Introduction to development of internationally acceptable guidance principles for testing and deployment of GM mosquitoes

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Introduction

Genetically modified (GM) mosquitoes are currently being developed for use in vector control related to human diseases, such as malaria and dengue, under individual institutional or national guidelines on research and biosafety. There is a lack of directed international guidance, however, on the development, testing and ultimate deployment of such GM mosquitoes. In this current situation, uncertainty over what practices are widely acceptable may cause delay in technical progress, may deter some investment in the technology, and may cause some national regulators to demand diverse and irrelevant data or to impose excessive safeguards. There is also a possibility that experimentation could proceed with inadequate safety in some countries with weaker regulatory systems, under pressure to act in the face of rising impacts from disease. While we may look for lessons learned, guidance aimed at GM technologies in general – historically drafted in the context of GM crops intended for consumption – are not universally applicable to the particular technologies or circumstances of GM mosquitoes. The potential application of GM mosquito technologies across the wide range of disease endemic countries suggests that a broad consensus on the regulatory approaches taken at each stage of testing and deployment ultimately will reduce the burden on national resources and address concerns of cross-border effects from intentional release of living modified organisms. WHO/TDR has recognised this need and funds a project (MosqGuide, www.mosqguide.org.uk) to present best-practice guidance as a step in establishing this consensus.

MosqGuide has partners in Asia, Africa, Europe and Latin America working in consultation with national and international groups involved in technical development, testing and guidance on best practices or regulation. The project is designed in modules related to sequential stages of bringing GM mosquito technologies from laboratory to field. Module 1 provides an overview of current technologies and ethical, social and legal issues that must be addressed. Module 2 focuses on research and production issues. Module 3 concerns pre-deployment country decisions. Module 4 is about field release data handling and environmental monitoring. Module 5 comprises a pilot field study of public and regulator attitudes about alternative control methods, including GM technologies. Module 6 interfaces with capacity-building curricula. Finally, Module 7 is a prototype issues/response tool to support national or regional decision-making in disease endemic areas regarding deployment of GM mosquitoes.

What guidance, by whom?

Users of GM mosquito technologies would benefit from guidance, guidelines, or standards for the full range of activities needed to bring the technologies into use, such as: laboratory practices and cage trials; site selection for field trials and first open releases; production and transport of GM mosquitoes; and operational and environmental monitoring in deployment. Angulo and Gilna (2008) suggest three objectives (in the context of self-dispersing GM insects): consistent problem definition, analysis and decision making; minimal conditions for "wise use"; and compliance instruments.

Efficiency and understanding would be helped if common formats are adopted for risk analyses, and particularly for communication of risk regarding GM mosquito technologies. Such formats are agreed internationally in plant health, animal health and some areas of human health. Some detailed international standards exist now for similar scenarios, such as the release of biological control agents and other beneficial organisms (IPPC, 2005), confined field release of GM insects (NAPPO, 2007), and quality control and shipment of mass-reared sterile insects (FAO/IAEA/USDA, 2003). Although focusing on plant and animal pests, these provide useful cases of current, widely accepted approaches to the issues and models for the process of reaching agreement. The Organisation for Economic Cooperation and Development has recently published a guide on preparation of consensus documents related to biotechnology (OECD, 2008). There may be a need to provide a comprehensive set of internationally accepted biological information on the particular species and the technologies for GM mosquitoes.

Language to describe uncertainty and conclusions based on uncertainty has been carefully described by the Intergovernmental Panel on Climate Change (IPCC, 2005). This covers sources of uncertainty, the use of expert judgement, the precision and calibration of terms, and the quantification of confidence and likelihoods. National frameworks also provide useful examples. The Australia/New Zealand Risk Management Standard 4360 (Standards Australia, 2004) is helpful in providing guidance on consistent qualitative descriptions of likelihoods

and consequences in risk analysis. The New Zealand Environmental Risk Management Agency (www.ermanz.govt.nz) applies consistent procedures across the range of potential new organism introductions it judges. The Great Britain Non-Native Species Risk Analysis Panel (www.nonnativespecies.org) uses a model risk framework which translates into easily-communicated graphics for managers on risk and uncertainty.

The complexity of risks potentially posed by GM mosquito technologies makes it difficult to put responsibility for guidance onto a single agency. Concerns include both environmental and health impacts and apply to both the Convention on Biological Diversity (www.cbd.int), particularly through the Cartegena Protocol on Biosafety (www.cbd.int/biosafety/), and WHO (www.who.int). Intensive consideration by the CBD on the single issue of transboundary movement of GM organisms (CBD, 2006) indicated a need for broad coordination of many standard setting agencies, and this is also likely in a comprehensive approach to guidance on GM mosquitoes. In the end, however, decisions regarding risks must be taken in the context of possible health benefits.

Estimates on the cost of preparing technical, international standards in plant protection started at nearly US$200,000 for more conceptual standards (IPPC, 2007), with particularly controversial or complex standards costing up to US$3 million. A recent standard on a previously unaddressed issue (wood packaging material, ISPM 15) involved at least 1,700 man-days of effort to reach agreement among the approximately 170 contracting parties (IPPC, 2007). Attempts to agree on common understanding of Social Responsibility have taken nearly a decade and will result next year in a guidance document (ISO/CD 26000) from the International Organisation for Standardization (www.iso.org), one of the few standard setting bodies to require broader stakeholder inclusion.

Despite these costs, international guidance saves the burden of similar efforts on the level of individual countries, while allowing adaptation to national realities. Implementation of international guidance can require significant national documentation in support of testing, production and deployment of GM mosquitoes. This need not be costly for the countries involved, however. Fees for approval of introductions of GM organisms into containment in NZ, which has a fairly vigorous regulatory cost recovery policy, are approximately US$6,500 and for field testing US$23,000 (current fees from ERMA New Zealand,www.ermanz.govt.nz). The cost of documentation and hearings to applicants would be substantially greater than the regulatory fees.

Conclusion

The application of GM mosquito technologies would benefit from a comprehensive set of international guidance or standards. While there are precedents among current standards for similar processes, there are also divergent approaches which would need to be rationalised. The complexity of the issues involved suggests that guidance will need to be coordinated among a wide range of specialist technical and standard setting agencies, rather than emanating from a single agency. This may add to the time and cost of guidance and present conceptual differences in approaches to risk, but will save time and resources in the disease endemic countries where decisions on deployment ultimately will be made.

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WHO Technical Consultation on use of GM mosquitoes to inhibit disease transmission

Jim Lavery

Thoughts on relationships with host communities for caged and open field release trials

A central theme in the history of research involving human subjects is the concern that researchers will enlist or use individuals in order to answer scientific questions without adequate safeguards to ensure that their participation is both voluntary and reasonable in terms of any potential harms or potential benefits.

Although common in the history of anthropology and other social sciences, the concept of community engagement has been slow to find traction in the biomedical sciences. But as advances in genetic technologies made it possible, and increasingly efficient, to characterize whole subsets of a population as "at risk" for various types of cancer, or to be a particularly important reservoir for infectious disease, these communities have suffered very focused discrimination and stigma. The biomedical research community has been forced to re-think the ethical and social significance of interacting with communities in research.

Guidelines for the ethical conduct of research are evolving and have begun to reflect greater attention to the interests of communities and the corollary obligations of investigators and research sponsors. But even with this increasing interest, we have not yet developed adequate accounts of which community engagement practices are necessary, under what circumstances, and why.

Genetic and biologically modified mosquitoes raise a wide range of questions and concerns in communities in which caged field trials or open-release trials are being conducted or contemplated. At a fundamental level, it is not clear even what precise harms might befall communities as a result of hosting such trials. Similarly, although we have well-honed (though often questionably effective) procedures to limit harm for individuals engaged in research (e.g., informed consent), we are at the very early stages of determining what procedures might be most useful, effective, and appropriate for protecting communities participating in these trials, or even for gauging their willingness to participate in the first place.

My comments at the meeting will focus largely on our growing experience with community engagement in a wide variety of research contexts around the world and some preliminary thoughts about what might constitute effective community engagement in research involving modified mosquitoes. An initial framework for community engagement that a number of us developed and proposed as a starting place for community engagement activities related to caged field trials of GM mosquitoes in southern Mexico, has informed the continued evolution of the framework.

The key dimensions of the framework are (a) an explicit set of procedures or operating principles that provide a general architecture for how community enagement might be approached in practice (the rows of the framework); and (b) an explicit set of ethical commitments or principles that provide the underlying rationale for why various approaches might be considered "good" or "effective".

The framework itself is not the end product. Instead, it serves as an evolving "theory" of the effectiveness of community engagement and provides a convenient platform for collecting and analyzing a wide range of empirical insights from a range of research contexts, not limited to GM mosquitoes.

A key interest for our research group at the moment is how we might build partnerships to allow our emerging framework to serve as a "collaboratory", an open-source, interactive resource that will allow the on-going consolidation of insights about community engagement in global health in a way that will permit progress in our understanding.

Our ultimate aim is to develop an approach to community engagement that is equally valuable to investigators and communities and that lays out a shared set of commitments that can then serve as the basis for negotiations and specific decisions about initiating trials.

WHO meeting
4-6 May 2009
Geneva

Formulating an ecological risk assessment
David A. Andow

You will be hearing from the other speakers in this session of two contrasting approaches to ecological risk assessment of genetically engineered insects. These approaches may be useful models for some countries considering the introduction of GM mosquitoes, but other countries may find that their circumstances to be different. Hence there is a need to consider the ecological risk assessment (ERA) problem from general principles.

The most challenging aspect of ecological risk assessment is the formulation of problem. It is more of an art than a science. For GM mosquitoes, the main issue has been identifying potential harms or adverse effects on which to focus the ERA. Without this focus, one is left with a rather unsatisfying effort of trying to assess some kind of "average" effect on "biodiversity", which even if it could be measured well, is of questionable significance. In technical terms, this has been called a hazard assessment or a safety assessment.

Another challenging aspect of the problem is that almost no one likes mosquitoes. This creates a challenge because most people are willing to simply dismiss the possibility that harm could come from genetically changing or eliminating some of them. In addition, the diseases that they transmit are so devastating to human populations that we often simplify the problem to either them or us. But ecological systems are not Manichean. Species are not either good or bad; they are some blend, even when they are mosquitoes. In some ways, if we can address ecological risks of mosquitoes in the deft way demanded by ecological systems, then we will have a model for much less demanding introductions, be they exotic species or some other genetically engineered animal.

I have no easy suggestions.

The goal is to identify possible risk hypotheses. A risk hypothesis is a causal chain connecting a stressor to an endpoint. Here the stressor includes the processes of producing and releasing GM mosquitoes as well as the released mosquito itself. In other words, concomitant alterations of infrastructure and management should be considered in addition to the mosquito as a possible source of harm.

An assessment endpoint is a technical term, but to oversimplify a bit, it can be thought of as the bad thing that could happen. Change per se, it not bad. It is bad because the change runs against a social or cultural norm, or because a large group of people agree that it is bad. Bad is a reflection of human values. Consequently, in controversial cases, these values should be made clear, and the people who hew to them should be identified. As an aside, it is possible to introduce notions of environmental justice at this point.

For GM mosquitoes, there is one guidepost by which to organize the search for risk hypotheses. The kinds of possible adverse effects are known. These are adverse effects on biological diversity, adverse consequences of gene flow, and adverse environmentally mediated effects on human health. However, even this has not been that enlightening, because it is easy to get stuck simply repeating the category without being able to progress to specifics.

There are several ways to move to the specifics. Hayes et al. (2004, Env. Biosafe. Res. 3:109) illustrate a hierarchical holographic model. I will discuss two simpler approaches. The first is fault tree analysis. Fault tree analysis starts by listing all possible harms, whether they are caused by the stressor or not. The exercise is to work backwards up the causal chain, from the harm, and at each step inquire if it is possible that the stressor connects. Fault tree analysis comes hand-in-hand with event tree analysis, which starts with the stressor and tries to connect causal chains to a harm of any kind. Most of the published inquiry on the ecological risks of GM mosquitoes has implicitly taken the event tree approach.

To conduct a fault tree analysis, harms must be identified first. Sometimes we may lack the originality to imagine harms, and sometimes our imagination is overly fertile and there are too many to address. There are many ways to address the second possibility, using transparent, expert-driven, qualitative prioritization processes to limit the numbers. For GM mosquitoes, the first possibility seems more likely.

Another way to move to specifics is through a stakeholder process. I find the Mitchell et al. (1997, Acad. Manage. Rev. 22, 298) typology of stakeholders to be quite useful for thinking about selecting stakeholders. Once a group of stakeholders is identified, they can be asked to describe their environmental values. Within these descriptions are characterizations of harm. Fortunately this kind of work has already been initiated for GM mosquitoes, although it is called an ethical or social issue at this meeting.

If we can specify significant environmental harms, it will be possible to conduct fault tree analyses to generate possible risk hypotheses. The existence of a risk hypothesis does not imply that it is a significant causal pathway. Indeed, even a demonstrably significant pathway does not imply the risk is unacceptable. Although there is some distance between the risk hypothesis and a decision, without clear, specific risk hypotheses, a risk assessment is no better than watching leaves blow in the wind.

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1. Driving Y chromosomes. A driving Y chromosome could be used by itself to produce a male-baised sex ratio in a self-sustaining manner. Alternatively, a driving Y could be used in conjunction with a resistant X chromosome -- release of both would lead first to the spread of the driving Y, and then to spread of the resistant X. Any transgene linked to the resistant locus (e.g., a gene that blocks parasite development in the mosquito) would then spread as well. The data from Windbichler et al. (2008) showing over-transmission of the Y-chromosome due to PpoI-mediated cleavage of the X chromosome suggests that a driving Y chromosome could be made by putting the B2-PpoI construct on the Y chromosome.

2. Homing. Homing endonuclease genes (HEGs) are drive systems that occur naturally in fungi and other microbes. They bias their own transmission to the next generation by encoding an endonuclease that specifically cleaves chromosomes where they are not present; they then get copied across to the cut chromosome as a by-product of the repair process. A gene drive systems in mosquitoes that was based on homing could be used in a number of ways, including (i) knocking-out female fertility genes, thereby imposing a genetic load on the population and reducing the numbers of mosquitoes; (ii) knocking-out a gene needed for malaria transmission; or (iii) knocking-in a gene that disrupts malaria transmission. Recently we have put the SceI homing endonuclease gene from yeast and its recognition site into *Anopheles* to test whether homing would occur. We have found strong over-transmission of the SceI HEG, and confirmed with molecular data that at least some of this over-transmission is due to classical recombinational-repair mediated homing.

Large indoor trials of genetically engineered mosquitoes may be useful before outdoor field releases, and we are currently developing facilities for conducting such experiments in Italy.

Conventional Sterile Insect Technique against *Anopheles arabiensis*

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Project description

This project uses conventional sterile insect technique (SIT) against the mosquito *Anopheles arabiensis*. It is composed of two parts, technology development and field feasibility studies. The IAEA's role is primarily in technology development, but it also plays a supporting role assisting the national efforts in their field feasibility studies.

Two field sites are being studied for the initial feasibility studies: Nile valley in the Northern State of Sudan and La Reunion island. In both instances, *A. arabiensis* is the sole malaria vector.

Current status

The technological aspects include four components: sex separation methods, sexual sterilization using gamma irradiation, mass production and release methods. We expect to have at least rudimentary methods for performing all of these by the end of 2009. The sex-separation is based on a translocation which links dieldrin resistance (*Rdl*) to the Y chromosome. The relationship of irradiation to sexual sterilization has been described, and to a large extent, studies of its effect on male competitiveness have been completed. Mass production trays, cages, racks, pupa separation and blood-feeding apparatus are undergoing construction, modification and refinement.

The Sudan SIT project is now completing the design of a production facility, and trial releases are underway. Extensive studies of the distribution of larval breeding sites have been conducted, and these will be used to determine the seasonal dynamics of *A. arabiensis* that will be included in a model of adult populations. The project has been reviewed by the Sudan National Biosafety Framework, and they have expressed no significant concerns about the safety of the activities.

The La Reunion project has just entered into studies of the wild population abundance and genetics as a preface to determining whether to proceed with a feasibility study. No specific plans for a production facility or release numbers etc. is expected for at least 3 years.

Issues

In the context of this meeting, there are issues that have not been satisfactorily addressed, but neither are they are not unique to this technology. (1) If the program eliminates *A. arabiensis*, what will be the long-term consequences of creating a malaria-naïve population? (2) Is it possible that the introduction of an insecticide resistance allele will make conventional control more difficult (this is being addressed by laboratory experiments to some extent)?

Challenges

There are a myriad of logistic, financial and technical challenges in this program. They are not particularly related to human health or regulation. To test any technology on a large scale with little experience in a developed country is difficult. To do so in a developing country is more so. The availability of trained personnel, materials and infrastructure present country specific difficulties, some of which cannot be anticipated and for which remedies will be difficult.

The technical challenges of the SIT have been met and solved to a limited degree by previous SIT programs. They used techniques that increased the scale of laboratory culture, but the specialized devices they developed are no longer available. Therefore, the Agency program is attempting to learn from the previous efforts and to develop improved versions using modern knowledge and materials. The program is also attempting to develop technology that is scalable to larger production numbers. Therefore, thought is being given to mechanization, cost, efficiency and secondarily use with *Aedes* spp. All of the equipment has not been completed and tested, so there is no certainty that the current systems will be suitable.

Field Feasibility Studies Challenges

The density of mosquitoes in Sudan is sufficiently low that it is difficult to obtain good estimates of adult populations. Therefore, two years of monthly larval surveys will be the basis for estimating adult populations. This is a significant, but not insurmountable, biological modeling problem.

Aerial release technology is not available for mosquitoes, but for the first releases in Sudan, we believe that ground and river bank releases of pupae will be suitable. Devices for doing this must be devised and tested. In La Reunion, it seems certain that aerial release will be necessary since the terrain is uneven, complex and somewhat inaccessible by ground vehicles. Aerial release technology developed for other delicate insects must be adapted for mosquitoes.

Additional Needs

Planning vector release programs will be facilitated by the development of general mathematical models of SIT that are updated and modified to include characteristics such as larval density dependence, reduced mating competitiveness, species bionomics and semi-sterility. These programs should be accessible via a simple interface to end-users who are planning release programs, not only software developers or modelers.

Surveillance methods for *Anopheles* at very low population densities will be challenging. During the eradication of *A. arabiensis* from Egypt, the response of populations to ceasing control efforts was used, but this will not be suitable for routine assessment of program effectiveness. Sensitive methods and methods such as attractants are needed.

Conclusions by expert independent ethicists and public health practitioners should be reached about several ethical and environmental issues that have been raised but not resolved. These include: the desirability of inevitably creating of naïve human populations, desirability of area-wide control methods in an environment in which community based interventions are popular, eradication of genetic forms, and defining situations when informed consent is and is not applicable.

Recommendations

1. Provide funding to develop the general mathematical model described above. While there are several efforts to develop models for specific technologies, these are neither sufficiently flexible nor accessible by project planners to make them of value.

2. Provide funding for sensitive methods for *Anopheles* surveillance. These must be capable of being applied over large areas and robust.

3. Provide funding for development of aerial release equipment. The same equipment can likely be used for all genetic release programs. Even if it is not used immediately, it will provide the basis on which the spatial extent of vector releases can be realistically considered.

4. Create a panel of experts who will develop a landmark document of conclusions regarding the ethical and environmental issues such as those described above. This panel must be of high scientific caliber, completely independent and recognized by international bodies such as the WHO.

A Precedent for Genetic Engineering; the Environmental Impact Statement on Transgenic Fruit Flies and Cotton Pink Bollworm

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"Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs" is the title of the world's first Environmental Impact Statement (EIS) on any kind of transgenic organism, either plant or animal, prokaryote or eukaryote. This programmatic EIS is also a major part of the world's first official government regulatory process on any transgenic insect. It was published October 2008 and is on the Internet at: http://www.aphis.usda.gov/plant_health/ea/geneng.shtml
It was published by the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) and consists of 334 pages. Two complete Environmental Assessments (EAs) on transgenic pink bollworms preceded this EIS.

This EIS is of major value for genetic markers and *Aedes*, possibly *Anopheles*, sterile insect technique (SIT) population suppression using repressible lethal genetic constructs instead of radiation to sterilize the insects. This EIS also has some applicability for population replacement strategies for *Aedes* spp. or *Anopheles* spp. control using gene introgression/driver mechanism strategies.

When planning an environmental risk assessment for transgenic mosquitoes, it is imperative to determine what laws, as well as guidelines and regulations, apply in each country. Following applicable laws and regulations avoids prosecution and penalties, as well as court injunctions and lawsuits, whether frivolous or not. In the USA, either an EIS or EA may be required by the National Environmental Policy Act of 1969 (NEPA) 42 U.S.C. 4321 et seq. The three species of fruit flies and pink bollworm are plant or crop pests and fall under the jurisdiction of the Plant Protection Act of 2000. Mosquitoes that bite livestock, as well as humans, are under of jurisdiction of the Animal Health Protection Act of 2002. The NEPA was made law to ensure that the environmental impacts of any Federal or federally funded action are available to public officials and citizens before decisions are made and actions taken. NEPA applies within the USA and elsewhere in the world. Similar laws are now enacted in over 100 countries.

The EIS is a public and transparent process with stakeholder participation at the following phases of the EIS:
1. At publication of Notice of Intent to do an EIS
2. At scoping; several public meetings are held to plan the EIS extent and schedule
3. At information gathering and consultation in drafting the EIS
3. At the notice of availability of draft EIS for public comment

Other public stakeholder comment may be received and considered:
4. At the notice of availability of final EIS with public comments and their analysis
5. At the publication of the Agency Record of Decision

NEPA prescribes the following basic EIS components:
• Statement of Purpose and Need
• The Alternatives, including the No Action & Proposed Alternative
• Description of the Affected Environment
• Environmental (RISKS) Effects/Impacts/Consequences of Each Alternative
• Draft EIS, Final EIS, and Record of Decision

The species of the first transgenic insect EIS are:
• Mexican fruit fly, *Anastrepha ludens*
• Mediterranean fruit fly, *Ceratitis capitata*
• Oriental fruit fly, *Bactrocera dorsalis*
• Cotton pink bollworm, *Pectinophora gossypiella*

The alternatives presented in the EIS are:
1. No action; continue programs as they are
2. Expansion of existing programs; SIT and other methods, including pesticides
3. The preferred alternative of integration of genetically engineered insects into programs

The biotechnologies of the preferred alternative are:
• Mass-rearing of either males and females or only male fruit flies with a marker gene, and then sterilized by radiation before release
• Genetically sterilized male-only fruit flies that have a marker gene and that compete more effectively for mates than radiation-impaired fruit flies
• Fruit flies that produce only male offspring, which carry a repressible heritable sterility trait resulting in only males that carry the trait and no female offspring in the field
• Mass-rearing of male and female pink bollworms with a marker gene and are sterilized by radiation before field release (The only GM insect technology currently in large-scale, open-field trials.)
• Mass-rearing of male and female pink bollworms that are genetically sterile without radiation exposure producing males that are more competitive in mating with wild female bollworms than radiation-impaired males

Important appendices of the EIS referenced in the body of the EIS are the following:
• Appendix B. Cooperation, Review, and Consultation
• C. Analysis of Repressible Lethal and Marker Genetic Engineering (biotechnology description)
• D. RISK ASSESSMENT Criteria and Analysis
• E. Summary of Public Comments on Draft EIS
• F. Analysis of impacts on Endangered and Threatened Species in Program Areas

Appendix D. Risk Assessment Criteria and Analysis, was modified after: IAEA-TECDOC-1483, 2006, Proceedings of a technical meeting 8-12 April 2002,

http://www-pub.iaea.org/MTCD/publications/ResultsPage.asp
This report addresses GM arthropods that affect both plants and human health. Only limited risk assessment criteria in IAEA/FAO report were addressed since not all are applicable because of immobilization of the transposable element to mitigate horizontal gene transfer, biological containment (SIT function), and short-term presence in the environment.

In regard to the phenotype of the GM organisms compared to the unmodified organism, many of the phenotypic characteristics considered to have hazard in the IAEA/FAO report are contingent upon some increased biological fitness or horizontal gene transfer (flow) occurring. However, for the GM-sterile insects of the EIS, biological fitness approaches 0% for sterile males, or sterile males plus females. For a heritable female lethal system, biological fitness would approach 50% for first generation of a female lethal system, in which all females die, but not males that carry the female lethal gene, but approaches 0% as females disappear. Important biological fitness factors become biological performance factors in SIT applications for the following reasons:

- Improved suitability for mass-rearing
- Improved mating effectiveness, competitiveness, and longevity over radiation use
- Stability of the repressible lethal and marker constructs over multiple generations of mass-rearing

In regard to horizontal gene flow in the EIS, exchange of genetic material between insects of different species and between insects and other organisms may be possible over evolutionary time, but it is not practical to quantify in the laboratory. The transposable elements (*piggy*Bac) are removed or deactivated in the biotechnology addressed by the EIS to insure against horizontal gene transfer.

The EIS RISK ASSESSMENT conclusions are that the environmental consequences of the preferred alternative were found to have no more adverse environmental impact than the continuation or expansion of present SIT fruit fly and pink bollworm control programs, which use gamma radiation to sterilize insects and include other control and monitoring measures.

Population replacement strategies for dengue virus vector control (*Aedes*)

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Introduction

Population suppression strategies aim to reduce the number of vector mosquitoes in the target area. This is in contrast to population replacement strategies, which aim to alter (reduce) the vector competence of the mosquito population with only incidental effects on the total number of mosquitoes.

Current status

Relatively few genetics-based population suppression strategies have been proposed. Most represent variants on the Sterile Insect Technique (SIT). Several such strategies, including at least one specifically based on the use of GM mosquitoes, are either in field testing or expected to move to this phase in the near future.

SIT is a species-specific and environmentally non-polluting method of insect control that relies on the release of large numbers of sterile insects (Dyck et al., 2005; Knipling, 1955). Mating of released sterile males with native females leads to a decrease in the females' reproductive potential and ultimately, if males are released in sufficient numbers over a sufficient period of time, to the local elimination or suppression of the pest population.

Highly successful, area-wide SIT programs have eliminated or suppressed a range of major veterinary and agricultural pests around the world. These programmes can succeed on very large scales – the largest rearing facility alone produces around 2 billion sterile male Mediterranean fruit flies per week (~20 tonnes/week), primarily for use in California and Guatemala. For these pests, SIT is a proven, cost-effective strategy for eradication or suppression of target populations, or to protect areas against invasion or re-invasion.

For mosquitoes, the situation is less clear. Field trials in the 1970s and 1980s demonstrated that the SIT could also be made to work against mosquitoes, even with the technology then available (Benedict and Robinson, 2003; Klassen and Curtis, 2005). For example, *Anopheles albimanus* was successfully controlled in a trial in El Salvador, using chemo-sterilised mosquitoes (Lofgren et al., 1974).
The fundamental properties of SIT are still highly attractive for mosquito control. This has led to a resurgence of interest in recent years, with several research groups trying to mitigate or bypass some of the technical limitations which prevented conventional SIT from becoming a widespread approach following the early trials. Two issues in particular may be addressed by genetics

Sterilisation

Recent advances allow several potential improvements over the methods available in those early trials. All current SIT programs use radiation to sterilise the insects. However, it has proven difficult to irradiate mosquitoes to near-complete sterility without significantly weakening them (Andreasen and Curtis, 2005; Helinski et al., 2006). Two groups are trying to develop radiation-based SIT for mosquitoes, with some success: Romeo Bellini (Italy, *Aedes albopictus*) and the IAEA group currently led by Mark Benedict (IAEA/Austria, primarily *Anopheles arabiensis*) (Bellini et al., 2007; Helinski et al., 2008).

Early mosquito SIT trials used a range of sterilisation methods, for example chemosterilants in the case of the *Anopheles albimanus* program in El Salvador. Cytoplasmic incompatibility (CI) was also used successfully in field experiments with *Culex pipiens fatigans* (Laven, 1967). CI is caused by maternally transmitted, intracellular bacteria of the genus *Wolbachia* and refers to the strongly reduced hatch rate in matings between infected males and females that are either uninfected or harbour a different infection type (Dobson et al., 2002). Therefore, infected males released into an uninfected population are sterile without need for radiation. Interest in this approach, termed Incompatible Insect Technique (IIT) has recently revived (Brelsfoard et al., 2008; Dobson et al., 2002). However, infected females are fertile with infected males, so inadvertent release of infected females could lead to replacement of the target population with a *Wolbachia*-infected form, rather than suppression. For a species such as *Aedes aegypti*, not naturally infected by *Wolbachia*, this infection could spread beyond the release area, potentially worldwide, and may be irreversible. For a naturally infected species, such as *Aedes albopictus*, such replacement should not spread far beyond the release area and is theoretically reversible, if desired, by mass-release of wild type females. Modified strains showing CI with wild type have been constructed for *Aedes aegypti*, *Ae. albopictus* and *Ae. polynesiensis* (Brelsfoard et al., 2008; Xi et al., 2005; Xi et al., 2006).

Recombinant DNA methods are also being developed for sterilisation. It has been proposed that 'genetic sterilisation' could be achieved by use of engineered insects in a strategy termed RIDL (Release of Insects carrying a Dominant Lethal gene or genetic system, Alphey et al., 2007; Thomas et al., 2000). Released males would mate wild females; all their progeny would inherit the lethal gene and consequently die. The lethal gene needs to be conditional, so that the strain can be bred under permissive conditions prior to release. It has been suggested that adjusting the time of death, from the embryonic lethality characteristic of other methods, to later, e.g. pupal, lethality, would be advantageous. This is to mitigate the effects of the density-dependent population dynamics thought to be characteristic of some mosquito species such as *Aedes aegypti* (Alphey et al., 2007; Phuc et al., 2007). It has also been suggested that by restricting the lethal effect to females, provide both sex separation and genetic sterilisation could be provided by the same genetic system (Alphey et al., 2007; Thomas et al., 2000).

Sex separation

It is highly preferable to release only sterile male mosquitoes, not a mixture of males and females. Sterile females may bite and potentially transmit disease, and may also 'distract' co-released sterile males from seeking out wild females (Rendón et al., 2004). For Culicines, sex-separation has been achieved by physical methods based on pupal size, with 99-99.9% accuracy (e.g. Ansari et al., 1977; Focks, 1980). Several strains suitable for genetics-based separation have also been constructed using classical genetics. These were based on the translocation to the Y chromosome of a selectable marker, for example insecticide resistance (e.g. Seawright et al., 1978).

It has been proposed that engineered repressible female-specific lethal genes could provide effective genetic sex separation (Alphey et al., 2007; Thomas et al., 2000). Such strains have been developed for Mediterranean fruit fly (Fu et al., 2007) and for *Aedes aegypti* (Alphey, unpublished). Another proposed use of recombinant DNA methods is to engineer sex-specific expression of a visible marker, such as a fluorescent protein, allowing fluorescence based sorting (Catteruccia et al., 2005; Condon et al., 2007). This method by be limited by the speed of available fluorescence-based sorters.

Other traits and approaches

Several other useful traits are available through genetic engineering, such as a heritable marker to distinguish engineered and wild type insects, and various ways of mitigating accidental releases of insects.

Driving lethal genes into populations

On the border between population suppression and population replacement is the notion of driving deleterious genes into the target population. Methods to do this have been proposed, using homing endonuclease genes (HEGs: Burt, 2003; Burt and Trivers, 2006; Deredec et al., 2008). These would tend to spread and impose a genetic load on the population; mathematical model suggests that this might lead to extinction. The same approach could be used to drive anti-pathogen effectors or mutations and the issues and challenges around this approach are much more akin to population replacement. It has also been suggested that HEGs could be used to provide "RIDL-with-drive"; an augmented version of RIDL with greater persistence of the sterility gene in the wild population, though without the long-term persistence or spread characteristic of most population replacement approaches (Deredec et al., 2008).

Issues

Some of these issues are common to many control strategies, including other genetic control strategies:

- Intervention is species-specific
    - Multiple vector species in target area?
    - Mating barriers between populations / cryptic species?
- Relationship between number of mosquitoes and epidemiology may not be clear
    - Population suppression aims to reduce the number of vectors. But what level of success is required to give what level of benefit (i.e. reduction in human morbidity/mortality)?
- Community engagement
    - Some level of disquiet about any release of mosquitoes
        - Sterile-release methods require release of large numbers
    - Use of modified mosquitoes may induce additional concerns
    - Communicating mosquito and disease biology, and the risks and benefits associated with specific novel control strategies, are resource-intensive and have no obvious endpoint

Challenges

Economic cost-benefit analysis, which is needed to support use of novel interventions, is difficult because of lack of reliable data on economic burden of disease for dengue and other neglected tropical diseases, and because of uncertainty around development and implementation costs. Ideally it would be possible to analyse not only the cost-effectiveness of the stand-alone novel strategy, but also to compare it with existing alternatives and to model its incorporation in integrated vector management (IVM) programmes, and indeed integrated disease management programmes including drugs and vaccines, where available.

As genetics-based population suppression moves from lab to field the lack of a clear regulatory framework for field use of modified mosquitoes is a significant challenge. (This issue is not restricted to developing countries, or to strategies dependent on the use of recombinant DNA technology). Even once regulatory frameworks are in place, risk assessments and public consultation will be lengthy processes due the novelty of the technologies and lack of experience in regulatory agencies.

The route to implementation of control programmes based on these technologies is not obvious. Agricultural SIT programs have generally been established and operated by governments, though there is limited private sector involvement. Existing vector control programmes are generally government funded and operated, though they purchase vector control products and services from the private sector. The development of new vector control approaches is generally in the private

sector. The current genetic-based technologies are perceived as too "high risk" at this time for large companies to bring them into their portfolios. This risk is a combination of the technical and regulatory risk of bringing the technologies to market and the market risk, that is the uncertainty around customer and price. For example, the NGO sector has encouraged the private sector to develop and produce bednets to prevent malaria by guaranteeing certain purchase quantities. This model does not exist for novel strategies and dengue. There is one small private sector entity involved today but the technologies need to be "de-risked" before the larger companies that would be so useful for large scale implementation get on board. This de-risking could be in the form of funding for development and/or in conditional purchase guarantees.

Additional needs

In addition to addressing issues and challenges already mentioned above:

- Field demonstration
    - While there is much further work to be done in the lab, the pressing need is for field data on the performance, effectiveness and cost-effectiveness of the various population suppression strategies for which prototype strains have been developed. Convincing demonstration of suppression of wild target mosquito population(s) by one or more of these strategies would be transformative.
- Improved large-scale rearing methods and cost estimates for these
    - Implementation costs of genetics-based population suppression strategies depend in large part on the costs of rearing and distributing sterile mosquitoes. While methods exist from early trials, there is considerable scope for improvement and cost reduction.
- Better understanding of mosquito ecology and behaviour, especially for males, and the effect of vector numbers on epidemiology of relevant diseases.
- Developing guidance for use of modified mosquitoes in genetics-based population suppression strategies. The self-limiting nature of sterile insects (including IIT, RIDL, etc) tends to make the issues related to field use of these somewhat less challenging than for self-spreading systems characteristic of population replacement strategies, also they are closer to field use, so might be appropriate to consider first. WHO/TDR funding for capacity building and guidance development, and this Technical Consultation, are all steps in the right direction.

Recommendations

Recommendations relate to meeting the issues, challenges and additional needs identified above. More generally, we are on the brink of having a suite of new tools for field testing and, ultimately, program use. The development costs and timescales are very attractive relative to other approaches such as new drugs, vaccines or insecticides yet beyond the reach of individual research groups. Though there is currently considerable momentum behind the development of genetic control methods, this requires ongoing support, particularly during the transition from lab to field and into the first large-scale implementation programs.

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Population replacement strategies for dengue virus vector control (*Aedes*)

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Introduction

Population replacement refers to vector control strategies in which populations (or species) of mosquitoes capable of transmitting pathogens are replaced by those that can not (Curtis and Graves, 1988). As envisioned originally, a number of approaches could be used to achieve this end, and recent work has focused on testing strategies based on linking genes conferring pathogen resistance (effector genes) to systems designed to spread the desirable traits through the vector population (James, 2005; Sinkins and Gould, 2006). Work to develop these strategies with Culicine mosquitoes (specifically, species within the genera *Aedes* and *Culex*) is motivated by the need to control the transmission of arboviruses and filarial worms. A framework for testing population replacement was proposed that defines the context in which experiments are designed and establishes parameters by which success is measured (Collins and James 1996: James *et al.*, 1999). Three research areas were recognized, the first of which addresses the need to demonstrate in the laboratory that it is possible to engineer genetic resistance to pathogens in mosquitoes. The goals of this area are to discover and develop the expressed portions of effector genes that target specific infectious agents, identify functional control DNA fragments that direct their stage-, tissue- and sex-specific expression, and develop transgenesis so that the effector genes could be integrated stably into the genome of the target species. The second area of research emphasizes the development of means for moving laboratory-tested effector genes into wild populations and requires the adaptation or invention of genetic mechanisms, defined collectively as gene drive systems, for increasing the frequency of specific genes in those populations within an epidemiologically-relevant timeframe. Furthermore, the efficiency of the gene drive system must offset the impact of introduced genes on the fitness of the transgenic mosquito and spread despite any load associated with the genomic integration of novel DNA. The third area of research involves comprehensive investigation to define and characterize target vector populations. These efforts are essential to understanding the scope of the proposed intervention, optimizing development and deployment of application protocols, and adopting and testing end-point parameters for success. The ultimate measure of success of a population replacement effort is a decrease or elimination of human morbidity and mortality due to the altered vectorial capacity of the vector.

Current status

Excellent progress has been made in the laboratory development of virus-resistant *Aedes aegypti*. Effector portions of genes based on RNAi or ribozymes can disable dengue viruses resulting in reduced mean intensities of infection and prevalence (Franz *et al.*, 2006; M.J. Fraser, personal communication). In addition, single-chain antibodies targeting a conserved epitope of dengue envelope proteins have promise for preventing virus entry into insect tissues (K.E. Olson, personal communication). Multiple antiviral effector approaches are expected to mitigate selection for resistance to any single mode of action of an engineered gene. The recent successes were made possible by the use of control DNA sequences from mosquito-derived genes that direct expression and localization of effector molecules in the midgut, hemolymph and salivary glands, and the development of transposon-mediated transformation technologies (Moreira *et al.*, 2000; Kokoza *et al.*, 2000; Adelman *et al.*, 2002, Mathur

and James, unpublished). These approaches can be applied to target other arboviruses, including Yellow Fever, Chikungunya and West Nile, and notably, transgenesis has been achieved with *Culex quinquefasctiatus*, *Ae. fluviatilis* and *Ae. albopictus* (Allen *et al.*, 2001; Rodrigues *et al.*, 2008; L. Alphey, personal communication). No reports have been published yet that describe explicit efforts to develop effector molecules that target filarial pathogens.

A number of fundamentally-different genetic mechanisms are being researched for adaptation to gene drive systems for Culicine mosquitoes (James 2005, Sinkins and Gould, 2006). Safety and efficacy requirements are addressed in the design of two-component genetic mechanisms comprising one part that imposes a lethal outcome following its expression, and another that rescues that lethality. Systems based on under-dominance and Killer/Rescue gene combinations, and *Medea* are being developed for *Ae. aegypti* (Gould *et al.*, 2008; Bruce Hay, personal communication; L. Alphey, personal communication). *Medea* holds the most immediate promise because proof-of-principle has been demonstrated in the vinegar fly, *Drosophila melanogaster* (Chen *et al.*, 2007). Linkage of the effector genes to the components of these systems fixes the gene in the target populations. An additional approach seeks to exploit the ability of Class II transposable elements to spread rapidly through populations by linking antipathogen effector genes to them (Adelman *et al.*, 2007). Careful design of self-mobilizing transposons that excise and integrate autonomously is needed to ensure their safe and efficient spread through target populations while minimizing risks of any impact on non-target organisms (James, 2005). While it has proven to be straightforward to design and introduce engineered autonomous constructs into mosquito genomes, no unequivocal data exist yet demonstrating that the inserted DNA is able to remobilize (Sutheraman *et al.*, 2007; Wilson *et al.*, 2003).

Characterization of mosquito populations in disease-endemic regions is in progress. Target locales vary from densely-populated urban to low-density rural areas, and the specifics of mosquito bionomics in each need determining before it is possible to predict the likelihood of success of a population replacement intervention. Research on dispersion, mating competitiveness, population structure, seasonal and spatial density, oviposition behavior and insecticide resistance is expected to benefit population replacement strategies. The results of this work provide information on how far introduced mosquitoes might move, what are the selective advantages or disadvantages of carrying transgenes, what are the extents of the local demes, how many mosquitoes are in target areas, where the sub-adult stages are, and what are are the frequencies of insecticide-resistant genes in the target areas. All of these data are important when planning introgression of effector genes into local populations.

An important contribution to all phases of this work is the development of mathematical models that allow the comparison of different gene drive systems for the impact of fitness costs of the transgenes on gene spread and fixation, optimal release ratios, drive strength, environmental conditions, competition, and other factors (Sinkins and Gould, 2006; Marshall *et al.*, 2007; Rasgon, 2007; Marshall, 2008). This modeling permits critical decisions about which systems are more likely to have an impact on pathogen transmission, and aid significantly in the design of experiments and the establishment of end-point parameters for determining success. Other efforts in support of population replacement strategies include a document that provides guidance for contained field-testing of mosquitoes carrying active gene drive systems (Benedict *et al.*, 2008). This report outlines key issues associated with containment, regulation and experimental design for testing the safety and efficacy of gene drive systems, and makes recommendations on phased testing, risk assessment practices and oversight responsibilities. Detailed protocols must be developed for specific test situations based on the topics in the guidelines.

Issues

The issues listed here represent technical activities for which specific solutions are needed. Proof-of-principle must be demonstrated for a mosquito-adapted, gene-drive system and mechanisms for recall developed and tested. The latter will be useful to re-cycle the system with new effector genes or to remove those that are either no longer needed or not effective (Chen *et al.*, 2007). Effector genes are needed that achieve near-zero prevalence of pathogens in transgenic mosquitoes. This may require the use of combinations of effector genes, especially to prevent emergence of resistant pathogens. An empirical definition of a "pathogen-resistant mosquito" must be established that reflects the true transmission potential (vectorial capacity). A reliable vertebrate animal model for transmission would be an asset in this regard. Additional field research is needed to obtain a clear understanding of vertical transmission and its potential impact on epidemiology. The local significance of sylvatic and zoonotic cycles of transmission also must be known and if necessary, addressed. More work is required on the basic biology, most notably, mating behavior, and population structure of the target mosquito species in order to model the potential of population replacement.

Further refinement of molecular genetic technology will help to address some of the remaining challenges. Improvements in transgenesis and gene-targeting are needed to make them more efficient and mitigate issues with position effects and epigenetic gene silencing. We need to understand what is required to stabilize the expression of effector genes so that they remain effective throughout the control program, and this may be facilitated by transgenic lines receptive to site-specific integration (Nimmo *et al.*, 2006). Long-term storage of transgenic lines remains problematic. Some of the broader issues shared with research on Anopheline mosquitoes include the need for the sequencing additional genomes (in this case, *Ae. albopictus*), and more genomics-based work on gene expression (including developmentally-regulated gene expression with respect to sex-, tissue- and stage-specificity and responses to pathogen infection) to support the identification of target genes and novel intervention approaches., Additionally, it would be helpful to have realistic methods for comparative studies and information transfer from other insects to mosquitoes.

Challenges

These challenges elaborate aspects of population replacement approaches that are wider in scope and where issues are unknown or remain to be defined. Social and political barriers to the use of genetically-engineered insects must be identified, and where appropriate, steps taken to offer specific solutions to mitigate the barriers. Practical aspects include implementation of contained and open-field trials, and identification of the necessary regulatory requirements and community engagement activities needed for the trials. The immediate and long-term success of these efforts depends on awareness and discussion of their benefits, and therefore, communication channels among scientists, institutional administrators, government agencies and the public need to be established and maintained. Risk assessment procedures need codifying. The development of project plans with concrete performance measures and evaluation parameters is necessary.

For longer range decisions about development and deployment of population replacement methods, data-gathering methods are needed for accurate estimates of disease costs (including surveillance, treatment, prevention, and categories of lost revenue [for example, tourism]) in different endemic areas,

and robust models are needed for analyzing these data for cost-benefit projections of various control strategies. Scale-up plans from small testing to manufacturing levels, including the incorporation of good manufacturing procedures, need development.

Additional needs

Despite a significant increase in interest in research in population replacement strategies (Beaty *et al.*, 2009), there still is an urgent need for more specialists to be involved in all aspects of this work. This is true particularly for scientists from disease-endemic countries. Established scientists, postdoctoral fellows and graduate students are needed with expertise in basic and applied sciences, and in the disciplines that contribute to the cultural, ethical and social issues of using genetically-engineered organisms for disease control. Training opportunities, including short- and long-term courses, workshops and meetings, are needed for junior researchers and more established scientists contemplating moving into the field. The need is urgent for establishment of field sites for testing transgenic mosquitoes. These field sites need to reflect the diversity of the transmission areas that make up the range of endemicity of arboviruses and be coupled to facilities that can monitor the impact of the control efforts on disease epidemiology. Finally, it is essential that efforts by funding agencies be made to ensure the maintenance of basic science (discovery) while promising products are moved to development.

Recommendations

General recommendations include the need to endorse planning activities that envision how population replacement strategies will be integrated into control programs that include other infection-suppressing and disease-control measures to achieve maximum cost-effectiveness. Arguments have been made that integration should be extended from considering only vector control efforts to include the impact of clinical interventions (for example, vaccines and drugs) and public health interventions that can alter dengue transmission (Luckhart *et al.*, 2009). Sufficient thinking in advance is needed so that research modules (laboratory, field, regulatory, etc.) are working towards common goals optimized by mutual input. This broader thinking and planning is essential if we expect to have a sustained impact on disease prevalence. Consistent with this, vector control efforts are multidisciplinary and earnest efforts should be made to maintain the pipelines of activities that allow virologists, molecular biologists, field ecologists, ethicists, regulatory experts and others to contribute their expertise when and where it is needed. This is a non-trivial effort, especially as population replacement products move from the laboratory to field testing. More specific recommendations include the research topics addressed in the **Issues** and **Challenges**. In addition, guidelines for the transport, testing and use of genetically-engineered vectors must be standardized and meet the concerns of stakeholders. The WHO/TDR Technical Consultation, of which this report is part, is a move in the right direction for this need.

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