The invasion and spread of the bacterial endosymbiont *Cardinium* in *Encarsia pergandiella* (Hymenoptera: Aphelinidae)

by

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B.Sc., University of Lethbridge, 2005

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ABSTRACT

A large and extremely diverse number of insects harbour maternally transmitted bacterial symbionts. Some symbionts manipulate host reproduction in order to benefit their own fitness, and the most common of these reproductive manipulations is cytoplasmic incompatibility (CI). In CI, uninfected females produce few or no viable progeny when mated to infected males.

The bacterial endosymbiont *Cardinium* causes CI in its host, *Encarsia pergandiella* (Hymenoptera: Aphelinidae). I used population cages with varying initial infection frequencies to test a model of CI invasion. *Cardinium* was found to spread rapidly in all populations, even in cases where the initial infection frequency was well below the predicted invasion threshold frequency.

Male age can also be an important factor in CI dynamics. I tested the effect of male age on the level of incompatibility induced by *Cardinium* in *E. pergandiella*. Male age was found to have a negligible effect on CI strength.

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Chapter I: Introduction to insect symbionts and cytoplasmic incompatibility

Symbiosis can be defined as a long-term, intimate interaction between organisms of different species. As many as 70% of all insects have microbial symbionts, and these symbionts can play important roles in the ecology and evolution of their hosts (Ishikawa, 2003). There is a plethora of diversity among symbionts, ranging from mutualists that provide essential vitamins and nutrients to their hosts, to commensal gut microbes and reproductive parasites. Many symbionts are mutualists, and some of these are essential to the function of their insect hosts. For example, the aphid symbiont *Buchnera* is so critical to its aphid host that its removal results in reduced growth or host sterility (Houk and Griffiths, 1980). In addition to mutualistic symbionts, there are several parasitic symbionts, including *Wolbachia*, *Cardinium*, *Spiroplasma* and *Microsporidia*, which manipulate host reproduction in a variety of ways to benefit their own transmission.

Insect symbionts fall into two main categories: primary symbionts and secondary symbionts. Primary symbionts are obligate, and are typically housed in specialized host derived cells (Buchner, 1965; Moran, 2001). They are often associated with insects that have nutrient poor diets, such as blood, sap and wood feeders, where they provide their hosts with essential nutrients (Buchner, 1965). Secondary symbionts are typically facultative, and thus are not necessary for host survival and reproduction. They generally have a more varied distribution within the insect, and their effects on hosts are more diverse, ranging from mutualistic to parasitic (Moran, 2001).

The aphid symbiont *Buchnera* is probably the best described primary symbiont. Aphids feed exclusively on plant sap, which is rich in carbohydrates but limited in amino

acids. *Buchnera* is able to synthesize and provide these essential amino acids for its aphid hosts (Sasaki and Ishikawa, 1995). In exchange, the aphid provides *Buchnera* with a favourable living environment. This ancient symbiont lineage has been found in 80 million year old amber deposits, and is estimated to be 160 to 280 million years old (Moran and Baumann, 1994). Phylogenetic studies suggest that there was a single origin of *Buchnera*, and that faithful vertical transmission has allowed it to diverge in parallel with its aphid hosts (Wernegreen, 2002).

While secondary symbionts are generally not restricted to specialized cells within insects, they are similar to primary symbionts in that they are vertically transmitted from mothers to their offspring. Secondary symbionts have evolved a number of strategies to ensure their transmission to the next generation. Some of these symbionts accomplish this by directly benefiting their hosts, for example, by providing their hosts with protection from parasitoids (Oliver et al. 2003). Other secondary symbionts, known as the reproductive manipulators, enhance their transmission by manipulating host reproduction in ways that benefit their own fitness. Because these symbionts are transmitted only through females, they can spread in the population if infected females produce more daughters than uninfected females. There are currently four known types of reproductive manipulation, and these will be discussed in more detail subsequently.

The reproductive manipulators *Wolbachia* and *Cardinium*

The α Proteobacterium *Wolbachia* is well known as a master manipulator of arthropod reproduction (Stouthamer et al., 1999). Molecular screening assays indicate that *Wolbachia* is found in at least 16% to 24% of all arthropod species (Werren et al.

1995; Weeks et al. 2003; Zchori-Fein and Perlman 2004), and extrapolations estimate that 1.7 to 5.1 million insect species are infected globally, perhaps making it the most abundant parasitic bacterium worldwide (Werren et al., 1995). In fact, a recent meta-analysis estimates *Wolbachia* prevalence in insects to be even higher, infecting approximately 66% of all species (Hilgenboecker et al., 2008). *Wolbachia* is extremely widespread, and has been found to infect all of the major insect orders, other arthropods, and filarial nematodes (Werren et al., 1995; Lo et al., 2007). Interestingly, in filarial nematodes, *Wolbachia* is found at 100% prevalence, and acts as a mutualist (Bandi et al., 2001). While the effects of *Wolbachia* on arthropods are largely unknown, it has generally been found to be a reproductive parasite in these hosts (Lo et al., 2007). Strains of *Wolbachia* have been found to induce male killing, feminization, parthenogenesis, and CI in their hosts.

Wolbachia was first discovered by Hertig and Wolbach in 1924 in the mosquito *Culex pipiens* (Hertig and Wolbach, 1924), and in 1971, it was discovered to be the causative agent of cytoplasmic incompatibility in mosquitoes (Yen and Barr, 1971). Conventionally, researchers have referred to all closely related bacteria as strains of *Wolbachia*, although some strains show greater than 3% difference in 16S rDNA sequences (Lo et al., 2007). Recently it has been suggested that, to avoid confusion, all strains should be referred to as *W. pipientis* (Lo et al., 2007), although this remains controversial (Werren et al., 2008).

Unlike primary symbionts such as *Buchnera,* the phylogeny of *Wolbachia* is not congruent with that of its hosts, indicating that horizontal transmission events do occur. Indeed, closely related strains of *Wolbachia* have been found in distantly related

arthropods, some of which are ecologically related. For example, closely related *Wolbachia* are found in the parasitoid *Nasonia* and its host *Sarcophaga* (Werren et al., 1995). Similarly, the moth *Ephestia kuehniella* and its parasitoid wasp *Trichogramma bourarache* harbour closely related strains of *Wolbachia* (van Meer et al., 1999).

Although distantly related, *Cardinium* bears a strong resemblance to *Wolbachia* in its reproductive manipulations and ecology. *Cardinium* is a gram-negative, rod-shaped bacterium found in the phylum Bacteroidetes (Zchori-Fein et al., 2004). Its closest relatives are the recently described symbiont of plant parasitic nematodes, *Paenicardinium endonii* (Noel and Atibalentja, 2006), and a symbiont of acanthamoeba, *Amoebophilus asiaticus* (Zchori-Fein et al., 2004). *Cardinium* has been found to infect four arthropod orders: Acari, Hymenoptera, Hemiptera, and Araneae, and molecular surveys indicate that it is found in 6-7% of arthropods (Weeks et al., 2003; Zchori-Fein and Perlman, 2004; Duron et al., 2008). Interestingly, screening studies have detected several species with double infections of *Wolbachia* and *Cardinium* (Weeks et al., 2003; Zchori-Fein and Perlman, 2004). These double infections occur approximately twice as often as would be expected randomly, suggesting that *Cardinium* and *Wolbachia* may be interacting in some way (Weeks et al., 2003).

Cardinium is similar to *Wolbachia* in that rare horizontal transmission events are thought to play an important role in the infection of novel species (Zchori-Fein and Perlman, 2004). While closely related *Cardinium* strains are found within closely related host species, for example within the *Encarsia* wasps, distantly related species have also been found to be infected with closely related *Cardinium* strains (Zchori-Fein and Perlman, 2004). Additionally, *Cardinium* is similar to *Wolbachia* in that closely related

strains are found in several host-parasitoid pairs, indicating that the infection may be transmitted among ecologically related species (Zchori-Fein and Perlman, 2004). For example, the whitefly host *Bemisia* and its parasitoid wasp *Encarsia* harbour closely related strains of *Cardinium*, as do the scale insect *Aspidiotus*, its parasitoid wasp *Aphytis*, and its hyperparasitoid *Marietta* (Zchori-Fein et al., 2004). Despite being phylogenetically distant, *Cardinium* and *Wolbachia* clearly share similarities in the ways that they are transmitted and the reproductive phenotypes they induce. These similarities, in addition to the fact that *Cardinium* and *Wolbachia* co-occur in many host species (Weeks et al., 2003), has led to speculations that they may share some regions of genetic similarity, perhaps as a result of lateral gene transfer. Since lateral gene transfer has been found to be widespread among *Wolbachia* and their insect and nematode hosts (Hotopp et al., 2007), it seems plausible that it could also have occurred between these two bacteria.

Male Killing

Several microbial insect symbionts cause male killing, in which the sons of infected females die before reaching maturity (Hackett et al., 1986; Hurst et al., 1997; von der Schulenburg et al., 2001; Hurst et al., 2000; Werren et al., 1986). This can occur during embryogenesis, in the case of early male killing, or just prior to pupation, in the case of late male killing (Hurst, 1991). Because the infection is transmitted only through females, male hosts are an evolutionary dead end to the symbiont (Hamilton, 1967). Consequently, any strategy that allows infected females to produce more infected daughters is expected to be selected for by the symbiont. In the case of male killing, it is speculated that killing sons of infected females may be advantageous for the symbiont when it increases resource availability for infected daughters (Hurst, 1991).

Although male killing can be induced by many different symbionts, it is the only reproductive manipulation that *Wolbachia* is capable of and *Cardinium* is not. This is in spite of the fact that a recent study suggests that CI and male killing share molecular similarities, since a *Wolbachia* strain causing CI in its native host switched to male killing when introduced into a novel host (Jaenike, 2007). However, it is also quite possible that male-killing strains of *Cardinium* do exist and have simply not yet been discovered.

Feminization

Another strategy used by reproductive manipulators to increase the number of females in a population is feminization, in which genetic males are converted into phenotypic females. This is beneficial to the symbiont because the phenotypic females can often reproduce as females, and therefore can transmit the symbiont (O'Neill et al., 1997). The mechanism of this manipulation is unknown for many insects; however, in the isopod *Armadillidium vulgare, Wolbachia* is thought to suppress the development of the androgenic gland, which is responsible for the production of male hormones (Legrand et al., 1987). Feminization has been found to be induced by microsporidia (Bulnheim and Vavra, 1968; Terry et al., 1997), *Wolbachia* (Stouthamer et al., 1999) and *Cardinium* (Weeks et al., 2001).

Parthenogenesis Induction

Parthenogenesis induction (PI) occurs when genetic males are converted into genetic females. This reproductive manipulation has been found to be induced by *Wolbachia, Cardinium,* and *Rickettsia* (Stouthamer et al., 1993; Zchori-Fein et al., 2001;

Hagimori et al., 2006). PI is similar to feminization in that it increases the number of infected female offspring, thereby enhancing the symbiont's transmission. PI is known only in haplodiploid insects (mostly in the Hymenoptera) (Stouthamer et al., 1990; Arakaki et al., 2001, Weeks et al., 2001). The majority of hymenopterans reproduce by haplodiploidy, in which unfertilized (haploid) eggs become males, and fertilized (diploid) eggs become females. When a symbiont causing PI is present, these haploid eggs are converted into diploid females. Therefore, the presence of a PI inducing microbe is often associated with an all-female population, which should revert back to male production upon treatment with antibiotics (Stouthamer et al., 1990).

Cytoplasmic Incompatibility

Cytoplasmic incompatibility (CI) is the most common reproductive phenotype induced by *Wolbachia*, and it is also caused by *Cardinium* (Stouthamer et al., 1999; Hunter et al., 2003). In CI, infected males are reproductively incompatible with females that are uninfected or that do not harbour the same CI-causing strain. In contrast, females that are infected with the same CI-inducing strain do not experience these reproductive losses. Consequently, infected females benefit from the infection by avoiding incompatible matings, and uninfected females experience a reproductive disadvantage relative to infected females.

Cytoplasmic incompatibility can take either the form of unidirectional or bidirectional incompatibility (Werren, 1997). Unidirectional incompatibility is the simplest form, in which few or no progeny are produced from crosses between an infected male and an uninfected female. Bidirectional incompatibility occurs when two

populations harbour different CI strains, making them mutually incompatible (Werren, 1997).

Wolbachia has been found to cause CI in mites (Acari) and in an isopod (Isopoda) as well as in several insect orders, including Coleoptera, Diptera, Hemiptera, Hymenoptera, Orthoptera, and Lepidoptera (Stouthamer et al., 1999). Thus far, *Cardinium* has been found to induce CI in only two species: in the whitefly parasitoid *E. pergandiella* (Hymenoptera: Aphelinidae) and in the spider mite *Eotetranychus suginamensis* (Arachnida: Acari) (Hunter et al., 2003; Gotoh et al., 2007).

Mechanism of Cytoplasmic Incompatibility

Despite numerous cytological and molecular studies, the mechanism of CI remains enigmatic. Nevertheless, research in the past decade has provided several new insights into how CI might occur. The mechanism of CI is thought to follow a model of modification and rescue. In this model, developing sperm are somehow modified in the testes of an infected male. Only infected females are capable of "rescuing" the modified sperm, which is necessary in order to produce viable offspring (Werren, 1997). When an infected male mates with an uninfected female, the cross is incompatible because the egg is unable to rescue the modified sperm. This results in improper condensation of paternal DNA, and interference with the first mitosis of the zygote (Tram et al., 2003).

The effects that these paternal DNA abnormalities have on affected offspring are somewhat variable. In diploid insects, offspring of incompatible crosses die early in embryogenesis (Stouthamer et al., 1999). In haplo-diploid insects, females are normally produced by diploid, fertilized eggs, whereas males are produced by haploid unfertilized

eggs. Most commonly, CI in haplo-diploid species also causes early death in fertilized eggs (Bordenstein et al., 2003). However, in some species, fertilized eggs from incompatible matings do not die, but instead develop into haploid males (Bordenstein et al., 2003). Recent evidence suggests that the fate of incompatible eggs in haplo-diploids is determined by the way in which paternal DNA segregates during the first mitosis (Tram et al., 2006).

Cytological analysis of fertilization and karyogamy has provided a better understanding of how sperm and egg incompatibilities may occur. Normally when a sperm and egg fuse, paternal DNA decondenses, DNA replication occurs, and maternal and paternal chromosomes condense in preparation for first mitosis (Stouthamer et al., 1999). However, in incompatible crosses, paternal DNA condensation appears to be delayed, resulting in entangled and decondensed masses of DNA aligning on the metaphase plate (Callaini et al., 1997). Consequently, DNA is improperly segregated during anaphase, resulting in the formation of aneuploid or haploid nuclei (Callaini et al., 1997). Tram and Sullivan (2002) found that these mitotic abnormalities were the result of timing differences between maternal and paternal pronuclei. They observed asynchrony in nuclear envelope breakdown as well as in the activation of cyclin dependent kinase, an important cell cycle regulator, in incompatible crosses. However, this was not observed in crosses between infected males and infected females, indicating that an egg from an infected female is somehow capable of rescuing these abnormalities (Tram and Sullivan, 2002). Interestingly, Ferree and Sullivan (2006) observed abnormal development of the paternal pronucleus without the influence of the maternal pronucleus, suggesting that asynchrony is caused by factors in the egg cytoplasm (and not in the

maternal pronucleus) or that CI is instead caused by developmental abnormalities solely within the paternal pronucleus. In the latter case, it may be that developmental abnormalities in paternal DNA trigger a slow-down in paternal mitotic processes, resulting in the observed asynchrony between maternal and paternal pronuclei.

Molecular studies indicate that *Wolbachia*-induced protein imbalances may be responsible for the delayed entry of paternal DNA into mitosis (Clark et al., 2006; Xi et al., 2008). For example, Xi et al. (2008) recently compared differences in transcription between *Wolbachia*-infected and uninfected *Drosophila* cells in culture. They found that infected cells showed down-regulation in several heat shock proteins, as well as in *sex lethal (sxl)*, an important gene in sex determination (Xi et al., 2008). Furthermore, angiotensin converting enzyme (*ance)* was found to be up-regulated in infected females and down-regulated in infected males of both *D. simulans* and *D. melanogaster*, suggesting that it, too, may play a role in modification and rescue (Xi et al., 2008). While several studies have focused on the CI mechanism in *Wolbachia*, little is known about the mechanism in the more recently discovered *Cardinium*. While CI appears to be very similar between *Wolbachia* and *Cardinium*, molecular and cytological comparisons may prove an important step in better understanding this complex process.

The Invasion and Spread of a CI Symbiont

In some cases, CI symbionts have been found to spread rapidly in host populations, and they are often found at or near fixation in host populations. For instance, Turelli and Hoffmann (1991) documented the spread of a *Wolbachia* strain across California populations of *D. simulans* within a few years. Rapid spread of a

symbiont such as that observed in this study could be useful in future pest and disease control applications. For example, it could be used to drive the spread of a gene of interest, such as a gene that makes vectors resistant to disease transmission (Beard et al., 1993). The potential that CI symbionts may have in these important applications makes it crucial to understand factors affecting their invasion and spread within a population (Rasgon, 2008).

CI symbionts benefit their hosts by allowing them to avoid incompatible matings, and the extent of this benefit will depend on the symbiont's prevalence in the population. For example, when a CI symbiont is present at high frequencies, an uninfected female bears a high risk of encountering an infected (and therefore incompatible) male. Thus, in a population where the CI symbiont is found at high prevalence, the benefit to having the infection is high. On the other hand, if the symbiont is relatively rare in the population, there is low risk of an uninfected female obtaining an incompatible mating. Consequently, the benefits of the infection (avoiding incompatible males) increase with increased prevalence of a CI symbiont in a population (Turelli, 1994).

While the benefits of harbouring a CI symbiont are frequency-dependent, the fitness costs associated with an infection are often expected to remain constant, regardless of the infection frequency within a population. CI symbionts can have positive (Dobson et al., 2002; Weeks et al., 2007), neutral (Bordenstein and Werren, 2000), or negative (Hoffmann et al., 1990) effects on host fitness measures such as fecundity.

A CI symbiont is predicted to invade and spread in a population if the benefits of the infection exceed the costs (Turelli, 1994). Since the benefits of infection are

frequency-dependent, and the fitness costs are not, spread of the symbiont is expected to occur only once it reaches a certain frequency within the population, termed the invasion threshold frequency (Turelli, 1994). When the frequency of infection is higher than the invasion threshold, the infection will spread until it is at or near fixation. Alternatively, if the infection frequency is lower than the invasion threshold, the infection will be lost from the population (Turelli, 1994). The invasion threshold is thought to depend in particular on three crucial parameters: the maternal transmission rate (i.e. how faithfully the symbiont is transmitted from mother to offspring), the CI strength (i.e. the relative reduction in offspring caused by the incompatible cross) and the fitness costs of the infection (Turelli, 1994; Turelli and Hoffman, 1995; Vavre et al., 2003).

Factors Influencing the Invasion of a CI Symbiont

Several factors have been found to influence CI, including infection densities, temperature, male age, nonrandom mating, food quality, larval density, naturally occurring antibiotics, and male development time (Stevens and Wicklow, 1992; Turelli and Hoffman, 1995; Clancy and Hoffmann, 1998; Champion de Crespigny et al., 2005; Yamada et al., 2007). It is critical to understand how these factors may operate in the field in order to obtain a better understanding of how CI symbionts behave in their natural environments (Turelli, 1994).

Infection Density

Several studies have examined the role that symbiont density may play in the strength of CI induced by *Wolbachia*. For example, Noda et al. (2001) found that while *Wolbachia* induced complete CI in *Laodelphax striatellus* (ie. no viable offspring

produced by the incompatible cross), only partial CI was induced in *Sogatella furcifera* (ie. some offspring produced by the incompatible cross). Real-time PCR indicated that *L. striatellus* had ten times more *Wolbachia* than *S. furcifera*, suggesting that infection density may be positively correlated with CI strength (Noda et al., 2001). *Wolbachia* density has also been found to be correlated with CI strength in *Drosophila* (Bourtzis et al., 1996; Poinsot et al., 1998). For example, Bourtzis et al. (1996) found the *Wolbachia* strains could be separated into two groups according to whether they induced low or high CI, and that within these groups, CI strength was positively correlated with bacterial density. In addition, higher *Wolbachia* density within the sperm cysts has been found to induce stronger CI in *D. simulans*, *D. melanogaster* (Clark et al., 2003), and in the flour moth *Ephestia kuehniella* (Ikeda et al., 2003).

Temperature

In some cases, temperature has also been found to have an effect on CI strength, and this may be related to changes in infection density. For example, Clancy and Hoffmann (1998) found that *Wolbachia* induced much stronger CI at 19°C than at 27°C in *D. simulans*. They also found that *Wolbachia* density in embryos was significantly higher at 19°C than at 25°C, leading them to suggest that the observed decrease in CI strength with increasing temperature may be related to temperature-induced changes in *Wolbachia* density (Clancy and Hoffmann, 1998). In contrast, Mouton et al. (2006) observed no effect of temperature on CI strength in *Wolbachia* infected *Leptopilina heterotoma*, even though temperature did influence bacterial density.

Temperature has also been found to influence host fitness effects of *Wolbachia*. For instance, *Wolbachia* infected *D. melanogaster* experienced a fecundity cost to the infection in tropical climates, but not in temperate climates (Olsen et al., 2001). Furthermore, Reynolds et al. (2003) found that a virulent strain of *Wolbachia* increased *D. melanogaster* mortality at 25°C but not at 19°C.

Male Age

The age of infected males at the time of mating may also be an important factor influencing the strength of CI. For example, the strength of CI in *Wolbachia*-infected *D. simulans* males has been found to decrease with increasing male age (Turelli and Hoffmann, 1995). Similarly, Reynolds et al. (2003) observed that CI was stronger when young *Wolbachia*-infected *D. melanogaster* were used compared with older infected males. In contrast, male age had no effect on CI strength in the mosquito *Culex pipiens* (Rasgon and Scott, 2003). Interestingly, Kittayapong et al. (2002) reported no effect of male age on CI strength in *Aedes albopictus* infected with two strains of *Wolbachia*; however, there was a correlation between male age and CI strength when males were infected with only one of the *Wolbachia* strains. This suggests that male age may influence CI strength only below a certain infection density threshold (Kittayapong et al., 2002). The effect of male age on CI strength will be discussed in more detail in Chapter 3.

Assortative Mating

In a polymorphic population, in which some individuals are infected and some are not, it would seem highly adaptive for an uninfected female to prefer to mate with an

uninfected male, so that she does not suffer from incompatible matings. Of course, this would require that a female could somehow detect the infection status of a male before mating. This kind of assortative mating could influence the rate at which a CI symbiont spreads (Champion de Crespigny et al., 2005).

Conflicting results have been obtained on whether or not *Wolbachia* infection can influence mating preference. In *Tetranychus urticae* infected with *Wolbachia*, Vala et al. (2004) found some evidence that uninfected females were more likely to mate with uninfected males than infected males. Infected females, on the other hand, showed no preference between infected and uninfected males (Vala et al., 2004). In contrast, no consistent mating preference or assortative mating was observed in *D. simulans* or *D. melanogaster* infected with *Wolbachia* (O'Neill, 1991; Hoffmann et al., 1990).

Nutrition and Larval Crowding

Under conditions of low nutrition, *Wolbachia*-infected *D. simulans* males induced weaker CI compared to males that were provided with normal amounts of food (Clancy and Hoffmann 1998). In contrast, in the Asian tiger mosquito *A. albopictus*, food limitation caused a reduction in egg hatch, but this reduction was not related to CI (Islam and Dobson, 2006).

The effect of larval crowding is likely related to nutrition levels, as crowded larvae have access to fewer resources, which may result in smaller adult body size (Sinkins et al., 1995). Sinkins et al. (1995) studied the effects of larval crowding on *D. simulans* infected with two strains of *Wolbachia.* Infected males that were reared in crowded conditions were found to induce weaker CI than those reared in uncrowded

conditions. Additionally, rearing infected females in crowded conditions reduced their maternal transmission of the symbiont. Sinkins et al. (1995) suggest that these effects may be the result of lower bacterial densities in stressed individuals (Sinkins et al., 1995). In contrast, Clancy and Hoffmann (1998) did not find that larval crowding influenced CI strength in their study on *Wolbachia*-infected *D. simulans.*

Applications of Cytoplasmic Incompatibility

Widespread insecticide resistance has resulted in increased interest in using CI symbionts for control of insect pests and vectors of disease (Beard et al., 1993). The fact that CI symbionts infect a wide variety of arthropods makes them particularly interesting from an applied perspective. In fact, a recent survey detected *Wolbachia* in 46% of arthropod species relevant to biological control (Floate et al., 2006). *Cardinium* is also a good candidate for pest and disease control applications, being found in 6-7% of arthropods spanning a wide range of arthropod taxa (Weeks et al., 2003; Zchori-Fein and Perlman, 2004; Duron et al., 2008). Several potential applications of CI symbionts have been discussed, including mass releases of infected males, population replacement strategies, and using CI as a mechanism to drive desirable genes into a population (Stouthamer et al., 1999).

Releasing mass numbers of infected males has been proposed as an insect control strategy similar to sterile insect release (Laven, 1967). The released males would be reproductively incompatible with the target population, by being infected with a CI symbiont not present in the population. Zabalou et al. (2004) tested this strategy in populations of the Mediterranean fruit fly *Ceratitis capitata*, a worldwide fruit pest.

They transinfected hosts with *Wolbachia* naturally found in a related species, and introduced them to population cages along with uninfected individuals. They found that *Wolbachia* was effective at suppressing pest populations, reducing host populations by 99% in cages in which fifty infected males were added for every uninfected male and female (Zabalou et al., 2004). While such a massive introduction may be impractical from an insect control standpoint, some population suppression was noted even for ratios of one infected male for each uninfected male and female (Zabalou et al., 2004). The drawback to this control strategy is that if even one infected female is accidentally released into the population, the infection could spread, resulting in fewer incompatible matings and hence, less population suppression (Zabalou et al., 2004).

Another proposed arthropod control strategy is to introduce bidirectionally incompatible CI symbionts into a target host population, thereby increasing the number of incompatible matings (Dobson et al., 2002). One of the main advantages of this method is that infected females, as well as males, of each incompatible strain could be released, allowing for several generations of population control instead of just one (Dobson et al., 2002). Eventually, the CI symbionts would likely spread in the population. However, the host population could be controlled further by monitoring infection frequencies and re-introducing the CI symbionts that had become most rare in the population, resulting in further population suppression (Dobson et al., 2002).

Rasgon et al. (2003) have further suggested using CI strains to reduce the survival of disease vectors. This would be accomplished using a virulent strain of CI that induces severe host fitness costs, such as the *popcorn* strain of *Wolbachia,* which has been found to reduce the lifespan of *D. melanogaster* by 50% (Min and Benzer, 1997). Model

simulations indicate that under ideal conditions of high CI, efficient maternal transmission, and fitness effects present only after reproduction, an introduction of a CI symbiont could result in complete pest control (Rasgon et al., 2003).

Finally, it has been suggested that CI may be useful in introducing genes of interest, such as anti-parasitic or antiviral genes that decrease the ability of arthropods to act as disease vectors (Beard et al., 1993). Although genes that make insects refractory to disease transmission do exist, they are often not present at high frequencies due to associated fitness costs (Beard et al., 2003). Cytoplasmic incompatibility offers the advantage of being able to spread an associated gene into a population despite this fitness cost, because infected hosts benefit from avoiding incompatible matings. The biggest disadvantage of this control strategy is that if the gene of interest ever became dissociated from the CI symbiont, it would eliminate the driving force behind the spread of the gene in the population (O'Neill et al., 1997; Sinkins and Godfray, 2004). To overcome this risk, Sinkins and Godfray (2004) suggested linking the gene of interest with symbiont rescue genes introduced into the host nuclear genome, once rescue genes have been identified. This strategy is predicted to result in spread of the gene of interest, as long as maternal transmission of the symbiont is imperfect, or if the nuclear gene is better at rescuing CI than the CI symbiont (Sinkins and Godfray, 2004).

Several exciting strategies have been proposed for using CI symbionts to aid in the control of arthropod pests and disease vectors. Clearly, more research is needed to determine if these are viable and practical arthropod control strategies. In addition, most research to date has considered only the potential of *Wolbachia* for these pest and disease

control strategies. A comparison of the invasion dynamics of *Cardinium* with *Wolbachia* may prove useful in these potential applications of CI.

Future Directions in Cytoplasmic Incompatibility Research

A better understanding of the factors that influence CI is vital for its use in potential applications. Equally important is a better understanding of the fitness benefits and fitness costs induced by CI. Weeks et al. (2007) has demonstrated that host fitness effects can be dynamic, and appear to evolve within a surprisingly short time. The dynamic nature of CI makes it difficult to extrapolate experimental findings to natural populations. Consequently, there is often a discrepancy between the laboratory and the field in how CI symbionts interact with their hosts (Turelli, 1994). Thus, more work, both in the laboratory and in the field, is necessary to identify the factors responsible for this discrepancy.

In addition, very few experimental studies have examined the invasion or spread of *Wolbachia* (Johanowicz and Hoy, 1999; Reynolds and Hoffmann, 2002; Xi et al., 2005), and virtually nothing is known about the invasion and spread of *Cardinium*. While we know that CI symbionts have the potential to spread rapidly in the field (Turelli and Hoffman, 1991), it is vital that we obtain a better understanding of the conditions necessary for this rapid spread. The experimental testing of current models of CI invasion (Turelli, 1994; Turelli and Hoffman, 1995; Vavre et al., 2003) will allow for better predictions of how and when CI symbionts might spread, and this information will be critical in potential applications of CI.

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Chapter II: The invasion and spread of *Cardinium*, a bacterial endosymbiont inducing cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*

A large number of insect species harbour bacterial symbionts, and these symbionts have diverse effects on their hosts (Moran, 2001). Many symbionts are beneficial to their hosts, providing them with essential nutrients (Buchner, 1965; Baumann, 2005) or protection from parasitoids or pathogens (Oliver et al., 2003; Cardoza et al., 2006).

One class of endosymbionts is known as reproductive manipulators, and while these symbionts do not confer direct benefits to their hosts, they are still faithfully transmitted from mothers to offspring. The reproductive manipulators accomplish this using several different strategies, the most common of which is cytoplasmic incompatibility (CI). This strategy is particularly interesting because it can result in the rapid spread of a symbiont (Turelli and Hoffmann, 1991). This rapid spread could be useful for many potential applications, including using a CI symbiont to control pest populations, or to introduce beneficial genes into an insect population (O'Neill et al., 1997; Xi et al. 2005).

In CI, uninfected females produce few or no viable progeny when mated with infected males. Infected females, in contrast, can mate with either uninfected or infected males without experiencing large progeny losses (Figure 2.1). In this way, uninfected females experience a reproductive disadvantage relative to infected females, making it advantageous to be infected.

Different strains of two bacterial endosymbionts have been found to induce several reproductive manipulations, including CI, in arthropods. While the effects of the α Proteobacterium *Wolbachia* are mostly unknown, strains of *Wolbachia* have been found to induce several reproductive manipulations in arthropods. A survey of *Wolbachia* prevalence indicates that it is found in approximately 20% of all arthropod species (Werren et al., 1995). A recent meta-analysis suggests that *Wolbachia* prevalence may be much higher at approximately 66%, when survey estimates also consider species in which *Wolbachia* was likely undetected due to low infection frequencies (Hilgenboecker et al., 2008).

While the effects of *Cardinium* are unknown for many hosts, it too has been found to induce CI. *Cardinium,* a member of the phylum Bacteroidetes, has been found to cause CI in two hosts: in the parasitoid wasp *Encarsia pergandiella* (Hunter et al., 2003), and in the spider mite *Eotetranychus suginamensis (*Gotoh et al., 2007). Since its discovery, *Cardinium* has been found in four orders and approximately 6-7% of arthropods (Weeks et al., 2003; Zchori-Fein and Perlman, 2004; Duron et al., 2008).

Although the mechanism of CI remains enigmatic, it is thought to follow a model of modification and rescue (Werren, 1997). In this model, developing sperm are somehow modified in the testes of infected males. When an infected male mates with an uninfected female, the cross is incompatible, resulting in offspring failure. This incompatibility is thought to occur as a result of timing or developmental abnormalities in the paternal DNA (Tram and Sullivan, 2002; Ferree and Sullivan, 2006). Infected females, however, are capable of "rescuing" the modified sperm, and are therefore able to produce relatively normal numbers of offspring (Tram et al., 2003). While *Cardinium*

appears to be very similar to *Wolbachia* in the reproductive manipulations it induces, it is unknown if these symbionts share a similar CI mechanism.

The spread and long term equilibrium frequency of a CI-inducing symbiont within a population will depend on its relative costs and benefits. The symbiont's ability to induce incompatible matings will facilitate its spread within a population, since infected females experience a reproductive advantage relative to uninfected females. However, if there is a fitness cost associated with the infection, for example, reduced fecundity, this will also affect the symbiont's ability to spread. Consequently, the infection is only expected to spread when the benefits of the infection (i.e. avoiding incompatible matings) outweigh the fitness costs. While fitness costs, such as reduced fecundity, are not expected to vary with infection frequency, the benefits associated with the infection are frequency dependent. In a population with a high infection frequency, a female is very likely to mate with an infected male, and thus benefits greatly from being infected. However, in a population where the infection is rare, a female is not very likely to mate with an infected male, and therefore receives little benefit in harbouring the infection. The frequency-dependent nature of infection benefits raises the important question of how the symbiont is able to invade in a novel population.

Models have been developed to understand how CI can invade and spread in a population (Caspari and Watson, 1959; Turelli, 1994; Turelli and Hoffmann, 1995; Vavre et al., 2003). CI invasion and spread are thought to depend on three key parameters: the fidelity of maternal transmission (u), the fitness costs associated with the infection (F), and the relative losses in progeny experienced by the incompatible cross, or CI strength (H) (Turelli, 1994; Turelli and Hoffmann, 1995; Vavre et al., 2003). These models have

two key predictions. First, the equilibrium frequency of infection is predicted to be stable in a population at two points: when the infection is at or near fixation, and when it is completely lost from the population (Turelli, 1994). Second, an unstable equilibrium, also termed the invasion threshold, is found between these two stable infection frequencies. When the frequency of infection is higher than the invasion threshold, the infection is expected to spread until it is at or near fixation. Alternatively, if the infection frequency is lower than the invasion threshold, the symbiont will be lost from the population (Turelli, 1994).

Understanding the invasion and spread of CI symbionts is critical for several reasons. First of all, in order to be useful for applications of disease and pest control, the invasion and spread of the symbiont must be well characterized (Rasgon, 2008). Second, this is the first study to examine the invasion of a CI-causing *Cardinium*, and as such, it allows for a comparison with *Wolbachia* invasion. Finally, if current CI models prove inaccurate, it will suggest that other factors may be influencing CI spread, such as population subdivision or cryptic fitness costs or benefits (Egas et al., 2002). For instance, Xi et al. (2005) monitored the spread of a *Wolbachia* strain causing CI in *Aedes aegypti*. They found that *Wolbachia* did not spread until the initial infection frequency was ~20%, suggesting that there was some sort of cryptic fitness cost associated with the infection (Xi et al., 2005).

In my study, I predicted the invasion threshold frequency necessary for *Cardinium* to spread in *E. pergandiella* by using established models of CI (Turelli, 1994; Turelli and Hoffmann, 1995; Vavre et al., 2003) and estimates of CI parameters for this system (Perlman et al., 2008). I established *E. pergandiella* populations with initial

infection frequencies at, above and below the predicted invasion threshold, and monitored infection frequencies in these populations for nine generations. I predicted that *Cardinium* would spread in *E. pergandiella* populations when initial frequencies were at or above the predicted invasion threshold.

Methods

Cultures

Encarsia pergandiella is a hymenopteran parasitoid of whiteflies. The infected line was collected from *Bemisia tabaci* hosts in the Rio Grande Valley in Texas in 2003. Since then, it has been maintained in the laboratory on *B. tabaci* reared on cowpea plants (*Vigna unguiculata)* and is fixed for *Cardinium* infection. An uninfected line was obtained by curing a sub-population of the infected line by treating adult wasps with 50 mg/ml rifampicin in honey for three generations.

Strength of Cytoplasmic Incompatibility

CI strength, or the reduction in offspring experienced by the incompatible cross, will strongly influence the invasion and spread of a CI symbiont (Turelli, 1994). To determine the strength of CI in *Cardinium*-infected *E. pergandiella*, freshly emerged uninfected virgin females were mated individually to 1-2 day old virgin males of known infection status. Each female was then transferred to a 35 mm petri dish containing 1% agar, upon which a cowpea leaf disk was placed. Females were allowed to oviposit on leaf disks infested with 50 to 100 whiteflies in the third to early fourth nymphal stage for 24 hours.

Following the oviposition period, infested leaves were incubated at 27°C until *E. pergandiella* pupae could be counted and removed (8 to 10 days). When an *E. pergandiella* egg fails to develop due to CI, the parasitized whitefly is developmentally arrested (Hunter et al., 2003). Therefore, the number of arrested whiteflies served as an indicator of CI. Whiteflies were considered developmentally arrested if, by the time of scoring, they had not developed eye spots or wing buds. The majority of whiteflies in the control treatment had already emerged at the time of scoring. Females that did not produce any pupae and produced <2 developmentally arrested whiteflies were presumed to be unmated, and were removed from the analysis.

The strength of CI (H) was measured as the average reduction in the number of pupae produced by the incompatible cross (infected male x cured female) relative to the average number of pupae produced by the compatible cross (cured male x cured female).

Invasion Models

The spread of *Cardinium* was modeled using the Hoffmann-Turelli model (Turelli 1994; Turelli and Hoffmann, 1995) which was modified by Vavre et al. (2000) for haplodiploid hosts with the female mortality type of CI:

$$f_{t+1}=\frac{Ff_t(1-u)}{Ff_t[1-u(1-H)m_t]+(1-f_t)[1-(1-H)m_t]}$$

$$m_{t+1}=\frac{Ff_t(1-u)}{Ff_t+(1-f_t)}$$

where f_t is the female infection frequency at time t, f_{t+1} is the female infection frequency one generation after time t, F is the fecundity cost of the infection, u is the maternal transmission efficiency, H is the CI strength, m_t is the male infection frequency at time t, and m_{t+1} is the male infection frequency one generation after time t.

A slight modification was made to the CI invasion models for haplodiploids (Vavre et al., 2003) so that an infection benefit could also be modeled, similar to that done by Dobson et al. (2002) for the Turelli-Hoffmann model. The modified model solved the equation in terms of α, where $0 \leq \alpha \leq 1$, and is defined as the fitness of uninfected females relative to the fitness of infected females. The resulting models were:

$$f_{t+1} = \frac{f_t(1-u)}{f_t[1-u(1-H)m_t] + \alpha(1-f_t)[1-(1-H)m_t]}$$

$$m_{t+1} = \frac{f_t(1-u)}{f_t+\alpha(1-f_t)}$$

The invasion threshold frequency was calculated using the following equation, which was also developed by Turelli (1994) and Turelli and Hoffmann (1995) and later modified by Vavre et al. (2000) for the female mortality type of CI in haplodiploids:

Invasion Threshold $= \frac{-B-\sqrt{(B^2-4AC)}}{C}$; where

in males: A = (1-H); B = 1(1-F+1-H); and C = 1-F(1-u);

and in females: A = (1-H)(1-Fu)(1-u)F+$(1-F)^2$; B = - (1-F+1-H)+(F-H)[1-(1-u)F]; and C = 1-F(1-u)

In a previous study, Perlman et al. (2008) found that *Cardinium*-infected female *E. pergandiella* produced 18% fewer offspring than uninfected females over a period of four days. Therefore, the fitness cost of the infection was estimated to be 18% (F=0.82). Perlman et al. (2008) found that maternal transmission efficiency was nearly perfect among field-caught females, so I used the conservative estimate of 99% (u=0.99). In the present study I found that, on average, the incompatible cross produced 38% of the offspring of the control cross (H=0.38). Given these parameter estimates, the invasion frequency required for *Cardinium* to spread was predicted to be 36% for females and 31% for males.

Population Cages

E. pergandiella wasps of known infection status were introduced to 50cm x 50cm x 50cm cages (4 cages per treatment) to establish populations harbouring initial infection frequencies below (15%), at (36% for females and 31% for males) and above (55%) the predicted invasion threshold frequency. The initial infection frequencies were established using three successive weekly introductions of 50 female and 24 to 33 male adult wasps. All wasps were 3-5 days old at the time of introduction. *E. pergandiella* is autoparasitic, meaning that while females develop on whiteflies, males develop exclusively on other parasitic wasps developing inside whiteflies (Hunter and Woolley, 2001). Wasps were thus allowed to mate and oviposit on cowpea plants infested with whitefly nymphs, as well as pupae of the whitefly parasitoid *Eretmocerus eremicus*. The majority of whiteflies were third instar nymphs at the time of plant introduction, while the majority of *E. eremicus* provided were early pupae. Each generation, wasps were

provided with two cowpea plants infested with *B. tabaci* and an additional plant infested with *E. eremicus* developing on *B. tabaci*. Plants were removed from the cages before adults emerged, and a subset of 50 female and 30 adult males from the new generation were re-introduced to the cage. In generation three, there was a shortage of males in some cages due to poor health of plants infested with *Eretmocerus eremicus*. Therefore, male introductions in cages 1 and 4 (below the predicted threshold), 5 and 7 (at the predicted threshold), and 11 (above the predicted threshold) were supplemented with 30, 30, 25, 13, and 8 males from the previous generation, respectively. Infection frequencies in each cage were sampled at generations 2, 4, 6, 8, and 9. Wasps were frozen or stored in 95% ethanol until DNA extraction.

DNA Extractions

In order to assess *Cardinium* infection, single wasp DNA extractions were performed on at least fifty female *E. pergandiella* wasps from each cage for each generation tested. DNA was extracted by grinding individual wasps in 3 μL of 20 mg/mL proteinase k, and adding the homogenate to 50 μL of 5-10% w/v chelex. Samples were incubated at 37°C for one hour, and then at 96°C for 8 minutes, with periodic vortexing. Samples were stored at -20°C.

Diagnostic PCR

Wasps were screened for the presence of *Cardinium* using diagnostic PCR. Two sets of *Cardinium* specific primers were used, Ch441F (GTACAGGAGCAAAACAATCCC) and either Ch665R

(TATTCTTAACTCAAGCCTAAT) or Ch1017R (ATTTTTCAAAGTAGCAAAATA), which amplified a ~200bp and 600 bp region of 16S rDNA, respectively (developed by Stephan Shmitz-Esser, University of Vienna). PCR conditions for Ch441F and Ch665R were a 3 min. initial denaturation at 94°C, followed by 40 cycles of 94°C for 30s, 53°C for 45s, and 72°C for 45s, and a final extension of 72°C for 5 min. PCR conditions for Ch441F and Ch1017R were a 3 min. initial denaturation at 94°C, followed by 40 cycles of 94°C for 30s, 56°C for 45s, and 72°C for 1 min, and a final extension of 72°C for 6 min. Samples which appeared to be negative for *Cardinium* were screened twice more. A positive control of an infected *E. pergandiella* and a negative control of a cured *E. pergandiella* were included in each run.

Samples determined to be negative for *Cardinium* were screened for single or double copy *E. pergandiella* genes (EF1α, opsin, or histone) as a positive control for the DNA extraction. A ~380bp segment of EF1α was amplified using EpergEF-F and EpergEF-R (Perlman et al., 2008) with PCR reaction conditions of: 95°C for 3 min., followed by 30 cycles of 95°C for 60s, 50°C for 60s, and 72°C for 90s, with a final extension of 72°C for 10 min. A ~1.5kb fragment of the opsin gene was amplified using LWRh-F and LWRh-R according to Mardulyn and Cameron (1999), and a faint band of ~700bp was often also observed, likely because there is a double copy of this gene in *E. pergandiella*. A ~400bp region of histone was amplified using H3Af and H3Ar (Colgan et al., 1998) with PCR conditions of 94°C for 3 min., followed by 40 cycles of 94°C for 45s, 65°C for 45s, and 72°C for 60s, with a final extension of 72°C for 6 min. A positive control of *E. pergandiella* and a negative no-DNA control were included in each EF1α,

opsin, and histone PCR reaction. Samples which appeared negative for *E. pergandiella* DNA were excluded from further analysis.

Statistical Analysis

A Mann-Whitney test was used to determine if wasp fecundity was significantly different between the predicted incompatible and compatible crosses. To determine if there were significant changes in infection frequency over the nine generation period, a replicated goodness of fit test was calculated. Logistic regression analysis was performed using the binomial response variable of the presence or absence of infection within individual wasps, where each population cage was nested within treatment. This analysis allowed for the identification of variables that were significant predictors of the probability of infection with *Cardinium*. The Hosmer and Lemeshow goodness of fit test was used to examine fit between the logistic regression model and the data. A linear regression was performed by comparing the average infection frequency in each cage at each generation tested with the corresponding frequency predicted by the model. If the predicted values perfectly matched the observed values, then linear regression of observed values against predicted values would generate a line with a slope of 1 and an intercept of 0. Thus, the fit of the regression to a line with a slope of 1 and an intercept of 0 was used as a measure of the model fit. Logistic and linear regression analysis was performed using SAS, version 9.0 (SAS Institute, Cary, NC, U.S.A.).

Results

Wasp fecundity was significantly lower when uninfected females were mated with infected males (ie. the incompatible cross) compared to when they were mated with uninfected males (ie. the compatible cross) (Figure 2.2; Mann-Whitney test; U=55.5; n=47; p=0.000). On average, incompatible crosses produced 38% of the offspring produced by the compatible crosses (H=0.38).

Cardinium was found to spread rapidly in *E. pergandiella* for all three initial infection frequencies (Figure 2.3). A replicated goodness of fit test demonstrated that the initial infection frequencies were significantly different from the infection frequencies at generation nine for the treatment below (G_p=111.37; df=1; p<0.001), at (G_p =257.45; df=1; p<0.001) and above the predicted invasion threshold (G_p =176.28; df=1; p<0.001). There was considerable variation in infection frequencies between treatments (Figure 2.3), and this was particularly true in generation 2, where the infection frequency of cage 5 was 29% higher than the average for the other cages in this treatment. Generation (p<0.001), treatment (p<0.001), cage (p<0.002), and the interaction between generation and cage (p<0.0001) were significant predictors of the probability of *Cardinium* infection. The logistic regression model generated using these parameters had good fit to the data (Hosmer and Lemeshow Goodness of fit test χ^2=9.01; df=8; p>0.34). The interaction between treatment and generation was not associated with the probability of infection (logistic regression; p>0.05).

In order to determine the infection parameter values that allowed for the best fit between the modeled and observed data, the fitness cost (F), CI strength (H) and maternal

transmission efficiency (u) were tested over a range of values. The fitness cost was varied first, as it has been found to be most influential in determining the invasion threshold (Perlman et al., 2008). The observed data best fit models with a fitness benefit of α=0.98 or F=1.00 (Figure 2.4). To determine the best overall model, a fitness benefit of α=0.98 was modeled using varying maternal transmission efficiencies. As expected, decreasing maternal transmission efficiency decreased the rate of spread of *Cardinium* in the host population (Figure 2.5). The optimal maternal transmission efficiencies for model fit were u=0.03 and u=0.01 (Figure 2.5). Similarly, a decrease in CI strength was found to slow down the spread of *Cardinium* in the population (Figure 2.6). A good model fit to the data was observed for CI strengths ranging from $0.38<H<0.48$ (Figure 2.6).

Discussion

Interestingly, the strength of CI in *Cardinium*-infected *E. pergandiella* was found to be much weaker at H=0.38 in the current study (Figure 2.2) than previous studies, where H ranged from 0.07 to 0.13 (Hunter et al., 2003; Perlman et al., 2008). Several factors have been found to affect the expression of CI, including bacterial density (Noda et al., 2001; Clark et al., 2003), temperature (Clancy and Hoffmann, 1998), male age (Turelli and Hoffmann, 1995) and host density (Sinkins et al., 1995). While temperature, male age and host density were likely constant between experiments (S. Perlman, personal communication), differences in infection density or plant quality could have been important factors affecting the change in CI strength.

It is also possible that the host or symbiont has evolved towards weaker CI. Although a decrease in CI strength over time has not, to my knowledge, been reported before, theoretical studies suggest that host selection will favour variants that induce weaker CI if they also exhibit reduced fitness costs in infected females (Turelli, 1994; Vavre et al., 2003). A recent study demonstrated rapid evolution in *D. simulans* infected with *Wolbachia,* with a shift from a 15-20% fecundity cost to a 10% fecundity benefit within 20 years (Weeks et al., 2007). Backcrossing experiments indicate that this change in fitness effects was not due to changes in host genotype, which therefore suggests that the *Wolbachia* strain had evolved (Weeks et al., 2007). It is therefore plausible that host-parasite evolution may also have resulted in a change in CI strength or host fitness in *Cardinium*-infected *E. pergandiella*. However, it seems unlikely that these differences would have evolved within a period of only five years. It would be interesting to compare laboratory and field populations of *E. pergandiella* to determine if there is indeed a difference in CI strength. Unfortunately, the original *E. pergandiella* population infected with CI-inducing *Cardinium* is now rare due to a number of exotic competing wasp species introduced for biological control of *B. tabaci* (M. S. Hunter, personal communication).

Cardinium was found to spread rapidly in *E. pergandiella* for all initial infection frequencies, indicating that the invasion threshold is much lower than the predicted value of 36% (Figure 2.3). While no previous studies, to my knowledge, have examined the invasion and spread of *Cardinium*, a few studies have documented the invasion and spread of CI *Wolbachia*. The rapid spread observed for *Cardinium* in *E. pergandiella* is similar to that found in populations infected with a CI-inducing *Wolbachia*. For example,

in their population cage study, Reynolds and Hoffmann (2002) found that a *Wolbachia* causing CI in *Drosophila melanogaster* increased in prevalence from 50% to at or near fixation within five generations. Turelli and Hoffmann (1991) also reported the rapid spread of *Wolbachia* in wild populations of *D. simulans* across California, where the infection was found to spread upwards of 100 km per year. However, *Wolbachia* infections have not always been found to spread, indicating that an invasion threshold does sometimes exist. For example, Johanowicz and Hoy (1999) found that a *Wolbachia* causing CI in the mite *Metaseiulus occidentalis* was not able to spread within 12 generations when started at an introductory frequency of 10%. This invasion threshold is likely due to high fitness costs of the infection in this host (Johanowicz and Hoy, 1999). Similarly, Xi et al. (2005) found that CI-inducing *Wolbachia* did not spread in *A. aegypti* when initial infection frequencies were below 20%.

Jansen et al. (2008) demonstrated that stochastic events may allow infections to spread even when the initial frequency is lower than the invasion threshold, particularly in the case of a small or sub-divided population. Stochasticity is likely responsible for some of the variation in infection cage frequencies in our study (Figure 2.3). It may also explain the large upsurge in infection frequency in one of the cages in the treatment at the predicted invasion threshold in generation 2 (Figure 2.3). This upsurge may have resulted in an over-estimation in infection frequencies for this generation. However, it is unlikely that stochasticity is responsible for an under-estimation of the invasion threshold in our study, since the infection was found to spread in all population cages.

The dramatic spread of CI-inducing symbionts demonstrates their potential for use in disease and pest control applications. These applications include releasing mass numbers of incompatible males into a pest population, thereby reducing offspring production (Laven, 1967). Additionally, CI symbionts could potentially be used to drive genes of interest, for example a gene that decreases the longevity of a vector, into a population (Beard et al., 1993). Alternatively, Sinkins and Godfray (2004) have suggested that, if the genes responsible for CI can be identified, it may be possible to incorporate symbiont genes directly into host genomes, allowing for the spread of desired host genomes. Our study suggests that, in addition to *Wolbachia*, the symbiont *Cardinium* may be a good candidate for use in these potential applications.

As expected, generation and treatment were good predictors of the probability of infection. Perhaps surprisingly, infection frequencies were not associated with an interaction between treatment and generation, indicating that *Cardinium* spread in a similar way in all treatments. However, the experimental time period of nine generations may have been too short to reveal treatment level differences in the way that *Cardinium* spread. Infection was found to be significantly associated with cage, which is logical, since cage frequency should depend on the frequency in prior generations, and there was a high level of variation in infection frequencies among treatments (Figure 2.3).

Changes in the host fitness effects of *Cardinium* had the greatest impact on fitting the model to the observed data (Figure 2.4). The best overall model fit to the observed data used a slight fitness benefit (Figure 2.4). In contrast, Perlman et al. (2008) reported that *Cardinium* infection reduced *E. pergandiella* host fecundity by approximately 18%

within the first 4 days of reproduction. In that study, Perlman et al. (2008) provided female *E. pergandiella* with high-density arenas of unlimited whitefly hosts. It is possible that this observed fitness cost is not realized in circumstances where hosts are more scarce. In addition, cryptic fitness benefits of the infection, for example an increased ability to find hosts, would not have been detected in the confined arenas of the laboratory study, while these effects may be detected in the more natural environment of the population cages. A study on *Wolbachia* infected *D. simulans* also found that fitness costs of CI symbionts may be higher in laboratory than in field populations (Hoffmann et al., 1990). The discrepancy between laboratory and more natural populations in the fitness costs of infection could have important consequences in accurately predicting CI symbiont invasion and spread. As such, further work should be done to help determine the underlying cause of this discrepancy.

A decline in the modeled maternal transmission efficiency resulted in a predicted decline in the spread of *Cardinium* (Figure 2.5). The model fit the observed data optimally at the maternal transmission efficiencies of u=0.03 and u=0.01, which is consistent with the estimated value for this parameter based on the findings of Perlman et al. (2008). Consequently, the estimate of near-perfect maternal transmission of *Cardinium* in *E. pergandiella* is further supported by our study. A decline in CI strength also resulted in a reduced rate of modeled spread of *Cardinium* (Figure 2.6). The best fit to the observed data was observed for H=0.43; however, the fit was similar for values of H ranging from 0.38 to 0.48. This is consistent with the measured CI strength of H=0.38.

Based on infection parameter values of no fecundity cost (F=1.00), near-perfect maternal transmission (u=0.01) and a CI strength of H=0.38, the predicted invasion threshold is 1.64%. By incorporating a slight fitness benefit (α=0.98), the invasion threshold is eliminated, meaning the infection is able to spread at any initial infection frequency. It would be useful to test this prediction by studying *E. pergandiella* populations with very low (~1-2%) initial infection frequencies to determine if *Cardinium* is able to spread. The stable equilibrium for both of these models (F=1.00 or α=0.98, u=0.01 and H=0.38) is 99.4%. Since the *Cardinium* infection frequency was able to increase from 55% to an average of 96% within nine generations, a stable equilibrium frequency of ~99% seems plausible. In addition, *Cardinium* appears to be fixed in laboratory populations of *E. pergandiella* (Hunter et al., 2003), lending further support to a stable equilibrium near fixation. Additionally, the predicted stable equilibrium infection frequency of ~99% is only slightly higher than the estimate of 92% (95% confidence interval of 81.4-97.9%) infection frequency for *E. pergandiella* in the field (Perlman et al., 2008).

Overall, the revised models (using F=1.00 or α=0.98, u=0.01 and H=0.38) describe the observed invasion and spread of *Cardinium* very well. To my knowledge, this is the first study examining the invasion and spread of *Cardinium*, and my results indicate that its infection dynamics are remarkably similar to those of *Wolbachia*. In addition, the ability of *Cardinium* to rapidly spread within a host population demonstrates its potential for future CI applications.

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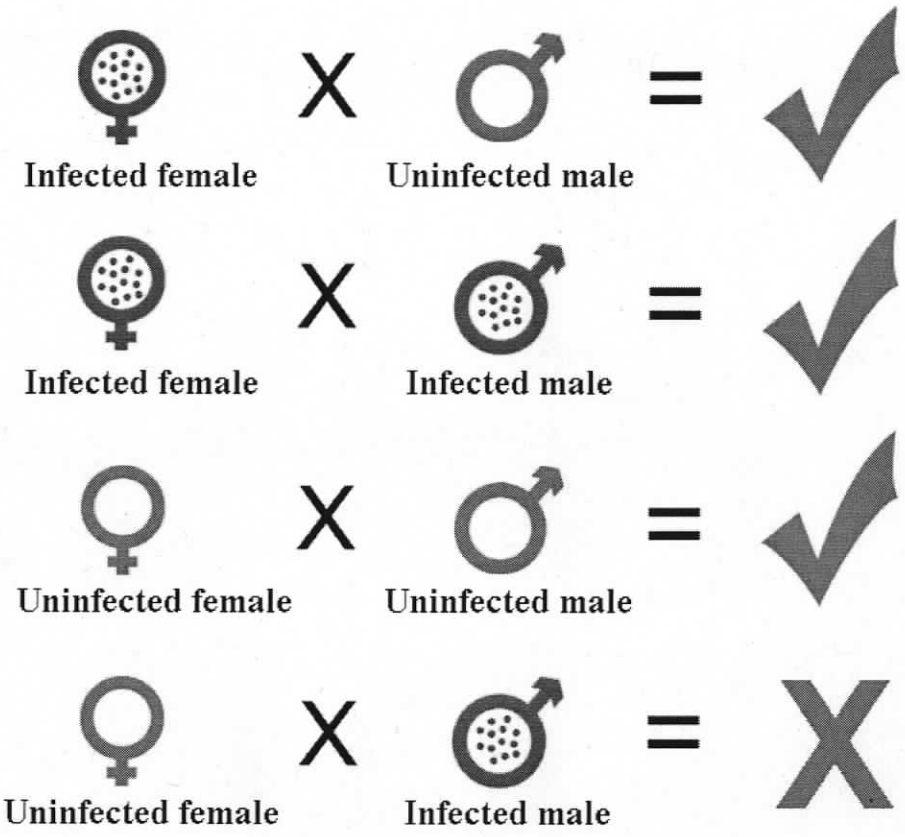

Figure 2.1. Cytoplasmic incompatibility. When an infected male mates with an uninfected female, the cross is incompatible and few or no offspring are produced.

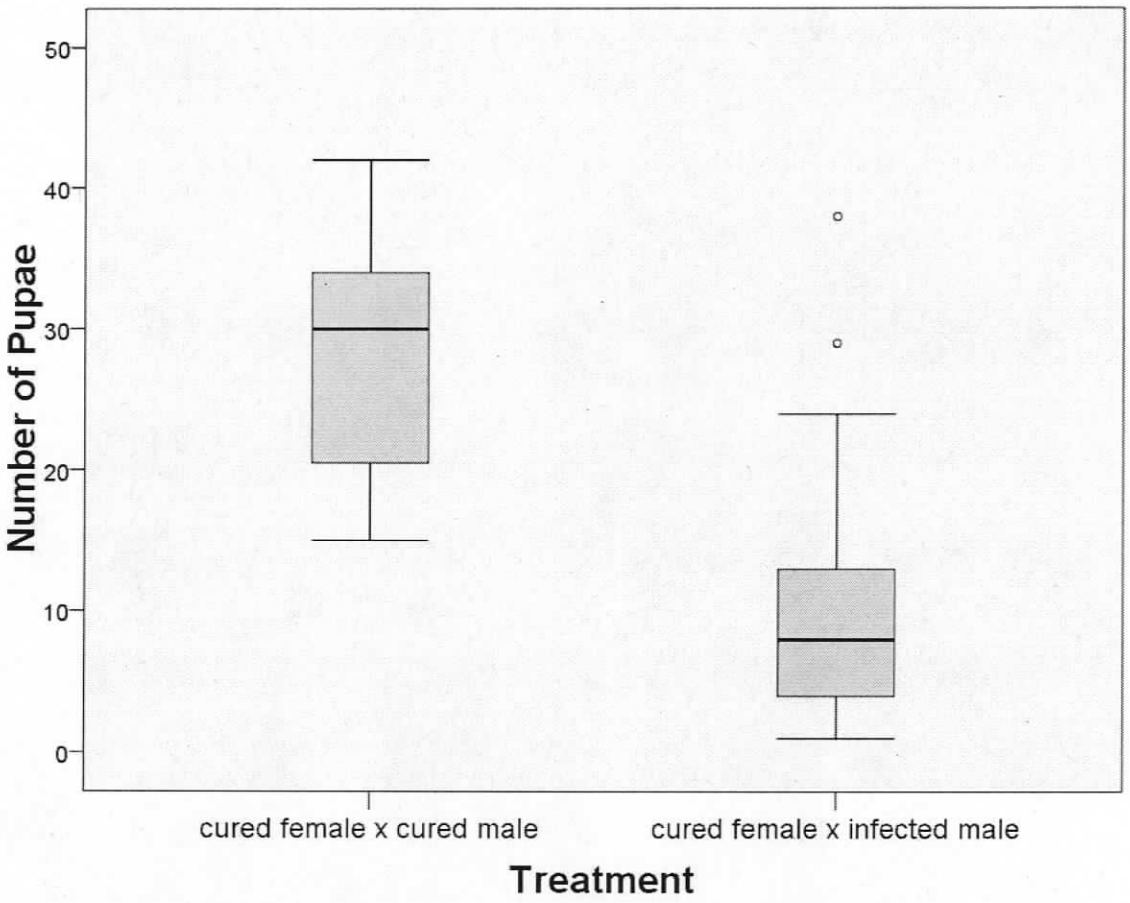

Figure 2.2. Number of pupae produced by the control (cured female x cured male; n=23) and incompatible (cured female x infected male; n=24) *E. pergandiella* crosses. All pupae were female.

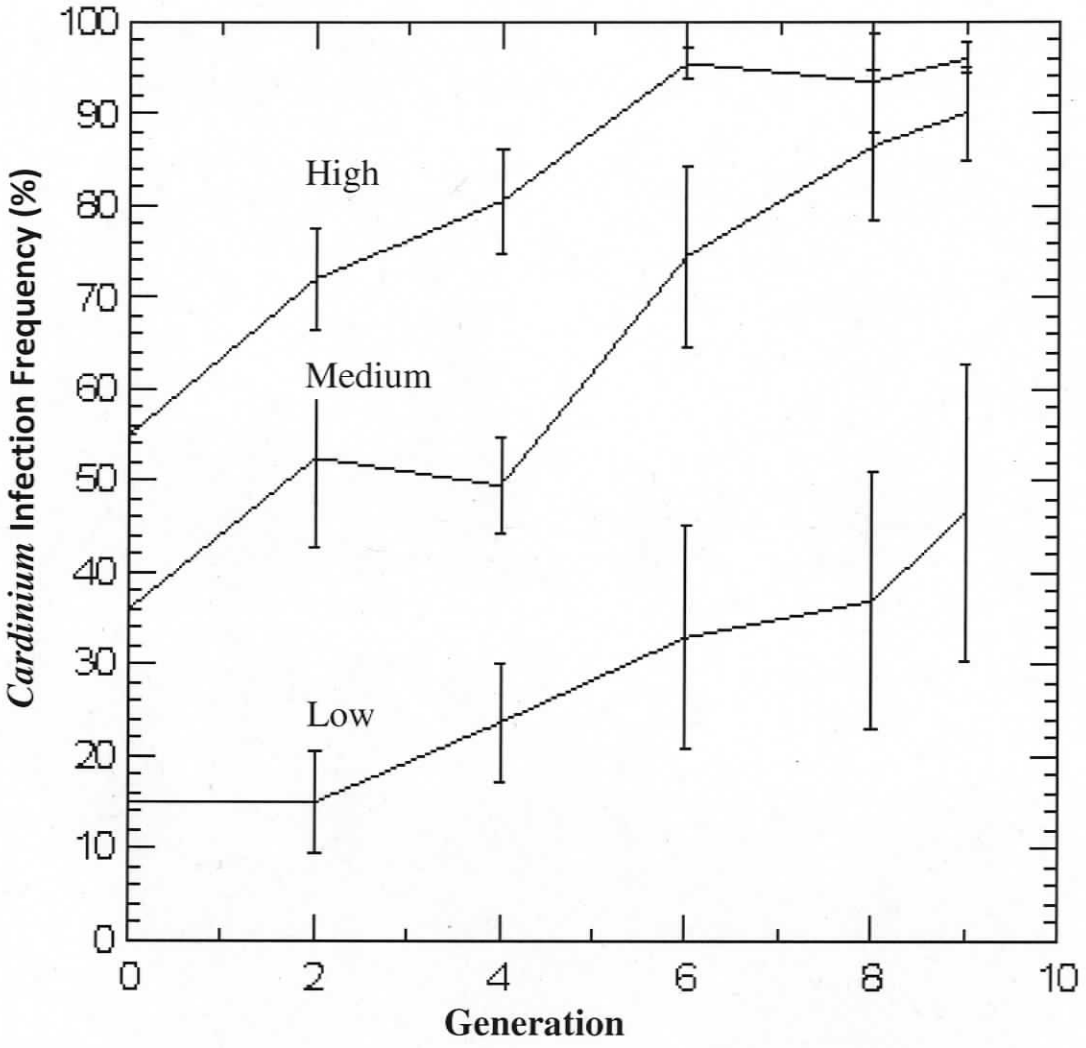

Figure 2.3. Spread of the bacterial symbiont *Cardinium* in *E. pergandiella* population cages with initial infection frequencies of 15%, 36% and 55%. Data represent the mean ± standard error, n=4.

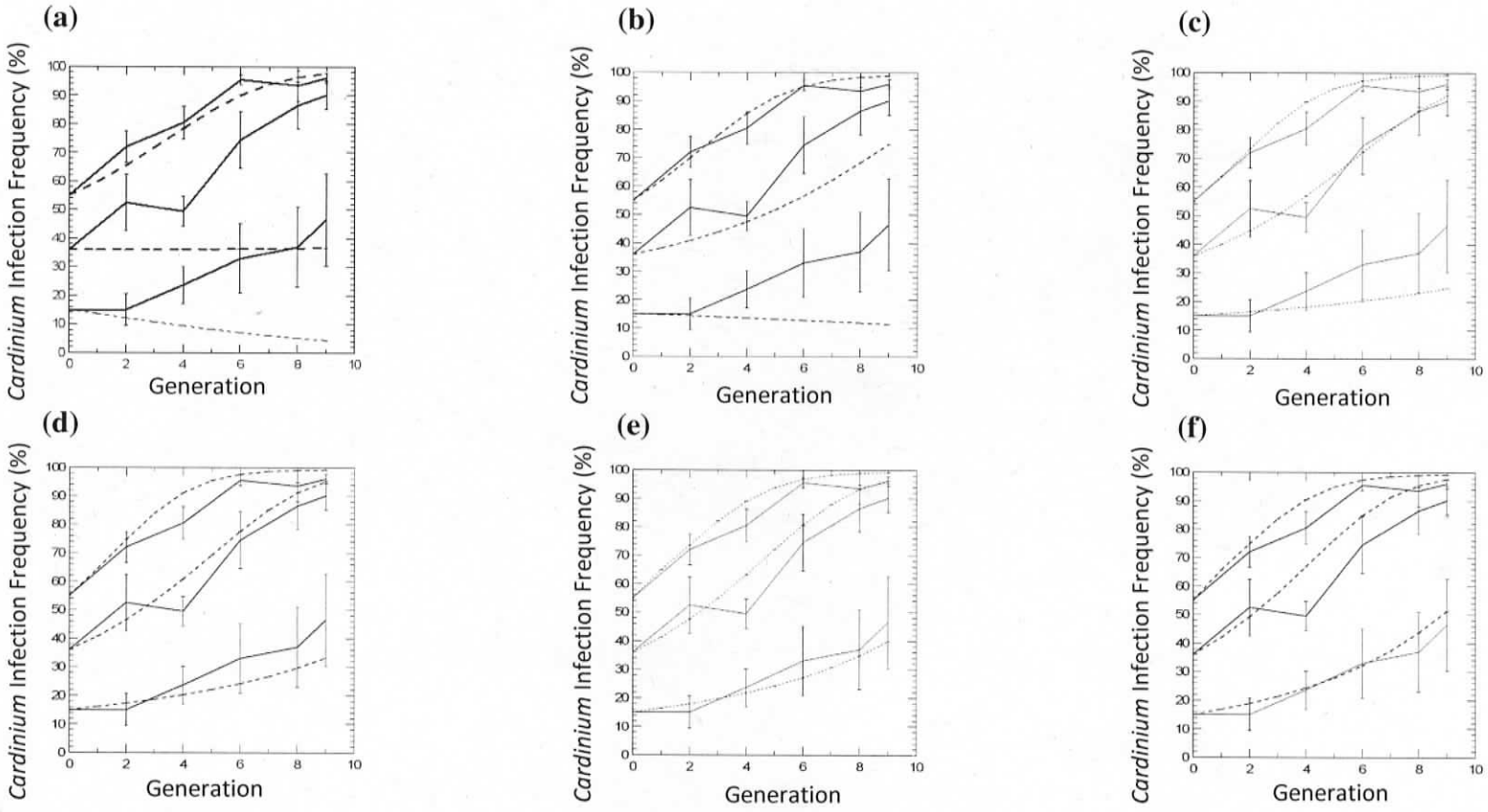

Figure 2.4. Effect of fitness cost F or fitness benefit α on modeled invasion of *Cardinium* (dashed lines) as compared to observed frequency data (solid lines) where maternal transmission efficiency u=0.99, CI strength H=0.38 and (a)F=0.82; (b)F=0.90; (c)F=0.97; (d)F=1.00; (e)α=0.98; and (f) α=0.95. Observed data represent the mean ± standard error, n=4. The fit of modeled data vs. observed data to a slope of 1, and the residual sum of squares R^2 between the modeled frequencies and the observed frequencies were (a) $F_{1,70}$=7.01; p=0.01; R^2=53 153; (b) $F_{1,70}$=1.59; p=0.21; R^2 =22 914; (c) $F_{1,70}$=0.56; p=0.46; R^2 =13 574; (d) $F_{1,70}$=0.91; p=0.34; R^2 =11 932; (e) $F_{1,70}$=2.02; p=0.16; R^2=11 273; and (f) $F_{1,70}$=3.79; p=0.06; R^2 =12 245.

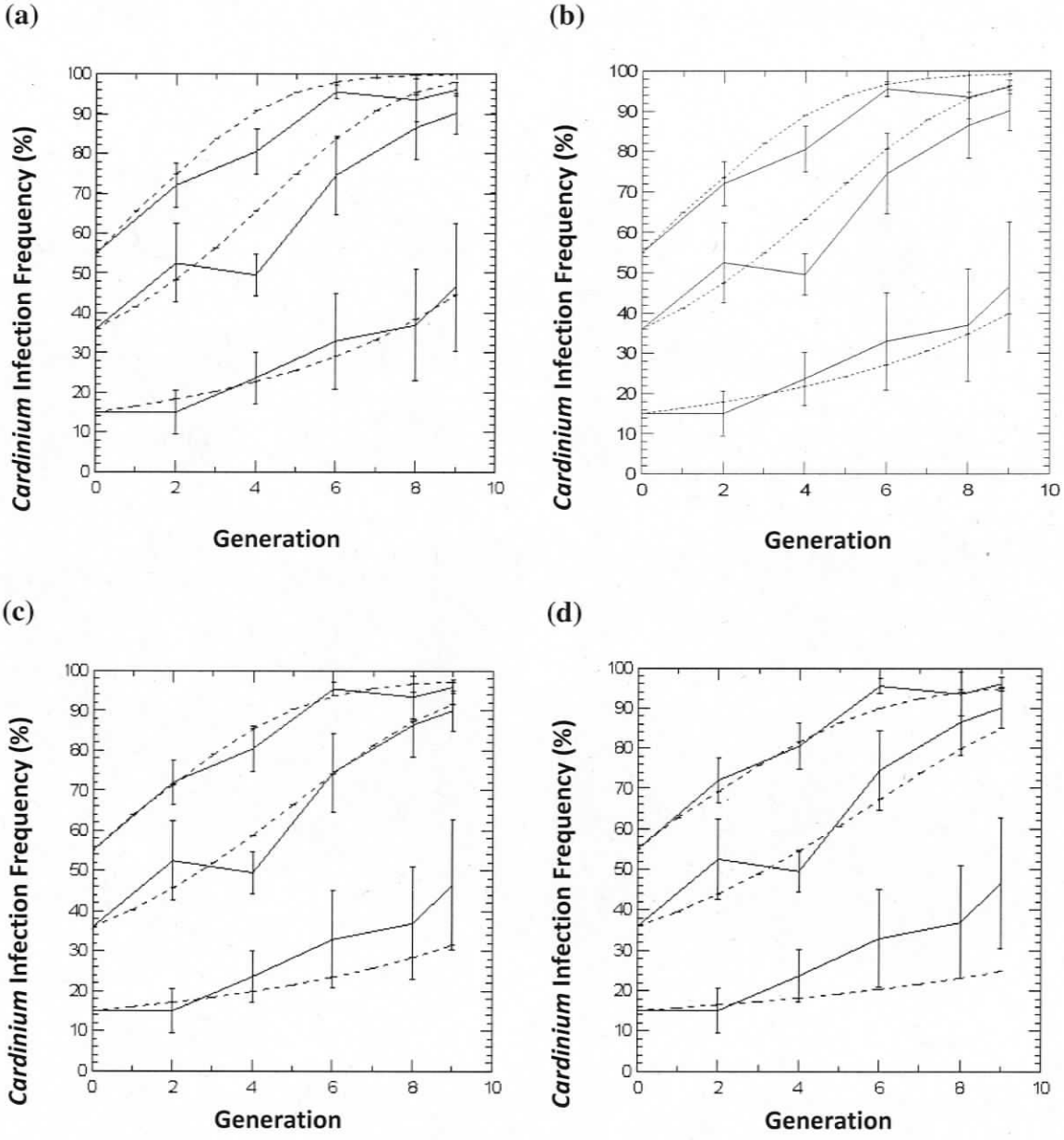

Figure 2.5. Effect of maternal transmission efficiency u on modeled invasion of *Cardinium* (dashed lines) as compared to observed frequency data (solid lines) where the infection fitness benefit α=0.98, CI strength H=0.38 and (a) maternal transmission efficiency u =0; (b) u =0.01; (c) u =0.03; and (d) u=0.05. Observed data represent the mean ± standard error, n=4. The fit of modeled data vs. observed data to a slope of 1, and the residual sum of squares between the modeled frequencies and the observed frequencies were (a) $F_{1,70}$=1.78; p=0.19; R^2 =11 893; (b) $F_{1,70}$=7.01; p=0.01; R^2 =11 273; (c) $F_{1,70}$=3.25; p=0.08; R^2 =11 416; and (d) $F_{1,70}$=6.24; p=0.01; R^2 =13 559.

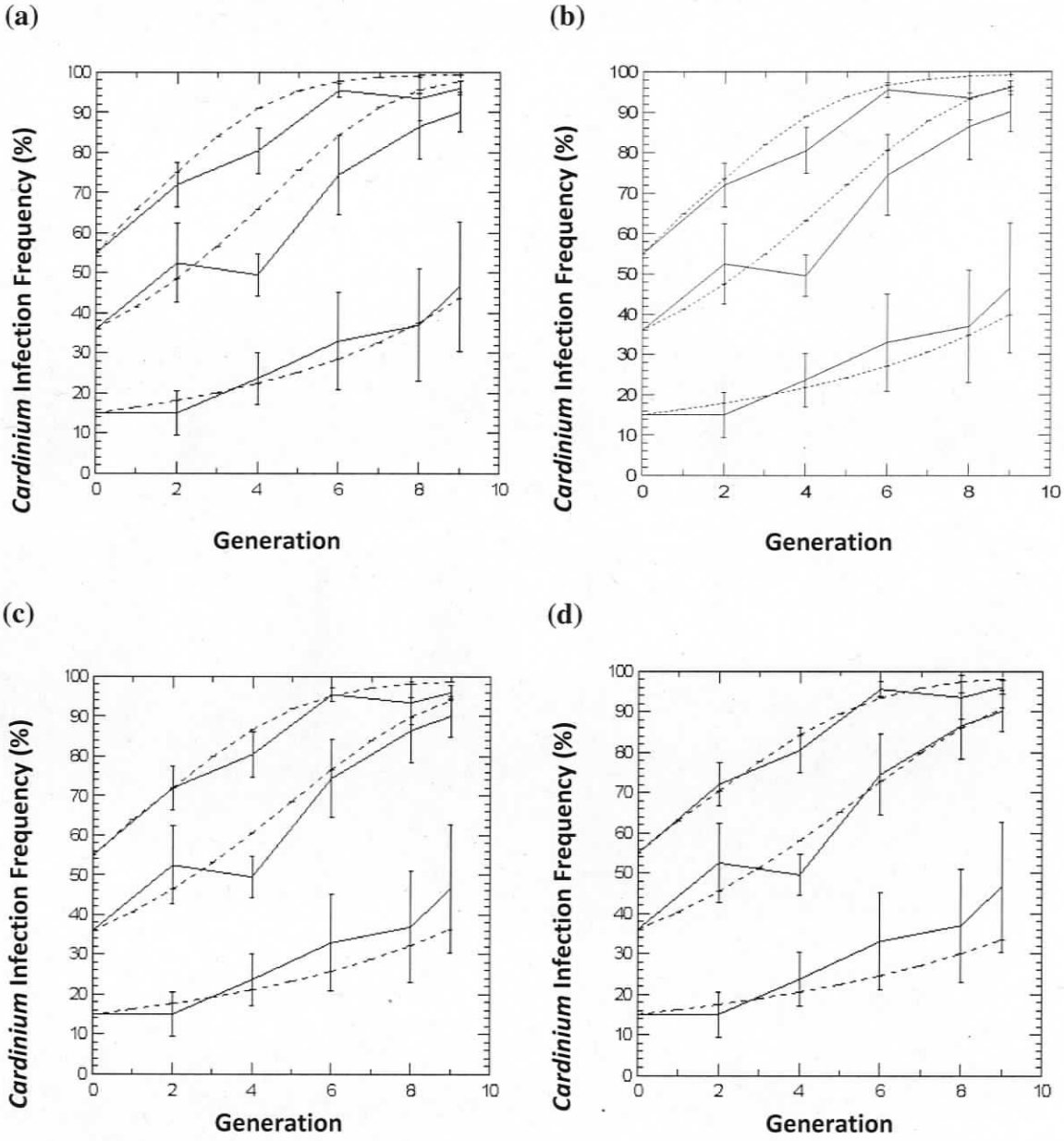

Figure 2.6. Effect of CI strength (H) on modeled invasion of *Cardinium* (dashed lines) as compared to observed frequency data (solid lines) where the infection fitness benefit α=0.98, maternal transmission efficiency u =0.01 and (a) H =0.33; (b) H=0.38; (c) H =0.43; and (d) H=0.48. Observed data represent the mean ± standard error, n=4. The fit of modeled data vs. observed data to a slope of 1, and the residual sum of squares between the modeled frequencies and the observed frequencies were (a) $F_{1,70}$=1.62; p=0.21; R^2 =12 040; (b) $F_{1,70}$=7.01; p=0.01; R^2 =11 273; (c) $F_{1,70}$=2.86; p=0.10; R^2 =10 891; and (d) $F_{1,70}$=4.45; p=0.04; R^2 =10 989.

Chapter III: The effect of male age on cytoplasmic incompatibility induced by *Cardinium* in the parasitoid wasp *Encarsia pergandiella*

Bacterial endosymbionts of arthropods interact with their hosts in a variety of ways, and these interactions range from parasitic to mutualistic (Moran, 2001). One class of bacterial symbionts is known as reproductive manipulators, and these manipulate host reproduction in ways that benefit their own fitness. These symbionts are vertically transmitted, passed on from mothers to their offspring. Consequently, the symbiont's fitness is linked directly to the number of infected daughters produced by an infected female. It is therefore in the interest of the symbiont to increase the production of infected daughters, and there are several ways in which the reproductive manipulators accomplish this, the most common strategy being cytoplasmic incompatibility (CI).

In CI, uninfected females, or females infected with a different symbiont strain, suffer reduced numbers of viable offspring when mated with infected males. Infected females, on the other hand, do not experience this reproductive loss, regardless of the infection status of the males they mate with. Consequently, female hosts benefit from the infection by avoiding incompatible matings. By increasing the relative fitness of infected females, the bacterial symbiont is often able to spread very rapidly in host populations. For example, the CI symbiont *Wolbachia* was documented to spread in California *Drosophila simulans* populations at an astounding rate of 12 km per generation (Turelli and Hoffmann, 1991).

While the mechanism of CI is not fully understood, it is thought to follow a model of modification and rescue (Werren, 1997). In this model, the sperm of infected males is somehow modified within the testes, rendering it incompatible with eggs from females that

are not infected with the same CI strain. This incompatibility is thought to be caused by abnormalities in paternal DNA condensation and segregation (Tram et al., 2003). These abnormalities are somehow rescued when an egg is infected with the same CI symbiont, resulting in viable offspring.

Two bacterial endosymbionts have been found to be able to induce CI in arthropods. *Wolbachia,* in the α proteobacteria, is the most well known reproductive manipulator, infecting at least 16% to 24% of all arthropod species (Werren et al., 1995; Weeks et al., 2003; Zchori-Fein and Perlman, 2004). While the effects of *Wolbachia* infection are unknown for many hosts, it has been found to induce a wide variety of reproductive phenotypes, including CI (Stouthamer et al., 1999). *Wolbachia* has been shown to cause CI in several insect orders, including Coleoptera, Diptera, Hemiptera, Hymenoptera, Orthoptera, and Lepidoptera, as well as in mites and isopods (Stouthamer et al., 1999). The more recently discovered symbiont *Cardinium* is found in the Bacteroidetes group. Since its discovery, *Cardinium* has been found in four orders and approximately 6-7% of arthropods (Weeks et al., 2003; Zchori-Fein and Perlman, 2004; Duron et al., 2008). Like *Wolbachia*, the host effects of *Cardinium* infection are largely unknown, although it, too, has been found to induce several reproductive phenotypes (Zchori-Fein and Perlman, 2004). *Cardinium* has been shown to induce CI in the parasitoid wasp *Encarsia pergandiella* (Hunter et al., 2003), and in the spider mite *Eotetranychus suginamensis (*Gotoh et al., 2007).

The strength of CI, or the reduction in offspring experienced by the incompatible cross, is a critical factor in the invasion and spread of a CI-inducing symbiont (Turelli, 1994). Several factors have been found to influence CI strength, including temperature (Clancy and Hoffmann, 1998), bacterial density (Noda et al., 2001; Bourtzis et al., 1996), male

development time (Yamada et al., 2007) and the age of male at the time of mating (Turelli and Hoffmann, 1995; Reynolds et al., 2003). Learning more about these factors will contribute to a better understanding of the CI mechanism, as well as the invasion and spread of these symbionts in the field.

The reason older males induce weaker CI is thought to be related to differences in the bacterial density or the number of infected sperm cysts present in the testes of infected males. For example, Binnington and Hoffmann (1989) used transmission electron microscopy to demonstrate that in *Drosophila simulans,* older males were less likely to harbour *Wolbachia*. Bressac and Rousset (1992) later found that 87% of the sperm cysts of younger *D. simulans* males were infected, compared to only 3% in older males. Additionally, there is some evidence that the density of *Wolbachia* in infected sperm cysts is positively correlated with CI strength in incompatible crosses (Clark et al., 2003). For example, Poinsot et al. (1998) infected *D. simulans* with a *Wolbachia* strain normally found in *D. melanogaster*. They found that ten times as many sperm cysts in *D. simulans* were infected, and observed CI to be much stronger in *D. simulans* (98% vs. 18-32% embryonic mortality).

Male age has been found to be a major factor influencing CI strength in several hosts. For example, Hoffmann et al. (1990) found that male age had a large impact on CI strength in *D. simulans*. When mated with uninfected females, one day old male *D. simulans* infected with *Wolbachia* produced 0.2 to 1.4% of the number of offspring of the compatible cross. At 12 days old, the incompatible males produced 42% of the number of offspring produced by the compatible cross, and this number increased to 74 to 77% when males were 21-24 days old (Hoffmann et al. 1990). A similar reduction in CI strength with increasing male age was

reported in two different populations of *D. simulans* and in *D. melanogaster* (Turelli and Hoffmann, 1995; Reynolds and Hoffmann, 2002).

Studies involving *Wolbachia* in mosquitoes have been less conclusive about the effects of male age on CI strength. Jamnongluk et al. (2000) found that *Wolbachia* in *Armigeres subalbatus* mosquitoes reduced egg hatch by 50% in incompatible crosses when young males were used, whereas older infected males did not induce CI at all. An examination of mating swarms in the field suggested that 25-63% of the males were older, which helped to explain why *Wolbachia* seemed to have no effect on *A. subalbatus* hatch rates in the field (Jamnongluk et al., 2000). On the other hand, male age had a negligible effect on CI strength in *Culex pipiens* infected with *Wolbachia* (Rasgon and Scott, 2003).

Our study examined the effect of male age on the strength of CI induced by *Cardinium* in the parasitic wasp *Encarsia pergandiella*. While several studies have examined the effect of male age on CI induced by *Wolbachia*, little is known about how male age may affect CI strength in *Cardinium*. Understanding how male age may influence a novel CI inducing symbiont will contribute to a better understanding of the interactions between these symbionts and their hosts. These interactions could be important in predicting the likelihood and rate of spread of a CI causing symbiont. In addition, studying factors that affect CI strength may lead to new insights on similarities or differences in how these symbionts modify sperm in their male hosts.

Methods

Encarsia pergandiella cultures

Encarsia pergandiella is an aphelinid (Hymenoptera) parasitoid of whiteflies. Infected lines were collected in the Rio Grande Valley in Texas in 2003 and 2006 and are fixed for *Cardinium*. The uninfected lines were obtained by curing a sub-population of each infected line by treating adult wasps with 50 mg/ml rifampicin in honey for three generations. All cultures (cured and uncured) were maintained in the laboratory on *Bemisia tabaci* whiteflies reared on cowpea plants (*Vigna unguiculata*) for several generations prior to the study. The 2003 and 2006 lines were used in separate trials to determine the effect of male age on CI strength. All experiments were conducted at the University of Arizona in Tucson, Arizona.

Infection Status of Cured Culture

To confirm the infection status of the cured and uncured lines, 25 female wasps from both the 2003 and 2006 cured lines and ten females from both the 2003 and 2006 infected lines were screened for *Cardinium* using PCR. DNA was extracted by grinding five wasps from the cured culture in 5 uL of 20 mg/mL proteinase k, and adding the homogenate to 50 uL of 5-10% w/v chelex. Samples were incubated at 37°C for one hour, and then at 96°C for 8 minutes, with periodic vortexing. DNA extractions were screened for infection using the *Cardinium* specific primers ChF (5'- TACTGTAAGAATAAGCACCGGC) and ChR (5'- GTGGATCACTTAACGCTTTCG), which amplified a ~450bp region of 16S rDNA (developed by Stephan Shmitz-Esser, University of Vienna). PCR conditions were a 3 min. initial denaturation at 94°C, followed by 40 cycles of 94°C for 1 min., 50°C for 1 min., and

72°C for 1.5 min., with a final extension of 72°C for 6 min. A positive control of an infected *E. pergandiella* and a negative control of a cured *E. pergandiella* were included in each run. Samples determined to be negative for *Cardinium* were screened for the single double copy *E. pergandiella* gene EF1α as a positive control for DNA. A ~380bp segment of EF1α was amplified using EpergEF-F and EpergEF-R (Perlman et al., 2008) with PCR reaction conditions of: 95°C for 3 min., followed by 30 cycles of 95°C for 60s, 50°C for 60s, and 72°C for 90s, with a final extension of 72°C for 10 min.

E. pergandiella strain comparisons

Both host nuclear background and CI strain can influence CI strength (Clancy and Hoffmann, 1996; Poinsot et al., 1998). Therefore, the fecundity and CI strength of the 2003 and 2006 populations were compared. Freshly emerged virgin uninfected females of each strain were mated to 0-1 day old cured or infected males of the same strain. Wasps were held at 27°C in mating vials for 24 hours, and then females were transferred to leaf disks containing 50 to 100 third to early fourth whitefly nymphs. The number of developing *E. pergandiella* pupae was scored as described below.

Effect of male age on cytoplasmic incompatibility

Freshly emerged cured virgin females were mated individually to young, old or very old virgin males of known infection status. Young, old and very old males were 0-2, 6-9, and 11-13 days old for the 2003 trial, and 1-2, 4-5, and 9-11 days old for the 2006 trial, respectively. In the 2003 trial, females were held in mating vials until visual confirmation of mating, which normally occurs within the first 10 seconds. Because this resulted in some females being unmated even after appearing to have mated, mating pairs were held in vials

for 24 hours for the 2006 trial. Each female was then transferred to a 35 mm petri dish with a cowpea leaf disk on 1% agar. Each leaf disk was infested with 50 to 100 third to early fourth instar whitefly nymphs (*B. tabaci*). Females were allowed to oviposit for 24 hours before being removed from leaf disks.

Following the oviposition period, infested leaves were incubated at 27°C until *E. pergandiella* pupae could be counted and removed (8-12 days). When an *E. pergandiella* egg fails to develop due to cytoplasmic incompatibility, the parasitized whitefly is developmentally arrested (Hunter et al., 2003). Whiteflies were considered to be developmentally arrested if, by the time of scoring, they had not developed eye spots or wing buds. Females that did not produce any pupae and had less than two developmentally arrested whiteflies on their leaf disk were considered to be unmated, and were removed from the data analysis.

Data analysis

The presence of CI in the 2003 and 2006 populations was confirmed by comparing the fecundity of the predicted incompatible and compatible crosses using a Mann-Whitney test. The strength of CI was measured by calculating the relative number of offspring compared to the average number of offspring produced by the compatible cross for each treatment. This effectively normalized the data for fecundity differences between compatible treatments. Fecundity and CI strength were compared among populations and age groups using Mann-Whitney and Kruskal-Wallis tests. All statistics were calculated using SPSS 16.0.

Results

E. pergandiella strain comparisons

Among the compatible crosses, the 2003 population of *E. pergandiella* was significantly more fecund than the 2006 population (Figure 3.1; Mann-Whitney test; U=140; n=43; p=0.028). Average fecundity of the predicted incompatible crosses was significantly lower than that of the compatible crosses for both the 2003 (Mann-Whitney test; U=55.5; n=47; p=0.000) and 2006 (Mann-Whitney test; U=32; n=45; p=0.000) populations. The incompatible crosses of the 2003 and 2006 populations produced 38% and 30% of the offspring of the compatible crosses, respectively (Figure 3.1). No difference was found in the level of incompatibility, or CI strength, between strains (Mann-Whitney test; U=279; n=49; p=0.674). A high level of variation was observed in the number of offspring produced by the incompatible crosses, and some of the incompatible mating pairs appeared unaffected by CI (Figure 3.1). For example, in two out of 23 females from the 2003 line, and in one out of 25 females from the 2006 line, the incompatible mating pairs produced more offspring than the average number of offspring produced by the compatible crosses.

Effect of male age on cytoplasmic incompatibility

There was no effect of male age on average fecundity among the compatible crosses for the 2006 population (Figure 3.2; Kruskal-Wallis test; $\chi^2_{1,2}$=3.881; p=0.144). In the 2006 population, CI strength was found to be weakest when females were mated with 4-5 day old males (Figure 3.3), and this difference was close to significant (Kruskal-Wallis test; $\chi^2_{1,2}$=5.844; p=0.054). Similarly, the fecundity of compatible crosses was not significantly

different between male age treatments in the 2003 population (Figure 3.4; Kruskal-Wallis test; $\chi^2_{1,2}$=5.59; p=0.061). In the 2003 population, a large proportion of males died before they were old enough to use in the very old male treatment. Additionally, many of the females mated to very old males produced no offspring and no arrested whiteflies, and were therefore presumed to be unmated. Consequently, these females were removed from data analysis, resulting in a sample size of three in the incompatible cross of the old male age treatment groups. Due to its limited sample size, the very old age treatment was removed from further analyses. However, no significant difference was found between the young and old aged males in CI strength in the 2003 population (Figure 3.5; Mann-Whitney test; U=100; n=31; p=0.514).

Discussion

E. pergandiella strain comparisons

Interestingly, the 2003 population of *E. pergandiella* had significantly higher fecundity than the 2006 population, even though both were collected from the same location (Figure 3.1). This may perhaps be explained by the fact that the 2006 laboratory culture was established from fewer females that the 2003 population (Martha S. Hunter, personal communication). Consequently, the 2006 population may have lower genetic diversity than the 2003 population, and this could have affected its fecundity. The 2003 line has also been in laboratory culture for three additional years, and it is possible that, in the absence of environmental factors that may have constrained reproduction, it has evolved toward greater fecundity. Although it is not known if the *Cardinium* strains infecting the 2003 and 2006

populations are identical, they appear to be causing a similar level of CI, regardless of the observed population differences in fecundity (Figure 3.1).

Interestingly, the strength of CI in this study was weaker than earlier findings, with incompatible crosses producing approximately 30 to 38% of the progeny that the compatible crosses produced. Previous studies on *E. pergandiella* report much stronger CI, with incompatible crosses producing only 7 to 12.5% as many offspring as compatible crosses (Hunter et al., 2003; Perlman et al., 2008). These differences in CI strength could be the result of differences in rearing conditions. For example, there could have been differences in larval density or food quality during wasp rearing, both of which have been shown to influence CI strength (Sinkins et al., 1995; Clancy and Hoffmann, 1998). It is also possible that there may have been evolution of either the host or the symbiont towards weaker CI. Modeling studies indicate that, while selection on the symbiont does not directly act to increase or decrease CI strength, the host should evolve to reduce CI strength (Prout, 1994; Turelli, 1994). It would be interesting to perform microinjection or backcrossing studies on laboratory populations as well as on field populations, to determine if host nuclear background or any strain differences in *Cardinium* influence CI strength.

Effect of male age on cytoplasmic incompatibility

Although not significant due to the high variability in fecundity, the 2006 old compatible treatment produced, on average, 30% fewer offspring than the very old compatible treatment (Figure 3.2). This reduced fecundity would have resulted in weaker CI strength estimates, which is likely to have caused the close to significant difference in CI strength among treatments (Figure 3.3). Similarly, considerable variation in fecundity was

observed in the 2003 population, although this was not significant (Figure 3.4). As in the 2006 population, male age was not found to influence CI strength in the 2003 population (Figure 3.5).

The majority of females mated to very old males in the 2003 study did not oviposit. Similarly, more females mated to very old males failed to produce offspring in the 2006 population. This could be the result of females refusing to mate with older males that may be less attractive. For instance, Kaltenpoth and Strohm (2006) found that, in the European beewolf (*Philanthus triangulum*), older males produce smaller amounts of a pheromone important in reproduction. In addition, there was high mortality of males in the very old treatments before they were mated. This suggests that the oldest male treatment group may not have been biologically relevant, since males of this age likely do not make up a significant proportion of the mating male population, especially in the field.

Our results suggest that there is no effect of male age on the level of CI induced by *Cardinium* in *E. pergandiella* (Figures 3.3 and 3.5). In contrast, several studies on *Wolbachia* have found that weaker CI is induced by older males (Hoffmann et al., 1990; Turelli and Hoffmann, 1995; Jamnongluk et al., 2000). However, this effect seems to be species-specific, as it was not observed in *C. pipiens* (Rasgon and Scott, 2003). The male age effect in *Wolbachia* has been attributed to differences in the proportion of sperm cysts that are infected in older males (Bressac and Rousset, 1992; Clark et al., 2003). Additionally, Clark et al. (2002) indicate that the density of *Wolbachia* within the sperm cysts and spermatids is correlated with CI strength, and this declines with male age in *D. melanogaster* and *D. simulans*. The lack of a male age effect on the CI strength in *Cardinium* infected *E. pergandiella* is best explained by either:

a) the fact that *E. pergandiella* males do not vary in the density or number of infected sperm cysts and spermatids as they age (at least not enough to significantly change CI strength); or
b) the level of incompatibility induced by *Cardinium* in *E. pergandiella* is not dependent upon the density of *Cardinium* within the sperm cysts and spermatids.

Some evidence suggests that male age may affect CI strength only when the infection density is below a certain threshold. For example, Kittayapong et al. (2002) found no effect of male age on CI strength for *Aedes albopictus* that were simultaneously infected with two strains of *Wolbachia.* However, older males induced weaker CI when the host was infected with only one of these *Wolbachia* strains (Kittayapong et al., 2002). It is therefore possible that *Cardinium* reaches high enough densities in *E. pergandiella* that they are unaffected by male age.

It would be useful to confirm our findings of no effect of male age on CI strength in *E. pergandiella* using a larger sample size. In addition, future studies should aim to determine if CI strength is correlated with the density of *Cardinium* in male *E. pergandiella* hosts. It would also be useful to determine if *Cardinium* localization within males is similar to that of *Wolbachia.* Understanding *Cardinium* infection and localization patterns may help to explain why male age affects CI strength in many hosts infected with *Wolbachia*, but did not affect *E. pergandiella* infected with *Cardinium.*

It would also be useful to test the influence of male age on the strength of CI-induced by *Cardinium* in the spider mite, *Eotetranychus suginamensis* (Gotoh et al., 2007). Gotoh et al. (2007) did not test the effect of male age on CI strength, but they did notice a wide

variation in the level of incompatibility observed between different incompatible mating pairs. They hypothesize that this large variation might have been due to differences in male age, since they used males that ranged from one to five days old (Gotoh et al., 2007). However, a large variation in the level of cytoplasmic incompatibility was also found in the present study, with some females of incompatible crosses producing normal numbers of offspring. Since no effect of male age was found on the level of incompatibility in this study, it seems likely that some of this variation is due to other factors, such as infection density (Bourtzis et al., 1996; Noda et al., 2001), larval density (Sinkins et al., 1995), food quality (Clancy and Hoffmann, 1998) or male development time (Yamada et al., 2007). In any case, further studies on the effects of male age on cytoplasmic incompatibility caused by *Cardinium* will help determine how similar, or different, its infection dynamics are to those of *Wolbachia*.

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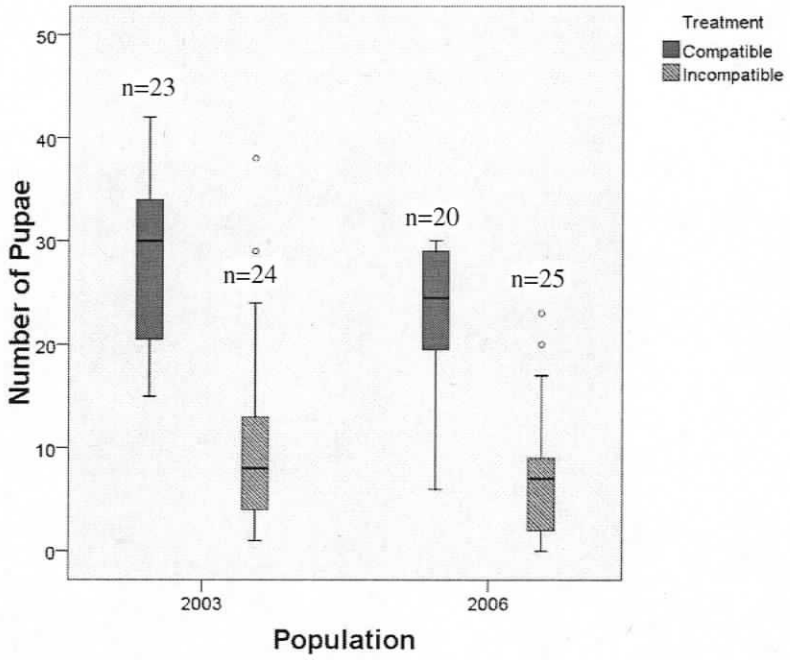

Figure 3.1. Comparison of fecundity and CI in the 2003 and 2006 *E. pergandiella* populations. Sample size (n) is indicated above each box.

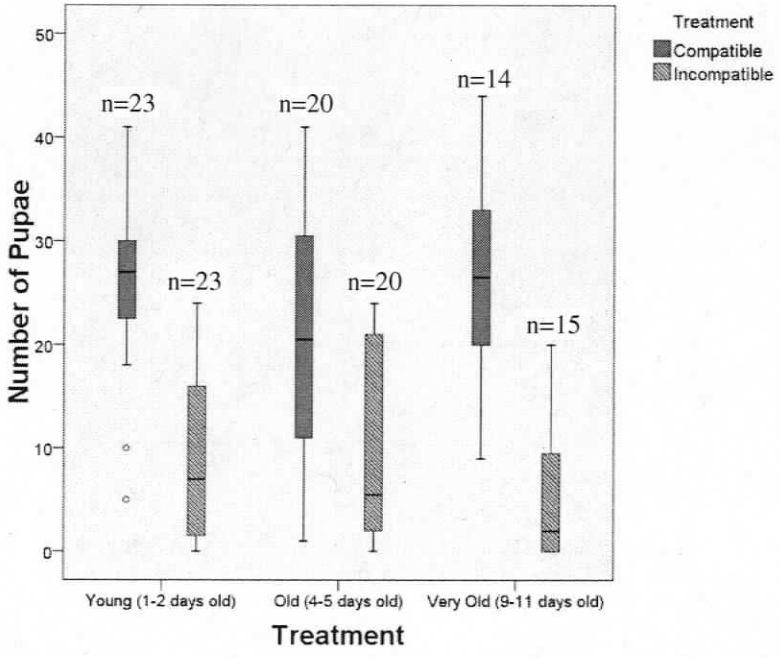

Figure 3.2. Effect of male age on the number of pupae produced by incompatible and compatible crosses of the 2006 population of *E. pergandiella.* Sample size (n) is indicated above each box.

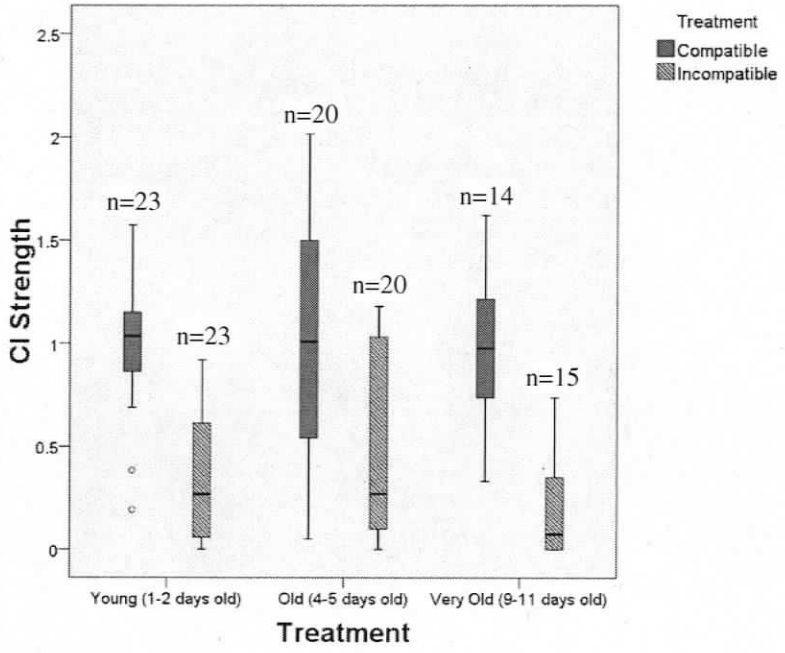

Figure 3.3. Effect of male age on the strength of CI observed for incompatible crosses of the 2006 population of *E. pergandiella*, with control crosses normalized to equal one. Sample size (n) is indicated above each box.

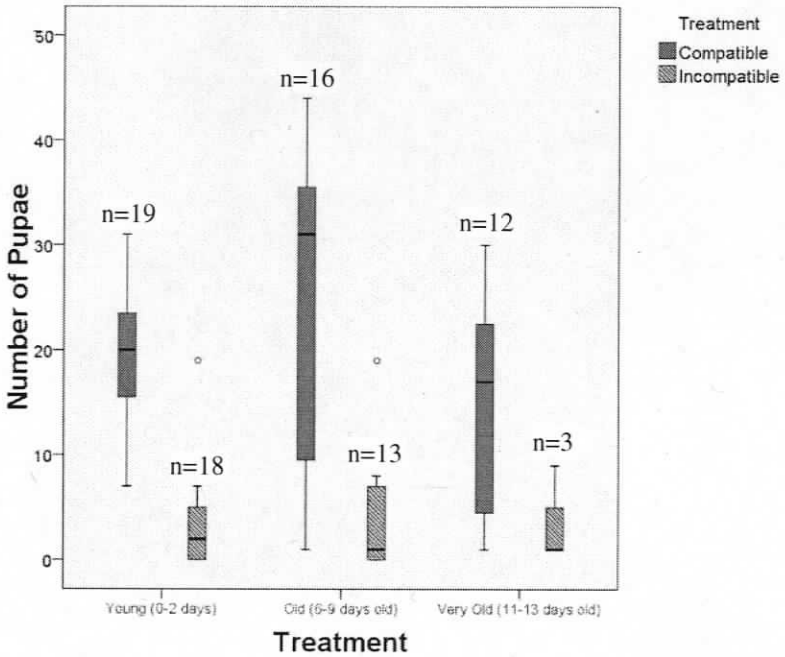

Figure 3.4. Effect of male age on the number of pupae produced by incompatible and compatible crosses of the 2003 population of *E. pergandiella.* Sample size (n) is indicated above each box.

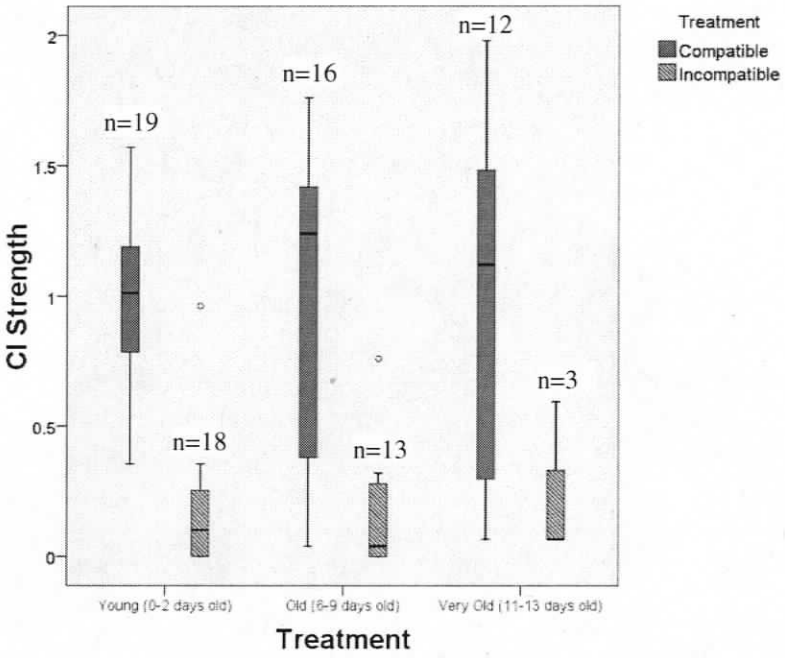

Figure 3.5. Effect of male age on the strength of CI observed for incompatible crosses of the 2003 population of *E. pergandiella*, with control crosses normalized to equal one. Sample size (n) is indicated above each box.