Redressing the sex imbalance in knowledge of vector biology

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The recent development of transgenic mosquitoes that are resistant to infection by the Plasmodium malarial parasite is a promising new tool in the fight against malaria. However, results of large-scale field releases of alternatively modified mosquitoes carried out during the 1970s and 1980s suggest that this approach could be difficult to implement in the field. These past attempts to control mosquito populations largely floundered as a result of our insufficient understanding of the behavioural ecology of released males. In spite of this, contemporary research on genetic control strategies has concentrated predominantly on molecular aspects, with little progress being made toward resolving key ecological uncertainties, male mosquito ecology being the most important. Here, we review the state of knowledge of male mosquito ecology, and highlight priorities for further research. Case studies of two crop pests, the Mediterranean fruit fly and melon fly, are given as examples of how knowledge of male ecology facilitates successful control in other species. Unless similar information becomes available for mosquitoes, any future genetic control strategy will risk failure.

Know thine enemy?
The most deadly member of the animal kingdom is not one with sharp claws, razor-sharp teeth, or a venomous bite. By far, the one responsible for the largest loss of human life is the Anopheles mosquito that transmits malaria (Plasmodium sp.). Each year, the infected bite of these mosquitoes kills 2–3 million people, and causes illness in many millions more [1]. Neither is the situation improving: since the early 1990s, the proportion of the world’s population exposed to malaria has increased by a quarter [2], and rates of malaria mortality in children living in East and Southern Africa have doubled [3]. Increasing incidence of insecticide and drug resistance might explain the deterioration of the situation [4,5], in addition to lack of funds and infrastructure to implement effectively robust control techniques, such as bed nets [6] and environmental management [7]. Clearly there is a need for new tools, and a refinement of existing ones, if the situation is to be improved.

One promising development is the recent creation of transgenic mosquitoes that are refractory (resistant) to infection by malaria parasites [8,9]. This innovation has bolstered hopes that Plasmodium transmission could be reduced by replacing parasite-susceptible vectors with genetically modified ones (GM control). Many scientific, logistical and ethical issues must be resolved before this strategy can be field tested [10,11]. However, the most substantial scientific barrier to progress is one that should have been addressed 20 years ago: an almost complete lack of understanding of the factors that govern Anopheles mating biology and male fitness. As we show here, this is of fundamental importance to the implementation of a transgenic-based control programme. The disastrous consequences of ignoring the details of male ecology and mating were vividly illustrated by the failure of field trials during the 1960s and 1970s. These trials involved the mass release of sterilized males of a variety of mosquito vector species in a bid to reduce the frequency of viable matings and thus ultimately population size. This outcome was rarely achieved, however, because of the poor mating ability of released males.

Disappointingly, little progress has been made in this area since the field trials of the 1960s and 1970s. Paradoxically, we have made substantially more headway in understanding the reproductive biology of species with no direct public health or economic importance, such as Drosophila, fur seals Arctocephalus spp. and blue tits Parus caeruleus, than we have done for this vector that kills millions. GM control attempts for malaria will undoubtedly flounder unless this knowledge deficit is remedied.

Genetic control of disease vectors: the importance of fitness
The idea that vectors and/or their disease transmission potential could be eliminated through genetic manipulation dates back to the 1940s [12]. Here, we define genetic manipulation as any strategy involving the release of insects that have been modified so that the genetic material that they pass on reduces disease transmission by either: (i) making offspring resistant to infection; or...
(ii) rendering females infertile. Under this definition, genetic manipulation encompasses current methods of introducing malaria-refractory genes, and earlier methods of inducing sterility by chemicals, radiation, or translocations that impede zygote formation Table 1. The success of both approaches relies crucially on the ability of modified individuals to live long enough to mate, locate a partner and successfully compete for them in the presence of wild types; traits we collectively refer to here as ‘fitness’.

Some argue that even if GM mosquitoes do have lower fitness than the wild type, the refractory genes that they carry will still spread as long as they are linked to an efficient genetic drive mechanism [13]. Genetic drive will increase the rate of gene invasion, but only if insemination occurs in the first place; and this is fundamentally linked to mosquito ecology. Furthermore, given that no appropriate drive mechanism has yet been identified, and that the ability to link an immune effector gene tightly to a drive mechanism is in question [14,15], the importance of mosquito ecology to GM control remains paramount.

Why focus on males?
It is the fitness of male Anopheles that will prove most important to GM control. This is because releases of transgenic and/or sterile mosquitoes must necessarily be restricted to this sex, as releasing females could increase the transmission of other pathogens (e.g. filariasis), and incur an additional biting nuisance that would compromise community acceptance. Studying anopheline mating behaviour alone is not sufficient, because additional knowledge of the ecological factors that mediate male longevity, energetic resources, dispersal, habitat use and predation risk are also required to predict whether mating opportunities will arise in the first place. Unfortunately, our base-line knowledge of male biology is poor, especially in comparison to what we know about female mosquitoes. Whereas most of the ecological factors listed above have been thoroughly investigated in free-living females [16,17], they remain unexplored in males. We do not even possess tools to sample free-living Anopheles males, never mind discern key life-history parameters, such as age and reproductive history, as is common practice with females (but see [18,19] for exceptions). Whereas detailed profiles of female Anopheles age structure, survival and fecundity under field conditions abound (e.g. [20–23]), we do not yet have a good understanding of where adult male mosquitoes live. This knowledge imbalance must be remedied if we are to make progress in the fields of GM research. Study of both GM and wild-type males will be required, as no minimum standards for the quality of GM males can be set without consideration of wild-type males.

Can GM mosquitoes compete in nature? Lessons from the past
Ecologists have reacted with cautious optimism to the development of GM mosquitoes, stressing the need for investigation of their competitiveness relative to wild-type conspecifics [24]. Consequently, three studies have recently been published that investigate the fitness of transgenic mosquitoes under laboratory conditions [25–27]. This work is certainly valuable, but is not the only source of information regarding the fitness of modified mosquitoes. Substantial research has been conducted on the effects of early types of genetic manipulation based on inducing sterility; including large-scale field releases coupled with detailed behavioural and ecological research (Table 1 [12]).

Information provided by these early studies is often ignored in contemporary discussions of transgenic mosquitoes. Indeed, only one of three recent studies examining the fitness of transgenic mosquitoes made any reference to this past work [26]. There is no a priori reason to believe that the fitness loss caused by transgene insertion should be fundamentally greater than that imposed by other kinds of modification, particularly because all modified mosquitoes – regardless of mechanism – need to be mass reared before release (and thus be exposed to the fitness costs that such mass rearing might imbue). These fitness costs could include the production of adults with small body and a correlated reduction in survival and mating success that frequently occurs under the high-density rearing conditions used in laboratories [28], or males that are unable to find wild-type females because they have been entrained on an artificial photoperiod in the laboratory that causes them to swarm at an inappropriate time [29]. Thus, reviewing the outcome of these field trials of sterile insects (SIT) can

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Table 1. Summary of defining characteristics of the two main methods of mosquito genetic control discussed in this article

<table>
<thead>
<tr>
<th>Basis</th>
<th>GM Control</th>
<th>Sterile insect technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aim</td>
<td>Passing on of genetic material that makes offspring resistant to parasites</td>
<td>Passing on of genetic material that makes offspring nonviable</td>
</tr>
<tr>
<td>Mode of action on malaria transmission</td>
<td>Mosquito population replacement</td>
<td>Mosquito population reduction and/or eradication</td>
</tr>
<tr>
<td>Release requirements</td>
<td>Direct: the probability of parasites infecting a mosquito is cut (mosquitoes are immune)</td>
<td>Indirect: the probability of parasites contacting a mosquito is cut (mosquitoes are less prevalent)</td>
</tr>
<tr>
<td>Evaluation of fitness consequences</td>
<td>A few releases could generate self-reinforcing spread of resistant genes through a population</td>
<td>Continual release of sterile males is required until the population is eradicated</td>
</tr>
<tr>
<td>Influence of procedure on male fitness</td>
<td>Lab studies of caged mosquitoes onlya</td>
<td>Laboratoryb, enclosed fieldc and field releasesd</td>
</tr>
</tbody>
</table>

aSee [27–29].
bSee [40,87].
cSee [88].
dSee [36,37,39].
eSee [21–23].
fSee [12,32–36].
provide valuable insight into the probable fate of GM mosquitoes on field release. In 2003, Benedict and Robinson [12] reviewed field trials that used SIT to reduce or eliminate populations of mosquito vectors. Of the studies that they detail, most were conducted during the 1970s. The near cessation of SIT-based control efforts during the past 20 years can be attributed to the limited success of these early trials [12].

Fifteen of the 27 field trials reviewed had the specific aim of reducing or eradicating a mosquito population [12]. Of these, only three could be categorized as successes [30–32], with the most common reason for failure in the others being the poor mating competitiveness of sterilized males compared with their wild-type counterparts. We define mating competitiveness here as the ability of sexually mature males to inseminate females on a given night relative to wild-type males.

Overwhelmingly, sterile males that were deemed to be competitive in the laboratory were feeble on field release. For example, although chemosterilized Aedes aegypti had approximately equal competitiveness to laboratory controls [33–35], they were almost ten times less likely to get a mate than were the wild type on field release in Kenya [36]. Similarly, both Culex with sterility-inducing translocations and chemosterilized Anopheles had equal or greater competitiveness compared with laboratory-reared controls, but competed poorly in the wild [37,38]. Here, poor competitiveness was possibly a consequence of mass rearing instead of the sterilization procedure itself, because it was evident only when modified males were compared with wild mosquitoes, but not to similarly reared laboratory controls. However, this observation is far from conclusive, and further research is required to separate the mosquito fitness consequences of mass rearing from those associated with the sterilization or transgenesis procedures themselves. Inbreeding depression combined with the loss of genetic diversity resulting from bottlenecks arising from the colonization process could explain why mass rearing could reduce mating competitiveness, as could the dietary and climatic conditions of the insectary.

This reduced mating competitiveness was repeatedly found to be a product of the rapid appearance of assortative mating under laboratory conditions; a phenomenon that can arise within just three generations of colonization [37]. For example, whereas both Culex tarsalis and Cx. tritaeniorhynchus sterile males were ‘supercompetitive’ for laboratory-reared control females, they failed to inseminate wild-type females because they preferred to seek out laboratory-reared female counterparts that were released with them [37]. If such assortative mating behaviour were to develop in GM mosquitoes, introduction of refractory genes would be difficult even if male competitiveness under laboratory conditions was high.

In addition to mating competitiveness, mass rearing and/or genetic modification is known to influence mosquito survival. Mass-reared sterilized Aedes aegypti and Cx. fatigans both had low survival on field release in addition to being uncompetitive for mates [39,40]. Thus, longevity as well as suboptimal environmental conditions at the release site [12,37] also contributed to the failure of early release trials. However, the ubiquity of poor mating competitiveness across studies suggests that this will be the primary challenge for future genetic control efforts.

Given that the primary stumbling blocks to early genetic control attempts (reduced mating competitiveness, survival and assortative mating of modified males) have been known for 25 years, one might expect this research area to have boomed. Although it is difficult to quantify objectively the pace of research, a telling indicator comes from analysis of a Web of Science literature search (http://wos.mimas.ac.uk) under the term ‘Anopheles gambiae’ – the most high profile target of transgenic modification (Figure 1). Of the 902 directly relevant records to come up under this term from 1980 to July 2004, only 19 were specifically focussed on any aspect of male mosquito ecology or mating biology (Figure 1). Furthermore, of those 19 studies, only five were conducted under field conditions [41–45].

Thus, in spite of the long-standing and fundamental need for more studies of male reproductive fitness, entomologists and/or funding agencies both seem to have avoided this area. Regardless of the reason for this, it is clear that we have wasted an enormous opportunity. Now, instead of being able to transfer transgenic technology confidently into field-ready implementation plans, the enterprise must be halted as vital data on male biology and gene flow are collected.

The importance of male biology: case studies of control success stories

We have highlighted the low success rate of early genetic mosquito control trials on account of the complexity of male ecology, but can the hurdles posed by male mating behaviour be overcome? Two examples from agriculture provide reason for optimism: in both, a small amount of information about male biology and mating behaviour had a radical impact on control. The first of these, the Mediterranean fruit fly Ceratitis capitata, is a global scourge on citrus plants. It has been successfully controlled by the mass release of sterile males in many countries, including Chile, Costa Rica, Guatemala, Peru and the USA. Hand-in-hand with control, much field and laboratory research has been conducted on the determinants of male fruit fly reproductive fitness, including its dependency on age, body size [46], genotype [47,48], diet [49], and pheromones [50]. Mass-reared sterile C. capitata males generally have poorer mating competitiveness than do the wild type [51], but this research identified two simple methods to minimize this problem. The first was to provide lab-reared males with a protein-rich diet before release [49]. A second was that flies exposed to ginger root oil, a substance with mate attractant properties, had greatly enhanced mating success. When ginger root oil was applied to lab-reared irradiated males, they out-competed the wild type on 75% of occasions [50].

A second example is the melon fly Bactrocera cucurbitae, another fruit pest that became established on several Japanese islands during the past century. In 1972, the Japanese Government initiated an intensive SIT campaign to eradicate the fly (reviewed in [52]). Unlike the fruit fly, sterile male melon flies had equal or greater
mating competitiveness compared with the wild type [52], a trait that should have ensured control success. However, 20 years after the onset of this programme, wild-type females on one island began to avoid sterile males [53,54], possibly as a consequence of behavioural evolution. This development had the possibility of not only eradicating the efficacy of control in Japan, but also spreading a ‘sterile-insect-resistant’ genotype of melon fly worldwide. However, because studies of the mating ability of sterile males were ongoing, this change was detected rapidly. In response, scientists increased the number of sterile males released into the area, causing rapid destabilization of the population. The melon fly elimination programme in Japan was successfully concluded in 1993, a feat that could not have been achieved without careful monitoring of the mating biology of males both before and during the control programme [52]. If we can achieve this success with insects that consume agricultural produce, there is no reason why we cannot also do so for insect vectors of human and animal disease.

Determinants of male mosquito reproductive success and their relevance to GM control
The above examples illustrate the importance of having good background knowledge of male fitness determinants to facilitate genetic control. If we are to follow a similar model with mosquito vectors, a useful starting point would be the compilation of pre-existing knowledge on mating success, as well as documentation of what uncertainties remain. To begin, we briefly review the biology of mosquito mating.

Reproductive physiology
The reproductive system of male mosquitoes consists of two testes connected to a pair of accessory glands and an ejaculatory duct [18]. Fluid in the accessory glands transports sperm and, in some species, it also influences the tendency of recipient inseminated females to remate, oviposit and blood feed [55–59]. Accessory gland fluid is also used to form a mating plug in anopheline mosquitoes, a physical barrier implanted in the female genitalia to prevent further copulation at least temporarily [60]. With repeated mating, seminal and accessory gland fluid can become depleted [61]. No recovery is possible in some mosquito species, whereas in others, fluid can be regenerated after a few days rest [18,61–63]. Little is known about how variation in the quantity, quality and regeneration cycle of seminal fluid influences male lifetime reproductive success. However, An. gambiae males with small accessory glands are less likely to induce monogamy in their mates than are those with larger glands [64]. Thus, for practical control purposes, researchers should examine how accessory gland size varies between transgenic and wild-type males, and test for differences in their ability to render females monogamous.

Mating system
In most mosquito species, mating occurs in swarms [65], with groups of up to several hundred males aggregating in flight over a stable visual marker around dusk [65–68]. Females are drawn to these swarms, although the mechanism of attraction is unknown. The cue is unlikely to be male flight tone, but perhaps is a common response to either the visual markers males use for swarming [65] or pheromone signals [69]. As a female flies through the swarm, males use her flight tone [70], and possibly also pheromones, to confirm whether she is the correct species and is receptive (i.e. uninseminated) [69,71]. Just as the application of ginger oil to sterilized fruit flies improved their mating competitiveness, identification of key male Anopheles mate attraction pheromones could greatly enhance the reproductive success of transgenics in the field [50].

To initiate copulation, a male uses its tarsal claws to grab the female, before adopting an end-to-end position
Other factors influencing reproductive success

Generally, female mosquitoes mate only once, using sperm from the first insemination to fertilize subsequent egg batches [55]. Field investigations suggest that some males mate many times during their lifetime, and others not at all [18]. The extent of this skew is unknown, but one study of An. culicifacies found that only 15% of mature males mated in one night [38]. Several intrinsic and extrinsic factors have been postulated as determining the probability and number of successful inseminations, but few have been tested.

One correlate of lifetime reproductive success could be survival, because studies across and within mosquito species suggest that males producing the greatest number of offspring have the longest life spans [18,63]. This could arise either because male lifetime reproductive fitness is more dependent on the number of mating opportunities than on success in any one mating attempt, or because traits that confer success in a swarm also promote longevity. Regardless of the reason, it suggests that, to increase the rate of refractory gene introduction, transgenics with good competitive ability within a swarm and also with good survival are required. Survival is also important in that it determines whether males will reach their peak reproductive age. Many studies have found that male insemination success varies with age [60–63,77], although a universal optimum has not been identified. Were this to be found, releasing transgenic males of only this age class could ensure a greater reproductive return from each released individual, and thus minimize the number of releases required.

Genetic factors might also mediate male fitness: males carrying insecticide-resistance genes have lower mating competitiveness than do wild-type males [78,79]. This implies that the proposal to increase the fitness of transgenic mosquitoes by endowing them with such genes [80] could backfire, especially in areas of low insecticide use. With respect to environmental factors, larval and adult nutrition might both influence male reproductive success. Starved female Aedes were less likely to produce eggs after mating with males starved as adults than they were after mating with males maintained on an ad libitum diet [81]. Larval nutrition might also influence male mating success because, among other things, it determines their emergence time [82]. Male emergence time, at least relative to that of competing males and females, influences their lifetime reproductive success [83]. Understanding how the larval nutrition of mass-reared mosquitoes could be managed to produce males with optimal development and body size would thus be useful for GM programmes.

Promisingly, many of the potential determinants of male mosquito reproductive success discussed here are heritable (e.g. development time, survival [84] and insecticide resistance [85]), suggesting that desirable traits could be selected for under laboratory conditions before the release of modified males. In addition to these published determinants of male reproductive success, numerous others have yet to be investigated. These include dispersal, pheromones, resource allocation, larval conditions, sperm quality (e.g. protein content, density, size polymorphism and utility), maternal and/or paternal effects, and female mate choice. The experiments required to address these are neither complex nor expensive, and could provide a wealth of useful information to guide renewed attempts at genetic control of vectors.

Priorities for future research

Of the biological uncertainties discussed here, which should be prioritized for research? All areas of male mating behaviour and ecology could yield useful information. However, those pertaining to mate selection, particularly the tendency to mate assortatively, are key, given their role in the failure of previous mosquito control attempts. Also vital are studies of the environmental and genetic determinants of male survival, dispersal and mating success, of the importance of male accessory gland substances and sperm competition to offspring fitness, and of the role of female traits in attracting males. Finally, we stress that studies of male mosquitoes should include both static (ecological) and dynamic (evolutionary) components of reproductive fitness. The appearance of female melon flies that avoided sterile males indicates that behaviour can evolve within the course of a control programme, and could radically alter the outcome as predicted by short-term ecological studies.

Conclusions

Transgenic mosquitoes that are refractory to malaria or other vector-borne diseases represent a promising new opportunity for the control of infectious disease. However, in pursuing this enterprise, it is crucial to take stock of the lessons from previous control trials using mosquitoes modified by different means (Box 1). The substantial body of work conducted during the 1970s and 1980s indicates that mass rearing of mosquitoes generally reduces mating competitiveness. This problem is likely to blight future attempts, and can only be overcome by renewed attempts to study male mating biology and ecology; fields of research that have all but disappeared during the past 25 years. The question of why medical entomologists have been slow to take up this challenge in contrast to their counterparts working in agriculture control looms large. One possibility is the relative paucity of public funds available for vector-borne disease research in contrast to those funds generated by the agricultural industry. Behavioural ecology research, however, is relatively
Box 1. Key lessons pertaining to male mosquito ecology and mating biology learned from field-release trials of sterile mosquitoes

- Sterilized, laboratory-reared males generally have lower mating competitiveness, and sometimes lower survival, than do wild-type males [12,34–38].
- Males found to have a strong mating ability under laboratory conditions often compete poorly in the field [12,36–40].
- The process of mass rearing is likely to reduce mosquito fitness, in addition to any further effects arising from the specific modification procedure (e.g. sterilization by chemicals or radiation). This is based on the fact that sterilized males frequently had equal fitness when compared with unmodified control males reared under the same laboratory conditions [33,34], and showed reduced mating competitiveness only in comparison with non-laboratory reared, wild-type males [37,40].
- The failure of laboratory-reared males to inseminate wild-type females was most frequently explained by the development of assortative mating behaviour under laboratory conditions [37,38].
- Colonization can lead to the rapid evolution of mosquito mating behaviour; with the ability to mate successfully with wild-type females being lost in as little as three generations of laboratory maintenance [37].

cheap and should not be restricted to the domain of cash crops. Indeed, increasing knowledge of male mosquito ecology would cost very little. Ten field-based MSc students from malaria endemic regions working simultaneously on behavioural and ecological aspects of male vectors could easily quadruple our knowledge of this area within a few years. Furthermore, the combined cost of their training and resources would probably be < US$50,000; a small portion of the total spent on transgenic research every year. Indeed, were a dedicated funding scheme to be established for this enterprise, it would both greatly increase the likelihood of successfully implementing a transgenic programme and increase the research capacity for medical entomology within the developing world [11].

Rather than downplaying the accomplishments made by molecular biologists in achieving transgenic disease vectors, our article is an open invitation to researchers to join the pursuit of fundamental determinants of male reproductive success, both at the genetic and molecular level. For example, if a transgenic could be made with both a gene for pathogen refractoriness and high mating competitiveness, the efficacy of control would be substantially enhanced. With this approach, it is possible that the problems of the past could be avoided and new successes obtained.

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