CHAPTER

Modifying Insect Population Age Structure to Control Vector-Borne Disease

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Abstract

Age is a critical determinant of the ability of most arthropod vectors to transmit a range of human pathogens. This is due to the fact that most pathogens require a period of extrinsic incubation in the arthropod host before pathogen transmission can occur. This developmental period for the pathogen often comprises a significant proportion of the expected lifespan of the vector. As such, only a small proportion of the population that is oldest contributes to pathogen transmission. Given this, strategies that target vector age would be expected to obtain the most significant reductions in the capacity of a vector population to transmit disease. The recent identification of biological agents that shorten vector lifespan, such as *Wolbachia*, entomopathogenic fungi and densoviruses, offer new tools for the control of vector-borne diseases. Evaluation of the efficacy of these strategies under field conditions will be possible due to recent advances in insect age-grading techniques. Implementation of all of these strategies will require extensive field evaluation and consideration of the selective pressures that reductions in vector longevity may induce on both vector and pathogen.

Introduction

Most pathogens vectored by arthropods, such as *Plasmodium* and dengue virus, must undergo an extrinsic incubation period (EIP) in the vector, before they can be transmitted to a new host. During this time arboviruses and parasites like *Plasmodium*, penetrate the vector's midgut, replicate in various host tissues and infect the salivary glands prior to transmission during subsequent bloodfeeding. The duration of the EIP is variable and is heavily influenced by ambient temperature. For many pathogens the duration of the EIP consumes a significant proportion of the vector's lifespan. As a consequence, only a small fraction of the vector population that is oldest is of epidemiological importance.

The significance of vector age as a target for disease control was first realized by MacDonald through his quantifications of basic case reproductive rate for malaria.[1] Garrett-Jones[2,3] devised the concept of vectorial capacity by isolating the entomological components of MacDonald's[1] formulation. Essentially, it is a statement that expresses the intensity of transmission due to physiological, behavioral and ecological aspects of the vector population. While vectorial capacity increases linearly with factors such as vector competence and vector density, it increases exponentially with increases in mosquito longevity. Consequently, vector lifespan is the most sensitive determinant of a vector population's capacity for pathogen transmission. It is not surprising then, that very successful control strategies, such as indoor surface spraying of houses, effectively target older mosquitoes in the vector population.

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Due to a number of complex social and biological factors, vector-borne diseases such as dengue fever are currently reemerging throughout the tropics.[4] With few exceptions, current control methods are not proving effective in stopping this alarming trend. In recent years a number of new approaches have been proposed that use biological agents to modify the age structure of mosquito populations. These include; (i) the use of virulent strains of the common bacterial endosymbiont *Wolbachia*,[5,6](ii) entomopathogenic fungi[7] and (iii) densonucleosis viruses (densoviruses).[8] These biological agents induce mortality effects late in adult life, and as a result skew vector population age structure towards younger individuals, thereby potentially limiting pathogen transmission without eradicating the mosquito population. As these strategies target mosquito longevity, even minimal reductions in lifespan could yield significant reductions in vectorial capacity.

Entomological Components of Pathogen Transmission

After a mosquito consumes a blood meal containing a pathogen, there is a period during which the pathogen replicates and disseminates in the mosquito's body before it can be transmitted. This period from pathogen ingestion to potential infectivity is termed the extrinsic incubation period and lasts between 10-14 days for dengue virus[9] and 9-14 days for malaria.[10] Consequently, a mosquito disease vector must live longer than the combined duration of the initial nonfeeding period (approx. 2 days) plus the EIP of the pathogen before contributing to disease transmission. This combined period is long relative to adult mosquito lifespan, therefore, the majority of disease transmission is due to the feeding activity of old individuals. Consequently, the vectorial capacity of an insect population is closely linked to its demography.[11]

Vectorial capacity is defined as the daily rate at which future human inoculations arise from a single infective case.[12] Mathematically presented, vectorial capacity (V) is:

$$V = \frac{ma^2 p^n b}{-\ln p}$$

where:
m = vector density in relation to host density
a = probability a vector feeds on a host in 1 day (= host preference x feeding frequency)
p = probability of vector surviving 1 day
n = duration of extrinsic incubation period (EIP) in days
b = vector competence
$1/(-\ln p)$ = duration of a vector's life, in days after surviving EIP.[13]

The probability of contact between vector and host depends on the vector population size (term m) and vector feeding preference and frequency (term a). Vector feeding behavior, specifically host preference and feeding frequency, combine to form term a in the vecterial capacity equation. Feeding behavior can have significant implications for epidemiological risk as it influences the probability of contact between vector and host. For example, female *Ae. aegypti* feed almost exclusively on humans[14] and take multiple blood meals during each gonotrophic cycle.[15,16] Consequently, *Ae. aegypti* is the most epidemiologically important dengue vector even though *Aedes albopictus* is a more competent vector for dengue viruses.

Vector competence (term b) refers to a vector's ability to acquire and transmit a pathogen. Extensive research efforts are currently being directed towards genetic manipulation of vector competence to block disease transmission.[17-23] Vector competence is a linear component of vectorial capacity and as such, theoretical predictions suggest that large reductions in vector competence would be required to generate significant reductions in disease transmission.[24]

Vectorial capacity is most responsive to reductions in daily survivorship (term p). This is a consequence of the duration of the EIP of a pathogen being long relative to vector life expectancy. The duration of the EIP of dengue has been estimated to be between 10 to 14 days from early research using human volunteers.[9] Long-lived vectors contribute most to

pathogen transmission and small decreases in vector life expectancy can cause large reductions in transmission rates.[2,11] This observation has attracted researchers to the possibility of utilizing different biological agents to shift the age structure of vector populations as a way to reduce disease transmission.

Wolbachia Pipientis

Wolbachia pipientis is an obligate intracellular bacteria that was first observed in the reproductive tissues of the mosquito *Culex pipiens*[25] and later described in the same insect by Hertig.[26] Since its original description, *Wolbachia* has been found in a diverse range of invertebrate taxa including insects, mites, spiders, terrestrial crustaceans and nearly all filarial nematode species. Several PCR-based surveys have indicated that *Wolbachia* chronically infects more than 20% of all insect species.[27,28]

Wolbachia are vertically inherited by transovarial transmission within host populations and only very rarely appear to move horizontally and infect new species.[29] They induce a number of intriguing reproductive abnormalities that account for their success. These include parthenogenesis,[30] feminization,[31] male-killing,[32] and cytoplasmic incompatibility (CI; the developmental arrest of insect embryos that result when females are mated to males that have a different infection status).[33] All these reproductive effects enhance *Wolbachia* transmission at the expense of the arthropod population that is not infected. This in turn results in the active spread of this endosymbiont into uninfected host populations even if it confers a fitness cost.[34]

The ability of *Wolbachia* to invade host populations using the most common of these phenotypes, CI, has been quantified and modeled in several arthropod species including *Drosophila simulans*[35,36] and *Ae. albopictus*.[37,38] Some strains of *Wolbachia* induce high CI, which leads to rapid population invasion. For instance, the *Wolbachia* strain *w*Ri has been shown to spread geographically at a rate of 100km/year into uninfected *D. simulans* populations through the action of CI.[35] While CI has been proposed as a major mechanism allowing certain strains of *Wolbachia* to invade and persist in host populations, mechanisms that underlie the spread of other *Wolbachia* strains are less well understood. Several *Wolbachia* strains, such as *w*Au from *D. simulans* and *w*Mel from *D. melanogaster*, have invaded natural populations even though they induce little to no CI under field conditions.[39] It is hypothesized that these strains may provide an as yet undetermined fitness benefit to their hosts.[34]

Life-Shortening *Wolbachia*

Recently, virulent *Wolbachia* strains that replicate to unusually high densities and shorten adult host lifespan have been reported.[40,41] For example, *Wolbachia* infection in the parasitoid wasp *Leptopilina heterotoma* induces a significant reduction in adult survival, locomotor activity, and fecundity.[40] Despite causing detrimental effects to their hosts, these life-shortening strains apparently persist because hosts are able to reproduce before death. Consequently the life-shortening strain spreads due to the reproductive fitness benefit that CI imparts to individuals carrying the infection.

To date, the best characterized and most virulent *Wolbachia* strain is *popcorn* (*w*MelPop) from *D. melanogaster*. Min and Benzer[41] discovered *w*MelPop during a screen for gene mutations that cause brain degeneration in *D. melanogaster*. They noticed that a particular X-chromosome deficiency strain; $Df(1)^{ct4b1}$ y^{1}*/Binsn* originally isolated by Hannah in 1947,[42] had drastically reduced lifespan compared with normal flies. The basis for this reduced lifespan was the presence of the *w*MelPop infection. Interestingly, *w*MelPop is present in low numbers during embryonic, larval and pupal stages, but in adults density increases in several tissues including the brain, retina and flight muscle. With progressing age, cells in these tissues become filled with bacteria causing overt host cell pathology. This effect culminates in early death of adults, with the lifespan of infected flies being reduced by about 50% relative to uninfected controls. At 29°C this equates to 100% mortality of infected flies by 14 days.

The degree of life-shortening induced by *w*MelPop is influenced by several factors, including temperature and host genetic background. Several reports have noted that the virulence of *w*MelPop increases at higher temperatures,[41,43,44] suggesting an active link between temperature and the life-shortening phenotype. Renyolds and coworkers[44] documented slight variations in *D. melanogaster* lifespan when *w*MelPop was placed over different genetic backgrounds at 25°C. In addition, they found no significant difference between the lifespan of infected and uninfected flies at 19°C.[44]

When transferred from *D. melanogaster* to *D. simulans*, *w*MelPop still caused life-shortening at comparable rates to those observed in *D.* melanogaster, indicating the over-replication of *Wolbachia* was a property of the microbe's genome.[43] Furthermore, when transferred to *D. simulans*, *w*MelPop induces strong CI and had little fecundity cost after adaptation to its new host.[43,45] The ability of *w*MelPop to induce strong CI and not induce large fecundity costs in a novel host indicates that this strain has the potential to be introduced into different insect species where it may induce significant life-shortening, yet potentially still invade uninfected populations.

One possible application of *w*MelPop would be to alter the age structure of *Ae. aegypti* populations to reduce dengue transmission.[6] By introducing life-shortening strains of inherited *w*MelPop into mosquito populations, old mosquitoes could be selectively eliminated, preferentially removing the segment of the vector population responsible for most disease transmission. Predictions from recent theoretical models indicate that this strategy could result in significant reductions (80-100%) in disease transmission.[5,46]

Experimental Transfer of *Wolbachia* into Disease Vectors

Wolbachia occurs naturally in several medically important species of sandflies,[47] tsetse flies,[48] and mosquitoes[49,50] including several vectors of dengue such as *Ae. albopictus*,[51] *Aedes polynesiensis*,[52] and *Aedes scutellaris*.[53] *Ae. albopictus* is naturally infected with two *Wolbachia* strains, *w*AlbA and *w*AlbB, and both of these strains are capable of inducing strong CI.[37,38,54-58] However, some of the major disease vectors are not naturally infected, including all anopheline mosquitoes sampled to date, and *Ae. aegypti*, the primary vector of dengue.[59-61] Transinfection of these species is a major research priority.

The first experiment specifically designed to move *Wolbachia* between two host species involved the transfer of *w*Ri from *D. simulans* to an uninfected strain of *D. melanogaster* by injecting infected cytoplasm between presyncitial blastoderm stage embryos of these species.[62] Since then, *Wolbachia* has been successfully transferred between several insect species[43,63-67] and crustaceans,[68,69] including transfer attempts between phylogenetically distant insect orders.[70,71] Transfer experiments that generate novel infection types have been used to answer questions relating to *Wolbachia*-host interactions,[43,45] as well as to develop population replacement and population suppression strategies for controlling harmful insect species.[72,73]

As *Wolbachia* are extremely fastidious microorganisms and cannot be maintained in cell-free media, methods typically used to transfer *Wolbachia* between hosts include the direct-transfer of *Wolbachia*-infected cytoplasm and transfer of embryo homogenate via embryonic microinjection.[62,63,70,74] With both techniques, *Wolbachia* is microinjected into the posterior end of early embryos, with the goal of infecting embryonic pole cells that will develop into germ tissues. More recently, successful transfer of *Wolbachia* has been achieved using adult microinjection where *Wolbachia* purified from hemolymph or embryo homogenate has been microinjected into the thorax or abdomen of adults. *Wolbachia* then proceeds to disseminate throughout the body and infect the ovaries.[75] This technique has been successful in establishing *Wolbachia* infections in arthropods where embryonic microinjection techniques are not well developed including isopods,[69,76] parasitoid wasps,[65] planthoppers,[77] and mosquitoes.[78]

A complicating factor associated with transferring *Wolbachia* between species is the stable maintenance of the new association. In some cases, transferred strains are extremely stable and maternally inherited at very high rates.[67] In other cases the new infection appears poorly adapted

to its new host and shows variable degrees of maternal transmission efficiency.[71,73,79] Several advances in the area of *Wolbachia* transfer and culture over the past few years may allow researchers to overcome these barriers. The ability to maintain *Wolbachia* in vitro in cell culture systems,[80-83] and the availability of cell lines from many recipient target species provides an ideal system to preadapt *Wolbachia* strains to the intracellular environment of novel host species, prior to transfer via embryonic or adult microinjection. It is possible that this preadaptation strategy may lead to higher initial levels of maternal transmission efficiency, than by directly transferring *Wolbachia* into a novel intracellular environment. An additional advantage of the use of cell culture systems as a source of *Wolbachia* for microinjection, is the ability to obtain large quantities of *Wolbachia* to optimize transinfection protocols and infective doses. Further, the suspension of purified *Wolbachia* in injection buffers that maintain viability and increase transinfection efficiency such as sucrose-phosphate glutamate buffer,[74] may allow researchers to establish infections in species previously thought refractory to *Wolbachia* infection.

Although *Ae. aegypti* does not naturally harbor *Wolbachia* in nature, the successful transfer of *w*AlbB from *Ae. albopictus* to *Ae. aegypti* using cytoplasm transfers between presyncitial blastoderm stage embryos indicates this species is not refractory to *Wolbachia* infection.[67] Further, *Ae. aegypti* has also been transinfected with a *w*AlbA and *w*AlbB double infection from *Ae. albopictus* using adult microinjections.[78] Following on from this initial success, *w*MelPop has recently been transferred to *Ae. aegypti* via embryonic microinjection. (Conor McMeniman, pers. comm.). The method used to establish this infection differs from all *Wolbachia* transfers to date in that it used *w*MelPop passaged for several years in the *Ae. aegypti* cell line RML-12, as a source of bacteria for microinjection. Several previous attempts in our laboratory to directly transfer *w*MelPop between *D. melanogaster* and *Ae. aegypti* using cytoplasm transfers and adult microinjections were unsuccessful, with infection being lost after one or two generations after establishment of the infection due to poor maternal transmission efficiency. In an attempt to preadapt *w*MelPop to the *Ae. aegypti* intracellular environment, to maximize the possibility that the new association would be stable, we established an in vitro infection in RML-12 before transfer to *Ae. aegypti*. Initial data indicates that maternal transmission efficiency of *w*MelPop in several newly generated lines is high, with the effect of *w*MelPop on parameters such as life-shortening and cytoplasmic incompatibility still to be determined. Importantly, pilot transfers of *w*MelPop from the RML-12 cell line back into its native host *D. melanogaster* indicate *w*MelPop has not attenuated in virulence during in vitro maintenance. (Conor McMeniman, pers. comm.).

Temperature and the Impact of Life-Shortening *Wolbachia*

As *w*MelPop virulence is temperature-dependent, the range of environmental temperatures encountered by *Ae. aegypti* is important for understanding the efficacy of this approach. In the field, temperature fluctuates on both a daily and seasonal basis. During summer when temperatures increase, the effect of *w*MelPop on *Ae. aegypti* longevity is expected to be most pronounced. Significantly, summer also corresponds to the period of peak dengue transmission in many regions.[84,85] During periods of sustained cooler temperatures, when dengue transmission risk is lowest, virulence of *w*MelPop in *Ae. aegypti* populations would also be predicted to decrease. Importantly, temperature will also influence the infection dynamics of *w*MelPop in *Ae. aegypti* populations. It is well documented that a trade-off exists between fitness costs generated by *Wolbachia*, and the unstable equilibrium point that must be exceeded for *Wolbachia* to actively spread into a population under the action of CI.[34] It would be expected that this point would be lower in cooler months and during this season the infection would spread more readily.

Layered upon the effect of seasonal changes in temperature on *w*MelPop in *Ae. aegypti*, are the daily thermal preferences of *Ae. aegypti* itself. Although little is known about the thermal ecology of mosquitoes, preference of *Ae. aegypt*i for microhabitats such as vegetated and dark sheltered areas are well documented.[86] Preference for different microhabitats have the potential

to alter the range of temperatures mosquitoes experience during the day. As such, to fully evaluate the impact of such environmental heterogeneity on the efficacy of this approach contained semi-field trails will need to be conducted. Quantitative measures from these field cages coupled with modeling will allow researchers to make biologically relevant predictions about the potential efficacy of this strategy.

Molecular Basis of Life-Shortening in *w*MelPop

Identification of putative loci that modulate life-shortening in *w*MelPop would be useful from an applied perspective. These loci may involve point mutations, insertions or deletions that may have occurred in genes or promoter regions of *w*MelPop involved in regulation of bacterial cell division or density, causing it to over-replicate in a temperature sensitive manner. It may be desirable to genetically modulate virulence of *Wolbachia* strains that naturally infect (or can be transinfected) into disease vectors. Currently, transformation technologies are still being developed for *Wolbachia*. Encouragingly, homologous recombination-based techniques have been used successfully to introduce point mutations and foreign DNA into the *Wolbachia* chromosome. (Iñaki Iturbe-Ormaetxe, pers. comm.). This alternative strategy may be useful if attempts to transinfect disease vectors with *w*MelPop are unsuccessful, or cytoplasmic incompatibility and fecundity effects of *w*MelPop in these host species are less than optimal.

To determine the molecular basis of the life-shortening phenotype the genome of *w*MelPop has been mapped on a number of levels. Initial studies focused on comparisons of the genome organization of *w*MelPop to the closely related, yet nonvirulent strain *w*Mel. Using southern hybridization with probes designed from the recently sequenced *w*Mel genome,[87] Sun et al[88] arranged restriction fragments of the *w*MelPop chromosome to create a comparative map of the *w*MelPop genome. Results indicated the genome organization of *w*MelPop was identical to *w*Mel with the exception of a single 150 kb inversion of its chromosome. This region has been further characterized using a finer scale genetic map of the *w*MelPop genome. This map, created by PCR-tiling across the entire genome has revealed that break points flanking the inversion occurred in noncoding regions. (Markus Riegler, pers. comm.). Therefore, it is unlikely that the inversion has disrupted operons that in turn influence the life-shortening phenotype. Mapping has also indicated minor size differences between the genomes of *w*MelPop and *w*Mel. When PCR fragments, 2-10 kb in length, were resolved on agarose gels, two differential IS5 transposon insertion sites and two separate variable number tandem repeats (VNTRs) in the *w*MelPop genome were identified. These minor genomic differences between *w*Mel and *w*MelPop were considered potentially informative until it was discovered that *w*MelPop shares these differences, including the orientation of the 150 kb chromosomal inversion, with a previously undescribed avirulent *w*Mel strain, called *w*MelCS.[89]

As these two strains share identical genome arrangements it is likely that *w*MelPop is a substrain of *w*MelCS, rather than *w*Mel. The *D. melanogaster* laboratory stock from which *w*MelPop was identified, $Df(1)^{ct4b1}$, y^1/*Binsn*, was isolated in 1947.[42] From the available literature concerning the origin of this strain, it is not possible to know whether *w*MelPop arose from *w*MelCS during the mutagenesis experiment used to generate this fly strain, or beforehand. Most other *D. melanogaster* stocks established around this time were infected with *w*MelCS.[89] To create the X-chromosome deficiency line $Df(1)^{ct4b1}$, y^1/*Binsn*, males from ring chromosome stock X^{c2} were exposed to an X-ray radiation source and the line selected by crosses to females from an un-specified X-chromosome marker stock.[42,90] Unless *w*MelPop was paternally inherited from males used is this study, a circumstance very rare in nature,[36] it most likely that *w*MelPop arose in females from the marker stock used in this study. As no stock name for this marker line was specified the circumstances surrounding the origin of *w*MelPop remain a mystery.

Currently, the genome of *w*MelPop is being sequenced in our laboratory. Once completed, the genome sequences of *w*Mel and *w*MelPop will be compared and possible point mutations in gene and promoter regions identified. These differences will then be verified by comparative PCR in *w*MelCS, and possible candidate genes for life-shortening identified. These candidates

will then be functionally tested using site-directed mutagenesis via homologous recombination to introduce mutations in *w*MelCS or *w*Mel genome to test these putative loci for their role in life-shortening.

Entomopathogenic Fungi

An alternative strategy that reduces vector lifespan using entomopathogenic fungi has recently been proposed. Reports have shown that several disease vectors, including tsetse flies and mosquitoes, are susceptible to a range of fungal entomopathogens.[91-96] Mosquitoes infected with fungal spores have increased mortality rates, with most individuals surviving to 10-14 days post infection. More recent studies have illustrated the potential of using commercially available oil-based formulations of these fungi to alter the daily probability of survival of anopheline mosquitoes for malaria control.[7,97]

In a laboratory-based setting, Blanford and coworkers[7] examined the survival and sporozoite burden of *Anopheles stephensi* exposed to an isolate of the fungus *Beauveria bassiana*. Results indicated that short periods of exposure of mosquitoes to cage mesh sprayed with oil-based formulations of *Beauveria* were sufficient to cause > 90% mortality by day 14 after contact (the approximate EIP for malaria). Importantly, exposure of mosquitoes infected with the rodent malaria *Plasmodium chabaudi* to surfaces sprayed with *Beauveria* spores reduced the transmission risk by a factor of 80.[7] At day 14 post-exposure, 31% of malaria-infected control mosquitoes were alive and able to transmit, compared with only 0.4% mosquitoes in the *Beauvaria* and malaria treatment. The potential of this approach for malaria control was further demonstrated by a field-based study in a Tanzanian village by Scholte and coworkers.[97] In their study, the ability of a different fungus, *Metarhizium anisopliae*, to infect and reduce the lifespan of wild *An. gambiae* mosquitoes after resting on fungus-treated cotton sheets suspended from the ceiling of huts was evaluated. Over the three weeks of the trial, 580 female *An. gambiae* were collected from treated huts and 132 of these mosquitoes were infected with the fungus (23% infection rate). Significantly, infected mosquito lifespan was reduced to 11 days post-contact. Using these results, a model to estimate the intensity of malaria transmission indicated that a 75% reduction in transmission could be achieved using this strategy. In addition, the authors noted that increasing the coverage of treated mosquito resting sites to infect 50% of mosquitoes would reduce transmission by 96%. Interestingly, further experiments to characterize the effect of *M. anisopliae* on *An. gambiae* have indicated that infection with this fungus reduces the propensity of females to blood-feed, as well as decreasing fecundity and total lifetime productivity of mosquitoes.[98]

The documented effects of entomopathogenic fungi reducing the probability of daily survival[7,97] and feeding frequency[98] of malaria vectors are extremely encouraging. Initial studies suggest that implementation of this method in an applied setting may cause large decreases in vectorial capacity in mosquito populations. However, like residual insecticides, the gradual loss of viability of entomopathogenic fungal spores on treated surfaces over time[97] will necessitate their repeated application for sustained disease control.

Densonucleosis Viruses

Mosquito densoviruses (Family *Parvoviridae*) are also being investigated as potential control agents for mosquito-borne diseases due to the reductions in adult mosquito lifespan they induce.[8] Densovirus infected mosquitoes show increased mortality during both immature and adult life stages.[99-102] The level of pathogenicity of mosquito densoviruses appears to be highly dependent on densovirus strain and infective viral dose.[101,103] Female mosquitoes infected with densovirus strains may also have reduced fecundity and egg viability.[103,104]

Mosquito densoviruses were initially isolated from *Ae. aegypti* colony material sourced from Southeast Asia.[8] In addition, densovirus strains have been isolated from mosquito colonies[105] lines,[106,107] and from natural populations.[100,108] Currently little is known about the maintenance of densoviruses in natural mosquito populations. It is believed that densovirus transmis-

sion occurs within larval habitats as infected larvae excrete virus particles in the water.[99,101] Vertical transmission of densoviruses has been reported, yet the mode of transmission is unknown.[100,103,104] Initial results from population cage studies suggest that vertical transmission will allow the virus to spread to new larval habitats. This may mitigate the need for repeated applications of densovirus formulations to larval habitats.

It is unclear to what extent densovirus-induced mortality will have on disease transmission. Modeling of the mortality effects of densovirus infection in *Ae. aegypti* predicts a reduction of approximately 76% of infectious mosquito days.[103] While preliminary, this result suggests that densovirus-based vector control strategies may lead to significant reductions in dengue transmission. However, extensive research is needed to develop mosquito densovirus formulations for widespread application to control mosquito populations.

Evaluating of the Efficacy of Strategies Targeting Vector Longevity

One challenge associated with the development of control strategies that reduce vector longevity relates to how the efficacy of these approaches will be evaluated under field conditions. Researchers will need to accurately monitor changes in age structure of mosquito populations with overlapping generations. As such, tools that provide accurate estimates of mosquito survival are needed. Various methods of varying utility exist that enable the estimation of mosquito longevity.

Daily survivorship can be assessed through horizontal life tables constructed by following a cohort of individuals. Mark-release-recapture techniques (MRR), reviewed by Service[109] and Hagler and Jackson,[110] are central to this approach. Periodic collections give an indication of the number of marked individuals surviving within the population. Reliable estimates of daily survival rate require numerous marked individuals per recapture, which requires large numbers of mosquitoes at the initial release. Consequently the approach is labor intensive and researchers undertaking MRR studies in disease endemic areas need to be mindful of contributing to epidemiological risk which may limit the application of this approach.[111,112]

Daily survival rates can alternatively be inferred directly from the age structure of a vector population. Russian scientists pioneered the first mosquito age-grading techniques during the 1950's (reviewed by Detinova[113]). Their work identified consistent changes in female ovarian anatomy with age. As a female mosquito ages and passes through successive gonotrophic cycles, permanent changes occur in the ovaries and associated structures. The simplest reproductive age-grading technique differentiates nulliparous (has not oviposited; <4 days of age) and parous (one or more ovipositions; >4 days of age) females on the basis of ovarian tracheations.[113] Tracheoles are small tubules of the insect respiratory system that form tightly wound structures (skeins) on the ovaries of nulliparous mosquitoes. In parous females the tracheoles have stretched to accommodate the growth of the ovary during the first gonotrophic cycle and form a tracheal net. The limitation of this method is that it only differentiates two age classes and this provides limited information of epidemiological importance.

An alternative reproductive age-grading method counts individual dilatations in the ovariolar pedicel that correspond directly to multiple gonotrophic cycles.[113] This allows more age classes to be determined, as dilatations result from distension and incomplete contraction of ovariolar walls with the passage of a mature egg through the ovariole.[113] This method requires delicate dissection and examination of multiple undamaged ovarioles to ensure an accurate estimate of parity.[114] Improved techniques of preparing ovarioles for examination have been developed,[115,116] however, the method remains technically challenging and time consuming, and its application to large-scale studies is limited. Further, ovariolar dilatations may not be appropriate for age-grading all mosquitoes of medical importance, such as *Ae. aegypti*.[114,117]

More recent approaches for age-grading insects have focused on age-related changes in the abundance of pteridines[118-120] and cuticular hydrocarbons.[121-124] Pteridines are fluoresent pigments that are synthesized in the fat body of insects and transported to various parts of the body where they accumulate. Pteridines have been used to determine chronological age in

several Dipterans.[119,125] Application of this method to mosquito age-grading was initially promising as laboratory studies observed inverse linear trends between pteridine abundance and age in *An. gambiae* and *An. stephensi*.[120] This association was, however, not apparent in *Anopheles albiminus* collected from the field.[119] Additional studies of pteridine fluorescence in *Aedes* and *Culex* spp. showed pteridine levels were below the detection limit of standard spectrofluorometers.[118]

The abundance of cuticular hydrocarbons (CHC) has been used to age-grade *Ae. aegypti*. Relative changes in the abundance of specific CHCs from *Ae. aegypti* legs were measured using gas chromatography and showed a linear association with age up to 15 calendar days.[122] A MRR experiment undertaken to validate this age-grading method under field conditions in Thailand demonstrated seasonal variation in CHC abundance.[124] Application of CHC profiles for age-grading several other mosquito species has shown promise.[121,126,127]

The most recent advance in mosquito age-grading has been the development of a transcriptional age-grading technique for *Ae. aegypti*.[128] This method quantifies age-related transcriptional changes of eight genes using quantitative reverse transcriptase PCR (qRT-PCR). Age predictions are derived for adult female *Ae. aegypti* using calibration data, transcriptional profiles quantified from mosquitoes reared to known ages under field conditions.[128] Field studies used to validate this method estimated the age of individual mosquitoes using both transcriptional and CHC age-grading methods. Mosquito legs were used for CHC analysis, while transcript abundances were quantified from the head and thorax of the same mosquito. This study demonstrated that transcriptional age-grading gave more accurate age estimates than those derived from CHC analysis.[128]

Cook et al[128] presented additional results showing age predictions derived from the three most informative genes. The subsequent calibration model resulted in a slight overestimation of age across most age classes, however this bias may be acceptable given the considerable simplification of the assay. It is expected that identifying additional genes of interest that are transcriptionally active in later age classes will remove this bias and increase the accuracy of age predictions in individuals older that 13 days of age. Preliminary findings suggest that appropriate candidates are available. Field-based evaluations of the transcriptional age-grading technique in different geographical locations and across different seasons are currently being undertaken.

Research is currently underway to transfer the transcriptional age-grading technique into other mosquito species. Transcriptional studies using *An. gambiae*[129] and *Drosophila*[130,131] microarrays have shown similar age-related trends in orthologs in two of the most informative genes used for *Ae. aegypti* age determination.[128] This may indicate the potential of the transcriptional age-grading method to be broadly applicable to other medically important Diptera.

Evolutionary Consequences of Strategies That Reduce Vector Longevity

Virulent *Wolbachia*, entomopathogenic fungi and densoviruses offer new and potentially effective vector control strategies that target mosquito lifespan. Given the rapid and widespread evolution of insecticide resistance, it would seem prudent to consider potential selection pressures that any of these life-shortening strategies may impose on both the pathogen and vector populations.

One concern is the possible development of resistance to these life-shortening agents by the mosquito vectors. At the present time little data is available to indicate how quickly mosquitoes might develop resistance. In the case of life-shortening *Wolbachia*, no signs of resistance have emerged in laboratory cultures of *D. melanogaster* since its initial description in 1997. It has also been suggested that mosquitoes may evolve mechanisms that limit the ability of entomopathogenic fungi to penetrate through the cuticle or replicate within the host.[132] However, to date there has been no reported cases of resistance to fungi used in insect pest

control. This is thought to be in part because fungi use several different effector molecules to attack the insect such as chitinases, proteases and toxins.[133]

Alternatively, reductions to vector lifespan may select for pathogens with faster development rates and shorter extrinsic incubation periods. Due to the limited lifespan of most disease vectors, pathogens must rapidly infect, proliferate and/or disseminate in the vector to ensure their transmission. Given this, it would be expected that most vector-borne pathogens would be under significant evolutionary pressure to maximize their ability to infect and proliferate within the vector or alternatively, adopt strategies that would increase vector survival.[134] This acceleration in development rate, within the vector, is not readily observed in natural vector-borne pathogen systems. As such, it likely that the duration of a pathogen's EIP is evolutionary constrained. Little is known about factors that may cause this limitation, however it would seem plausible that selective pressures imposed by the vector and vertebrate host may act in opposing directions. Research undertaken using murine malaria has demonstrated that higher parasite virulence can be selected relatively quickly when parasites are sequentially passaged through just a mammalian host.[135] However, selection is much less effective when parasites are alternated between mammalian and insect hosts.

Conclusion

Vector age is one of the most sensitive parameters influencing the epidemiology of vector-borne disease. Strategies that aim to reduce adult mosquito lifespan are expected to be very effective in reducing pathogen transmission. This is because vectorial capacity is most sensitive to changes in vector longevity. Entomopathogenic fungi, virulent *Wolbachia* strains and mosquito densoviruses all show the potential to modify insect vector population age structure to such a degree that significant reductions in disease transmission may result. Indeed, using these agents synergistically as part of an integrated approach reducing mosquito population age structure may yield the most effective results.

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