

***Wolbachia*-based strategies to control insect pests and disease vectors**

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Abstract. *Wolbachia* are a group of obligate intracellular maternally inherited bacteria that have been found in several arthropod groups including spiders, terrestrial crustaceans, and insects, in addition to filarial nematodes. It has been estimated that >65% of insect species harbor *Wolbachia*, making it one of the most ubiquitous intracellular bacteria discovered to date. In arthropods, *Wolbachia* behave as a reproductive parasite by manipulating host reproduction to enhance their vertical transmission. One reproductive modification, cytoplasmic incompatibility (CI), has received attention for use in applied strategies targeting economically important insect pests and disease vectors. The two proposed CI-based strategies are: (1) population suppression, analogous to the sterile insect technique (SIT) and (2) population replacement, using *Wolbachia* as a vehicle to drive desirable phenotypes into natural populations. Strategies are based upon the use of both naturally occurring infections and genetically modified *Wolbachia* strains. In this review, we summarize recent developments in *Wolbachia* research, specifically within the context of applied *Wolbachia*-based strategies used to suppress or modify natural insect populations.

Keywords: *Wolbachia pipientis*, cytoplasmic incompatibility, population replacement, sterile insect technique, biological control

INTRODUCTION

Insect-borne diseases impose an immense burden on global health, and insect crop pests greatly influence economic and agricultural productivity. For example, malaria alone is responsible for over a million deaths every year (Snow *et al.*, 2005). With the resurgence of vector borne disease, some have been pessimistic that conventional control measures, such as using insecticides for long-term periods will be effective. Furthermore, continued use of insecticides has led to concerns of negative environmental effects. Thus, the need for novel environmentally friendly control strategies has been suggested to complement current insect control measures.

Wolbachia are maternally inherited intracellular rickettsiae-like bacteria belonging to the α -Proteobacteria (O'Neill *et al.*, 1992; Werren, 1997; Werren *et al.*, 2008). The type species for the *Wolbachia* genus is *Wolbachia pipientis*, first described in the mosquito *Culex pipiens* (Hertig and Wolbach, 1924). Since then, *Wolbachia* has been found worldwide in numerous arthropod species, including: insects, mites, spiders, terrestrial isopods, as well as filarial nematodes (Werren, 1997; Zhou *et al.*, 1998; Lo *et al.*, 2002; Gotoh *et al.*, 2003; Cordaux *et al.*, 2004; Goodacre *et al.*, 2006; Hilgenboecker *et al.*, 2008; Werren *et al.*, 2008). A recent meta-analysis has

estimated that >65% of insect species harbor *Wolbachia*, making it one of the most ubiquitous endosymbionts on earth (Hilgenboecker *et al.*, 2008). Recently, *Wolbachia* has received attention as a potential bio-control agent that may yield novel insect control strategies.

In invertebrates, *Wolbachia* has been shown to manipulate cellular and reproductive processes (Hoffman and Turelli, 1997; Werren, 1997; Sinkins, 2004; Jeong and Suh, 2008; Werren *et al.*, 2008). In filarial nematodes, *Wolbachia* appears to behave as a mutualist (Taylor *et al.*, 2005). *Wolbachia* may provide metabolic pathways absent in filarial nematodes, which are important for the fecundity of its host (Taylor *et al.*, 2005). However, in arthropods, *Wolbachia* behaves more like a reproductive parasite by inducing: feminization of genetic males, parthenogenesis, male-killing, and cytoplasmic incompatibility (CI) (Werren, 1997; Werren *et al.*, 2008). These modifications typically give a reproductive advantage to infected individuals and allow for the spread of *Wolbachia* through a population (Turelli and Hoffman, 1991; Dobson *et al.*, 2002a; Dobson *et al.*, 2002b; Dobson, 2003; Xi *et al.*, 2005a).

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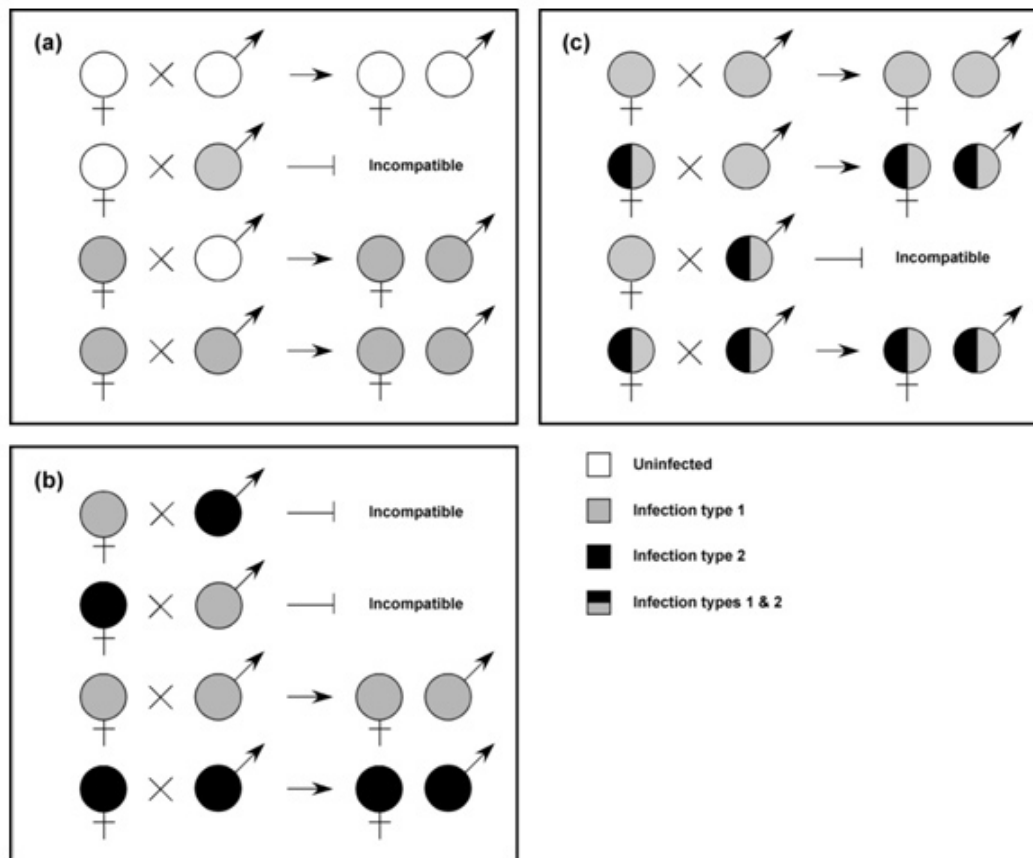


Figure 1. Examples of cytoplasmic incompatibility. (a) Uni-directional CI can occur between *Wolbachia* infected and uninfected individuals. Infected females are expected to have a reproductive advantage. Unlike their uninfected counterpart, they can mate with infected and uninfected males and produce viable offspring. As a result, the infection would be expected to spread through and replace the population. (b) Bi-directional CI can occur between individuals infected with different *Wolbachia* infection types. If this situation occurs, the strain that is in the majority or has a higher penetrance of CI is expected to replace the population. (c) If multiple infections (superinfections) exist in a population, an additive pattern of uni-directional CI can occur. Superinfected males are incompatible with females harboring only one infection type. In this case, superinfections would be expected to spread through a population harboring one infection type.

CI is the most widespread and the most well studied reproductive modification induced by *Wolbachia*. CI is a form of sterility composed of two distinct components: a *Wolbachia*-induced “modification” of sperm during spermatogenesis and a “rescue” of this modification in embryos infected with the same *Wolbachia* type (Werren, 1997). If the same *Wolbachia* strain is not present in the egg during embryogenesis, embryonic development is disrupted. Thus, if a male is infected with CI-inducing *Wolbachia*, sperm is “modified” such that an uninfected egg or an egg with a different infection type can no longer “rescue” the sperm, resulting in incompatibility. Thus, in populations that include infected and uninfected individuals, a pattern of uni-directional CI can result (Figure 1a). In uni-directional CI, infected females have a reproductive advantage and produce a greater number of offspring in a population, because they can mate successfully with both male types. Furthermore, since *Wolbachia* is maternally inherited, the frequency of infection increases with every generation. The same “modification-rescue” model can be applied when there are two different *Wolbachia* types in a population, resulting in a pat-

tern of bi-directional CI between individuals with different infection types (Figure 1b). Moreover, when multiple *Wolbachia* infection types exist in a population (superinfection), an additive pattern of uni-directional CI can result (Figure 1c). Although the effects of *Wolbachia* infection on vector reproduction are understood on some level, the exact molecular mechanism of CI is still unknown despite a substantial amount of research (reviewed by Poinot *et al.*, 2003).

To date, CI has been a focus of *Wolbachia*-based applied strategies for insect control. Strategies include: (1) using *Wolbachia*-induced CI as a form of sterility for a mass male release strategy, analogous to a sterile insect technique; (2) using the reproductive advantage afforded by *Wolbachia*-induced CI as a population replacement strategy to drive wanted phenotypes into natural populations. These strategies are discussed in this review as ways to suppress or modify insect populations, to aid in the control of insect pests and disease vectors. Not the focus of this review, *Wolbachia*-induced parthenogenesis is also discussed as a means to facilitate biological control programs using parasitic wasps.

Wolbachia Based Applied Strategies

Incompatible insect technique (IIT)

One applied strategy using CI is analogous to the sterile insect technique (SIT), where mass inundative releases of sterile males are used to suppress and/or eliminate natural populations (Knipling, 1959). A major requirement for the success of any SIT strategy is the ability to deliver to the field population large numbers of sexually active fit males (Benedict and Robinson, 2003). However, the technology used to generate sterile males (e.g., irradiation and chemosterilization) often results in loss of fitness of release males (Alphey, 2002; Benedict and Robinson, 2003). In a *Wolbachia*-based incompatible insect technique (IIT) strategy, female sterility is artificially sustained by repeated releases of cytoplasmically incompatible males. Since *Wolbachia* is not paternally transmitted, the infection type present in the release strain does not become established in the field. As the size of the field population decreases due to incompatible matings, the proportion of males of the release strain increases. Similar to conventional SIT, the increasing ratio of incompatible matings over time can lead to population suppression and possibly population elimination.

A notable IIT success was a field trial, in which an isolated population of the mosquito *Culex pipiens quinquefasciatus* was eliminated by releasing males harboring an incompatible *Wolbachia* infection type (Laven, 1967). Earlier work with *C. pipiens quinquefasciatus* was later followed by the development of an IIT program jointly by the World Health Organization (WHO) and Indian council of Medical Research (ICMR) directed against *C. pipiens quinquefasciatus* in India. Large field releases were ongoing, but were stopped, due to unsubstantiated claims that the research was part of biological warfare program (Curtis and Reuben, 2007).

Criteria that should be met before the implementation of an IIT strategy, include: (1) *Wolbachia* infections in the release strain should display high rates of CI (i.e., egg hatch resulting from incompatible matings low or absent); (2) the release strain should show high rates of maternal inheritance, so that infections are passed on to subsequent generations of release strains and release males will continuously harbor incompatible infections; (3) release strain fitness and male mating competitiveness should be tested in natural systems and be comparable to wild type males; (4) there should be little risk of unwanted side effects in the ecosystem caused by releases; (5) proposals for releases should be presented to the public and be met with public acceptance before release; (6) a crucial criterion is that only males can be released. If females of the incompatible strain were accidentally released, there is a risk of the incompatible infection type becoming established and replacing the natural population (Dobson *et al.*, 2002a; Dobson *et al.*, 2002b; Dobson, 2003; Sinkins, 2004; Xi *et al.*, 2005a). A particular concern with disease vectors is that the accidentally replaced population could be a more competent vector than the target population. Therefore, vector competence and the risk of accidental population replacement should be evaluated.

To decrease the risk of accidental population replacement, it has been suggested that irradiation could be incorporated with CI to sterilize any accidentally released females (Sharma *et al.*, 1979; Arunachalam and Curtis, 1985). Using the example of mosquitoes, males and females could be mechanically separated as pupae with a >99% accuracy (Sharma *et al.* 1972; Focks 1980), and the resulting pools of individuals could be exposed to low doses of radiation, to sterilize any females that might escape a sex separation process. While irradiated females are at a lower risk of initiating a population replacement event, an important consideration for insect vectors, such as mosquitoes, is that accidentally released females can transmit disease. Therefore, it would be necessary to assess the role any accidentally released irradiated females may play in disease transmission.

The cost effectiveness of rearing and separating large numbers of individuals should be evaluated before the initiation of an IIT strategy. However, the development of technologies, such as genetic sexing strains and improved mass rearing and mechanical sex separation equipment (Focks, 1980) could improve the feasibility of IIT strategies. The feasibility of genetic sexing strains has been demonstrated using Y-linked transgenes in the Mediterranean fruit fly, *Ceratitis capitata* (med fly) an important agricultural pest (Condon *et al.*, 2007) and the mosquito *Culex pipiens* (Krishnamurthy and Laven, 1976). The *Culex pipiens* genetic sexing strain was later used in an IIT field trial in Delhi, India (Curtis *et al.* 1982). Furthermore, transgenic strains of the mosquito *Anopheles stephensi* have been developed that express enhanced green fluorescent protein (EGFP) (Catteruccia *et al.*, 2005). Expression of the EGFP allows for the separation of the sexes either manually or using automated methods as early as the 3rd instar larval stage (Catteruccia *et al.*, 2005).

Recently, IIT strategies have received increasing attention. Zabalou *et al.*, (2004) established a stable *Wolbachia* infection from the cherry fruit fly, *Rhagoletis cerasi*, in the med fly. The infected strain showed high rates of CI when mated with uninfected strains. To test the concept of an IIT strategy for the med-fly, laboratory populations were set up with differing ratios of uninfected to infected males. High rates of suppression >99% (measured by egg hatch) were achieved at a ratio of 1:50 (uninfected: infected males).

An additional example is provided by the recent transfer of an incompatible *Wolbachia* type to the principle mosquito vector of lymphatic filariasis in the South Pacific, *Aedes polynesiensis*, via interspecific hybridization with the mosquito *Aedes riversi*. The result was a stably infected *A. polynesiensis* strain that was bi-directionally incompatible with naturally infected strains. Incompatible strains also showed 100% maternal inheritance of the introduced infection. To examine for male mating competitiveness and population suppression, differing ratios of wild type males and incompatible males were combined in laboratory cages. Incompatible males were equally competitive with wild type males and high rates of suppression >75% were observed in

cages with a ratio of 5:15 (compatible: incompatible males) (Brelsfoard *et al.*, 2008). Results support a novel IIT strategy for controlling *A. polynesiensis*.

Population replacement

Gene drive strategies. With advances in the ability to develop transgenic insects and to identify transgenes that block pathogen transmission, the prospect of replacing populations of insect disease vectors with genetically modified populations refractory to pathogen transmission is becoming more attractive (Olson *et al.*, 1996; Ito *et al.*, 2002; Franz *et al.*, 2006; Marrelli *et al.*, 2007). However, gene drive systems are a required component of population replacement strategies. Gene drive systems provide a mechanism for the autonomous spread of desired transgenes into the target population (Turelli and Hoffman, 1999; Aultman *et al.*, 2001; Sinkins and Gould, 2006; Adelman *et al.*, 2007; Speranca and Capurro, 2007; O'Brochta and Handler, 2008).

It has been observed in laboratory and field populations that *Wolbachia* has the ability to spread rapidly through populations as the result of the reproductive advantage afforded by CI (Turelli and Hoffman, 1991; Hoffman and Turelli, 1997; Dobson *et al.*, 2002a; Dobson, 2003; Xi *et al.*, 2005a). Thus, an anti-pathogenic transgene linked to a *Wolbachia* infection would be expected to spread into a targeted population following the seeding of the population with infected individuals. Two *Wolbachia*-based gene drive strategies have been suggested based upon this hypothesis.

The first is based upon genetic transformation of the *Wolbachia* (i.e., paratransgenesis). This strategy would involve the transformation of *Wolbachia* to express a particular anti-pathogenic product in its host. There are several advantages to using genetically transformed *Wolbachia* as a gene drive mechanism: (1) *Wolbachia* has been shown to reside in numerous tissue types in its hosts (Dobson *et al.*, 2002c; Chen *et al.*, 2005; Sinkins and Gould, 2006). Therefore, anti-pathogenic genes could be engineered to express in specific tissues to target pathogens; (2) because of its wide host range, transformed strains of *Wolbachia* may be able to be transferred to a variety of hosts (Sinkins and Gould, 2006; Hilgenboecker *et al.*, 2008); (3) compared to other strategies based on Mendelian inheritance (e.g., transposons), gene drive strategies using *Wolbachia* may only require a small seeding of transgenic individuals (Sinkins and Godfray, 2004; Sinkins and Gould, 2006). However, to date, transformation of *Wolbachia* has not been achieved, but several transformation strategies have been suggested and are currently being investigated (Iturbe-Ormaetxe *et al.*, 2007; Chauvatcharin *et al.* 2006).

A second strategy is that *Wolbachia* genes responsible for CI could be inserted into a host chromosome and driven into a population (Alphey *et al.*, 2002; Sinkins and Gould, 2006). Anti-pathogenic effector genes could be linked with the CI genes to disrupt pathogen transmission by the host insect. This strategy relies on the discovery of the *Wolbachia* genes responsible for CI. To date, genes responsible for the CI mechanism have not been identified. However, with the

publication of several *Wolbachia* genomes, the identification of genes responsible for CI is one step closer (Brownlie and O'Neill, 2005). *Wolbachia* genomes published include the wMel strain, which is found in *Drosophila melanogaster* (Wu *et al.*, 2004) and wPip, which is found in the mosquito *Culex pipiens* (Klasson *et al.*, 2008). Both strains induce CI in their hosts. CI genes could be cloned and inserted into a targeted host nuclear chromosome and spread in a manner similar to an under-dominant trait (Sinkins *et al.*, 1997; Turelli and Hoffman, 1999; Sinkins and Godfray, 2004). Models have predicted that CI genes can invade a population using this strategy. However, due to low efficiency of the nuclear CI gene drive mechanism compared to maternal inheritance, and if no fitness cost is associated with the gene, large initial releases would be needed, making the strategy impractical for most vector systems (Turelli and Hoffman, 1999; Rasgon, 2008). Furthermore, in many natural systems, *Wolbachia* transmission dynamics are not perfect, making the spread of nuclear genes difficult.

Sinkins and Godfray (2004) have suggested an alternative method that may reduce the need for large initial releases. They suggest cytoplasmic transgenes could be driven through populations if linked to a nuclear rescue gene capable of restoring fertilization in a population already infected with *Wolbachia*. Nuclear factors have been identified in *Culex* mosquitoes that appear to restore compatibility between mosquitoes infected with presumably incompatible *Wolbachia* types (Sinkins *et al.*, 2005). Nuclear factors involved in CI have also been identified in other taxa (Bordenstein and Werren, 1998; Poinot *et al.*, 1998). However, models demonstrate that the natural *Wolbachia* infection would have to show imperfect transmission and/or be less efficient at restoring incompatibility than the nuclear rescue gene. Despite these problems there are advantages to this method, with the previously demonstrated ability to transform insects (Beaty, 2000; Ito *et al.*, 2002; Catteruccia *et al.*, 2005; Marrelli *et al.*, 2007) and the imperfect transmission often observed in nature (Hoffman *et al.*, 1990; Hoffman *et al.*, 1998).

Replacement of populations with natural *Wolbachia* infections.

Several *Wolbachia* strains have been reported that are associated with a shortened adult lifespan of their hosts (Min and Benzer, 1997; Fleury *et al.*, 2000; Reynolds *et al.*, 2003). It has been suggested that life-shortening *Wolbachia* infections could be used to reduce disease transmission by insect vectors. The time required for development and/or replication of an insect-vector pathogen is known as the extrinsic incubation period. For example, this time period is approximately two weeks for both dengue (Watts *et al.*, 1987) and malaria (Kiszewski *et al.*, 2004). Therefore, life-shortening *Wolbachia* infections that impact the population age structure of the host insect, shifting it toward younger individuals, would reduce the percentage of adults that survive long enough to transmit the pathogen (Brownstein *et al.*, 2003). Models have suggested that reduced adult longevity alone is not enough to inhibit the spread of the *Wolbachia* infection

(Brownstein *et al.*, 2003; Rasgon *et al.*, 2003; Rasgon, 2008).

While old females are responsible for the majority of disease transmission, they contribute only a minority to egg production in a population. However, if there were a significant reduction of the reproductive fitness of its host, there would be a decrease in the rate of spread of *Wolbachia* and an increase in the numbers of required initial releases. Thus, there is the need to investigate host fitness parameters other than adult longevity. Furthermore, since it is currently not possible to 'design' *Wolbachia* for the optimal impact on host longevity, this strategy is dependent upon identifying naturally occurring *Wolbachia* infections with the appropriate characteristics (i.e., sufficient life shortening to impact disease transmission, but the absence of host fitness impacts that slow/negate *Wolbachia* spread). There is the need for additional research into the potential evolutionary responses of host, pathogen, and *Wolbachia* to this type of strategy.

Recently, a life-shortening infection (i.e., *wMelPop*) was introduced into the dengue vector *Aedes aegypti* (McMeniman *et al.*, 2009). Characterization of *wMelPop* infected *A. aegypti* strains showed high rates of maternal inheritance, complete CI, and a reduction of lifespan by half compared to uninfected controls. The results encourage an examination of fitness effects of *wMelPop* infections in *A. aegypti* in more field-like conditions. However, before the release of fertile *wMelPop* infected individuals into the wild, the risk of unintentional *Wolbachia* spread into non-target populations needs to be evaluated, along with the social, ethical, and regulatory issues related to releases.

It has been suggested that natural *Wolbachia* infections may confer resistance to virus infections in their host. In a recent study, a naturally *wMel* infected strain of *Drosophila melanogaster* was shown to be more resistant to RNA virus infections (i.e., *Drosophila C virus* (DCV), Nora virus, and Flock House virus (FHV)) (Teixeira *et al.*, 2008). *Wolbachia* infected *Drosophila* strains infected with DCV, Nora virus, or FHV showed increased survivorship when compared to strains without a *wMel* infection. Furthermore, *wMel* infected strains had lower titers of DCV and the Nora virus, but little effect on the titers of FHV were observed when compared to strains without a *wMel* infection. In a similar study, DCV, FHV, or Cricket Paralysis virus infected strains of *D. melanogaster* with *wMel* infections were shown to have an increase in survivorship when compared to strains without a *wMel* infection (Hedges *et al.*, 2008). Hypotheses suggested by Teixeira *et al.*, (2008) that address the observed effect of *Wolbachia* on virus infections include: (1) *Wolbachia* induces a pre-activation of the insect immune system, thereby increasing the resistance to a viral infection; (2) *Wolbachia* induces an inhibitory effect on the insect immune system, increasing the survivorship of the host; (3) *Wolbachia* inhibits virus induced cell apoptosis, either autonomously or systematically. Additional studies addressing the aforementioned hypotheses may help determine the exact mechanism by which *Wolbachia* is influencing virus infections in their insect hosts. From an applied perspective, populations could be replaced

with individuals harboring natural *Wolbachia* infections that confer resistance to viruses. Ultimately, these infections could aid in the control of human and arthropod viral diseases. For example, *Wolbachia* could provide resistance to arboviruses in insect disease vectors such as the dengue vector *A. aegypti*. In addition, *Wolbachia* could provide resistance to viruses that impact the survivorship of insects important for agricultural production (e.g., honeybees) (Teixeira *et al.*, 2008). However, additional study is needed to better understand the mechanisms of interaction between *Wolbachia* and pathogen, whether previously reported examples represent specialized or general examples and the applicability to applied needs.

Parthenogenesis

An additional example of biological control in agriculture relies upon wasps that parasitize specific pest species. Female wasps oviposit in their host and their young parasitize, and kill the host. Parthenogenesis-inducing *Wolbachia* may provide a benefit to biological control strategies that use parasitic wasps. If females reproduced parthenogenetically all offspring would be female. As stated by Stouthamer *et al.*, (1999) a potential advantage of a parthenogenesis inducing *Wolbachia* infected wasp strain is that infected wasps may have a higher population growth rate due to the reproductive advantage afforded by *Wolbachia*. As a result, infected wasps may be able to suppress the target pest population at a lower level, and the cost of production per female would be less. However, there is an expense to this type of reproduction. Due to lack of sexual reproduction and crossing over, there is a risk of accumulation of deleterious alleles and loss of genetic variation. If the accumulation of deleterious alleles could be avoided, biological control programs may benefit from parthenogenesis inducing *Wolbachia* infections.

CONCLUSIONS AND FUTURE PERSPECTIVE

As reviewed above, the prospect of using *Wolbachia* to control insects shows considerable promise. To date, a major obstacle for *Wolbachia*-based strategies has been the ability to transfer *Wolbachia* infections to novel hosts. However, this obstacle has been overcome by recently demonstrated successful transfers into novel hosts, using microinjection of cytoplasm into embryos (Zabalou *et al.*, 2004; Xi *et al.*, 2005a; Xi *et al.*, 2005b; McMeniman *et al.*, 2009). The mosquito *A. aegypti*, an important vector of dengue virus and yellow fever virus, was recently infected with a *Wolbachia* type from *Aedes albopictus* (Xi *et al.*, 2005a). The demonstrated successful transfections are encouraging for the likelihood of transfer to major economic pests and disease vectors lacking naturally occurring *Wolbachia* infec-

tions. Successful transfers promote the development of novel control programs using the *Wolbachia*-based strategies described in this review.

Further work is needed to define the underlying molecular mechanisms of *Wolbachia* induced reproductive modifications, particularly CI. Genome wide analyses using available *Wolbachia* genome sequences should help in the development of a transgenic system that will help define the mechanism(s) of CI. Understanding the mechanism of CI is important from both a basic scientific standpoint and to facilitate *Wolbachia* based control strategies, specifically gene drive strategies.

The release of non-transgenic incompatible males may be a logical segue before the release of fertile transgenic or paratransgenic insects, which may yield improved efficacy and/or cost. Furthermore, public acceptance of transgenic insect releases may be increased via an approach that is integrated with *Wolbachia*-induce CI. Specifically, if released transgenic males are cytoplasmically incompatible with the targeted population, the released transgene has a reduced probability of establishing in the field. This approach would allow for the examination of the dynamics of a transgene in a population with less risk.

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