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Engineering the genomes of wild insect populations: challenges, and opportunities provided by synthetic *Medea* selfish genetic elements

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Abstract

Advances in insect transgenesis and our knowledge of insect physiology and genomics are making it possible to create transgenic populations of beneficial or pest insects that express novel traits. There are contexts in which we may want the transgenes responsible for these traits to spread so that all individuals within a wild population carry them, a process known as population replacement. Transgenes of interest are unlikely to confer an overall fitness benefit on those who carry them. Therefore, an essential component of any population replacement strategy is the presence of a drive mechanism that will ensure the spread of linked transgenes. We discuss contexts in which population replacement might be desirable and the requirements a drive system must satisfy to be both effective and safe. We then describe the creation of synthetic *Medea* elements, the first selfish genetic elements synthesized de novo, with the capability of driving population replacement, in this case in *Drosophila*. The strategy used to create *Drosophila Medea* is applicable to a number of other insect species and the *Medea* system satisfies key requirements for scientific and social acceptance. Finally, we highlight several challenges to implementing population replacement in the wild.

Keywords

Selfish genetic element; mosquito; malaria; dengue; pest; *Medea*; population replacement; maternal

Insects play important roles as predators, prey, recyclers, pollinators, hosts, parasitoids, and as sources of economically important products, while pest insects destroy crops and serve as vectors for plant, animal and human disease. The genomes of many insects of economic or

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medical interest have been, or will be sequenced in the near future. This, coupled with knowledge generated through transcriptional profiling and the phenotypic characterization of the consequences of gain- or loss-of-function of specific genes through transgene expression or RNAi, will facilitate the identification of mechanisms by which insects carry out specific processes. Several advances in transgenesis technology enable this work. In particular, artificial zinc finger nucleases provide a universal technology for inserting modifications at any desired position in the genomes of organisms whose germplasm can be accessed (Beumer et al., 2008), and site-specific integrases can be used to repeatedly create modifications at specific positions in genomes into which target sites have been inserted (Groth et al., 2004; Venken et al., 2006; Nimmo et al., 2006; Schetelig et al., 2009). These developments place us at the beginning of an era in which we can realistically propose to create transgenic insects with targeted modifications in specific traits of economic and medical interest.

Genetic population management

One use of these new technologies is genetic population management, the deployment of transgenic insects to suppress or modify wild populations. Population suppression has the goal of removing, at least temporarily, a pest population (reviewed in Whitten and Foster, 1975; Dyck, 2005; Gould and Schliekelman, 2004; Catteruccia et al., 2009). In contrast, the goal of population replacement, the topic of this review, is to leave the insect population intact in its ecological niche, but to force those who occupy the niche to carry a trait of interest (reviewed in Whitten and Foster, 1975; Braig and Yan, 2001; Sinkins and Gould, 2006). Traits of interest for beneficial insects might include resistance to diseases, pests, or insecticides, or the acquisition of other adaptations to facilitate their beneficial functions or expand their range. In the case of pest insects, behaviors that make them less harmful to crops, or that make them unable to transmit disease to plants, animals or humans would be desirable. As an example of the latter, and the focus of this review, population replacement has long been seen as an interesting strategy for preventing mosquito-borne diseases such as dengue and malaria (Braig and Yan, 2001; Sinkins and Gould, 2006).

Current methods of preventing these diseases, transmitted through the bite of an infected female mosquito, involve the use of antimalarial drugs if plasmodium infection occurs (there are no similar anti-dengue drugs), and vector control through the removal of breeding sites, the use of biological control agents, chemicals that act as larvicides or adulticides, and bed nets (Beier et al., 2008; Greenwood et al., 2008; Ramirez et al., 2009. While each of these approaches can succeed, they are also costly and require continuous effort. The appearance of plasmodium resistance to drugs, and mosquito resistance to insecticides further limits the efficacy of these two approaches. The release of sterile males provides an approach to vector reduction that does not involve the use of chemicals or modification of the environment, but it still requires continuous investment since it removes the vector while leaving its niche intact, an ecologically unstable situation that allows immigrants of the same species – or perhaps a different, worse species – to take up residence. In addition, achievement of the desired goal – eradication of an indigenous species (though not an invasive species) - may have untoward ecosystem effects.

In contrast, population replacement does not result in gross changes to the environment, use chemicals, or require any treatment of, or contact with, the human population. The changes brought about are highly specific, and thus likely to have minimal ecological impact since the ecological niche remains filled with populations of the same species, albeit with individuals that have subtle modifications to specific traits. Because population replacement takes advantage of the insect's tendency to seek out conspecifics, it is well suited to targeting populations in hard to reach places, and sites such as complex urban centers and high

diversity ecologies where gross environment modification – such as drying it out - is not desirable, or is impractical. Perhaps most importantly, population replacement is self-sustaining, limiting the need for reapplication and resulting in prolonged protection for all people living in the covered area, perhaps leading to permanent or semi-permanent loss of disease vector status.

Population replacement and disease prevention

There are several ways in which population replacement can be used to prevent disease. The first focuses on engineering insects to kill or prevent the replication or dissemination of specific pathogens, or to die in response to becoming infected, thereby blocking transmission of a specific disease (Terenius et al., 2008). A second, related approach seeks to truncate the mosquitoes' lifespan, forcing them to engage in a "live a little bit faster, die a little bit younger" life history that should result in only modest decreases in vector fitness - because late reproduction is rare to begin with - while perhaps having a major impact on the ability of the insect to transmit the pathogen – because mosquitoes only become infectious following a lengthy period required for development and dissemination of infectious forms to the salivary gland (Cook et al., 2008; Read et al., 2009). This approach has the appeal that it could lead to reduction in transmission of multiple pathogens, and it requires limited specific knowledge of the pathogen being targeted (only the kinetics of its lifecycle in the insect).

Population replacement requires a gene drive mechanism

Each of these strategies begs the question that forms the topic of this article: how are we to force the spread of specific traits (more specifically the transgenes that underlie these traits) through wild insect populations? Evolution achieves this goal in large populations when specific alleles encoding the traits in question confer a fitness benefit (enhanced rates of transmission through the generations of genotypes carrying these alleles versus other alleles at the same locus) to those who carry them. However, there is no reason to believe that forced alterations in life history that decrease longevity come without a cost. In addition, enhancement of immune function in insects is usually costly, requiring tradeoffs with other life history traits such as longevity and fecundity that result in an overall fitness decrease (Schmid-Hempel, 2005; Tripet et al., 2008). Even if enhanced immunity came with little cost (or perhaps even a benefit in the context of infection; see Marrelli et al., 2007), there is little reason to believe that we have the ability to create genotypes that are fitter than wildtype in both infected (the minority) and uninfected (the majority) individuals, such that transgenes would quickly spread through natural selection (Lambrechts et al., 2008). This is particularly unlikely if, as is likely to be the case, mosquitoes are engineered to express multiple genes that promote disease resistance, acting through distinct mechanisms so as to prevent the appearance of pathogen escape mutants. Thus, it is generally thought that population replacement will require the utilization of genetic strategies to force, or drive, transgenes through wild populations. What would a successful drive system look like? Answers to this question are constrained by features a drive mechanism should have in order to be effective, specific, and controllable. Below, we elaborate briefly on each of these points, some of which have been discussed previously (Braig and Yan 2001; Christophides 2005; James 2005).

Requirements for a functional and safe drive mechanism

The drive mechanism

First, the drive mechanism, the vehicle that will carry a linked cargo - genes that block pathogen transmission or truncate lifespan (generically referred to as effector genes) - into a

population, must be understood in enough detail that it can be engineered and genes for disease resistance inextricably linked to it. The drive mechanism must also be fast, capable of driving population replacement in years, not decades, and powerful, able to spread genes whose presence is associated with a fitness cost. Drive should also lead to the elimination of wild-type individuals (potential disease transmitters) from the population. This is important because modeling and epidemiological studies indicate that in areas with high levels of disease transmission even a small number of disease-transmitting insects (<1%) can sustain significant levels of disease transmission (Anderson and May 1991; Boete and Koella 2002). Related to the above points, the drive mechanism should also be able to establish and maintain population replacement in the face of significant levels of wildtype immigration. Finally, maintaining disease refractoriness in the face of standing pathogen genetic diversity and mutation is likely to require multiple effectors, working through different mechanisms. In addition, genes associated with drive function and/or associated cargo will need to be present in multiple copies so as to limit the rate at which non-functional elements are created through mutation, thereby facilitating a long functional lifetime in the wild. In short, the DNA encoding the drive mechanism must be able to support the spread of multiple genes (10s of kilobases of DNA), and permit the expression of these genes at the appropriate time and place.

Longevity

Genes for disease refractoriness and components that make up the drive mechanism must remain tightly linked so that opportunities for the spread of drive vehicles that have lost cargo genes are minimized, particularly if the presence of an empty drive vehicle in the population hinders the spread of those carrying intact cargo genes (see Marshall, (2008) for an example). The functional lifetime of genes comprising the drive mechanism and cargo will be limited by the rate at which these components mutate to inactivity, particularly if inactivity is associated with a decreased fitness cost (a fitness gain with respect to a population composed of individuals carrying intact elements). Chromosomal genes that replicate only once per cell cycle are replicated with high fidelity, undergoing mutation at frequencies of about 8×10^{-9}/bp/generation (Haag-Liautard et al., 2007). In addition (and contrasted with the replication of nonessential genes in transposons (Carareto et al., 1997; Braig and Yan, 2001), there are no obvious costs associated with the presence of modest increases (10s of kilobases of DNA) in eukaryotic nuclear genome size. Thus, all other things being equal, a successful drive mechanism will replicate only using the chromosomal DNA replication machinery.

Specificity

Population replacement has the essential feature that it brings new genes and gene combinations, in the form of a subtly modified version of an indigenous species, into a pre-existing environment. One concern with population replacement (and more generally with respect to all transgenic organisms) has to do with the potential for gene flow from the target species into another species, and the consequences that this may have for the ecosystem as a whole. Gene flow can occur through two distinct mechanisms: matings between members of closely related species (whole genome mixing associated with incomplete reproductive isolation), and horizontal gene transfer (direct movement of a specific piece of DNA into the germline of a single individual of a species that may be quite unrelated to the donor species). Gene flow through partial reproductive isolation is presumably relatively common, though the details will depend on the species involved. In contrast, gene flow through horizontal transfer of snippets of DNA from a donor species into the germline of a recipient species, through a process that does not involve mating, is exceedingly rare in plants and animals, though not in prokaryotes (Keeling, 2009; Keese, 2008). That said, the case of the P transposable element (as well as recently documented cases of horizontal transfer and spread

of other transposons (Loreto et al., 2008)), which entered wild *Drosophila melanogaster* from *Drosophila willistoni* in the first half of the 20th century, and became ubiquitous in the wild within 30 years, shows that if horizontal transfer is coupled with a strong drive mechanism (in this case transposition), rare transfer events can have dramatic consequences for an entire species (Kidwell, 1983; Daniels et al., 1990). Therefore, while DNA transfer across species boundaries can never be prevented, it is important that the drive mechanism not be able to promote the spread of linked transgenes in non-target species. This can be achieved in two ways. First, some drive mechanisms only function as such when a significant threshold introduction frequency has been surpassed (see below), making it unlikely that rare horizontal transfers, occurring in single individuals, will lead to drive. Alternatively, the drive mechanism may be non-functional in non-target species, or be a very weak driver at low frequency, so that if horizontal transfer does occur, these genes do not have a strong transmission advantage that would allow them to spread. In the absence of selfish behavior (or even in the presence of an element that shows very weak drive at low frequency; see below), genes that enter a large population in small numbers of individuals, particularly if they do not confer a fitness benefit to carriers, are likely to be rapidly lost from the population (Hartl and Clark 1997; Marshall, 2009). That said, population structure and competition within family groups (discussed further below) can lower barriers to spread for elements with high thresholds and/or weak, frequency-dependent drive. Thus, all other things being equal, the use of drive elements that are species-specific is preferred.

Control

The movements of transgenic plants and animals are highly regulated in ways that are complex, and that differ dramatically from one state, country, or continent to another (Knols et al., 2007). Because of this, the fact that released insects do not respect these regulations, and our general lack of knowledge as to the fate of transgenes in wild insect populations, it is important that the drive mechanisms used initially for population replacement, and perhaps into the foreseeable future, not be so strong that very small numbers of individuals, perhaps released accidentally during testing, or transported through low level migration or hitchhiking to other environments following release into the field, be able to drive population replacement. In other words, there should be a threshold frequency, below which the element will not spread. This provides a mechanism for preventing movement of the genes mediating drive and disease refractoriness beyond regions in which large numbers of transgenic individuals are introduced, and that are linked by significant levels of migration. The existence of a threshold also provides, in principal (and with caveats related to considerations population structure and competition within the family), a method for eliminating transgenes from the population through dilution below the threshold frequency required for spread.

Finally, it is important that mechanisms be available to undo or modify specific transgenic characteristics of the replaced population. For example, it is possible, though probably very unlikely, that the presence of specific transgenes will facilitate the emergence of new pathogens and forms of disease, or result in ecological effects on target or non-target species. In this case one wants to be able to remove these genes from the population, perhaps through dilution below a threshold frequency required for spread or through more active measures (see below). A much more likely scenario is that, since genes encoding drive components and resistance to pathogen transmission (probably) do not provide a fitness benefit to the organisms in whose genomes they reside, they will eventually undergo mutational decay to inactivity and be lost from the population. Pre-existing diversity and mutation within the pathogen population may also contribute to the emergence of pathogen populations resistant to first generation disease resistance effectors. In these situations one wants to be able to carry out second or third rounds of population replacement with new and

improved elements, carrying perhaps new genes targeting a pathogen that has evolved resistance to first generation effectors. Ideally, elements used for second or third rounds of population replacement would have the effect of driving transgenes from previous generations out of the population at the same time as the current generation element was being driven in, thereby making the components from these earlier elements available for future use and minimizing the "transgenic footprint" within the population.

To summarize, a good drive mechanism should be associated with a low mutation rate, which can most easily be achieved if it is chromosomally located and replicates once per cell cycle, with the nuclear genome. It should also be able to support the expression of multiple genes, include methods for tightly linking drive and effector genes, only show drive behavior in the species of interest, require a significant threshold introduction frequency in order for drive to occur, and allow for multiple rounds of population replacement.

Gene drive mechanisms considered for population replacement

Selfish genetic elements have increased rates of transmission relative to the rest of the genome of the individual in which they appear, resulting in their spread within a population even if their presence is selectively neutral or associated with fitness costs to the organisms in which they reside (Burt and Trivers, 2006). Because of this, many people have proposed bringing about population replacement by linking genes for disease refractoriness with a selfish genetic element, the hope being that these genes will hitchhike with the selfish genetic element as it drives itself through the native insect population. A number of naturally occurring selfish genetic elements have been considered as vehicles for drive. These include transposons, male post-meiotic segregation distorters, homing endonucleases, B-chromosomes, *Medea* elements, and the intracellular bacterial symbiont Wolbachia (reviewed in (Braig and Yan, 2001; Burt and Trivers, 2006; Sinkins and Gould, 2006). Another set of approaches to bringing about population replacement involves the use of insects carrying genes of interest in association with engineered chromosomes: translocations, compound chromosomes, or pairs of unlinked lethal genes, each of which is associated with a repressor of the lethality induced by expression of the other lethal gene (a system known as engineered underdominance) (reviewed in (Gould and Schliekelman, 2004; Sinkins and Gould, 2006). In each of these systems, matings between strains carrying engineered chromosomes and wildtype produce progeny that are less fit (usually dead, or giving rise to fewer offspring) than either parent. Populations containing both genotypes are unstable, with one genotype or the other being lost from the population, depending primarily on their relative starting frequencies. When insects carrying the engineered chromosomes are present in excess (the exact ratios with respect to wildtype being determined by the specific system involved), engineered chromosomes spread at the expense of their wildtype counterparts. This occurs because wildtype individuals (being less abundant) are more likely to mate with individuals carrying the engineered chromosomes (producing dead progeny or progeny who have fewer viable offspring) than with each other (producing viable progeny), while the converse is true for individuals carrying the engineered chromosomes.

Engineered underdominance has not yet been implemented. Strains bearing compound chromosomes or translocations were developed using pre-transgenic technology (X-ray mutagenesis), and have been shown to drive population replacement under some conditions (reviewed in Foster et al., 1972; Asman et al., 1981; Baker, 1984; Gould and Schliekelman, 2004). However, the low fitness associated with many of these strains, and the difficulty in bringing disease refractory genotypes into the appropriate genetic backgrounds, kept these approaches from further development. An important feature of each of these approaches is that drive only occurs when the introduction frequency surpasses a very significant threshold, making them interesting candidates for use in field trials, where the ability to limit

the spread of transgenes and/or remove them from the population through dilution is a high priority. Given that we can now create targeted genome modifications, including translocations, inversions, and compound chromosomes, in any organism whose germline can be accessed (e.g. Golic and Golic, 1996; Egli et al., 2004), these older approaches bear revisiting.

Medea as a drive mechanism for population replacement

As an alternative approach to the development of a gene drive mechanism that meets the above criteria, we set out to design from the ground up a selfish genetic element. Our idea was that by creating a chromosomal selfish genetic element from purely synthetic components, using well-understood molecular, genetic, and developmental mechanisms, we would completely understand the drive mechanism, and thereby be able to exercise the maximum amount of control over selfish element behavior, and understand - and anticipate - ways in which it might evolve or fail. As a natural-world template for our work, we focused on *Medea* (for Maternal-effect, dominant embryonic arrest), one of the least studied naturally occurring selfish genetic elements.

Medea elements are members of a class of selfish genetic elements that enhance their transmission by causing the death of offspring that fail to inherit the element (Burt and Trivers, 2006). Naturally occurring *Medea* elements were first identified (and named as such) in the flour beetle *Tribolium castaneum*, through analysis of crosses between geographically isolated strains. They sit at a fixed genomic position, and have the feature that when present in females only progeny that inherit the element-bearing chromosome from either the maternal and/or paternal genome survive (Figure 1A). In contrast, *Medea*-bearing males give rise to wildtype and *Medea*-bearing progeny with equal frequency when mated to wildtype females (Beeman et al., 1992). One *Tribolium Medea, Medea*^{M1}, has been mapped and is associated with a composite Tc1 transposon insertion that includes a number of genes (Lorenzen et al., 2008). How this insertion and/or the flanking genes bring about *Medea* behavior is unknown, but genetic analysis suggests a model - to be returned to below - in which *Medea* consists of two tightly linked loci: one that encodes a toxin inherited by all progeny of *Medea*-bearing mothers, and a second that encodes an antidote active in the zygote (Beeman et al., 1992; Beeman and Friesen, 1999). A locus with these characteristics is predicted to show *Medea* genetic behavior (Figure 1B). Loci (which may involve significant chromosomal regions) with genetic behavior similar to that of *Tribolium Medea* have been described in mice, but nothing is known about their molecular nature (Hurst 1993; Peters and Barker 1993; Weichenhan *et al.* 1996; (Weichenhan et al. 1998).

Population genetics of *Medea*

Medea spreads by causing the death of alternative non-*Medea*-bearing homologous chromosomes (hereafter referred to as non-*Medea* alleles), thereby causing a relative increase in the population frequency of the *Medea*-bearing homolog (Beeman et al., 1992; Wade and Beeman, 1994). *Medea*-bearing individuals and alleles usually (for exceptions see below) experience no direct benefit from this killing, but non-*Medea* alleles experience *Medea*-dependent death (a fitness loss) in each generation that is dependent on the *Medea* allele frequency. Therefore, for a *Medea* with no fitness cost (and in deterministic population models), the *Medea* allele spreads to fixation regardless of the introduction frequency because its fitness is always greater than that of the average non-*Medea* allele. However, the rate of *Medea* spread depends dramatically on the introduction frequency, illustrated in Figure 2. In particular, when *Medea* is rare, drive is so weak (on the order of p^2 [with p being the *Medea* allele frequency]; (Wade and Beeman 1994) that the fate of *Medea*

approximates that of a new mutation, which is usually lost from the population even if beneficial (Hartl and Clark, 1997; Marshall, 2009).

The ability of a *Medea* element with no fitness cost to spread when introduced at very low frequency violates a condition for acceptability (that spread requires introduction above a threshold). However, as discussed above, it is likely that the expression of linked genes conferring disease resistance will result in a fitness cost to *Medea* carriers. Costs may also arise through tight linkage between *Medea* and a nearby deleterious allele, insertion-dependent effects on nearby genes, or as a result of gene expression associated with *Medea*'s mechanism of action (discussed below). If these costs are dominant (observed in heterozygotes; and fitness costs associated with expression of genes that prevent disease transmission are likely to be dominant), then *Medea* can only spread when introduced above a critical introduction frequency determined by the fitness cost (Hastings, 1994; Wade and Beeman, 1994; Smith, 1998; Chen et al. 2007). The relationship between fitness cost, introduction frequency, and the fate of *Medea* is illustrated in Figure 2, in which the lower bound of the curve with the black border represents the minimum number of homozygous *Medea*-bearing males that need to be released (expressed as the ratio of homozygous *Medea*/wildtype males present in the population) in order for *Medea* elements with different levels of a multiplicative fitness cost (fitness of homozygotes = heterozygote fitness^2) to spread. This curve describes an unstable equilibrium, the critical male introduction frequency (CMIF), at which *Medea*-dependent killing of non-*Medea* alleles is balanced by natural selection loss of fitness-compromised *Medea* alleles. If the frequency of *Medea* is below the CMIF, the *Medea*-dependent cost to the non-*Medea* allele is less than the cost experienced by the *Medea* allele (the non-*Medea* allele has a higher average fitness than the *Medea* allele), and *Medea* is driven out of the population. In contrast, if the *Medea* allele frequency is just above the CMIF, the non-*Medea* allele has a lower fitness (due to increased *Medea*-dependent killing) than the *Medea* allele, and the frequency of *Medea* increases over the generations. Local population structure and stochastic effects (drift, founder effects) will soften this boundary such that *Medea* will sometimes spread when introduced below the CMIF, and sometimes fail to spread when introduced above it, as recently modeled for the case of the *Wolbachia* drive system (Jansen *et al.* 2008). That said, the larger point remains: *Medea* elements with a fitness cost are unlikely to spread if introduced at frequencies significantly below the threshold required for drive. Recent modeling efforts, carried out specifically to address the fate of small numbers of *Medea*-bearing individuals released accidentally into a larger wildtype population, point to a similar conclusion (Marshall 2009).

The above points notwithstanding, it is important to introduce one caveat. These models assume that all progeny in a population compete with each other for resources. However, when the progeny of a *Medea*-bearing mother compete primarily with each other (and not with unrelated progeny) for resources, a context known as family-level soft selection (Wade, 1985; Kelly, 1992), the death of non-*Medea* offspring within the family of a *Medea*-bearing mother frees limited resources (food, protection, etc) for sibling *Medea*-bearing progeny, providing them with a real fitness benefit that increases their likelihood of survival. This fitness benefit can counterbalance a *Medea*-dependent fitness cost, allowing *Medea* to spread at lower introduction frequencies (Wade and Beeman, 1994; Smith, 1998). There is no evidence that mosquitoes show family-level selection. That said, we are not aware that the topic has been explored in any detail. A number of mosquitoes such as *Aedes aegypti*, an important vector of dengue fever, breed in small containers that are likely to often be resource-limited for larval growth (Clements, 1999). Such environments provide an opportunity for family-level selection, particularly if only one or a small number of females lay eggs in each container. Determining if family level selection is present and promotes the spread of *Medea* in mosquitoes or any other insect will require analysis of female behavior with respect to oviposition site choice, the number of females who use a particular site, and

the ecology of these sites with respect to resources available for larval growth, competition and predation.

What does a population that has undergone replacement with *Medea* look like? When males carrying an autosomal *Medea* with a fitness cost are introduced above the CMIR, *Medea* increases in frequency through *Medea*-dependent killing of non-*Medea* alleles to a stable internal equilibrium value, at which this killing is balanced by the natural selection-dependent loss of fitness-compromised *Medea* alleles. Therefore, these populations contain wildtype alleles (for an exception see the case of X-linkage below). Importantly however, whenever *Medea* spreads, and heterozygous *Medea* females cause the death of *all* their non-*Medea* progeny, non-*Medea* individuals are eliminated from the population, satisfying a key drive mechanism requirement (C.M. Ward and J.T. Su, unpublished). Non-*Medea* individuals disappear because in each generation the fraction of non-*Medea* females, which are required for the production of non-*Medea* offspring, is ratcheted into a smaller and smaller pool through matings between their wildtype mothers and *Medea*-bearing fathers, producing *Medea*-bearing female offspring who can only give rise to *Medea*-bearing progeny. As the frequency of *Medea* increases, so does the frequency of these matings (as compared to the frequency of matings between wildtype females and wildtype males). Non-*Medea* females eventually disappear when the last non-*Medea* female mates with a homozygous *Medea* male, producing only heterozygous *Medea* offspring. The rate at which *Medea* spreads and non-*Medea* individuals are eliminated from the population is a function of fitness cost and introduction frequency. This is illustrated in Figure 2, in which the regions between the colored lines indicate the number of generations required to bring the non-*Medea* genotype frequency below 1%, for different levels of a *Medea* fitness cost and introduction frequencies.

In contrast to the case of an autosomal *Medea*, when a *Medea* element is located on the X chromosome, in species in which females are XX and males X/Y, the non-*Medea* allele is driven to extinction, even when *Medea* carries a fitness cost (C. M. Ward and J.T. Su, unpublished). This occurs because in each generation X-linked non-*Medea* alleles present in heterozygous *Medea* female parents have a 50% probability of ending up in a male progeny, who is doomed to death because males cannot be rescued by a paternally-derived *Medea* allele. Thus, the non-*Medea* X allele experiences a minimum 50% probability of death in the next generation each time it finds itself in a heterozygous *Medea* female, and the probability of finding itself in a heterozygous *Medea* female (as opposed to a non-*Medea* female) increases as the frequency of *Medea* increases. The thresholds for introduction of an X-linked *Medea* are a bit higher, and the times required for replacement a bit longer than for an autosomal element with an equivalent fitness cost. However, there are several reasons this may be a price worth paying. First, X-linkage guarantees that following replacement each individual carries two copies of all effectors present in the *Medea*, which should extend the functional lifetime of the element in the population. X-linkage also creates a context in which second generation *Medea* elements can be used to drive first generation elements out of a population at the same time as they are driving themselves in (see below), providing a point of control over the fate of a *Medea* element in the population. In summary, when *Medea* spreads the wildtype genotype, and in some cases the wildtype allele, is eliminated from the population. *Medea* spreads quickly when introduced at high frequency, but spreads slowly, or drops out of the population when significant fitness costs are present and the introduction frequency is low. These features represent a compromise between invasiveness and control.

Finally, it is worth commenting on the introduction frequencies required, which represent a significant fraction of the total male population for *Medea* elements with modest (~5-10%) fitness costs (Figure 2). Releases of this magnitude are not unreasonable. Wild populations

of *Aedes aegypti* and some *Anopheles* species range from 10,000-20,000 adults per village (Scott et al., 2000; Taylor et al., 2001), which is small compared to the numbers associated with classical sterile male release in other insects; 68,000 per week in the case of the screw worm fly, and ~10^9 in the case of ongoing Mediterranean fruit fly suppression programs (Dyck, 2005). With respect to mosquitoes, weekly factory production of 1,000,000 *Aedes aegypti* could be achieved routinely in the 1960s (Knipling et al., 1968). Large numbers of *Anopheles* males have also been produced in factory environments using mid-twentieth century technologies (Asman et al., 1981) (Dyck, 2005).

Strategies for building synthetic *Medea* elements

How can we build synthetic *Medea* elements? Naturally-occurring *Medea* elements probably consist of two tightly linked functional units (each of which may consist of one or more genes); the first creates a toxin (or pre-toxic state) that *Medea*-bearing mothers provide their oocytes, and that results in embryonic arrest if left unopposed; the second creates a zygotically-expressed antidote that neutralizes or eliminates this toxin or toxic state. There are several general ways in which these principals might be used by Nature or man to build *Medea* elements. In the first, maternal expression creates a gain-of-function (a toxic protein or RNA) that must be neutralized in the zygote (Figure 1B). In the second, maternal expression creates a loss-of-function (loss of a maternally-expressed gene product essential for embryogenesis; the pre-toxic state) that must be restored in the zygote (Figure 3).

The gain-of-function approach requires that one be able to maternally express a molecule at levels high enough to guarantee embryonic lethality in progeny that fail to inherit *Medea*, but not so high that one copy of the antidote (in a heterozygous progeny) is unable to neutralize toxin generated by two copies of a maternal *Medea*. And of course, the toxin must also not kill oocytes or early embryos prior to the onset of zygotic transcription, something that can in principal be achieved either through translational suppression during oogenesis and early embryogenesis, or through the use of a protein (or RNA) whose expression is toxic to embryos after the onset of zygotic transcription, but not oocytes or early embryos. The antidote can be anything that neutralizes the toxin, promotes its degradation or sequesters it away from targets. The gain-of-function approach is challenging to engineer because it requires careful titration of toxin-antidote levels, and perhaps translational regulation. Gain-of-function toxins such as proteins can also easily undergo mutation to inactivity through disruption of the coding sequence. Loss of toxin activity creates antidote-only *Medea* alleles, which are insensitive to *Medea*-dependent killing. These $Medea^{ins}$ alleles will spread if they have a fitness advantage with respect to intact elements, leading to the reappearance of wildtype, disease transmitting, progeny (Smith, 1998). Multiple copies of the toxin gene can provide a level of redundancy that will decrease the frequency of elements that fail to express a functional toxin in the maternal germline, but this strategy then requires that zygotic antidote levels be adjusted upwards as well. The major advantage of the gain of function approach is that one needs only minimal knowledge of the developmental biology of the insect under study, primarily information relating to promoters and UTRs that provide control over RNA and protein expression. This makes the system potentially transportable from one insect species to another. That said, protein toxins may kill in other species as well, raising the possibility that (if the promoters are also functional) the element could drive in a non-target species.

Because *Medea* elements synthesized using a protein-based, gain-of-function strategy seemed likely to break down rapidly in response to mutation, and to be difficult to engineer, we focused our efforts on creating synthetic *Medea* elements using the loss-of-function approach. We first generated a *Medea* known as $Medea^{myd88}$ (Chen et al., 2007). A modified version of the maternal-specific bicoid promoter was used to drive the expression a

transcript encoding two synthetic microRNAs designed to silence the expression of myd88, a maternally-expressed component of the Toll pathway required for embryo dorso-ventral pattern formation (Charatsi et al., 2003; Kambris et al., 2003). Maternal expression of these small RNAs created the pre-toxic state, a loss of maternal myd88, which if left unopposed led to the death of all embryos. Zygotic rescue in this case was mediated by expression of an adjacent gene, encoding a microRNA-insensitive version of the myd88 transcript lacking target sites present in the endogenous transcript, driven by a transient early zygotic-specific promoter from the *bottleneck* (*bnk*) gene (Schejter and Wieschaus, 1993). Bnk-driven expression of this transgene occurs early enough in development that embryos inheriting it, but lacking maternal myd88, developed normally. These features, complete maternal-effect lethality, coupled with complete zygotic rescue, in the absence of other obvious fitness costs, define *Medea* behavior, and predicted that this element should drive population replacement, which was in fact observed: $Medea^{Myd88}$, introduced into a wildtype population at a 1:1 homozygous *Medea* male/wildtype male ratio, spread such that after 10-12 generations all individuals in the population carried at least one copy of *Medea*. Elements configured such that the maternaly-expressed microRNAs were located in an intron of the antidote also showed *Medea*-like behavior (Chen et al., 2007). This configuration is useful going forward because it prevents chromosome breakage and rejoining from creating *Medea* elements that lack the effector ($Medea^{\Delta eff}$), or antidote-only elements ($Medea^{ins}$), each of which can lead to the appearance of wildtype individuals (Smith, 1998) (Figure 4). More recently we have generated *Medea* elements that work through maternal silencing and zygotic re-expression of o-fucosyltransferase-1 (*o-fut1*; also known as *neurotic*) (Sasamura et al., 2003), a fucosyltransferase required for Notch signaling in some contexts, or discontinuous actin hexagons (*dah*) (Zhang et al., 1996), which is required for cellular blastoderm formation (C.H. Chen, H. Huang and B.A. Hay, unpublished). These observations suggest that it should be possible to create *Medea* elements through manipulation of a variety of signaling pathways. This last point is particularly important in the context of maintenance and control of *Medea*-based population replacement, discussed further below.

The above approach to *Medea* generation, illustrated in Figure 3, has several appealing features. First, no foreign proteins are being introduced into the insect; there is only a subtle shift in the expression of an endogenous gene from the mother to the early zygote. Second, The use of microRNAs to generate a pre-toxic state provides an important degree of redundancy because multiple microRNAs, each processed and functioning as an independent unit, can be linked into a polycistronic transcript (Chen et al., 2007). Similar considerations apply to the use of long double stranded RNA, in which the silencing function is distributed over the length of the RNA. In addition, multiple small RNA-expressing genes (each of which is capable of achieving silencing of the target transcript on its own) can be incorporated into the element so as to provide redundancy in terms of maternal promoter activity. Because the pre-toxic state represents loss of a maternally expressed transcript, and one can only decrease the levels of a transcript to zero, there can never be too much toxin, as with the gain of function approach. This eliminates issues related to titration of toxin and antidote levels, allowing one to focus rescue efforts simply on driving zygotic expression of a small RNA-insensitive rescuing transgene early in development, and at roughly wildtype levels so as to bring about normal development. The use of microRNAs as inducers of maternal-effect lethality also provides a simple basis by which *Medea*'s selfish behavior can be limited to the target species. *Medea* only shows drive when maternal silencing of a gene whose product is essential for embryogenesis creates an opportunity (embryo death) for zygotic rescue of progeny that inherit the element. Therefore, drive can be limited to the target species by using small RNAs that are species-specific in their ability to silence the maternally expressed gene of interest.

Creating synthetic *Medea* selfish genetic elements in other insect species

A modest number of components are needed in order to create *Medea* elements based on zygotic rescue of a maternal loss-of-function. These include promoters that drive maternal- or transient, early zygote-specific expression. These elements can be identified through transcriptional profiling of different life stages followed by transgenesis to identify specific DNA regions with promoter activity. Observations in *Drosophila* point to the existence of a cis-regulatory heptamer motif, CAGGTAG and related sequences (ten Bosch et al., 2006; De Renzis et al., 2007), important for early zygotic expression of multiple genes, and a maternally-supplied transcription factor, Zelda, has been identified that acts on these sites (Liang et al., 2008). DNA regions containing multiple copies of these motifs, located near genes with transient, early zygotic expression, make good candidates for early zygotic promoters (though it remains to be shown that Zelda-dependent transcriptional activation through these motifs is a conserved feature of early zygotic transcription in other insects). No similar motifs have been described as determining maternal-specific expression. However, a modest number of genes have been shown to be expressed in a germline-specific, or female germline-specific manner in mosquitoes and *Drosophila* (most genes have not been examined), and regulatory regions for some of these genes have been identified, providing starting points for further exploration (Sano et al., 2002; Cho et al., 2006; Adelman et al., 2007; Papathanos et al., 2009). Comparative analysis of genes with similar expression patterns and functions in related insects (e.g. Sieglaff et al., 2009) provides an additional path to identification of important cis-regulatory motifs. Synthetic microRNAs or long double-stranded RNAs that silence a specific maternal transcript are also needed. These are easily generated using standard tools of molecular biology (e.g. Chen et al., 2007), as are small RNA-insensitive, recoded versions of the maternally silenced transcript destined for use as the zygotically expressed, rescuing transgene. The major challenge to creating *Medea* elements based on zygotic rescue of a maternal loss-of-function is that one needs to know the identities of genes whose maternal expression is required for embryogenesis, but not oogenesis, and whose maternal loss cannot be rescued through zygotic expression of the endogenous wildtype allele. Homologs of *Drosophila* genes with these features are good candidates for closely related dipterans such as mosquitoes, and transgenesis and RNAi can be used to test these genes. However, for more distantly related species in which early developmental mechanisms may have diverged, acquiring this information through transgenesis and expression of small RNAs designed to silence the gene of interest could represent a significant challenge. Double-stranded RNA can be injected into larvae or adults, and used to trigger RNAi-induced gene silencing in adult tissues of many insects. In a number of cases this has been shown to result in phenotypes in ovaries or progeny of injected females, indicating that injected dsRNA can trigger RNAi in the female germline, and that this can be transmitted to progeny (Bucher et al., 2002; Liu and Kaufman, 2004; Lynch et al., 2006; He et al., 2006; Ciudad et al., 2006; Boldbaatar et al., 2008; Ronco et al., 2008; Mito et al., 2008; Lu et al., 2009). It may be possible to use this technique to more rapidly screen genes for loss-of-function, maternal-effect lethal phenotypes, though false negatives may result if silencing is incomplete.

Challenges from the wild gene pool

What is the fate of a population in which a *Medea* with a fitness cost is present in all individuals? It is possible, though probably unlikely, that the population will suffer a crash in numbers or undergo a range contraction in response to competition from another species that occupies the same area, an outcome more or less desirable depending on context. A more pressing set of concerns has to do with the question of how the presence of a fitness-compromising *Medea* will influence the gene (allele) pool of the population it is present in, and how these changes will in turn influence *Medea*. The problem is this. In a replaced

population *Medea* is a constant, present in all individuals. This creates opportunities for selection. Alleles at other loci that do well with *Medea* - that result in decreased fitness costs to carriers - should (all other things being equal) spread at the expense of alleles that do poorly in the presence of *Medea*. In response, fitness costs associated with *Medea* might be expected to decrease over time. If so, how will a decreased fitness cost manifest itself with respect to the core *Medea* functions of drive and disease refractoriness?

Generally speaking, there are two possible outcomes. First, alleles that compensate for *Medea* drive associated fitness costs, or costs associated with expression of genes conferring resistance to disease transmission, may come to predominate. These alleles might tweak aspects of early development so as to suppress defects in embryonic development associated with a *Medea*-driven shift from maternal to early zygotic expression, or they might subtly alter resource allocation or life history so as to compensate for activation of the immune system in a specific tissue such as the midgut, in all individuals, regardless of their infection status. These are acceptable outcomes because they leave the *Medea* functions of drive and disease refractoriness intact, though a decrease in the fitness costs associated with carrying *Medea* would make it more difficult to remove these elements through dilution.

Alternatively, alleles that decrease *Medea*-dependent fitness costs by shutting down essential *Medea* functions such as maternal-effect killing or effector expression, thereby allowing the reappearance of wildtypes and carriers of *Medea* in which genes for disease refractoriness have been silenced, may be selected for. A series of recent of observations in *Aedes aegypti*, in which a transgene expressing an anti-dengue effector was transcriptionally silenced over about 17 generations, serves to highlight this latter possibility, though the exact mechanism by which silencing occurred, and any selective pressures that might have contributed to in silencing, are unknown (Franz et al., 2006; Franz et al., 2009).

Can we prevent selection for modifiers that silence *Medea* functions in wild populations? Probably the best we can do is to try and wall off our transgenes from the effects of repressive chromatin that would silence the expression of genes associated with *Medea*. This can be achieved, at least to some extent, by flanking *Medea* (or any other drive element) with sequences that confer boundary/insulator function. Sequences with boundary function recruit proteins that protect regulatory regions from the effects of adjacent repressive chromatin such as heterochromatin, while those with insulator function recruit proteins that prevent enhancers (which may carry repressive functions) from acting on nearby genes (Bushey et al., 2008). Recent genome-wide analyses in *Drosophila* suggest that boundary and insulator activities are often found together, and are in particular associated with binding sites for the CTCF protein (Bushey et al., 2009; Negre et al., 2010). CTCF is found in most organisms, including all insects examined, though it is missing in a few other organisms such as *Caenorhabditis elegans* and *Arabadopsis thaliana* (Heger et al., 2009; Schoborg and Labrador, 2010). Importantly, CTCF-binding sites with insulator activity in *Drosophila* have been shown to have insulator function in mammalian cells (Moon et al., 2005), indicating a high degree of functional conservation and suggesting that insulator/boundary sequences from *Drosophila* will be functional in other insects. In any case, straightforward cell culture approaches are available to test insulator function (Ciavatta et al., 2007), though not boundary activity.

To summarize, the genetic diversity in wild insect populations is certainly very large, it is unknown, and in some sense unknowable because it continually changes in response to mutation, selection, migration and drift. Selection will act on genotypes that carry *Medea*, as it does on all genotypes with an effect on fitness. One of the greatest unknowns in the field of population replacement is the extent to which patterns of gene expression associated with *Medea* and its contents (or those associated with any other drive element/effector) can be

buffered from these selective pressures. We believe that effective buffering will be essential to the success of any population replacement strategy.

Controlling the fate of *Medea*-bearing populations

Populations subject to replacement will always need maintenance and modification over time. In particular it is likely that first generation effectors will mutate to inactivity, become silenced in response to selection, and/or lose effectiveness as pathogens adapt. Second and third rounds of population replacement can be carried out in populations carrying earlier generation elements so long as the new *Medea* element uses a novel toxin-antidote combination, and the new element can freely recombine with elements from earlier generations. The population simply ends up with all individuals carrying at least one copy of all elements.

Alternatively, one may want to place a second-generation element at exactly the same position in the genome as the first-generation element, so as to take advantage of a particularly favorable genomic environment in terms of stability of transgene expression and/or selfish element behavior. Second-generation *Medea* elements can be inserted at the same position in the genome as first generation elements using site-specific recombination. In such a scenario a first-generation *Medea* element, *Medea*^{n}, consists of toxin^{n}, antidote^{n} and cargo^{n} while the second-generation *Medea* element, *Medea*$^{n+1}$, consists of toxin^{n+1}, antidote$^{n+1,}$ cargo^{n+1}, and antidote^{n}. If *Medea*$^{n+1}$ is introduced into a population fixed for *Medea*^{n}, females transheterozygous for both elements will be generated. These females express both toxin^{n} and toxin^{n+1}. Homologous recombination during meiosis cannot move both elements onto the same chromosome because they sit at the same location. Therefore, the only progeny of transheterozygous mothers that can survive are those that inherit *Medea*$^{n+1}$, since only they inherit both antidote^{n} and antidote^{n+1}. As a result, *Medea*$^{n+1}$ spreads by causing the death of alleles (homologous chromosomes) carrying first generation *Medea*^{n} elements, and any non-*Medea* alleles, in the same way that *Medea*^{n} spread by causing the death of the non-*Medea* allele during the initial population replacement (Chen et al., 2007) (Figure 5). If the two *Medea* elements are located at a common position on the X chromosome, in a species such as *Anopheles gambiae*, a major vector of malaria in which males are the heterogametic sex, *Medea*$^{n+1}$ would be expected to spread while driving *Medea*^{n} completely out of the population, for all fitness costs associated with *Medea*$^{n+1}$ up to 0.5 (C. M. Ward, unpublished). This general strategy for cycles of population replacement has the added appeal that it leaves a very modest ‘footprint’ in the population gene pool: just a new element at the same position.

It is also possible, though probably unlikely, that the presence of a first generation effector will lead to unexpected effects that make it important to remove the element from the population. There are several ways removal may be achieved. First, if the presence of *Medea* results in a significant fitness cost, dilution of the replaced population with wildtype mosquitoes so that the frequency of *Medea* falls below the threshold required for spread can lead to loss of *Medea*. In a related approach, mosquitoes that bear a *Medea*-specific suicide transgene could also be introduced into the population. These would express, under the control of the zygotic promoter used to drive rescue transgene expression, small RNAs that target the zygotic rescue transcript (but not the endogenous transcript) for degradation. Progeny of *Medea*-bearing females that inherit *Medea* and this transgene will die, while progeny of wildtype females (or females carrying a *Medea* with a different toxin-antidote combination) will survive, again driving the frequency of *Medea* below the threshold for spread. Interestingly, a genetic element (molecular nature unknown) with some of the characteristics of a *Medea*-specific suicide gene has been described in *Tribolium* (Thomson and Beeman, 1999). For both of these approaches it may be possible to facilitate removal of

Medea by first decreasing the numbers of *Medea*-bearing individuals through the use of insecticides, or by introducing wildtypes/suicide gene-carriers at a time of year when the population has undergone a natural contraction. While the above approaches may work in uniform environments, spatial structure can make removal difficult to achieve if *Medea* remains at high frequency in small pockets, from which it can ultimately spread back into the wider population. Finally, one can take advantage of the strategies discussed above involving cycles of replacement, removing a first generation element through competition with a second generation element located at the same genomic position. This does not restore the population to its pre-transgenic state, but it does provide a method for decreasing the frequency of, or removing, specific transgenes from the population.

Summary and prospects for population replacement

The work discussed above shows that synthetic *Medea* elements, generated using cell biological and developmental principles common to insects and other animals, drive population replacement, eliminating wildtype genotypes from the population. *Medea* elements generated along these principles can be made robust to mutational inactivation, and they can carry multiple genes, suggesting that they can be engineered to have extended functional lifetimes in the wild. Importantly, gene drive with the *Medea* system depends strongly on introduction frequency and associated fitness costs such that *Medea* is only likely to spread when introduced above a threshold frequency. But, if *Medea* is introduced at high frequency it brings about rapid population replacement. Thus, the *Medea* system provides a balance between invasiveness and containment, which can be altered through modifications of fitness costs (perhaps) and release ratios. Finally, *Medea*-dependent drive can be made species-specific, and several strategies are available to either remove specific elements from the population and/or introduce new and improved elements. As a result, the *Medea* system has limited potential for genetic effects on other species, and allows a measure of control over the fate of these elements in populations subject to replacement.

These positive points notwithstanding, several hurdles must be overcome. First, it must be shown that synthetic *Medea* elements can be built along the above principles in insects of economic or medical interest, and that these elements still drive in the diversity of a wild genetic background, in complex ecological settings. This daunting task is made even more challenging by the fact that populations of interest are often complex, consisting of different chromosomal forms that are partially reproductively isolated, a condition that can slow, though not necessarily prevent, the spread of *Medea*. In addition to this basic problem of drive, the genomic sites at which *Medea* elements are located must consistently allow expression of drive and effector genes at the appropriate time and place, in the diversity of the wild genetic background. It remains to be seen if the expression of these genes can be buffered from the effects of alleles at other loci that gain a fitness advantage by silencing their expression. Each of these unknowns must be addressed, but it is important to remember that they are unknowns because we have not had access to a working drive mechanism with which to explore. Experiments are now possible, and this constitutes an important beginning.

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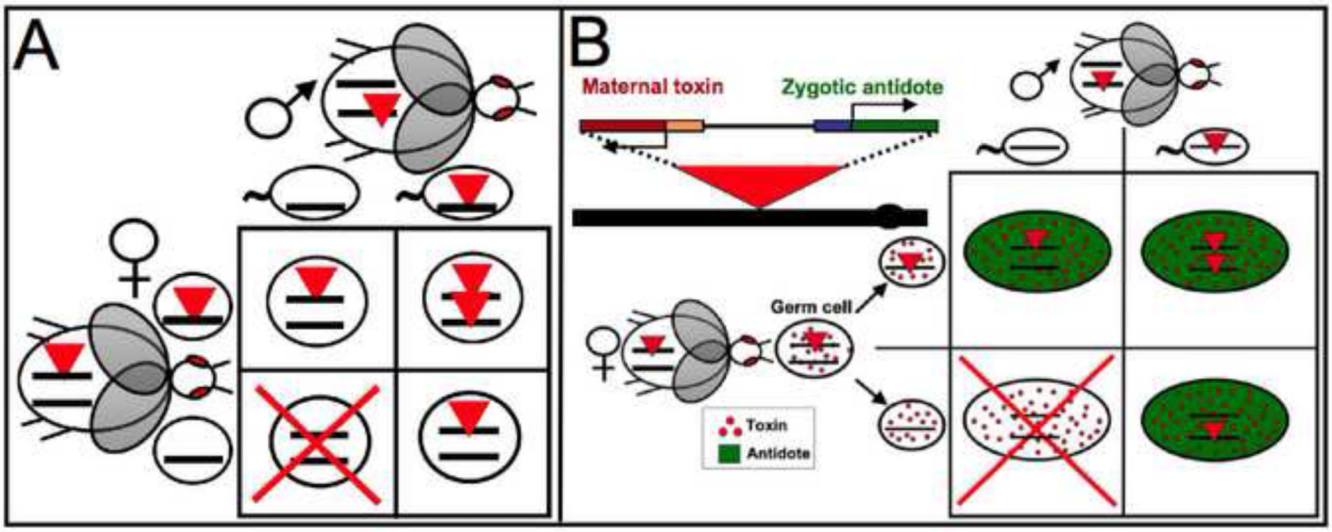

Figure 1.

Medea genetics, and a possible mechanism by which *Tribolium Medea* acts. (A) When *Medea* is present in a female, only progeny that inherit *Medea* from one or both parents survive. (B) The genetics of *Tribolium Medea*, in particular the isolation of mutants that have zygotic rescuing, but not maternal killing activity, suggest a model in which *Medea* consists of two tightly linked genes, a maternally-expressed gene (Maternal toxin) whose product (red circles) causes developmental arrest of all eggs, and a zygotically expressed gene (zygotic antidote) whose product (green background) is able to rescue the normal development of eggs that inherit the element from either parent.

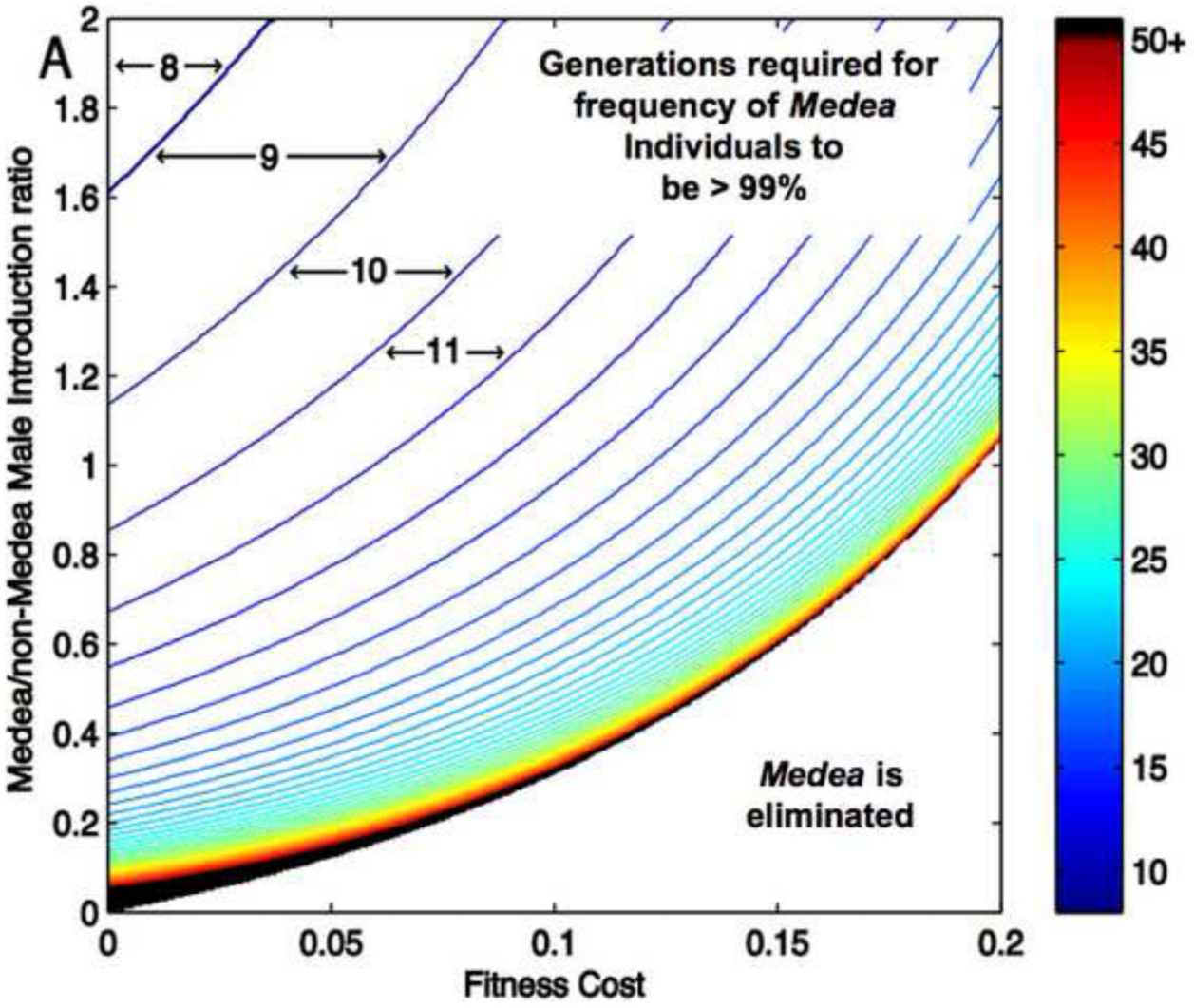

Figure 2.

Population genetic behavior of *Medea*. Plot describing the number of generations required for *Medea* to be present in >99% of individuals, for a *Medea* element with different levels of a multiplicative embryonic fitness cost. Homozygous *Medea* male:non-*Medea* male introduction ratios are indicated on the Y axis, and embryonic fitness cost on the X axis. Area between lines indicates regions of parameter space within which a specific number of generations (indicated by numbers and arrows) are required for the frequency of *Medea* individuals to reach a frequency of 99% or greater. Line color, shown in the heat map at right, provides a rough measure of how many generations are required. Black lines (50+) indicate that fifty or more generations are required. The border between the black-lined region and the lower unlined region defines the critical *Medea*:non-*Medea* male introduction ratio (CMIR), below which *Medea* will be eliminated from the population. The model assumes an infinite population size, discrete, non-overlapping generations, and random mating.

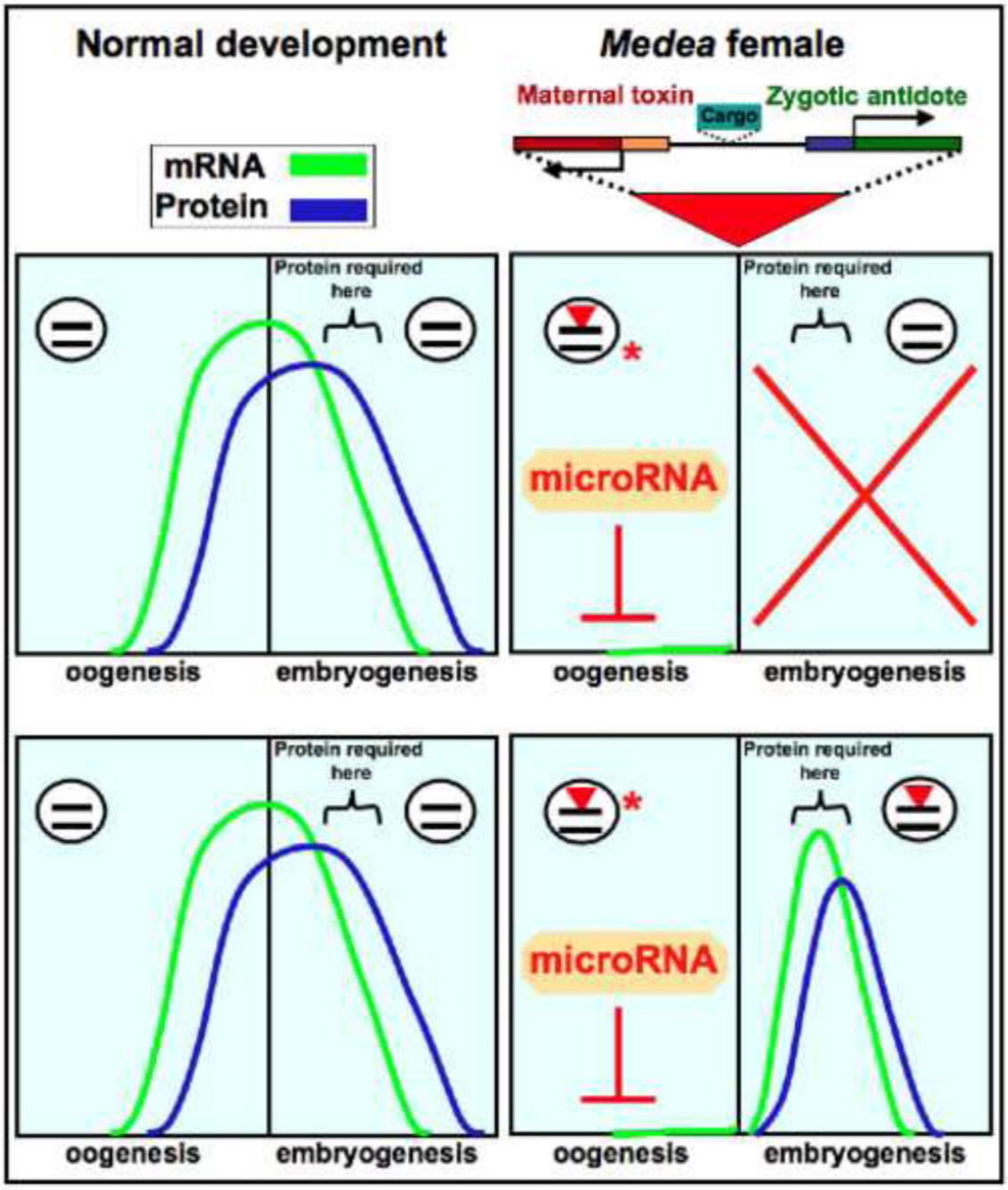

Figure 3.

Synthetic *Medea* elements result from zygotic rescue of a maternal loss-of-function that results in embryonic arrest. During wildtype oogenesis (left-side boxes, upper and lower) a maternal transcript is synthesized (green line). This transcript is translated during oogenesis, but the product is not utilized until early embryogenesis. When a female is heterozygous for *Medea* (red triangle; right-side boxes, upper and lower) a transgene drives maternal germline-specific expression of microRNAs that silence expression of the gene whose product is required for early embryogenesis. This results in inheritance of a lethal condition - the loss of an essential maternally deposited product - by all oocytes/embryos. Progeny survive the embryonic arrest thereby induced if they inherit from their mother (in this

example) a tightly linked transgene driving early zygotic expression of the maternally silenced gene just in time to restore embryo development (box in lower right), but they die if they fail to inherit it (large red X in box in upper right). Circles indicate adult females (left side of each box) and embryos (right side of each box). Black lines in these circles represent a pair of homologous chromosomes. *Medea* is indicated by a red triangle and the chromosome inherited by progeny by a red asterix.

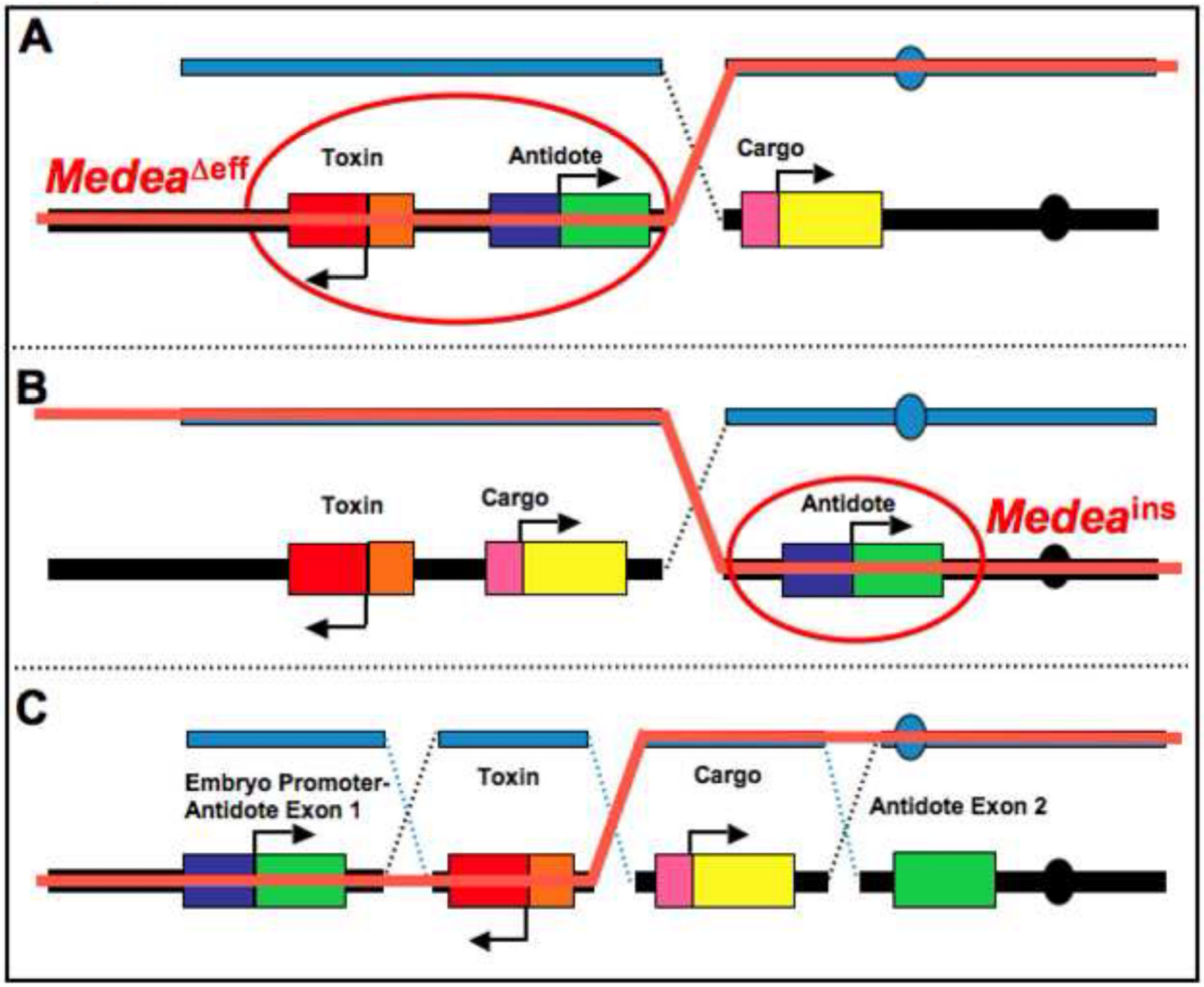

Figure 4.

Chromosome breakage and rejoining can create *Medea*$^{\Delta eff}$ and *Medea*^{ins} chromosomes, and can be prevented by placing the toxin and the effectors in an intron of the antidote. (A) Chromosome breakage and rejoining (illustrated by the dotted lines and the salmon-colored thick line) that separates the *Medea* element from its cargo results in the creation of a *Medea*$^{\Delta eff}$ element, which lacks cargo. (B) Placing the cargo between the toxin and antidote genes prevents breakage and rejoining from creating a *Medea*$^{\Delta eff}$ element, but it does not prevent the appearance of *Medea*^{ins}, an antidote-only element. (C) Splitting the rescue molecule into two, individually non-functional parts creates an element in which DNA breakage and rejoining events results in loss of rescue activity. The chromosomes thereby created cannot show maternal-effect selfish behavior, nor will they block the spread of intact *Medea* elements through the creation of rescue-only alleles.

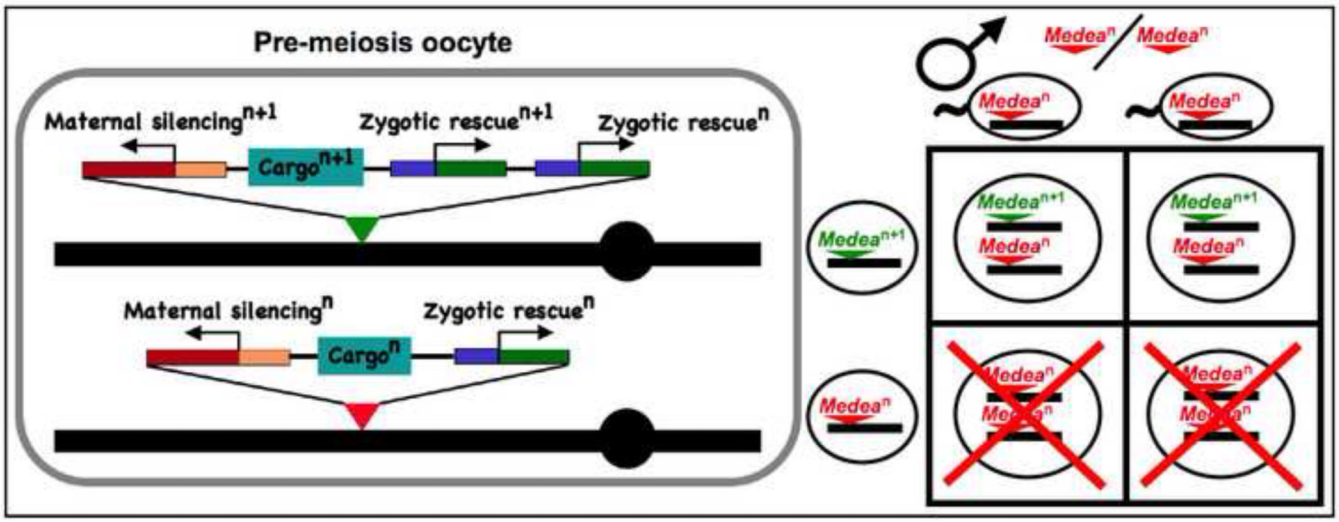

Figure 5.

Second generation *Medea* elements (*Medea*$^{n+1}$), comprised of a new toxin, a new antidote, a new cargo and the first generation antidote, can drive first generation elements (*Medea*^{n}) out of the population at the same time as they are driven in, if both elements are located at the same position in the genome.