3	F	1	0	0
Spain	Jan-12	ESP4	M	1	0	0
Spain	Jan-12	ESP5	M	1	0	0
Spain	Jan-12	ESP6	M	1	0	0
Spain	Jan-12	ESP7	F	1	0	0
Spain	Jan-12	ESP8	M	1	0	0

Spain	Jan-12	ESP9	M	1	0	0
Spain	Jan-12	ESP10	F	1	0	0
Spain	Jan-12	ESP11	M	1	0	0
Spain	Jan-12	ESP12	F	1	0	0
Spain	Jan-12	ESP13	F	1	0	0
Spain	Jan-12	ESP14	M	1	0	0
Spain	Jan-12	ESP15	F	1	0	0
Spain	Jan-12	ESP16	F	1	0	0
Spain	Jan-12	ESP17	F	1	0	0
Spain	Jan-12	ESP18	F	1	0	0
Spain	Jan-12	ESP19	F	1	0	0
Spain	Jan-12	ESP20	F	1	0	0
Spain	Jan-12	ESP21	F	1	0	0
Spain	Jan-12	ESP22	F	1	0	0
Spain	Jan-12	ESP23	M	1	0	0
Spain	Jan-12	ESP24	M	1	0	0
Spain	Jan-12	ESP25	F	1	0	0
Spain	Jan-12	ESP26	F	0	0	0
Spain	Jan-12	ESP27	F	1	0	0
Spain	Jan-12	ESP28	M	1	0	0
Spain	Jan-12	ESP29	F	1	0	0
Spain	Jan-12	ESP30	M	1	0	0
Spain	Jan-12	ESP31	F	1	0	0
Spain	Jan-12	ESP32	M	1	0	0
Pismo Beach	Feb-09	W1	F	1	0	0
Pismo Beach	Feb-09	W2	F	1	0	0
Pismo Beach	Feb-09	W4	F	1	0	0

Pismo Beach	Feb-09	W7	F	1	0	0
Pismo Beach	Feb-09	W8	F	1	0	0
Pismo Beach	Feb-09	W6	F	1	0	0
Pismo Beach	Feb-09	W10	F	1	0	0
Pismo Beach	Feb-09	W11	F	1	0	0
Pismo Beach	Feb-09	W12	F	1	0	0
Pismo Beach	Feb-09	W14	F	1	0	0
Pismo Beach	Feb-09	W15	F	1	0	0
Pismo Beach	Feb-09	W19	F	1	0	0
Pismo Beach	Feb-09	W20	F	1	0	0
Pismo Beach	Feb-09	W21	F	1	0	0
Pismo Beach	Feb-09	W22	F	1	0	0
Pismo Beach	Feb-09	W23	F	1	0	0
Pismo Beach	Feb-09	W24	F	1	0	0
Pismo Beach	Feb-09	W25	F	1	0	0
Pismo Beach	Feb-09	W26	F	1	0	0
Pismo Beach	Feb-09	W27	F	1	0	0
Pismo Beach	Feb-09	W29	F	1	0	0
Pismo Beach	Feb-09	W30	F	1	0	0
Pismo Beach	Feb-09	W31	F	1	0	0
Pismo Beach	Feb-09	W33	F	1	0	0
Pismo Beach	Feb-09	W101	M	1	0	0
Pismo Beach	Feb-09	W102	M	1	0	0
Pismo Beach	Feb-09	W103	M	1	0	0
Pismo Beach	Feb-09	W104	M	1	0	0
Pismo Beach	Feb-09	W105	M	1	0	0
Pismo Beach	Feb-09	W106	M	1	0	1

Pismo Beach	Feb-09	W107	M	1	0	0
Pismo Beach	Feb-09	W108	M	1	0	0
Pismo Beach	Feb-09	W109	M	1	0	0
Pismo Beach	Feb-09	W110	M	1	0	0
Pismo Beach	Feb-09	W111	M	1	0	0
Pismo Beach	Feb-09	W112	M	1	0	0
Pismo Beach	Feb-09	W113	M	1	0	0
Pismo Beach	Feb-09	W114	M	1	0	0
Pismo Beach	Feb-09	W115	M	1	0	0
Pismo Beach	Feb-09	W116	M	1	0	0
Pismo Beach	Feb-09	W117	M	1	0	1
Pismo Beach	Feb-09	W118	M	1	0	0
Pismo Beach	Feb-09	W119	M	1	0	0
Pismo Beach	Feb-09	W120	M	1	0	0
Pismo Beach	Feb-09	W121	M	1	0	1
Pismo Beach	Feb-09	W122	M	1	0	0
Pismo Beach	Feb-09	W123	M	1	0	0
Pismo Beach	Feb-09	W124	M	1	0	0
Santa Barbara	Nov-09	SB146	M	1	0	0
Santa Barbara	Nov-09	SB103	M	0	0	0
Santa Barbara	Nov-09	SB131	M	1	0	0
Santa Barbara	Nov-09	SB123	M	1	0	0
Santa Barbara	Nov-09	SB167	M	1	0	1
Santa Barbara	Nov-09	SB133	M	1	0	0
Santa Barbara	Nov-09	SB159	M	1	0	0
Santa Barbara	Nov-09	SB64	M	1	0	0
Santa Barbara	Nov-09	SB58	M	1	0	0

Santa Barbara	Nov-09	SB73	M	1	0	0
Santa Barbara	Nov-09	SB111	F	1	0	0
Santa Barbara	Nov-09	SB106	F	1	0	0
Santa Barbara	Nov-09	SB140	F	0	0	0
Santa Barbara	Nov-09	SB155	F	1	0	0
Santa Barbara	Nov-09	SB67	F	0	0	0
New Zealand	Jan-11	NZ1	F	1	0	0
New Zealand	Jan-11	NZ2	F	1	0	0
New Zealand	Jan-11	NZ3	F	1	0	0
New Zealand	Jan-11	NZ4	F	1	0	0
New Zealand	Jan-11	NZ5	M	0	0	0
New Zealand	Jan-11	NZ6	M	1	0	0
New Zealand	Jan-11	NZ7	M	1	0	0
New Zealand	Jan-11	NZ8	M	1	0	0
New Zealand	Jan-11	NZ9	M	1	0	0
New Zealand	Jan-11	NZ10	M	1	0	0
New Zealand	Jan-11	NZ11	M	1	0	0
New Zealand	Jan-11	NZ12	M	1	0	0
New Zealand	Jan-11	NZ13	M	1	0	0
New Zealand	Jan-11	NZ14	M	1	1	0
New Zealand	Jan-11	NZ15	M	1	0	0
New Zealand	Jan-11	NZ16	M	1	0	0
Hawaii	Nov-09	HW1	M	1	0	0
Hawaii	Nov-09	HD9	M	1	0	0
Hawaii	Nov-09	HJ8	M	0	0	0
Hawaii	Nov-09	HJ1	M	1	0	0
Hawaii	Nov-09	KK2	M	1	0	0

Hawaii	Nov-09	HL10	M	1	0	0
Hawaii	Nov-09	HO6	M	1	0	0
Hawaii	Nov-09	HE2	M	1	0	0
Hawaii	Nov-09	HE4	M	1	0	0
Hawaii	Nov-09	HF2	M	1	0	0
Hawaii	Nov-09	HP6	M	1	0	0
Hawaii	Nov-09	HO1	M	1	0	0
Hawaii	Nov-09	HQ2	M	1	0	0
Hawaii	Nov-09	HD4	M	1	0	0
Hawaii	Nov-09	HJ11	M	1	0	0
Hawaii	Nov-09	HK5	M	1	0	0
Hawaii	Nov-09	HI1	M	1	0	0
Hawaii	Nov-09	HM4	M	1	0	0
Hawaii	Nov-09	HM8	M	1	0	0
Hawaii	Nov-09	HM7	M	1	0	0
Hawaii	Nov-09	HI9	F	1	0	0
Hawaii	Nov-09	HI2	F	0	0	0
Hawaii	Nov-09	HU8	F	1	0	0
Hawaii	Nov-09	HO8	F	0	0	0
Hawaii	Nov-09	HM9	F	1	0	0
Hawaii	Nov-09	HT5	F	1	0	0
Hawaii	Nov-09	HB1	F	1	0	0
Hawaii	Nov-09	HI12	F	0	0	0
Hawaii	Nov-09	HI4	F	1	0	0
Hawaii	Nov-09	HS2	F	1	0	0
Hawaii	Nov-09	HO9	F	1	0	0
Hawaii	Nov-09	HH1	F	1	0	0

Hawaii	Nov-09	HR2	F	0	0	0
Hawaii	Nov-09	HM4	F	0	0	0
Hawaii	Nov-09	HT1	F	0	0	0
Hawaii	Nov-09	HQ1	F	1	0	0
Hawaii	Nov-09	HJ1	F	0	0	0
Hawaii	Nov-09	AK3	F	1	0	0
Hawaii	Nov-09	HO5	F	1	0	0
Hawaii	Nov-09	HF3	F	1	0	0

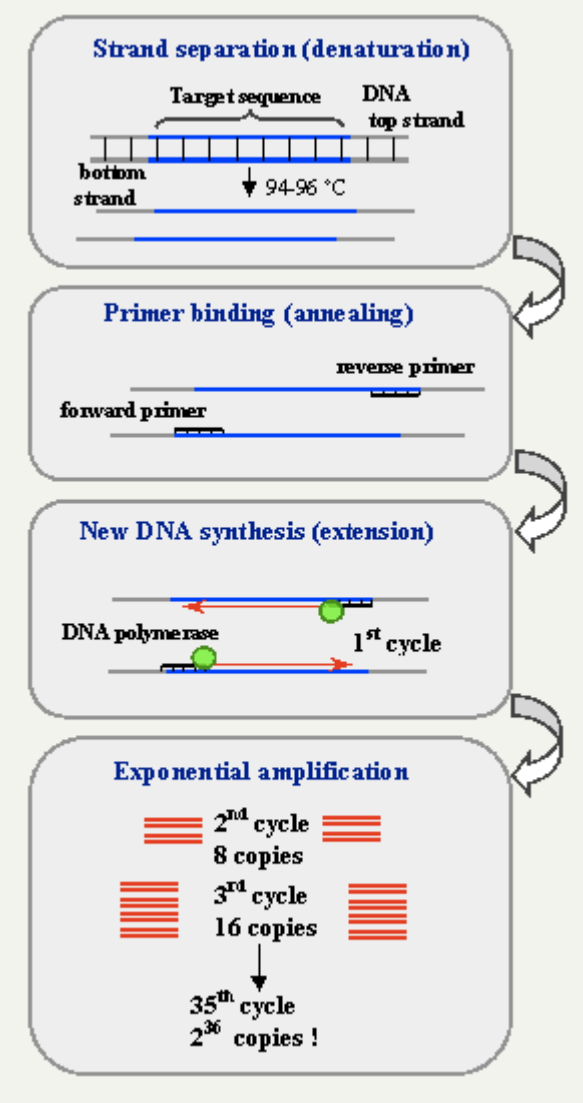
Appendix B

PCR explained

Each PCR reaction usually contains the following:

- Target DNA sequence that needs to amplified
- Primers: 2 strands of nucleic acid that are complementary to the 3' ends of each double-stranded DNA template. These are required to identify the target and provide the first nucleotide necessary for DNA replication, because the enzymes that are responsible for adding new base pairs can only do so to existing DNA.
- Taq Polymerase/DNA polymerase: Responsible for the elongation of the new strand of DNA complementary to the target.
- Buffer: Required for the stability of the DNA polymerase and provides a suitable chemical environment for the reaction to take place.
- Nucleotides (dNTPs): The building blocks or base pairs A,C,G,T required for new DNA

Fig 1. How PCR works



Initialization: The reaction mixture is heated to 94-96°C. This activates the DNA polymerase.

Denaturation: This is the first step of the PCR cycle, where the high temperature melts the double stranded DNA template to generate two single strands.

Annealing: The reaction temperature is lowered (50-65°C), depending on the annealing temperature set, to allow the primer to bind the single-stranded DNA molecules. This will only occur if the primer sequence very closely matches the target DNA sequence.

Elongation: The reaction temperature in this step depends on the DNA Polymerase used, but generally 72°C works. The DNA Polymerase synthesizes a new strand of DNA by adding dNTPs complementary to the template

This continues for several cycles.

Final elongation: This is the last step after the last PCR cycle to ensure all the DNA strands have been extended.

The reaction is held at 5-15°C; **it can be stored at this temperature till ready for use.**

Appendix C

UltraClean™ Tissue and Cells DNA Isolation Kit – MO BIO Laboratories, Inc.

(Copied Protocol from the Kit)

Detailed Protocol (Describes what is happening at each step)
Please wear gloves at all times

1. Shake to mix Solution TD1. If you are isolating from tissue, to the **Dry Bead Tubes** provided, add 700 µl of **Solution TD1**. Then add 1-25 mg of tissue.

What's happening: Solution TD1 is required for tissue or cultured cell homogenization and cell lysis. Solution TD1 is also a high concentration salt solution required for binding DNA.

2. **Proteinase K** Digestion (Optional)

For soft tissue samples a **Proteinase K** digestion is not required. A 20 mg/ml Proteinase K Solution is included in this kit for processing tough tissue samples.

What's happening: Proteinase K helps break down tough tissues and facilitates cell lysis. It is an endopeptidase enzyme that catalyzes the hydrolysis of proteins.

3. Secure **Dry Bead Tubes** horizontally using a Vortex Adapter (MO BIO Catalog# 13000-V1) or adhere to a flatbed pad with tape, then vortex at maximum speed for 10 minutes.

What's happening: Vortexing is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical lysis and mechanical shaking. The vortex action is typically all that is required, however, more robust bead beaters may also be used.

4. Remove tubes from adapter and make sure the **Dry Bead Tubes** rotate freely in the centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 1 minute at room temperature.

What's happening: Cellular debris is sent to the bottom of the tube while DNA remains in the supernatant.

5. Avoiding the beads, transfer the entire volume of liquid sample to a **Spin Filter** (provided) and centrifuge at 10,000 x *g* for 30 seconds at room temperature.
What's happening: DNA is selectively bound to the silica membrane of the Spin Filter. Contaminants pass through the filter membrane, leaving only the DNA bound to the membrane.

6. Discard the flow through.
What's happening: The flow through contains non-DNA organic and inorganic waste.

7. Add 400 ml of **Solution TD2** and centrifuge at 10,000 x *g* for 30 seconds at room temperature.

What's happening: Solution TD2 is an ethanol based wash solution used to clean the DNA that is bound to the silica membrane of the spin filter. Solution TD2 removes residual salts, cellular debris, and proteins while allowing the DNA to stay bound to the membrane.
8. Discard the flow through.

What's happening: The flow through contains non-DNA material washed away by Solution TD2.
9. Centrifuge again at 10,000 x *g* for 1 minute at room temperature to remove residual **Solution TD2**.

What's happening: The second spin removes residual Solution TD2. It is critical to remove all traces of the wash solution because the ethanol can interfere with many downstream applications such as PCR, restriction digests, and gel electrophoresis.
10. Carefully place the **Spin Filter** in a new clean **2 ml Collection Tube** (provided).

11. Add 50 ml of **Solution TD3** to the center of the white filter membrane.

What's happening: Solution TD3 is an elution buffer. Placing Solution TD3 in the center of the small white filter membrane will ensure that the entire membrane is wetted. This will result in a more efficient and complete release of DNA from the silica Spin Filter membrane. When Solution TD3 passes through the silica membrane, DNA that was bound in the presence of high salt is now selectively released by Solution TD3 which lacks salt.

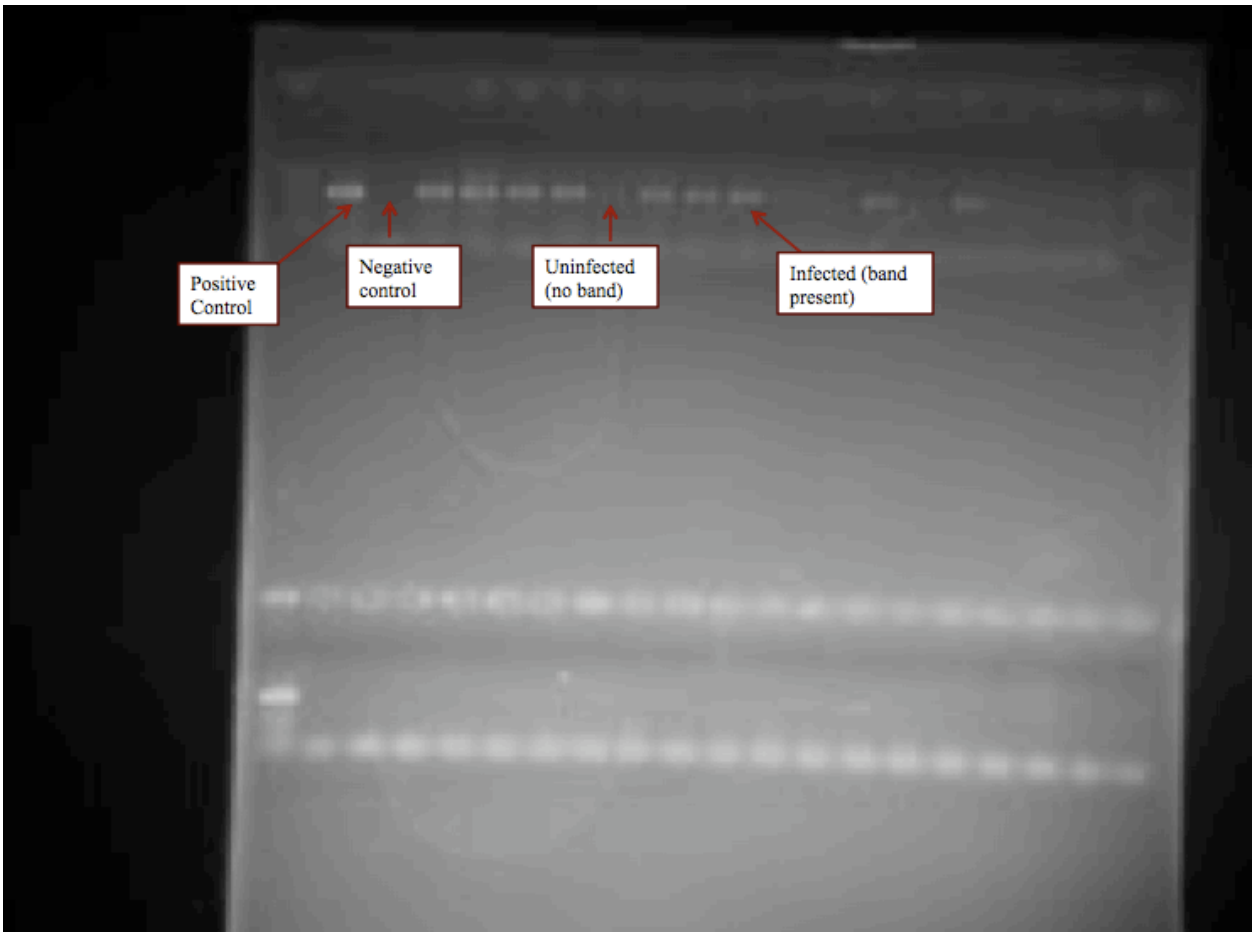
12. Centrifuge at 10,000 x *g* for 30 seconds at room temperature.

13. Discard the **Spin Filter**. DNA in the **2 ml Collection Tube** is now ready for any downstream application. No further steps are required.

Appendix D

Gel Images

1. **Gel without double bands**



2. Gel from Hawaii population