16

Fitness studies: developing a consensus methodology

Thomas W. Scott[1], Jason L. Rasgon[2], William C. Black IV[3], and Fred Gould[4]

Abstract

In the near future, population biologists will be increasingly called upon to assess the potential of a large number of different genetically modified mosquito (GMM) strains to reduce pathogen transmission by natural mosquito populations. Adopting a standardized methodology for GMM fitness assessment will allow researchers to compare results from different laboratories and rapidly identify constructs and GMM strains that are most likely to be of applied use in the field. In this article we provide an operational definition for fitness, review the complexity of fitness, discuss lessons that can be learned from past genetic-based mosquito control programmes, and propose a methodology for rapidly and effectively assessing the fitness of GMMs compared to wild-type mosquitoes. Fitness is best understood as success at producing offspring. Because it can vary across identical genotypes, fitness is often considered as the average contribution to succeeding generations. Herein, we refer to the *relative fitness* of GMMs because they will be compared to their wild-type counterparts. Fitness is dynamic and measuring it is complicated. It can be influenced by variation in environment and genetic background. Based on conclusions from past mosquito population reduction projects, mating competitiveness and processes by which the size of populations are regulated will be important considerations for population replacement strategies. An examination of published results from fitness assessment of three transgenic mosquito lines indicates that to avoid the effects of inbreeding and fitness depression, transgenic lines should be outbred with wild-type strains before measuring fitness, and that transgenes may not necessarily confer a fitness cost. As a methodology for assessing GMM fitness we advocate three phases of cage competition experiments, beginning in the laboratory and ending in large field enclosures. For all three we recommend introgression of transgenes into the genetic background of the proposed target field population. Control cages should be included to assess common environmental effects. Relative fitness can be estimated from the frequency of transgene genotypes in subsequent generations. In the first phase, outbred GMMs would be introduced into laboratory cages at equal frequencies with

[1] Department of Entomology, University of California, Davis, CA 95616, USA. E-mail: twscott@ucdavis.edu

[2] The W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins Malaria Research Institute, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205, USA. E-mail: jrasgon@jhsph.edu

[3] Department of Microbiology, Immunology, and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA. E-mail: wcb4@lamar.colostate.edu

[4] Department of Entomology, North Carolina State University, Raleigh, NC 27695, USA. E-mail: fred_gould@ncsu.edu

mosquitoes from the target field population. Phase two would be the same experiment, except that cages would be held at the proposed release site and GMMs would compete against mosquitoes collected directly from the field. In the third phase, mosquitoes would be released into large replicate outdoor enclosures and competed against field-collected conspecifics. The process would begin with many GMM candidate lines and end with one or very few lines that will be seriously considered for use in disease prevention.
Keywords: fitness; genetically modified mosquitoes; mosquito; genetic control; dengue; malaria

Introduction

Assessing fitness of GMMs will be a critical component of genetic programmes for control of disease vectors and prevention of vector-borne disease. It is assumed that in most cases genetic modification will incur fitness costs. This could undermine a population reduction strategy by rendering the released insect non-competitive for wild-type mates. In a population replacement approach, genetic drive mechanisms are used to spread desirable genes into a population, but if insects and their offspring with the desirable genes are less fit than wild mosquitoes, the drive mechanism may not be strong enough to offset the impact of the fitness cost and the desirable genes may be lost from the target population. A goal for both strategies, therefore, will be to minimize fitness disadvantages associated with genetic modification. The probability of a fitness advantage resulting from modification is considered low, but if it should occur it would be expected to promote success of either intervention strategy. Consequently, for the development and deployment of GMMs it is of paramount importance that the concept of fitness be fully understood and that a consensus is reached on how best to predict the fitness of GMMs relative to the wild-type mosquitoes they will be intended to eliminate or replace. Herein we (1) define the concept of fitness, (2) explain the complexities that will make measuring the fitness of released GMMs a challenge, (3) review research that highlights the importance of mosquito fitness for genetic-control strategies, and (4) propose a methodology for predicting the fitness of GMMs released into a natural environment.

Definitions

Fitness is one of the most controversial concepts in evolutionary biology. There is a large body of literature defining fitness, how it varies in different situations, and how best to measure it (Beatty 1992; Hartl and Clark 1997). Its etymology is believed to have been from Darwin's reference to survival of the fittest. Following the development of population genetics during the 1920s and 1930s the term evolved to its present form, which is "success in producing offspring, irrespective of the causes of that success" (Paul 1992). To be more fully appreciated the concept requires three important qualifications. First, because production of progeny can vary due to factors other than genotype – e.g., differential environmental effects across different individuals – fitness is often expressed as the average contribution of individuals, genotypes or alleles to the next or succeeding generations. Second, the potential for contributions to the subsequent generations are often expressed as rates of population increase. Two commonly used measures of the capacity for a population to grow are net replacement rate (R) and *per capita* instantaneous growth rate (r). R is the sum across all ages of the products of the portion of the population alive at age x (l_x) and

production of offspring at age x (m_x), such that $R = \sum l_x m_x$. Because the rate at which offspring are produced can offset the total number produced, *r* takes into account average generation time – average time from the birth of an individual and the birth of its first offspring. Third, in practice fitness often can be gauged only by comparing measures of survival, reproduction and population expansion between or among different genotypes. When this is done, it is referred to as *relative fitness* (Hartl and Clark 1997; Futuyma 1998).

Complexities of fitness

The definition provided above is possibly too simple for such a remarkably complicated and dynamic process. Definitive characterization of the causes of changes in fitness is a formidable challenge because fitness can be modified by a long list of biotic and abiotic factors, many of which are difficult to measure or disassociate empirically. Complicating issues centre on the observation that fitness can be significantly influenced by variation in environment and genetic background. Moreover, fitness is dynamic. It can change, for the same genotype, as the environment changes and as the structure of populations change.

Key components for assessing fitness are the environment in which it is measured and the number of individuals studied. A mosquito that is fit in one environment where vertebrate hosts are abundant and defenceless may be unfit in another where rare hosts repel host-seeking mosquitoes. Likewise, due to random effects, three mosquitoes with an identical genotype in the same environment may not necessarily produce equal numbers of offspring; one may be eaten by a bird, the other may take only a partial blood meal and thus produce few eggs, and the third may imbibe a full blood meal and lay a large batch of eggs. The concept of *average contribution* to the next generation addresses these random sources of variation in fitness. In other words, mosquitoes that are on average more fit in a particular environment will *tend* to do better in that setting than those that are less fit.

Due to the potentially strong and differential effects of environment on fitness of distinct mosquito genotypes, it is not justifiable to assume that relative fitness values obtained from mosquitoes studied in the laboratory can be extrapolated to the field or *vice versa* (Tabachnick 2003). Therefore, analyses in laboratory cages, in large outdoor enclosures or with colonized strains of mosquitoes may provide little insight to the relative fitness of released mosquitoes that must compete with their wild-type counterparts in a natural environment. Environment-dependent fitness differences may be weakly expressed or not expressed at all in controlled cage trials but could be strongly expressed in the field and would undermine the success of a genetic-control strategy. Similarly, in the natural environment measures of relative fitness at one site are not necessarily representative of a mosquito genotype's performance at a different location or at a different time at the same site. Sources of variation among mosquito genotypes in fitness are potentially extensive and difficult to define precisely. Examples include, but are not limited to, survival and development time of immatures, mating success, blood-feeding success, predator avoidance, adult survival, age of first reproduction, oviposition behaviour and lifetime reproduction. Each of these fitness components can be further broken down. For example, issues associated with mating behaviour could include the age when a male or female becomes sexually active or receptive, the capacity to locate mates, competition for mates, mate choice, sperm depletion and sperm utilization. These kinds of factors could act independently,

in concert or in antagonistic ways to influence an individual genotype's relative fitness.

Genetic background – all of the genes in an organism other than the transgene – of GMMs can affect their fitness in at least three ways. First, if a genotype used for transformation is substantially different from the wild-type population into which GMMs will be released, potential selective advantages or disadvantages may be due to the relative fitness of the parental genotype rather than the transgene or transformation. Second, because creation of a strain from a single transformed insect results in homozygosity of a large number of genes that are linked to the transgene, there is potential for inbreeding depression in fitness due to low fitness of one or more of the alleles in homozygous condition. Third, genes do not always function in an independent or additive fashion. When the relationship between genotype and phenotype is not additive, interactions between alleles at two or more loci can affect fitness in ways that are different from the sum of the loci considered separately (Futuyma 1998). This kind of non-additive interaction between different genes is referred to here as epistasis and is something that will need to be taken into consideration when evaluating fitness of GMMs. For example, theory predicts that a consequence of epistasis is that through time and space populations may have different responses to natural selection. Depending on the size of a population or frequency of alleles in it, populations may respond differently to selection even if they are in identical environments. Thus, relative fitness of GMMs may change as the size of the target population changes – i.e., population expansion during the rainy versus contraction during the dry season – or as allele frequencies change during the process of a transgene spreading.

Research on GMM fitness

Below we review six research projects that included assessment of components of GMM fitness. The initial three took place during the 1970s and included field releases (Curtis 1977; Reisen 2003; Lounibos 2003). Three recently published reports – 2003-2004 – concern fitness of transgenic mosquitoes (Catteruccia, Godfray and Crisanti 2003; Irvin et al. 2004; Moreira et al. 2004). There are important lessons to be learned from each of these programmes.

A large, multinational project in India was unfortunately terminated, based on totally unfounded media reports that the project was a cover for work on biological warfare agents (see Chapter 2). At the time when the project ended project scientists had already done careful evaluations of fitness of *Aedes aegypti* and *Culex pipiens fatigans* that were sterilized by chromosomal translocations (Curtis 1977; Gould and Schliekelman 2004). For both species, mating competitiveness of sterilized males with wild females was considered very adequate (Grover et al. 1976a; 1976b). A similar result was reported for a genetic-control programme with *An. albimanus* in Central America (Dame, Lowe and Williamson 1981). However, it was determined that density-dependent survival of immature *Cx. p. fatigans* could be problematic (Rajagopalan et al. 1977). Results from experimental studies indicated that depending on the time of year and the proportion of egg sterility, releasing sterile adults could free larvae from density-dependent competition and result in production of more adults than if no control was attempted.

Population reduction and replacement strategies were studied in Lahore, Pakistan for *Anopheles culicifacies* and *Cx. tritaeniorhynchus*, and in California, USA for *Cx. tarsalis* using chromosomal rearrangements, chemosterilants and irradiation (Reisen

2003). All three programmes failed because released and wild-type mosquitoes did not mate randomly with one another. In the laboratory, sterilized males were highly competitive at mating with laboratory-reared females that possessed a similar genetic background. Conversely, in the field few released males mated with wild-type females. When the cause of this disparity was investigated for *Cx. tarsalis*, it was discovered that wild-type males swarmed above the vegetation and sterilized males swarmed close to the ground where they seldom encountered wild-type females. Apparently, laboratory colonization had selected for colonized males with different swarming behaviour than most wild-type males.

Along the East coast of Kenya three release experiments were carried out with male *Ae. aegypti* that were sterilized by heterozygous or homozygous translocations (Lounibos 2003). In two experiments release of sterilized males that were derived from mosquitoes collected at the study area resulted in significant reduction in fertility but no detectable decrease in the population size of adult wild-type *Ae. aegypti*. Similar to the studies of *Cx. p. fatigans* in India, it was concluded that density-dependent larval mortality compensated for short-term reductions in fertility. The third experiment was carried out with sterilized males derived from a strain of *Ae. aegypti* from New Delhi, India. A genetic marker indicated that the released genotype had increased frequency in the egg stage but not the pupal stage. Subsequent studies indicated the low prevalence of the released exotic strain was associated with low fertility, larval development time, survival of larvae and adults, and mating competitiveness.

The first fitness assessment of a transgenic mosquito was done with *An. stephensi* carrying a fluorescent marker (Catteruccia, Godfray and Crisanti 2003). Inbred transgenic lines homozygous for a genetic marker were established in a 1:1 ratio in the same cage, with mosquitoes from a long-established laboratory colony. Interstrain crossing was permitted but not ensured. Females were allowed to blood-feed on mice to obtain the nutrients necessary to develop eggs. In two experiments the frequency of transgenic alleles fell rapidly and they were lost in 4 to 16 generations. The low relative fitness of transformed mosquitoes was attributed to the cost of transgene expression, transgene insertion in chromosomes or inbreeding depression fixation of deleterious alleles during inbreeding to establish homozygous transgenic lines. It is likely that the low fitness of the transgenic strain resulted from inbreeding rather than the transgene (see also Chapter 5).

Fitness of transgenic *An. stephensi* was examined in a life-table experiment and by cage competition (Moreira et al. 2004). Prior to conducting these two experiments, two distinct transgenes were independently introgressed into the genetic background of a non-GM strain by 16 repeated backcrosses of transgenic males with non-transgenic females from an undefined source strain. Transgenic females were fed mouse blood at undefined time intervals. Measures of fitness for one transgenic construct were not different from the source-strain control. The other construct conferred a significant fitness cost under the laboratory conditions used. It was concluded that detection of a fitness load depends on the effects of the expressed transgene and that transgenes will not necessarily confer a fitness cost.

Finally, fitness of transgenic *Ae. aegypti* was assessed using a life-table approach (Irvin et al. 2004). From mosquitoes that had been in colony since 1961, three lines homozygous for the transgenes were established and maintained for 2-3 years. Transgenic lines and the parental colony were housed separately. Mosquitoes in each cage were fed mouse blood 12-14 days after emergence. Survivorship, longevity, fecundity, sex ratio and sterility of the transgenic lines were compared with the

control laboratory colony. Life-table data were used to determine population growth parameters (*R*, *r*, generation time and doubling time). These growth parameters were significantly diminished in the transgenic mosquito lines. Fitness reduction in transgenic lines was attributed to insertional mutagenesis, detrimental expression of transgenes or inbreeding depression. Inbreeding was likely an important factor in the reduced fitness of the transgenic lines.

Recommendations for measuring fitness

The following recommendations are based on consideration of the preceding material, the applied goal of genetic vector control strategies, and the intent of developing a meaningful and practical methodology for assessing relative fitness of GMMs.

If researchers choose to carry out life-table studies, we encourage them to analyse data in a rigorous way using standard life-table parameters and rates of potential for population expansion as described by Carey (1993) and reported by Scott et al. (1997), Harrington, Edman and Scott (2001) and Irvin et al. (2004). Although a life-table approach will generate valuable data for comparing GM to wild-type mosquitoes, it may not be necessary for high-throughput assessment of relative fitness. For that purpose, we advocate competition experiments.

We foresee three phases of competition experiments involving cage populations, beginning in the laboratory and ending in field enclosures. Building upon previous experience we recommend introgression of transgenes into the genetic background of the proposed target field population. Assuming that this is successful, in the first phase, the strain bearing the introgressed transgene would be placed in equal frequencies with mosquitoes from the target field population. Control cages containing only mosquitoes from the target population or the transgenic strain can be included to assess common environmental effects. Results from these experiments would be used to eliminate transgenes with major impacts on fitness. In the second phase, the same cage experiment would be performed but this time at the proposed release site, using mosquitoes collected directly from the field. This would expose GMMs to the local environment and place them in competition with field mosquitoes. Strains that survived phase two would, in the third phase, be released into large replicate outdoor enclosures, like the ones described by Knols et al. (2003). GMMs would be released in equal frequencies with mosquitoes collected directly from the field. It is our intention that this approach will reduce the likelihood discussed earlier of generating GMMs for field release that are competitive in the laboratory but not in the field (Reisen 2003). An assumption made in this design is that a transgene that causes fitness reduction in the laboratory will also cause fitness reduction in the field. Given our early discussion of environment-dependency of fitness, it is possible that a transgene that caused fitness reduction in the lab would not cause fitness reduction in the field. However, we feel that the data from previous fitness experiments indicate that this is unlikely.

We recommend competition experiments because they are an efficient way to assess rapidly the relative fitness of different genotypes and because in any field release there is expected to be competition between the transgenic and field strains. Different parental genotypes, which can include parents from a field population, are placed in a cage and transgene frequencies are determined in subsequent generations. Understanding that genetic drift alone may cause shifts in transgene frequencies, replication becomes an essential component of this experimental design. We

recommend using a minimum of three cages for each treatment that are examined under identical conditions. In cases where a researcher wants to understand the factors leading to competitive differences between transgene-bearing and non-transgene-bearing mosquitoes it is advisable to set up control cages (GMM alone and wild-type alone) to assess environmental effects for respective genotypes. Relative fitness is estimated from the observed frequencies of transgene genotypes (transgene homozygotes, heterozygotes and wild-type homozygotes) in each subsequent generation. This approach can be used over multiple generations with randomized selection of a subset of offspring to advance to the next generation to estimate components of fitness and predict genotype frequencies of natural populations (Prout 1971a; 1971b; Manly 1985; Endler 1986). We recommend examination of at least five continuous generations. When performing multiple-generation experiments the competing strains are expected to mate with each other. In such experiments it is critical to keep track of genotypes instead of strains. In the case of work with GMMs we are mostly interested in the impact of the transgene on fitness, so monitoring the change in frequency of the transgene within competition experiments should be the major goal.

A drawback of competition experiments is that unlike life-table analyses they do not allow one to examine complex parameters such as mortality trajectories and the ways in which they differ between various genotypes. On the other hand, their advantage is that one can examine relatively large numbers of mosquitoes in replicate cages in a reasonable period of time, all life stages of the mosquitoes can be examined, and the performance of different genotypes is directly compared. One can, therefore, determine *in the defined study environment* whether the GMM strain is neutral (no frequency change), less fit (frequency decrease) or more fit (frequency increase) than wild-type mosquitoes. Another advantage of competition experiments is that in a real release there will be competition between the transgene-bearing mosquitoes and wild-type. Because relative fitness of two lines maintained separately versus in direct competition may give different results, pure-line experiments are likely to be less relevant to the field than the competition experiments. The line that does best in the absence of interstrain competition may do worst when there is interstrain competition, which will occur if mosquitoes are released into the natural environment and interact with wild-type conspecifics.

The probability of detecting relatively small fitness differences and determining when during the life history of a GMM a fitness effect is most likely occurring will be increased by examining GMMs over multiple generations and sampling different life stages. Investigators will need to justify their choice of setting up experiments having overlapping versus non-overlapping generations. In order to avoid unnatural population build-ups and crashes in overlapping-generation studies, procedures will need to be developed and justified in which some but not all of the eggs laid are used to initiate subsequent generations.

We recommend that competition experiments be done in a pragmatic and systematic way. Although interesting scientific questions may arise in the course of these kinds of studies, in our opinion the applied goal of reducing disease is not consistent with tangential research projects that would divert resources and personnel from our primary aim. In our suggested approach, fitness evaluations move progressively from the lab to the field in increasingly larger cages that are placed in settings that increasingly mimic those of the target population. We envision a process that starts with many GMM candidate lines and ends with one or very few lines that will be seriously considered for use in disease prevention. Systematic screening will

progressively eliminate GMM lines that possess inferior fitness characteristics. If a fitness deficit is detected, that line is excluded from further analysis. In most circumstances, we do not advocate trying to determine the underlying cause of lower fitness nor do we suggest trying to remedy it.

Prior to releasing GMMs purposely into a natural environment, the potential for modifying the genetic structure of endemic mosquito populations or pathogen transmission will be a critical consideration. Caution must be taken when conducting fitness studies in the field. Outdoor cages should provide proper containment to prevent accidental release of GMMs. Field releases should never be done without proper biosafety and ethical approval. In the process of obtaining that kind of approval we expect that different gene drive systems will be assigned different risks. A system like underdominance (Curtis 2003), which theoretically requires exceeding relatively high thresholds for the construct to spread, likely will be considered less of a risk than a transposable element. In theory, the latter could spread over an extensive geographic area following the escape or release of a single GMM.

Mosquito mating behaviour, which will be an essential component of any genetic-control strategy, will be another important field-related consideration. Previous research demonstrated that because GMMs can mate competitively in the laboratory does not mean they will also mate competitively with wild-type mosquitoes in the field (Reisen 2003). Field studies may need to be carried out with GMMs to insure mating competitiveness. Evaluations between marked-released GMMs and wild-type mosquitoes, like the ones described by Grover et al. (Grover et al. 1976a; 1976b) and Dame, Lowe and Williamson (1981) for population reduction strategies, may not be sufficient for a population replacement programme because they could underestimate the importance of assortative mating. If wild-type mosquitoes do not mate randomly and there is a selective advantage to avoiding GMMs, assortative mating by wild-type mosquitoes could undermine a population replacement approach over time. An outcrossing protocol like the one discussed below is a way to try to avoid GMM mating deficiencies.

The genetic background of the mosquitoes studied for fitness differences will be of paramount importance. Wild-type mosquitoes from the area where control ultimately will be directed should be used for GMM fitness studies. Laboratory colonies should be avoided because the process of colonization is expected to select for genotypes that are not representative of the target population (Reisen 2003). Colonization is a founding event in which rare alleles are lost, heterozygosity decreases (Munstermann 1994; Mukhopadhyay et al. 1997), and inbreeding depression can lead to reductions in fitness. Studies will need to be done to determine how long GMM colonies can be maintained without suffering from inbreeding depression and random genetic drift. Colony maintenance will be easier with *Ae. aegypti* than anophelines because eggs of the former can be stored for extended periods of time, whereas anopheline eggs cannot be stored. Maintaining an appropriate genetic background can be accomplished by using an outcrossing scheme like the one described by Moreira et al. (2004), except that, rather than crossing GMMs with a laboratory strain, crossing should be done with wild-type mosquitoes from the field site. Depending on the strategy used to create the GMM, this may require introgression of transgenes into a wild-type background prior to fitness assessments. This approach will avoid confounding data analysis with questions about genetic background and inbreeding depression. Analyses can instead focus more definitively on the effects of genetic modifications.

In the third phase the environment in which GMM fitness is assessed should be as close to that of the target population as is possible. This will include temperature,

relative humidity, photoperiod, feeding frequency and diet. Frequent and preferential feeding on human blood and carbohydrate versus no carbohydrate has been shown to affect the fitness of anthropophilic mosquito vectors of dengue and human malaria (Scott et al. 1997; Harrington, Edman and Scott 2001; Gary Jr and Foster 2001). Although rodent or avian hosts are more convenient sources of blood in the laboratory than humans, the chemical composition of their blood differs from that of humans. The complications of providing human blood to mosquito species that naturally imbibe it will need to be addressed.

Although technological advances have recently refocused attention on using genetic strategies to control insect disease vectors, this is not a new approach (Gould and Schliekelman 2004). Past efforts indicate that fitness will be a critical component in the success or failure of strategies employing GMMs for disease control. The adoption of a standardized, consensus methodology for GMM fitness assessment will allow researchers to compare results from different laboratories and rapidly identify constructs and GMM strains that are most likely to be of applied use in the field. Rapid and accurate assessment of GMM fitness will be a cornerstone in the development, evaluation and application of novel transgenic technologies for effective vector-borne disease prevention.

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