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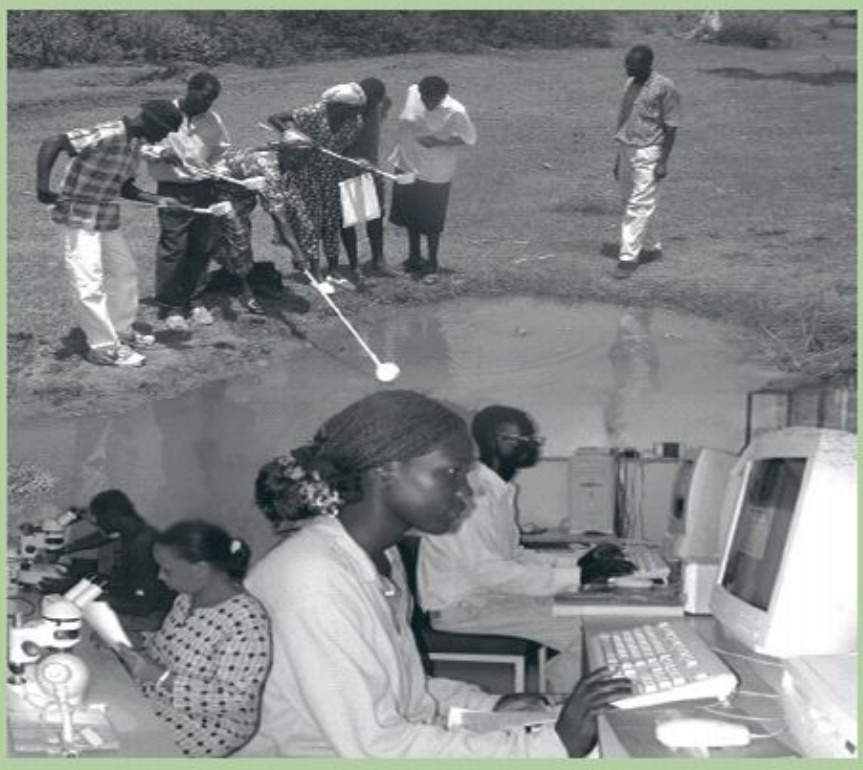
Bridging Laboratory and Field Research for Genetic Control of Disease Vectors

Edited by
B.G.J. Knols and C. Louis



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Edited by

B.G.J. KNOLS

International Atomic Energy Agency (IAEA), Seibersdorf, Austria and Laboratory of Entomology, Wageningen University and Research Centre, Wageningen, The Netherlands

and

C. LOUIS

Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Crete, Greece

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Integration

Preface

Novel scientific ideas have historically been met with resistance from parts of society, best exemplified by Galileo Galilei and, more dramatically, Giordano Bruno. It is, thus, not surprising that even in the 21st century objections are expressed by many when the genetic modification of organisms and its use in potential applications are brought into discussion outside the strict scientific community. The potential use of genetically modified insects in the control of vector-transmitted diseases is no exception. The aim of this book, stated immediately at the beginning of this preface, is not to help overcome these objections but, rather, assist scientists in finding answers to these arguments and possible misunderstandings. It is hoped that this can be achieved by improving collaboration and cross-talk between them.

The diffusion and adoption of modern biotechnological innovations, from laboratory settings to full implementation in 'real life' is a complicated process. With many as yet unknown variables influencing the speed at which this may occur there are six general factors affecting the adoption process of novel methodologies aiming at controlling vector-transmitted diseases:

1. The technology should have a relative advantage or be of a complimentary nature to existing tools available for these diseases.
2. The approach should be consistent with the values, experience and needs of the society in places where the technology is to be applied.
3. The technology should be easy to understand, develop and implement without the need for significant investment in knowledge and skills acquisition.
4. The technology should be able to be trialled or piloted on a limited basis.
5. The technology should deliver noteworthy public-health benefits
6. Last but not least, the technology should be shown to pose no threats to either local or global environment.

With regards to the current status of the development of new genetic control methods for disease vectors, none of the above points are fully met or they remain simply unknown, making adoption highly unlikely. As governments and agencies responsible for vector control in disease-endemic countries have not seen any outcome of transgenic approaches beyond laboratory experimentation, it will at present be hard to justify a commitment of their limited resources to such new approaches rather than to tools known to deliver certain public-health gains. At present, therefore, no advantages of modern genetic control tools can be seen, pressing the need for all parties involved to establish proof-of-principle beyond laboratory boundaries. This book is the first of its kind to describe the opportunities to do so, and stresses throughout that this will depend on active and genuine collaborative efforts between those working at the bench and those conducting field research.

A huge gap has arisen between those developing molecular-genetic approaches to render disease vectors incapable of transmitting disease and those practicing vector research, and control in the field and mechanisms for bridging this gap are lacking. University curricula promote specialization rather than diversification, hindering opportunities to bridge existing gaps. Students in developing countries lack the resources needed to acquire the high-tech laboratory skills available in the North.

Beyond experiencing this for a limited duration (e.g. during a PhD project or coursework in northern laboratories) they are limited to basic and often rudimentary infrastructural facilities and end up practicing skills acquired prior to their 'high-tech' experience. It is somewhat surprising that five years after the first successful germline transformation of *Anopheles* no single laboratory in sub-Saharan Africa has developed

the research infrastructure to conduct similar research. Yet, as mentioned above, equitable partnerships and collaborative research are at present the most likely drivers of the adoption process.

Developments over recent years have been dramatic and the prospects of using genetic control methods to reduce vector-borne disease are becoming, albeit in the long-term, real. The most challenging part of this endeavour is the transition of technology from the laboratory to field settings, which, no doubt, will be a lengthy and difficult process. This book provides key insights into this process, lists many examples of research components for which the transition may soon be undertaken, and summarizes thoughts, ideas and opportunities drafted jointly by both laboratory and field scientists who met in Nairobi, Kenya in July 2004.

It is our sincere hope that this volume may facilitate the adoption process of modern biotechnological approaches to combat vector-borne diseases and may ultimately expedite the full evaluation of these approaches in terms of public-health benefits in the developing parts of the world.

Acknowledgements

This working group meeting was co-sponsored and organized by the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), the National Institute of Allergy and Infectious Diseases/US National Institutes of Health (NIAID/NIH), the International Atomic Energy Agency (IAEA) and Frontis (Wageningen University and Research Centre).

We are indebted to the outstanding administrative and organizational support of the International Centre of Insect Physiology and Ecology (ICIPE), in Nairobi Kenya, in particular the local organizing secretariat (Ms. Nelly Gitonga, Ms. Faith Kyengo, Mr. Fredrick Makhulo, Ms. Margaret Ochanda and Ms. Lucy Theuri), the WHO Representative's Office, Kenya (Dr. P. Eriki and Dr. Joyce Onsongo), Ms. Dorcas L. Appiah Agbogla (WHO/TDR), Dr. Rob Bogers and Ms. Petra van Boetzelaer for editorial support, Mr. Hugo Besemer and Ms. Paulien van Vredendaal for facilitating the lay-out process, and the authors for their contributions.

The editors,

Bart G.J. Knols
Christos Louis

Seibersdorf / Heraklion, June 2005

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Executive summary

Working-group participants

A scientific working group[1] convened in Nairobi, Kenya, from 14 to16 July 2004, to further discussions[2] on the potential use of genetically modified vectors (GMVs) for the control of vector-borne diseases, particularly malaria and dengue. The meeting was jointly sponsored and organized by the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), the National Institute of Allergy and Infectious Diseases/US National Institutes of Health (NIAID/NIH), the International Atomic Energy Agency (IAEA) and Frontis (Wageningen University and Research Centre). The group specifically focused on how to increase involvement of disease-endemic country (DEC) scientists in this endeavour as well as enhance collaboration between field and laboratory researchers.

A comprehensive research agenda and strategic plan to bridge laboratory and field research was presented along four thematic sessions. Historical applications of genetic vector control, population biology and vector genomics were discussed first. These were followed by descriptions of the state-of-the-art and future needs in laboratory and field sciences, with emphasis on how to integrate these two branches of entomology. The third part described the vector-borne-disease situation in different geographical regions with a focus on malaria and dengue vectors, while the final session included twelve key areas for research involving both laboratory and field sciences, structured to a) describe the state-of-the-art of each topic, b) identify salient issues and challenges, c) state research opportunities and d) propose future directions for research and capacity/partnership building.

Seven key points with recommended actions were recognized that would provide a basis for TDR to define its own research programmes, whilst taking into account its comparative advantages in both research and capacity-building activities.

Recommendations

The goal of the meeting was to provide technical and scientific guidance for addressing issues and challenges about genetic control of disease vectors and to develop a strategic plan to bridge laboratory and field research. Particular emphasis was placed on identifying research opportunities that merge interests from both branches of medical entomology and that can significantly advance the GMV approach for disease control.

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[1] List of participants, see elsewhere in this volume.

[2] For previous meeting reports see Alphey, L., Beard, C.B.; Billingsley, P., et al., 2002. Malaria control with genetically manipulated insect vectors. Science, 298 (5591), 119-121; Takken W. and Scott, T.W. (eds.). Ecological aspects for application of genetically modified mosquitoes. Kluwer Academic Publishers, Wageningen UR Frontis Series, Vol. 2.

To this effect, the participants recognized the following seven key points with recommended action:

1. Genetic modification of insects could be used to control vector-borne diseases

Replacement of existing populations with resistant or refractory strains is expected to contribute to the control of disease. Recent successful and stable genetic transformation of important vectors has underlined the need to optimize and standardize transformation technology. The search for systems that incapacitate pathogen development in the vector should be strengthened. Additional endogenous and/or synthetic effector genes, conditional lethals, and novel phenotypes should be searched and characterized. Tissue-specific promoters for such systems need to be identified and adequately engineered to ensure complete refractoriness based, ideally, on multiple effector genes in order to prevent pathogen adaptation. Finally, recent studies are showing promise towards paratransgenic approaches requiring the identification of suitable bacterial strains and the study of subsequent delivery in field populations after genetic engineering.

2. Develop techniques for driving effector genes that interfere with disease transmission into wild insect populations

Proof of principle has been established for implementation of anti-pathogen constructs, but appropriate drive mechanisms are still to be developed. Genetic stability of effector and drive mechanisms and their associated fitness cost must be researched, including genetic phenomena that alter segregation ratios to spread specific genes or alleles through populations. Such systems should first work in the laboratory, subsequently progress to the semi-field, and be subjected to appropriate risk assessment. Systems must then be introgressed into recent field-derived mosquito strains and evaluated for reproductive and competitive abilities in semi-field systems, also assessing potential horizontal transfer among closely related species. DECs are best suited for carrying out this research, and their inclusion in these activities is essential.

3. Studies of vector field populations with respect to potential future release of a GMV

Sufficient data on target vector populations (e.g. population structure, gene flow, and behaviour and ecology) are needed to model and predict the behaviour of introduced genes, providing the required baseline information in terms of vectors and the diseases they transmit.

Selection of field locations should focus on settings with manageable complexity in terms of vectorial system, pathogen transmission, and adequate isolation. A framework for this needs to be developed and its implementation will have to be guided by a coordinating body (see below under 7). The acquisition of additional knowledge on vector populations and disease transmission requires further development of sampling methods and molecular tools.

Correlates between field populations and disease transmission parameters should be based on the use of integrative models and be tailored towards the assessment of both efficacy (e.g. measuring sporozoite rate) and effectiveness (e.g. disease incidence) of GMV introductions. Suitable entomological parameters for measuring dengue transmission are in need of development.

Potential fitness effects of genetic transformation and mating success will directly influence the outcome of GMV releases, thus standardized procedures that enable comparative evaluation of fitness effects are needed. Whether fitness costs can be

compensated for by a highly efficient transgene drive mechanism remains unknown. Pertinent laboratory research is still preliminary and further investigations in more realistic environments are required.

Mosquito mating behaviour remains understudied, and application of new approaches (e.g. genomics) is yet to commence. Research should primarily assist in assessing whether or not released transgenic males can effectively compete for females of the wild vector populations.

Key issues underpinning the success of GMVs, such as recombination and subsequent impact on transgene drive, may only be studied through modelling. Models should focus on defining threshold levels in terms of system efficacy in order to attain maximum epidemiological impact in both spatial and temporal dimensions.

DEC activities should include efforts to comprehensively identify and characterize potential field sites and research to assimilate better knowledge on the relationship between transmission intensity and disease outcome.

4. Development of processes dealing with the ethical, legal and social issues (ELSI) of the use of GMVs

Various biotechnological and implementation challenges remain to be addressed in order to make GMVs a control approach applicable in the public-health arena. There is consensus that the introduction of GMVs must result in a predictable and positive public-health outcome. The process of mobilizing active support and involvement at all levels in society needs to be put in place to provide a proof of efficacy and safety (i.e. minimizing risk) of the use of GMVs for disease control, required to initiate the process of formalizing ethical, legal and social issues.

Effective strategies to communicate with the media and policymakers are crucial. A long-term effort to clarify the scientific uncertainties under different experimental conditions and with the involvement of DEC investigators is mandatory, as is the development of guidelines and principles for minimum-risk field research that includes environmental risk management.

5. Enhanced involvement of scientists and institutes in DECs

Closer involvement and participation of DEC scientists in the GMV endeavour is essential. The challenge of fighting vector-borne diseases is international in nature and needs an inclusive, open research community that exploits the expertise of all scientists. DEC scientists are frontline stakeholders and their laboratories should be engaged in equitable partnership in the pertinent research including research areas such as post-genomics and bioinformatics. Frequent courses, workshops and meetings could strengthen partnership and collaborative efforts. The inter-relationship between academic and implementation communities in DECs should be reinforced

6. Inclusion of GMVs in disease control programmes

GMVs have to be aligned with existing control strategies already adopted and implemented by health authorities in DECs. Appropriate mechanisms for evaluating the impact and cost–benefit relation of GMVs against a background of ongoing control strategies (e.g. insecticide-treated bednets) will have to be developed. Integration of GMVs into Integrated Vector Management (IVM) policy frameworks is foreseen and deserves consideration.

7. Coordinating and follow-up mechanism for GMV research and implementation

The complexity of issues related to GMV development and implementation requires a multi-disciplinary effort and a coordinating board should be put in place. This will focus on the broader dissemination of scientific progress to stakeholders and the facilitation of collaborative efforts and partnership strengthening within and beyond the scientific community.

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Report of the working-group meeting

Christos Louis[#] and Bart G.J. Knols[##] (rapporteurs)

Introduction

The continued threat of vector-borne diseases calls for both reactive and proactive efforts to mitigate the significant morbidity and mortality they cause. To anticipate future demands for novel vector control strategies, the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) formalized a work plan to evaluate genetic-control strategies for prevention of the transmission of selected vector-borne diseases. The original plan focused only on malaria, but was later expanded to include dengue fever. The plan calls for research to determine if genetics, and specifically genetically modified mosquito vectors, could be used to supplement established control methods that rely on chemicals and environmental management. Buoyed by support provided by TDR, the John D. and Catherine T. MacArthur Foundation, the Wellcome Trust, the Burroughs Wellcome Fund, National Institutes of Health (USA) and other funding agencies, research on genetically modified vectors (GMVs) has resulted in the proof-of-principle demonstration that malaria-resistant and dengue-resistant mosquitoes can be produced. It remains to be demonstrated that laboratory-derived engineered mosquito strains can be deployed effectively and safely in the field.

Following the laboratory achievements in genetically modifying mosquitoes, three workshops (London 2001, Atlanta 2001, Wageningen 2002) began a process to discuss issues of benefits and risks in the use of GMVs. A number of recommendations were proposed at the different workshops and are summarized here:

Laboratory-based research

There is a need for continued research in the laboratory-based development of GMVs for disease control. This research includes improvement in transgenesis technology for *Anopheles gambiae* and other relevant anopheline species, as well as for *Aedes aegypti*.

Research on the validation of anti-pathogen effector molecules must be continued. Specifically, the predictive value of animal model systems for what might be expected with human pathogens needs to be determined. Research is needed to determine the potential for development of pathogen resistance to effector molecules. The genetic load imposed on vectors as a result of carrying effector molecules must be assessed using rigorous standards for fitness. The long-term stability of effector gene constructs in transgenic mosquitoes must be evaluated.

[#] Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Crete, Greece. E-mail: louis@imbb.forth.gr
[##] International Atomic Energy Agency (IAEA), Seibersdorf, Austria and Laboratory of Entomology, Wageningen University and Research Centre, Wageningen, The Netherlands.
E-mail: B.Knols@IAEA.org

Long-term preservation of laboratory strains will become increasingly important. These will include reference and transformed strains of specific insects.

There is a need to develop additional targets of genetic manipulation that may result in non-biting vectors, those with altered host preferences and other mechanisms that may not target specifically the pathogens.

Translational research

There is a need for increased interplay between laboratory and field-based research, so that advances from the laboratory are moved to the field and insights from the field help shape increasingly rigorous laboratory-based investigations. Basic research must be done on target populations, which could include topics like behaviour (reproduction, host location and host preference); population size, structure and dynamics; and physiology (reproductive and feeding). Research must be undertaken that measures the impact of field conditions on GMVs. Examples include, but are not limited to, the effects of colonization on fitness, behaviour, physiology and pathogen transmission. An increased effort must be directed toward identifying useful mechanisms to manipulate gene flow (gene drive) in target populations. This should include Mendelian systems, non-Mendelian mechanisms, and symbionts, including paratransgenesis. To meet these goals field sites for long-term study must be identified, characterized, staffed by personnel with appropriate expertise and resources, and designed to facilitate training as well as integrative research.

Safety and efficacy research

There is an urgent need to identify and define biological factors that will be important for risk assessment. These include horizontal transfer of genetic material, modified vector competence and vectorial capacity, and issues associated with the release of nuisance vectors.

Communication and education research

There is a fundamental need for collaborative work with other committees and public policy groups to evaluate decision-making processes associated with the social, ethical and legal aspects of using genetics to control vector-borne diseases.

In order to substantially elevate the prospects for successfully applying genetic tools to control vector-borne diseases, there is a need to develop consensus opinions regarding key areas that integrate field and laboratory research. The following sections describe the outcome of deliberations held in an interactive workshop format. It was structured to respond specifically to previous recommendations and prioritize laboratory and field research areas.

Objectives and expected outcomes

The SWG was to set a research agenda to address issues and challenges about genetic control of disease vectors and develop a strategic plan to bridge laboratory and field research. This would provide a basis for TDR to define its own vector research programme, taking into account its comparative advantages. The objectives of the meeting were to:

- identify research areas that will integrate molecular and non-molecular research orientated towards the use of genetics in the control of vector-borne diseases;

- identify key areas of research that take into consideration the ecological, behavioural and other field-related parameters that will affect the strategies for using genetics to control vector-borne diseases;
- establish priorities for TDR and partners-funded research to achieve a meaningful incorporation of ecological factors in genetic control;
- identify training opportunities that will result in researchers who are able to consider the laboratory-based advances in relation to the relevant ecological context.

A dialogue between the various disciplines involved in vector-borne-disease control and GMV development (e.g. molecular biologists, geneticists, physiologists, population biologists and ecologists) was expected to result in identification of key research areas for the application and evaluation of genetics to control vector-borne diseases. The production of this workshop report would subsequently provide guidelines for the TDR Molecular Entomology Committee work plan and research agendas of its partners. A strengthened research portfolio should then stimulate research in key areas related to GMV development through solicitation for research proposals.

The widening gap between laboratory and field-based research on disease vectors has been noted with concern. This meeting was the first to bridge this gap and facilitate future collaboration and training of scientists with multidisciplinary expertise in genetic control of vector-borne diseases. Successful development, evaluation and refinement of genetic strategies to control vector-borne diseases can only be expected if both laboratory and field-based scientists merge their efforts and strategically focus their impetus in this endeavour.

Genetic disease-vector control: past, present and future

Historical perspective of application of genetics to vector control

The concept of genetic control of insect pests and vectors was formulated independently three times in the 1930s-40s, obtaining encouraging results with releases of radiation-sterilized male tsetse. This was followed in the 1970s by work on mosquitoes using sterile males and systems, which could potentially be developed further to be used for driving genes into populations. Chemosterilized male *Anopheles* separated from females by a genetic system were successfully released in El Salvador. In India chemosterilization, cytoplasmic incompatibility, translocations and meiotic drive were tested with culicine mosquitoes in field cages, for mating competitiveness in the field and in some cases in village-wide release trials. The field tests in India of *Culex quinquefasciatus* and *Aedes aegypti* with various genetic-control systems indicated adequate mating competitiveness for females of wild origin. However, corresponding tests with *Cx. tarsalis* in the USA gave evidence for assortative mating. Corresponding tests with promising GM strains would be important. During the earlier stages of testing these could be radiation-sterilized to ensure that any unexpected adverse effects of the GM constructs would not be propagated in the wild population. In India, a town-wide eradication attempt planned in 1975 with *Ae. aegypti* was stopped due to spurious claims about biological warfare.

The historical examples mentioned above have in common a reduction of the mosquito populations through the use of either sterilized males or strains that lead to an unbalanced male-female ratio. In other words, the sterile-insect technique (SIT) in a wider sense of the term, an approach that has proven successful in the control of other insect pests such as, for example, the Mediterranean fruit fly and the New World

Screwworm. Although approaches of this kind are not to be excluded from consideration in the control of disease vectors, the focus of attention today is different: it entails the use of GMVs that will, ideally, not be able to transmit particular disease agents. Naturally, genetic modification can also be considered for the production of so-called sexing strains, i.e. strains that produce males only and that can, therefore, be used in SIT approaches. This strategy, though, was not discussed further at the meeting and, thus, emphasis will be put on the development of genetically modified, refractory vector strains that, after release in the environment, will eventually replace the resident populations leading to an end of disease transmission.

Anticipated impact of genetic tools in vector control and expected benefits from genomics

Presently, research efforts are concentrated on developing all the required tools for such an endeavour and providing proof of principle. Although the idea of replacing wild populations with refractory ones was already suggested in the 1980s, the compulsory germ-line transformation techniques were only developed, initially for *Aedes*, in 1998. More disease vector insects followed in the next few years, most prominently *Anopheles gambiae* in 2001. Even if the molecular toolbox has not yet been filled with all the required devices the availability of germ-line transformation made it possible to start working towards answering the crucial question of whether vector strains can be engineered to produce insects that are not able to transmit pathogens. Based on a series of experiments that used either a *Plasmodium gallinaceum/Aedes aegypti* or a *Plasmodium berghei/ Anopheles stephensi* system, it is clear today that, such an approach is, at least in principle, possible. Therefore, the task that is now faced by the research community is to expand the required techniques so that the strategies can be tested under conditions that come closer to the actual interventions in the future.

The expansion of the toolbox will have to encompass a variety of entomological and general molecular methods and approaches, such as the effector gene construct(s) to be used (including the actual gene and its entire control machinery) and the drive system that will be employed to spread the GMV population and replace the existent ones. Care will have to be taken to ensure ‘fool-proof’ mechanisms: an early failure may not only have negative effects on the future of the approach, one should also be aware of the fact that a malfunction of the system may lead to a complete permanent breakdown of the method depending on the individual components chosen. Therefore, genetic stability of such constructs is something that demands extreme attention.

To help prevent a complete failure, it will also be necessary to provide answers to basic questions that deal both with the GMV disease-refractory strains to be used (e.g. fitness, long-term stability of the engineered constructs, etc.) and the populations that these strains will be replacing. It becomes obvious that a shift in research is emerging. This shift does not reflect a major change in the long-term objectives but, rather, it encompasses the spread of new methodologies that allow for a much faster acquisition of data from the field. For example, the lack of easily scored markers (n.b.: the term ‘easy’ reflects speed, availability of resources, cost, etc.) made the study of mosquito populations somewhat tedious. The advent of nucleotide sequence markers and RFLPs, and especially the first generation genomic tools such as microsatellites, RAPDs and other ‘anonymous’ markers, provided new approaches to analyse mosquitoes and other vector insects at the population level. It should be stressed here that the use of microsatellites in population studies makes it easier to move a larger

part of the respective research to laboratories located in disease-endemic countries (DECs). Only a few years later, whole genome sequence (WGS)-derived genetic markers, such as SNPs are now bound to speed up the collection of data from field research, although the use of these very powerful markers relies on the availability of a WGS for the organism of interest, and is still relatively costly. Finally, it should be stated that an ever-growing pool of sophisticated software for population analysis accompanies the modern molecular techniques.

A major thrust in knowledge acquirement is expected from the availability of additional WGS of disease vectors. The new era was initiated by *An. gambiae* but genome projects are under way for different vector arthropods, including *Ae. aegypti* and tsetse. Considering the fact that the *An. gambiae* WGS has only been available for less than two years, it is highly encouraging to see the wealth of information that has emerged from its use. This wealth relates to an increased knowledge on gene systems that are, directly or indirectly, linked to diseases (e.g. immune system), on comparative genomics and, thus, on specific evolutionary aspects, and last but not least, the aforementioned development of specific tools. It is clear that with more genomes being analysed, the benefit for the research community will also increase, given all potential applications that will materialize. These applications will have to include tools for monitoring genetic structure and stability of vector populations, also aiding studies on dispersal (gene flow) of vector populations. Moreover, on the level of actual genomic research, emphasis will have to be given to postgenomic research on the level of general and specific RNA profiling, but also on fields that are underrepresented in vector biology today, such as proteomics.

What is needed?

The shift mentioned earlier can also be described as a move from descriptive population genetics to experimental and applied population genetics. This is a development that is a prerequisite for the successful use of GMV in attempts to control disease. Although at a purely scientific level the chances of success for this are becoming better through the availability of improved techniques, a few more items are required. These include a push to improve partnerships and create or expand existing networks between DEC scientists and laboratories in the North. This has been recognized as a *conditio sine qua non*, given the particular interest of DECs in solving the problem, combined with the fact that the problem is, in reality, international.

Current state and future needs of laboratory and field sciences

Laboratory science: progress and bottlenecks in GMV development

Although proof of principle has been achieved and vectors that are refractory to parasites already exist in the laboratory, releases of GMVs, even at a small-scale experimental level, are not anticipated in the near future. Critical laboratory-based research must be carried out to solve problems that have already been identified. This needs to include the following:

1) Identification of new promoters. A number of vector promoters (tissue-, sex-, stage-specific promoters) from *Anopheles gambiae* and *Aedes aegypti* have now been characterized and are ready for use in GMVs. However, further research is needed to identify and characterize new promoters. For example, the identification of *Anopheles gambiae* salivary-gland promoters that function in adult female salivary glands is urgently needed.

2) Development of new effector genes. The SM1 peptide does not interfere with *Plasmodium falciparum* entry into midguts or salivary glands of *Anopheles gambiae* and new effectors must be designed specifically for this parasite–vector system. Therefore, additional genes will have to be tested for their ability to suppress the transfer of aetiological agents by the insect vectors. It will probably be necessary to design multiple effector-gene strategies to target the parasite and prevent development of pathogen resistance to a given GMV approach through their very close linkage. This will also be true for ongoing endeavours to develop GMVs resistant to dengue viruses. For instance, homology-dependent RNAi approaches are now being developed that express virus-derived double-stranded RNAs that target and destroy multiple regions of a dengue-virus RNA genome.
3) Characterization of genetic drive systems. A major area of research that urgently needs to be addressed is the development of methods to drive effector genes into mosquito field populations. While several approaches are being considered, such as transposable elements, *Wolbachia*, meiotic drive and paratransgenesis, their feasibility remains to be demonstrated. Each approach has both potential benefits and problems as drive mechanisms. Transposable elements have made genetic transformation of vectors possible and have for a long time been considered good gene-drive candidates, but to date, little has been done to test whether current transposons can act as drive mechanisms. Natural transposons carrying 'foreign' genes do exist but their spread is extremely slow, if detectable at all. In addition, fully loaded transposons, which will have to carry effector genes and transformation markers, have neither been tested for their ability to move nor for stability. These types of experiments are urgently needed to assess the feasibility of GMV approaches, in addition to a thorough molecular investigation of the non-transposon-based spread mechanisms mentioned above.

Many other laboratory-based issues remain to be solved for the successful population replacement of competent vectors using GMV approaches, but major milestones have been reached. It should be stated that there exists no universal consensus that the GMV strategy will provide the long-sought solution to the problem of disease-vector control. For example, many meeting participants expressed confidence that major advances have taken place in the laboratory-based GMV research and remain very optimistic about the role of GMVs in arthropod-borne-disease mitigation. In contrast, some felt that GMVs may not present the ultimate, but they recognize that they may also have an important role in vector-population reduction strategies, particularly in producing large quantities of males (e.g. *Aedes aegypti*) for SIT strategies. Indeed, GMV lines with SIT applications have apparently been generated that may lead to field trials much sooner than GMVs developed for population replacement approaches. If so, this could provide important information on release strategies and ethical, legal and social issues (ELSI) for all GMV approaches.

Field science: current thoughts about genetic applications and integration of field and laboratory science

Laboratory-based research for developing a GMV approach will have to be complemented with field-based research to understand the ecology of vectors and arthropod-borne-disease transmission in DECs. Research areas, in which field-based research is necessary to complement ongoing laboratory-based research, have to be identified; investigations need to start now to provide critical insights into GMV-based control of both malaria and dengue. It should be noted that some of these field

studies are currently proceeding in selected DECs, but much more needs to be done and new relationships between DECs and non-DECs need to be formalized. These field-based studies include:

1) The determination of spread and stability of a transgene in vector populations by developing appropriate models of mosquito mating behaviour.
2) The definition of the genetic structure of mosquito populations to determine gene flow and probability of transgene spread.
3) The definition of vector population size with seasonal fluctuations.
4) The definition of factors controlling population regulation, and a number of other parameters for eventually designing GMV releases.

Field-based studies must also be designed and implemented to determine the evolutionary consequences of transformation, including fitness costs, phenotypic variation in effector-gene expression, and whether GMVs will effect transmission of other pathogens or ongoing vaccination, drug or vector control programmes. Obviously, the latter will have to be initiated in the laboratory. Field-based studies are also needed to develop mathematical models that can identify knowledge gaps in our understanding of disease transmission, thereby allowing researchers to better predict outcomes of potential release scenarios. A number of these studies can (and must) be initiated now in selected DECs. There have been ongoing efforts to formalize interactions of DEC scientists with laboratory- and field-based scientists of non-DECs in the context of the Gates Grand Challenges in Global Health initiative. Funding of some of these proposals should help in cementing these relationships, but it is clear that success of GMV approaches depends heavily on developing close working relationships with DEC scientists and other DEC partners.

The challenges

While approaches based on GMVs represent potentially useful solutions for mitigating malaria and dengue, there remain gaps in our knowledge that have slowed or prevented the development of genetic-control methods. These gaps exist between the state-of-the-art laboratory development of novel transmission-blocking tools and knowledge of field properties of mosquitoes that will affect their use, and between scientists in the developed world and the DEC scientists who would be responsible for implementing the technology. Further gaps exist among scientists and the agencies that would be responsible for the deployment of any genetic-control strategy, and in policies and procedures for evaluating how genetic-control methods fit into the overall strategy of existing or planned control programmes. Finally, gaps exist between the enthusiasm of scientists for these genetic methods and the level of awareness of potential end-users for the risks and benefits of using them for controlling malaria/dengue transmission. The challenge is to close these gaps in knowledge sooner rather than later.

Regional situation reports

As malaria and dengue/yellow-fever vectors (various anopheline species, *Aedes aegypti* and *Ae. albopictus*) are currently at the forefront of studies concerning genetically modified mosquitoes, the situation reports presented below focus mainly on these species. It is realized, however, that in due course vectors of other human pathogens will be included as well. These concern vectors of African trypanosomiasis, Chagas disease, onchocerciasis, leishmaniasis, filariasis and arbo-viral diseases other

than dengue and yellow fever. Several species of vectors of these diseases are already being considered for genetic modification and GMV strategies.

Africa

In recent years, work on African malaria vectors has concentrated much on investigations of species and population genetics associated with malaria transmission. In addition, studies have focused on the emergence of insecticide resistance and molecular tools for rapid assessment of such resistance. In much of tropical Africa, *Plasmodium falciparum* is the dominant malaria parasite, with *P. malariae* occurring at an incidence of 10%.

Studies on species complexes and population genetics

Important malaria vectors in Africa are members of the *Anopheles gambiae*, *An. funestus*, *An. nili* and *An. moucheti* complexes. These complexes consist of closely related sibling species with often distinctly different behaviours and ecological adaptations. Whereas *An. gambiae s.l.* and *An. funestus* are widely distributed across the continent, the latter two species complexes are mostly found in forest areas of Central and West Africa. All of these species express high heterogeneity in genetic make-up as well as in environmental adaptation, and this affects their significance as malaria vectors. Notably, not all sibling species within these complexes are vectors because of different feeding preferences.

Anopheles gambiae complex

Currently seven members of the complex have been described, of which *An. gambiae s.s.*, *An. arabiensis*, *An. melas*, *An. merus* and *An. bwambae* are malaria vectors, in this order of significance. *An. gambiae s.s.* and *An. arabiensis* are mostly panmictic across tropical Africa while *An. melas*, *An. merus* and *An. bwambae* have restricted distributions. In West Africa *An. gambiae s.s.* exhibits great genetic variability, appearing in five chromosomal forms (Savana, Bamako, Mopti, Bissau, Forest). In East and Southern Africa, only one chromosomal form (Savana) of *An. gambiae s.s.* can be found. Chromosomal forms Bissau and Forest are more often associated with humid zones, whereas the Mopti form appears to have adapted to dry zones. Recently it was shown that *An. gambiae s.s.* consists of at least two distinct molecular forms, S and M, which are characterized by their rDNA haplotype. Ongoing research shows that both molecular forms are widely distributed across the continent, with the exception of East and Northeast Africa, where so far only the S form has been found. Cytotaxonomic and molecular-genetic studies suggest that *An. gambiae s.s.* may be a sibling group expressing incipient speciation. This phenomenon has been associated with environmental changes such as irrigated agriculture and deforestation.

Anopheles funestus complex

Currently 11 species of this complex have been described, of which 10 are present in Africa. Of these only *An. funestus s.s.* is considered a malaria vector. This species is found across tropical Africa and an important malaria vector due to its high degree of anthropophily and endophilic character. Two chromosomal forms of *An. funestus s.s.* have been found: Kiribina and Folonzo. These forms are present at least from Senegal to Cameroon. From separate studies in Southeast and West Africa it was shown that genetic heterogeneity of *An. funestus* increases with geographical distance.

Anopheles nili complex

This group of malaria mosquitoes currently exists of 4 different taxonomic groups, *An. nili s.s*., *An. carnevalei*, *An. ovengensis* and *An. somalicus*. The first three species are efficient malaria vectors, with a high degree of anthropophily, whereas *An. somalicus* is zoophilic and not a vector.

Anopheles moucheti complex

Two members have been recognized, *An. moucheti s.s*. and *An. bervoetsi*. Both are sympatric in distribution, mainly restricted to central Africa. The species are morphologically identical. Locally, *An. moucheti s.l.* can be an important malaria vector.

From recent investigations it appears that members of these 4 complexes can now all be distinguished by PCR. Microsatellites are considered useful genetic markers to establish heterogeneities within a species as well as for genetic fingerprinting. Some studies using SNPs have been initiated with *An. gambiae s.s*., but they are still rare and costly.

Studies on insecticide resistance

Indoor residual spraying for malaria control has been widely applied in Africa, but was abandoned in many countries in the nineteen sixties, often for non-scientific reasons. However, in many countries resistance against DDT and dieldrin was reported. Locally, resistance against carbamates and malathion (an organophosphate) has also been reported. Studies with insecticide-treated nets (ITNs) began as of 1980 and were rapidly expanded on an experimental scale in several countries. The nets were impregnated with synthetic pyrethroids, mostly permethrin and deltamethrin. Because of the great success of these studies in reducing child morbidity and mortality by factors varying from 20 to 60% (depending on local epidemiological conditions), ITN technology was officially adopted by WHO as one of the main strategies for malaria control, through the Roll Back Malaria programme. Resistance of *An. gambiae s.s*. against pyrethroids was first observed in the Ivory Coast in 1993; since then resistance frequencies as high as 80% have been reported in this country. In 1994 resistance was also detected in *An. gambiae s.s*. from Kenya. The resistance reported from Ivory Coast has not been associated with the use of ITNs or indoor spraying, but with agricultural use of insecticides, notably for cotton production.

A special case of insecticide resistance in malaria vectors was reported from South Africa. In 1996 this country, having used DDT for malaria control since 1950, switched to pyrethroids for indoor spraying. Since then malaria incidence rapidly increased. The principal malaria vector, *An. funestus s.s*., was found to be highly resistant against the pyrethroids. The resistance was based on a metabolic mechanism of mixed-function oxidase. For these reasons South Africa resumed DDT spraying in 2001, and malaria cases have dropped since then. However, *An. arabiensis* developed resistance against DDT (dieldrin resistance of *An. arabiensis* had been reported earlier). The regional situation is complicated as Mozambique does not allow DDT spraying and continues to use pyrethroids.

Malaria-vector control in Africa

Currently, most countries have adopted ITNs as the main strategy for malaria intervention. Nine countries (in East and Southern Africa) use indoor residual spraying. In spite of the ITN strategy and implementation of the Roll Back Malaria strategy, on average only 2% of all children are covered effectively by an ITN, with

notable better exceptions in The Gambia and Sao Tomé (20% coverage). These strategies are sporadically and locally augmented by source reduction and larval control. Therefore, vector control for prevention of malaria is in urgent need of expansion.

Aedes aegypti in Africa

Aedes aegypti was the vector of yellow fever, particularly in urban centres. Efficient vaccination campaigns in the last century made yellow fever almost disappear. Such wide-scale vaccinations have been largely abandoned, and many people are non-immune against the virus.

Ae. aegypti in Africa can be distinguished into two different forms: *Ae. aegypti aegypti* (East Africa) and *Ae. formosus* (West and Central Africa). The species express different vectorial competence for dengue virus. *Ae. albopictus* has been recorded from Cameroon and South Africa. Recent studies in Cameroon show that *Ae. albopictus* is replacing *Ae. aegypti*, as the species is spreading eastwards from the coast. This species replacement is very similar to that observed in southern USA. There is no reported control of *Ae. aegypti* or *Ae. albopictus* in Africa.

Southeast Asia

This report on vectors and vector control of prevention of malaria and dengue in Southeast Asia is limited to Vietnam, Cambodia, Democratic Republic of Laos, Thailand, Malaysia and Singapore.

Malaria vectors and control

Malaria vectors in Southeast Asia belong to four species complexes, *Anopheles dirus*, *An. minimus*, *An. maculatus* and *An. balabacencis*, all made up of variable numbers of sibling species. The species occur in a wide diversity of habitats, from lowland rainforest to upland sunlit farmland (rice cultivation). The most important malaria species are *Plasmodium falciparum* and *P. vivax*. Overall, the malaria risk is relatively low, and is experienced by all age groups. However, forest workers in the age group of 30-39 years are most at risk.

Insecticide resistance is widespread in all vector species, presumably as a result of extensive application in agriculture, and in some areas as a result of decades of indoor residual spraying. There are good results with ITNs and this is, in most countries, the main tool for malaria prevention (notably in Vietnam, where some 11 million people are sleeping under ITNs).

Recently the first studies on population genetics of malaria vectors in Southeast Asia have been published, and it is expected that other such studies will appear soon, providing more information on the genetic make-up of Southeast-Asian malaria vectors.

Dengue vectors and control

In Southeast Asia dengue is highly prevalent and an important vector-borne disease. The main vectors are *Aedes aegypti* (urban centres) and *Ae. albopictus* (rural areas). Long-term studies on the biology, vector competence and ecology of *Ae. aegypti* are being conducted in Thailand. Recent studies on *Ae. albopictus* demonstrated high infection rates with *Wolbachia*. However, such infections were absent from *Ae. aegypti*. Therefore, translocation of *Wolbachia* from *Ae. albopictus* to *Ae. aegypti* using microinjection appears promising. Obviously, the use of *Wolbachia*

in control strategies has not yet been developed to a level where it could be considered for immediate use.

The incidence of dengue is increasing throughout the region, presumably due to deforestation (and other environmental changes) and rapid urban growth. Vector control occurs locally and mostly ad hoc, using fumigation of residential areas and larval control.

Latin America

In this region malaria is less prevalent compared to Africa and even lower than in Southeast Asia. Dengue outbreaks have been reported from the Caribbean since 1634, but in the last three decades serious outbreaks of dengue have been reported from Cuba, Peru and Brazil.

Malaria vectors and control

Malaria is present in the entire zone, with the exception of Chile. Both *Plasmodium falciparum* and *P. vivax* occur, in a ratio of 1:2. In 2003 more than 800,000 cases of malaria were reported, 40% of these from Brazil. The entire Amazon region is endemic with malaria. Vectors are *Anopheles albimanus*, *An. darlingi*, *An. pseudopunctipennis* and *An. nuñetzovari*. The former two species are most abundant and widespread, *An. albimanus* in Central America and along the west coast of South America. *An. darlingi* is a forest species, occurring from southern Mexico till southern Brazil. Unlike the Southeast-Asian and African anophelines, the American anophelines appear to be relatively homogeneous, showing far less genetic heterogeneity compared to the anophelines from the other continents.

Malaria control in Latin America is predominantly based on indoor residual spraying (notably Mexico), use of ITNs and focal larval control. The control of *An. darlingi* appears problematic, as this species exhibits high and varying degrees of exophilic and exophagic behaviour, and larval habitats can be huge and unsuitable for larval control. Malaria transmission by *An. albimanus* can be successfully prevented with ITN use.

Dengue vectors and control

In 2003, PAHO reported 380,000 cases of dengue in Latin America. To-date dengue transmission in Latin America is considered to be vectored by *Aedes aegypti*. This species was virtually eradicated from South America in the nineteen fifties, but has reinvaded all its previous habitats. Large epidemics of dengue have recently been recorded from Rio de Janeiro (Brazil) and Iquitos (Peru). Brazil has established a special dengue control programme, based on larval vector control. This is done with the insecticide abate and with biological agents such as *Bacillus thuringiensis var. israeliensis* (Bti). During epidemics, focal fogging of infected zones is also being performed. Other countries in the region are establishing dengue control programmes, with emphasis on larval control.

Key areas for research involving both laboratory and field sciences

Transition from the laboratory to the field

Current state of the art

It is essential that groups of researchers separated by virtue of differing interests, scientific disciplines or geographical separation interact with one another if GMV

development and application is to proceed satisfactorily. By sharing the same general goal of trying to reduce morbidity and mortality caused by vector-borne diseases, gaps relate to reciprocal lack of scientific skills and experience and the difficulties to communicate effectively and learn from one another.

Issues and challenges

The challenge is to develop an open, interactive research community that exploits the expertise of all scientists, empowers those in DECs and results in application of genetic-control methods that have a real and measurable impact on disease transmission. Communication, funding, clear strategic planning and development of genuine partnerships among all scientists are important ingredients for eliminating the barriers between DEC priorities and global research initiatives.

Opportunities and future directions for research and capacity/partnership building

The GMV endeavour is clearly dependent on the development of a scientific culture in which sharing of funds, building of intellectual partnerships, collaborative infrastructure development and sharing of technological achievements is commonplace. Another key aspect is the bridging of the 'language gap', requiring reciprocal training between field and laboratory biologists so that they can understand and appreciate one another's contribution towards GMV developments. Existing courses (like the BDV course) should grasp the opportunity to extend its training portfolio to include aspects not or only marginally covered previously (e.g. ecology and behaviour of disease vectors).

Drive mechanisms

Current state of the art

The hypothesis behind the development of drive mechanisms is that increasing the frequency of a gene (or allele) in a population of mosquitoes that interferes with the development or propagation of a pathogen will result in the reduction or elimination of transmission of that pathogen to humans. The expectation from a successful demonstration of this hypothesis is that a reduction in transmission will result in less disease. Research is needed in three areas, laboratory-based work to demonstrate that it is possible to use molecular tools to transform mosquitoes that would normally transmit a pathogen into ones that do not, laboratory and field-based work to develop mechanisms for moving genes made in the laboratory into wild mosquito populations, and the systematic assembly of information about target vector populations and disease transmission dynamics that is needed to model and predict how anti-pathogen genes will affect the epidemiology of a disease in a specific endemic region, to test this hypothesis.

Proof-of-principle advances in animal model systems of malaria and the epidemiological relevant dengue virus/*Aedes aegypti* combination confirm that much progress has been made to demonstrate the function of isolated promoters that can be used to drive the expression of anti-pathogen effector genes, the development of transgenesis technologies to integrate stably the effector genes in mosquito strains, and the synthesis of a variety of anti-pathogen effector genes that interferes with parasite and virus transmission. While consolidation and transfer of these efforts to human malaria parasites and their vectors is needed, it is appropriate now to engage the next major challenge and develop gene drive mechanisms.

Issues and challenges

The core features of a gene drive system are that is should be safe (minimum risk) and efficient. Safety refers to an evaluation of the risk factors associated with a particular system, and efficiency is assessed by how well it works to drive the gene through the population and the subsequent impact on transmission dynamics. Specific issues to be addressed are:

- the development of design criteria that mitigate scientific, environmental and epidemiological issues that might factor into safety and efficiency;
- development of a comprehensive approach for utilizing population replacement strategies that targets all the important vectors within a given area;
- development of procedures that introgress genes into strains of mosquitoes that are as similar as possible to the targets in the field.

Meeting participants discussed the desirable characteristics of drive systems and reviewed the potential effects of even low levels of recombination between the drive system and the effector gene(s), or back-mutation of these genes. In combination with (even) low levels of fitness cost associated with heterozygosity or homozygosity of the effector, only the drive system may become fixed in the target population. It is, therefore, essential to ensure extremely reliable linkage of the driver and effector, and genetic stability and full fitness of the effectors.

Research and control opportunities

Research on drive mechanisms will follow a path of studying natural genetic phenomena that alter segregation ratios to spread specific genes or alleles through populations. From these phenomena, specific drive systems must be designed that incorporate the features identified in the preceding section. Such drive systems must be demonstrated first to work in laboratory experiments and progress to semi-field evaluation. Eventually the systems will be introgressed into field-derived mosquito strains and evaluated for their reproductive and competitive success.

Future directions for research and capacity/partnership building

Future research must bridge gaps between the:

- state-of-the-art laboratory development of novel anti-pathogen tools and knowledge of field properties of mosquitoes that will affect their use;
- scientists in the developed world and the scientists living in DECs who would be responsible for implementing the technology;
- scientists and the agencies that would be responsible for the deployment of any genetic-control strategy;
- policies and procedures for evaluating how genetic-control methods fit into the overall strategy of existing or planned control programmes;
- enthusiasm of scientists for these genetic methods and the level of awareness of potential end-users for the risks and benefits of using them for controlling dengue and malaria transmission.

Participants emphasized that all future work should highlight the enlistment and involvement of DEC personnel to foster the level of awareness required for workable guidelines for the evaluation and application of genetic-control programmes.

A research agenda that requires a DEC component includes experiments to:

- introgress laboratory-derived systems into recent wild-derived target populations;
- evaluate drive mechanisms in semi-field settings;

- demonstrate that these drive mechanisms can spread an effector through semi-field populations;
- determine the effects on life-history traits of a drive mechanism in semi-field environments;
- determine the possibility of movement in semi-field cages of drive mechanisms between closely related species

Population genetics

Current state of the art

Over the last years, a number of excellent molecular tools have been developed that will now facilitate the characterization and monitoring of target field mosquitoes before and after releases. These tools also provide capabilities for having genetic markers that will assist the evaluation of aspects of competition between mosquitoes with and without transgenes. These tools include Multiple Displacement Amplification (MDA), which allows a 100-400-fold amplification of whole mosquito genomes, Single Nucleotide Polymorphisms (SNPs), and microarray chips.

Issues and challenges

The core features of theses tools should be that they are easily adapted for application in field research in the DECs. A major issue is that some of these tools are still highly expensive and therefore may drain limited resources rapidly. Training of personnel who can exploit these technologies deserves special consideration, given the adequacy of funds for this type of work. Specific scientific issues arise for the present compositions of microarray chips, which are based on expressed (EST) DNA sequences. Although useful, a better reagent would be a whole-genome tiling arrays.

Research and control opportunities

Participants noted the above challenges and placed emphasis on the following opportunities:

- meaningful capacity-building should be based on sharing of scientific material and biological specimens and move beyond the mere involvement of DEC partners in the collection and shipping of material to northern laboratories. It involves collaborative research, sharing of ideas and concepts, and population-genetics studies form an ideal target for this;
- some of the recently developed methods require sampling and preservation of specific life stages of vectors. The development of specific sampling protocols that provide nucleic acids (DNA and RNA) of mosquitoes that can be used to address major questions about gene flow, population structure etc., lends itself for joint involvement.

Future directions for research and capacity/partnership building

The rapid progression in identification of suitable genetic markers for mosquito population studies requires training that emphasizes hypothesis formation, data analysis and publication skills. The availability of these tools, as such, may not be sufficient to cover all aspects needed for GMV application, and it was recognized that indiscriminate application does not necessarily serve the purpose of the GMV endeavour. It was also noted that research to explore the adaptability of specific data sets for broader or different scientific questions is needed.

Effector molecule identification

Current state of the art

A number of endogenous refractory phenotypes have been recognized, and these include the melanotic encapsulation and lytic destruction of malaria parasites, and the RNAi-mediated impact on viruses. In addition, there are a number of other mechanisms, the specifics of which are unknown, which could include a lack of host factors (atreptic immunity). Synthetic refractory phenotypes include the construction of genes that have parasite anti-ligand effector molecules, host mosquito anti-receptor activities, toxins that kill the parasites, RNAi constructs that interfere with pathogen or vector genes, genes that lead to over-expression of pathogen antagonists, and inhibitors of parasite or viral gene expression. However, the paucity of molecules currently available hinders progress.

Issues and challenges

More work needs to be done in the identification/construction of effector/transgenes that target human malaria parasites, evaluations of dominance/recessiveness in endogenous mechanisms, and incomplete penetration and variable expression of transgenes. In addition, issues must be addressed of phenotypic variation in response to environmental conditions and selection by the transgene effector molecule of resistance or increased virulence of the pathogen. The prevalence (frequency) of the transgenes in the population will be important and threshold levels of refractoriness needed to interrupt transmission need to be determined. Field tests of specific genes and outcome evaluation parameters also are needed. With respect to this latter issue, assays for evaluating refractoriness to endogenous malaria pathogens are important and in need of development. Transmission-biting assays based on membrane feeding with gametocytemic blood derived from patients in transmission zones also need development.

Research and control opportunities

The issues and challenges can be met by evaluating specific genes in:

- Laboratory-based work
  - dominance/recessiveness
  - incomplete penetration and variable expression
  - selection of resistance
  - selection of virulence
  - combinations of effector mechanisms
- Field-based work
  - phenotypic variation in response to environment
  - field tests of performance and stability in cages
  - outcome evaluation
- Laboratory- and field-based
  - prevalence in mosquito population
  - threshold levels

Future directions for research and capacity/partnership building

Research is needed to identify:

- key qualitative and quantitative endpoints for the efficacy of transgene construct
- routine and reliable methods for transmission-blocking assays (membrane feeding) that do not involve feeding on children

- protocols for human subjects
- additional studies of natural refractoriness and its potential to adapt as a 'death-on-infection' effector mechanism.

Mosquito fitness

Current state of the art

Assessing fitness of GMVs will be a critical component of genetic programmes for control of disease vectors and prevention of vector-borne disease. It is assumed that in most cases genetic modification will incur fitness costs. This could undermine a population reduction strategy by rendering the sterile released sex non-competitive for wild-type mates. In a population replacement approach, genetic drive mechanisms are used to spread desirable genes into a population, but if insects with the desirable genes are less fit than wild mosquitoes, the drive mechanism may not be strong enough to offset the impact of the fitness cost and the desirable genes may be lost from the target population. A goal for both strategies, therefore, will be to minimize fitness disadvantages associated with genetic modification. The probability of a fitness advantage resulting from modification is considered low, but if it should occur it would be expected to promote success of either intervention strategy. Consequently, for the development and deployment of GMVs it is of paramount importance that the concept of fitness be fully understood and that a consensus is reached on how best to measure the fitness of GMVs relative to the wild-type mosquitoes they will be intended to eliminate or replace.

Of the research projects that included assessment of components of GMV fitness, three took place during the 1970s and included field releases and three recently published reports concern fitness of transgenic mosquitoes. Among these studies of transgenic mosquitoes, two reported reduced fitness of transformed mosquitoes, but their experimental design made it difficult to separate inbreeding depression from transgene effects. In the third study inbreeding depression was overcome by crossing the transformed strains with a genetically diverse laboratory strain. It was concluded that detection of a fitness load depends on the effects of the expressed transgene and that transgenes will not necessarily confer a fitness cost.

Issues and challenges

Clearly defining GMV fitness and accounting for the complexity of fitness as a concept needs to be addressed, given the fact that fitness remains one of the most controversial concepts in evolutionary biology. There is a large body of literature debating precisely what fitness is, how it varies in different situations, and how best to measure it. Its etymology is believed to have been from Darwin's reference to survival of the fittest. Following the development of population genetics during the 1920s and 1930s the term evolved to its present form, which is "*success in producing offspring, irrespective of the causes of that success*".

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

Suggested future directions

Fitness of selected strains can be evaluated by conducting competition experiments among different parental genotypes. Relative fitness can be measured in subsequent generations as frequencies of transgene genotypes (transgene homozygotes, heterozygotes and wild-type homozygotes). The advantages of this approach are that large numbers of mosquitoes in replicate cages can be examined in a reasonable period of time, all life stages of the mosquitoes can be included in the analyses, and the performance of different genotypes can be directly compared. Such evaluations should preferably be undertaken with recently transformed lines from the target population and be adequately replicated and include appropriate controls.

A three-phase process for fitness evaluation of GMVs was proposed:

- **Phase I:** Under standard laboratory conditions, a transgenic line is paired with equal frequencies of mosquitoes from the target field population. Fitness evaluations can be used to eliminate lines with major negative impairment.
- **PhaseII:** As Phase I, but at proposed release site, with freshly collected wild-type material. GMVs will also be exposed to the ambient environmental conditions.
- **Phase III:** Strains that survive Phase II are released into large replicate outdoor semi-field systems, in equal frequencies with freshly collected wild-type material from the proposed release site.

Research should be carried out on the effects of colonization and mass rearing because adaptation to a laboratory setting can alter the genetic make-up of colonized material, modify their behaviour and reduce their fitness. It remains unknown what requirements or remedial actions will be necessary to minimize loss of fitness and altered phenotypic expression associated with colonization and mass rearing. Fitness studies will greatly benefit from the availability of life-table analyses of the species that have not already been studied. Likewise, advanced (molecular) age-grading tools are in need of further development.

Future directions for research and capacity/partnership building

By adopting a standardized, consensus methodology, results from fitness assessments in one laboratory can be compared with results from another, which will help to rapidly identify appropriate constructs and GMV strains most likely to be successfully applied in the field. The transition in fitness assessment from the laboratory (Phase I) to the field (Phases II and III) offers multiple opportunities for collaboration between laboratory- and field-based researchers. Rapid and accurate assessment of GMV fitness will be important for development, evaluation and application of novel transgenic technologies.

Mosquito mating behaviour

State of the art

Of the critical behaviours that characterize the vector life histories, mating is probably the least well understood and most understudied. Yet, as disease vectors depend on sexual reproduction for species maintenance, this aspect of vector biology should receive the highest attention when seeking new avenues for genetic control and interventions of vector-borne disease. Which behavioural steps need to be considered when mating is concerned? How do vectors use their reproductive resources (sperm

vs. eggs) and what governs those choices? How can contemporary molecular tools be applied to provide improved precision on these important questions?

Issues and challenges

Mating in most disease vectors remains poorly understood. Yet, successful mating is critical for the success of proposed strategies for vector-borne-disease control using SIT or GMVs. Results from previous studies are not conclusive and the conceptual framework for understanding vector reproductive biology (especially mosquitoes) has lagged behind what has been revealed for a variety of other organisms. For instance, little attention has been paid to the mating behaviour of *Aedes aegypti* since the 1960s and 1970s. Only recently has new progress been made with studies on anopheline mating behaviour under field conditions (in São Tomé and Mozambique). Unfortunately, such studies are few, and do not address the question how mating is accomplished and by which factors this is regulated.

Suggested future directions

Because insemination of wild female mosquitoes by released transgenic or sterile males is obviously a requirement for any genetic-control programme, it is proposed that research focuses on the following aspects of mating behaviour:

- Assortative mating and polyandry
- Factors that effect frequency of mating
- Mate location (male and female)
- Cues that control male swarming (including site selection)
- Mate choice (male and female)
- Male fitness and feeding behaviour
- Pre- and post-mating behaviour
- Sperm production and depletion by males
- Sperm utilization by females
- Frequency of multiple-species swarming
- Genes that affect mating behaviour
- Mating studies of captive mosquitoes (lab to large outdoor enclosures)
- Alternative mating strategies
- Dispersal behaviour

The above list of topics is extensive, and not all aspects may be of importance for genetic-control trials to be implemented. The overall intention of mating-behaviour studies should be to aid in the interpretation of relatively simple field studies of whether or not released male GMVs can effectively compete for females of the wild vector population.

Capacity partnership building

The above aspects appear critical for a proper understanding of mosquito population biology and genetics and offer opportunities for interaction among molecular geneticists, vector ecologists and people modelling genetic vector control strategies. For instance, in population modelling of the behaviour of gene transfer between GMV and wild populations, the frequency of wild versus GMV matings should be well understood in order to predict the number of GMV released individuals required for effective results. Also, SIT programmes require a constant monitoring of wild versus sterile matings to adjust the release rate over time. Finally, any driving mechanism of foreign DNA into wild populations requires normal mating behaviour,

and can only be evaluated once it is properly understood. Many of these aspects can *only* be addressed through field studies, offering many opportunities for collaborative research between northern and DEC scientists.

Pathogen evolution

State of the art

Research on the interaction of *Plasmodium* and dengue virus with their arthropod and human hosts, indicates that the capacity to evolve resistance to GMVs may exist. The observations that *Plasmodium* can modulate the immune response of its mosquito vectors, and that there is an absence of sterilizing immunity in human infections, besides occurrence of drug resistance, are all well-documented phenomena that are consistent with the notion that parasites may evolve resistance to barriers intended to prevent transmission by GMVs. Similarly, dengue, like other RNA viruses, lacks a proof-reading mechanism to correct errors during replication. Consequently, dengue has a high mutation rate and the capacity to change rapidly due to drift or selection in response to changes in the environment in which it reproduces.

Issues and challenges

An important issue in addressing pathogen evolution relates to consensus building of exactly what is meant when one refers to resistance and virulence. Definition of these terms will avoid confusion and aid in the development of unified conclusions across different research groups studying those topics.

It will be critical to develop non-onerous means for assessing the capacity for parasites to avoid interference from GMVs and to characterize virulence of resistant parasites. A system that is operationally practical needs to be developed, should provide this essential information, and be standardized across different research groups and laboratories.

An undesirable outcome from a GMV release would be the evolution of resistant parasites that are more virulent than their predecessors. A rich body of literature on the evolution of virulence demonstrates that increased virulence is not necessarily disadvantageous to parasites. If the efficiency of transmission is linked to virulence in a way such that increasing virulence similarly increases the basic reproductive rate of the parasite, virulent phenotypes will have a selective advantage over those that are less virulent.

Suggested future directions

A practical strategy to assess and/or monitor evolution of parasite resistance in the mosquito and virulence to humans needs to be developed. Research should be carried out on the effects of genetic variation in mosquito and parasite populations on vector–parasite interactions, the evolution of resistance, and virulence characteristics of resistant parasites.

The requirements necessary for minimizing the adverse effects (e.g. mosquito colonization and parasite culturing) of carrying out vector–parasite interaction studies in the laboratory need to be defined. A deepened understanding of environmental effects on vector–parasite interactions will be beneficial in the study of GMVs.

Capacity partnership building

Expertise on parasite resistance and evolution of virulence needs strengthening and may be acquired from outside the discipline of vector biology.

Chapter 2

Modelling

State of the art

Models have had a profound impact on understanding processes of pathogen transmission and reducing disease. In general, models make two vital contributions to the development and application of disease-prevention technology. First, they help identifying knowledge gaps and second, they enable predicting outcomes of scenarios of interest. Past contributions of modelling to genetic-control strategies for vector-borne diseases and future opportunities were recently reviewed. It was noted that "during the era of classical genetic-control research there appears to have been incredibly good communication and cooperation between theoretical and empirical researchers. Indeed much of the empirical work was inspired by results of population genetics studies. There has been a tendency for the sophistication of modern science to isolate researchers involved in molecular work from those doing ecological and population genetics studies. More interaction between these scientists at the very early stages of genetic-control projects may increase the chances of producing useful strains and ushering in a new and long-lived, golden era of genetic control".

Issues and challenges

Although it is uniformly agreed that models will be an integral part of developing, evaluating, and deploying genetic strategies for vector-borne-disease control, there is debate regarding how they can be best constructed and used. For example, the relative merits of emphasizing generality, realism or precision remain questionable. The precise role and involvement of empirical researchers in the development, validation and application of models of genetic vector control need to be clarified. Accessibility and user-friendliness for non-specialists need to be addressed, as does the adaptation of models for field application. Existing models of genetic vector control illustrate the relative merits of different approaches. Some provide estimates for spread of transposons and associated genes across all species, but are applicable only to specific species. Others account for spatial heterogeneity but do not explain movement of transposons within genomes, which could profoundly influence their spread within a population.

Suggested future directions

Models should be used as a quantitative framework that can assist researchers and guide policymakers in more accurately assessing the field-implementation costs and potential for success of a variety of transgenic approaches. Modelling efforts should transcend simulation of events retrospectively and be more productively directed at predicting outcomes of proposed interventions. Predicting outcomes will require close collaboration among modellers, laboratory-based molecular geneticists, vector ecologists, and epidemiologists. This kind of reciprocal interaction is essential to obtain the necessary insights to advance vector-based genetic control. Complexity and generality of models will depend on their intended purpose, which will need to be determined based on interaction between the people developing models and those using their output. Models should be constructed in ways that make them easily accessible to users. Finally, suggested modelling topics could include efficiency of drive mechanisms, epidemiological impact on disease, etc.

Capacity partnership building

Construction, validation and application of models constitute one of the best examples of how people with different kinds of expertise can work together in mutually beneficial ways. For example, modellers can provide specific information to people creating transgenic mosquitoes regarding the kind and extent of transposon movement that is best suited for driving transgenes through target populations. They can also make recommendations for the best GMV release strategies. Vector ecologists can provide essential information for modelling mosquito population and pathogen transmission dynamics. Molecular geneticists can provide empirical information on transformation and transposition processes.

Identification and characterization of field sites

State of the art

The ongoing development of GMVs for application in genetic-control programmes against major tropical diseases such as malaria and dengue is gradually advancing to a stage where scientists involved are planning future field trials or, more in the short term, semi-field evaluations. The transition of research efforts from the laboratory to (semi-)field environments raises a number of important issues like ELSI (ethical, legal and social issues; addressed elsewhere), and also the selection of appropriate field sites. Once identified, target pest populations need to be characterized. At present, field sites for future genetic-control interventions are being selected, based on the biological characteristics these should possess. There is, however, no coordination of these activities (see section 6).

A priori, it can be noted that previous genetic-control trials (e.g. employing cytoplasmic incompatibility (CI), chromosomal translocations, the sterile-insect technique, etc.) have addressed many analogous problems related to present-day site selection and characterization, albeit without the genetic-engineering component that adds new and unique concerns. The following sections focus on GM mosquitoes, though most concepts apply similarly to other disease vectors.

Issues and challenges

The transition of laboratory research on GM mosquitoes to full programmatic implementation in disease-endemic settings encompasses a series of steps, each with its own unique challenges. Historical genetic-control attempts focused mainly on two key aspects affecting the potential for success, i.e. knowledge of the local vector population, and partial to full isolation of the target population.

CI trials in Myanmar were conducted in a village surrounded by rice fields, where the target pest did not occur. In Kenya, genetic-control trials against *Ae. aegypti* in the 1970s focused on villages and a small area surrounding them. The necessity for applying genetic control against isolated populations remains valid today. It has been proposed to target *Anopheles arabiensis* populations in urban areas surrounded by *An. gambiae s.s.* or urban *An. stephensi* populations surrounded by *An. culicifacies*. Others have suggested going beyond 'ecological islands' described above, and move to physical islands. Genetic-control trials have delivered dramatic successes through eradication of target pests, such as the eradication of *Glossina austeni* from the island of Zanzibar by 1997.

With regard to the application of GM approaches for disease-vector control, further containment (in terms of selecting isolated populations) is needed, to overcome potential adverse effects of the introduction of GM insects. The choice for physical islands, far from mainland populations, seems the best option in that regard

Beyond (1) geographic isolation, there are several more key factors affecting the selection of a field site, such as (2) occurrence in a narrow geographic range and (3) appropriately sized area (small enough to be manageable, large enough to be convincing); presence of (4) panmictic populations of (5) one vector species (although another closely related non-transmitting species may be useful as a 'control'); the target species occurs in relatively (6) low density and can be suppressed with existing vector control tools, and (7) disease transmission, in order to measure a public-health impact of the intervention.

These biological pre-requisites should serve as the first criteria when selecting a field site. Secondary criteria include (8) accessibility of the site and availability of research infrastructure, and the availability of (9) detailed entomological and epidemiological information.

Suggested future directions

Specific research topics related to field site selection and characterization should follow an evaluation of the existing human resources and scientific/public-health infrastructure at the chosen sites, and include:

- Collection of basic ecological and biological data of the target species, including relative population densities, adult and larval distribution patterns and an assessment of the role of the target species in disease transmission.
- Assessing the degree to which the target population is genetically isolated from surrounding populations and a description of the genetic structure of the population.
- Creating a geographic information system that describes the ecology of each site and is fully integrated with information from the ecology and population-genetics studies.
- Baseline studies on parasite transmission and disease epidemiology.
- Developing a system for oversight of research activities at field sites.
- Semi-field research in countries with established research and scientific collaborations.

Capacity partnership building

Perhaps the most significant challenge in terms of GMV development and implementation is the full participation of and genuine collaboration with partner institutions in DECs that meet the above criteria. In all likelihood, the suitability of island settings will be countered by the absence of appropriate institutional frameworks and local competence. It will be important to develop general guidelines for GMV implementation irrespective of the field site/country involved. A regulatory framework residing under a larger umbrella such as the WHO seems to be best suited for this (see Section 6).

Integrated disease management

Current state of the art

Most NMCPs focus on early diagnosis and treatment, personal protection, health education and vector control. While anticipated, vaccines and GMVs are currently not available. Current vector control measures emphasize personal protection by use of ITNs or IRS, and to a lesser extent source reduction by environmental management (drainage, filling), biological control (larvivorous fish) and larviciding (Bti, *Bacillus sphaericus* (Bs), temephos).

Issues and challenges

It was recognized that lack of sufficient knowledge on the ecology, behaviour and genetic background of vectors (in most disease-endemic areas at least two or more vectors occur sympatrically) hinders conceptualization of effective integrated approaches to disease-vector control. Given the adoption of GMVs in existing disease and vector control programmes, it remains unknown how the impact of GMVs on disease epidemiology will be gauged in a background of other control activities. Moreover, it remains unclear how GMVs will compare with other control strategies in socio-economic terms. Much effort is currently being undertaken to upscale the use of ITNs at community level and enhance sustainability of this intervention. If transgene spread occurs at similar rates in low-density or high-density populations, then both GMVs and ITNs may be applied concurrently.

Research and control opportunities

Future directions for research and capacity/partnership building relate to the integration of GMV development in ongoing vector research programmes in DECs. This requires significant expansion of both capability and capacity of DEC partner institutions. It was suggested to identify centres of excellence in DECs to tackle GMV development as a local solution rather than an adopted one from the North, advocating a transition in problem ownership. A real opportunity for DEC partners is advocacy of the GMV approach to policymakers, communities and NGOs, to promote GMVs as an integral component of IVM. A change in mind-set, from operating at household level (IRS, ITNs etc.) to community-wide and area-wide scales will require the development of approaches consistent with health-policy frameworks operating at such scales (e.g. district level).

Epidemiological impact assessment

Current state of the art

At least two frameworks exist to evaluate the entomological impact of a genetic-control approach for malaria. The vectorial capacity equation and the entomological inoculation rate (EIR) could, in principle, provide empirical measures of the effects of introduction of GMVs. For dengue, such measures are not available.

Issues and challenges

Challenges include evaluating the relevance of reproductive and physiological fitness to transgenic engineering. An important property of a GMV is its reproductive fitness compared to that of wild-type conspecifics. Transgenic mosquitoes must be as physiologically fit as the wild types. Transformation strategies must not compromise fitness. A major emphasis in genetic-control strategies is blocking the sporogenic cycle. The current genetic-modification strategies that probably have the least effects on fitness are blocking the penetration of the midgut and salivary glands by sporozoites. The result would be females that cannot support the sporogonic cycle. The possibility of reducing anthropophagy seems initially attractive. The approach would be to change the blood-feeding preference of *An. gambiae* from man to animals. Some of the sibling species of the *An. gambiae* complex such as *An. arabiensis* and *Anopheles quadriannulatus* can be highly zoophilic and it may be possible to introgress the relevant genes to reduce anthropophagy.

Research and control opportunities

The endpoint for any introduction of a GMV should be to measure a public-health benefit. To date, however, it remains unknown what entomological thresholds need to be attained before an epidemiological impact becomes measurable. These issues have been similar when measuring the impact of other interventions (e.g. ITNs) but become more complicated when introducing GMVs in areas where other interventions are practiced concurrently (see 5.10). Entomological monitoring tools that are efficient in collecting GMVs for research purposes are urgently required.

Future directions for research and capacity/partnership building

Participants agreed that GMV evaluation, if, for instance, based on *Plasmodium* sporogony interference, should have as its prime target the sporozoite rate. This can be done by collecting large numbers of females in the field and subjecting them to ELISA assays. Given that a GMV release programme yields a substantial reduction in the sporozoite rate within the targeted population, it would then be justified to proceed to measures of impact related to disease, e.g. incidence, morbidity and mortality.

Ethical, legal and social issues

Current state of the art

The technical feasibility of the development of transgenic mosquitoes unable to transmit malaria and dengue pathogens has been demonstrated in the laboratory. However biotechnological and implementation challenges remain to be addressed in order to make this approach a control method applicable as a public-health tool in disease-endemic settings.

Issues and challenges

The principle challenge is to provide a proof of efficacy and safety of the use of GMVs for disease control. This proof is required to initiate addressing the ELSI of the potential use of GMVs.

Research and control opportunities

There is a commitment from WHO/TDR as part of a wider network of research institutions and funding agencies to advance the area of genetic control of vectors. In addition it has been recognized that major initiatives (e.g. the Gates Grand Challenges in Global Health initiative) accommodate vector-research components and provide new research opportunities.

Future directions for research and capacity/partnership building

The working group noted that this area has not received sufficient attention over the last decade, but recommended specific areas for research:

- To provide proof-of-principle and biosafety assessment (risk management) of the use of GMVs for disease control by:
  - conducting studies on the efficacy, biosafety and risk/benefit evaluation through long-term efforts to clarify the scientific uncertainties under different experimental conditions and with the involvement of DEC investigators;
  - providing the basis for collection of data on vector biology, ecology, behaviour and genetics addressing efficacy and safety in the field;
  - developing guidelines and principles on the design and performance of efficacy and minimum-risk field research;

  - developing criteria and test methods for environmental monitoring;
  - developing criteria to identify and prepare field sites for approach evaluation.
- To ensure the public that this goal is desirable, feasible and can be accomplished safely, by:
  - developing a strategy to make the information available to the public and the media such as to raise their awareness and address their concerns about possible environmental and human-health risks;
  - bringing all parties together on common ground that can lead to objective, scientific, legal, ethical and social-based decisions by policymakers, bearing in mind that many people may not endorse the scientific efficacy and risk analyses.
- To develop a plan to gather all the information necessary for legal and regulatory approvals, particularly documentation related to biosafety and ethical review and national/local-authority approval.
- To enhance capacity in DECs: for biosafety assessment, risk/benefit evaluation, environmental monitoring, and data collection on the vector biology, ecology, genetics and behaviour.
- To promote South-South and North-South research collaboration based on well-defined ethical, equitable and scientific standards.
- To develop mechanisms for dissemination of information to researchers, decision-makers, affected communities, the public and the media, and develop procedures for obtaining informed community consent.

Coordination and follow-up mechanism

The development and implementation of GMVs is a multidisciplinary endeavour that requires ever-widening circles of involvement within society. Alhough it is recognized that many activities will remain laboratory-based for some time to come, involvement of field-orientated scientists is becoming increasingly important. This, in turn, raises issues related to communities living in such field settings, the political climate in the affected country, etc.

The need to establish a coordinating board to engage in the provision of guidance and steering to all levels within the molecular-science – society continuum, was noted and endorsed.

Although the constituency and mandate of this body need further deliberation, it was agreed that:

- it should take a platform function, communicate to all stakeholders in the GMV endeavour, and in particular sustain a mechanism for addressing media enquiries;
- it should engage in constant contextual analysis of the external environment in order to monitor developments and perception changes in the public and scientific community alike;
- it should play a catalytic and proactive role in establishing, securing and strengthening partnerships among stakeholders in the GMV field.

Membership of this board will be based on rotation, vested interest and adequate expertise, and should strive for an appropriate balance between members from DECs and developed nations.

3

Review of previous applications of genetics to vector control

Chris F. Curtis[#]

Abstract

The idea of genetic control of insect pests and vectors was invented independently three times in the 1930s-40s. Results with releases of radiation-sterilized male tsetse have been encouraging. Much work was done in the 1970s on mosquitoes with sterile males and systems that could potentially be used for gene driving. Chemosterilized *Anopheles* separated from females by a genetic sexing system was successfully released in El Salvador. In India chemosterilization, cytoplasmic incompatibility, translocations and meiotic drive were tested with culicine mosquitoes in field cages, for mating competitiveness in the field and in some cases in village-wide release trials. However, a town-wide eradication attempt with *Aedes aegypti* was stopped due to spurious claims about biological warfare. This experience underlines the need for very careful attention to relations with journalists, politicians and the general public. Transgenic sterile males, which are now available, should have considerable advantages over radiation and chemosterilization and would seem well suited for eradication of urban vector populations.
Keywords: sterile male tsetse; chemosterilized mosquitoes; cytoplasmic incompatibility; translocations, meiotic drive; mating competitiveness

The pioneers

The concept of genetic control was invented independently in three very different environments in the 1930s-40s (Klassen and Curtis in press). In the order in which their ideas were published the inventors were:

A.S. Serebrovskii

Serebrovskii (1940) had worked at Moscow State University with H.J. Muller, the discoverer of radiation-induced mutations, including dominant lethals, in *Drosophila*. Serebrovskii proposed the use of chromosome translocations as a source of inherited partial sterility with which to suppress pest populations. He realized that because they caused semi-sterility in heterozygotes, but not in homozygotes, translocations would be selected for in a population if sufficiently released to constitute a majority. However, he did not go on to suggest them as a means of driving genes tightly linked to the translocation and causing inability to transmit disease into populations. Curtis (1968) made this proposal after independently realizing that translocations, a common form of radiation-induced mutation, might have a role in genetic control. Curtis

[#] London School of Hygiene & Tropical Medicine, London WC1E 7HT, UK. E-mail: chris.curtis@lshtm.ac.uk

(1971) also showed that translocation heterozygotes and homozygotes could be produced in tsetse flies, despite the laboriousness of rearing such viviparous insects.

F.L.Vanderplank

Vanderplank (1947; 1948) worked as part of the remarkably active British colonial effort to control tsetse. He and others had found that one could cross closely related species of tsetse and that the crosses were partly sterile and produced partly sterile hybrids. He therefore proposed the collection of thousands of pupae in the breeding range of one species (*Glossina morsitans*) and their release into an isolated sector of the breeding range of another (*G. swynnertoni*) in Tanzania. It was realized that this would only have any useful effect if *G. morsitans* could compete for mating in the field with *G. swynnertoni*. Jackson (1945) released both sexes of both species and found that when couples were collected, during their two-hour copulation, all four combinations of the species could be found at frequencies indicating no behavioural barriers to cross-mating. Therefore Vanderplank initiated a field trial in June 1944, like another more famous enterprise. Over the next two years, releases of about 100,000 *G. morsitans* first led to virtual elimination of the resident *G. swynnertoni*, a phase lasting about a year in which significant numbers of hybrids could be found, and then to replacement by *G. morsitans*. However, in the long term this species could not maintain itself in the arid climate of a *G. swynnertoni* habitat and the introduced *G. morsitans* also declined toward extinction. After two years tsetse densities were so low that local farmers, whose activities completed tsetse eradication, could safely occupy the area. Thus, not only was Vanderplank the first person to release insects causing sterility but he also demonstrated a deliberate population replacement by a conditionally lethal genotype. Unfortunately Vanderplank only published a very brief account of his work. He gave detailed data on this remarkable trial to the present author in 1970 (see Klassen and Curtis in press) by which time release trials of irradiated tsetse were already in progress.

E.F.Knipling

Knipling (1955; 1959) observed in the US Dept. of Agriculture's Screwworm Fly laboratories that Screwworm (*Cochliomya hominivorax*) females are monogamous. It was apparently this observation that set him thinking about the possibility of the Sterile Insect Technique (SIT) if only some means of sterilizing male insects could be found. In the 1940s Knipling and Bushland made contact with H.J.Muller who pointed out that, based on 20 years of experience in *Drosophila* (Muller 1927), irradiation with gamma rays or short-wavelength X-rays readily induced dominant lethals (broken chromosomes) in sperms killing the embryos when mitosis begins after fertilization. A dominant-lethal rate of virtually 100% could readily be obtained by irradiation of Diptera at a dose far less than that needed to kill a pupa or adult insect in which development, including the radiation-sensitive process of mitosis, had already been completed. Knipling soon realized that, because radiation induced sterility in Diptera via dominant lethals, which did not inactivate sperm, his original observation of female monogamy in Screwworm was by no means a requirement for the SIT to work – an interesting example of being right for the wrong reasons! The belief that monogamy is a requirement for the SIT is still entrenched among journalists and, judging by recent comments of a referee on a grant application, among some biologists who ought to know better! Knipling, Bushland and the USDA team quickly showed that Screwworm pupae could be given a radiation dose that would ensure lifetime male sterility, that mass-rearing was possible and that, after

release, sterile males could compete for mates. They demonstrated eradication from the island of Curaçao and then from Florida. The programme expanded to achieve complete eradication of Screwworm from the southwestern states of the USA, Mexico and Central America as far south as Panama and of an accidentally introduced population of *C. hominivorax* from Libya (Wyss 2000; Lindquist 1993). A programme of aerial releases which moved steadily forward was able to deal with the problem of immigration by ensuring that females were engaged in sterile matings before beginning their migratory flights, achieving eradication of this major veterinary pest, which can also cause myiasis in humans, at a cost that was rapidly repaid by elimination of the cattle losses caused by the Screwworm. At one point it was claimed that behavioural barriers existed between different sub-populations of Screwworms (Richardson, Ellison and Averhoff 1982), but this was never supported by many data (Krafsur 1998) and the successful eradication all the way to Panama with flies reared from a single captive population indicates that this kind of biological complexity was not a real problem.

Induced sterility in vectors

Tsetse

Combined trapping and SIT programmes were carried out against the riverine sub-group of tsetse in Burkina Faso (Politzar and Cuisance 1984) and Nigeria (Takken et al. 1986). More recently *G. austeni*, the only tsetse species in Zanzibar, has been eradicated by SIT from that island (Msangi et al. 2000). In a few of the trials, late pupae have been irradiated but in most cases adults have been irradiated. Because of the extraordinarily low natural fertility of tsetse, the numbers that can feasibly be reared are far less than with Screwworm but, conversely, far lower sterile:fertile male ratios are presumably needed to initiate a downward population trend. In tsetse one can collect predominantly males for release from the pupae deposited on a given day, as the males emerge first and the females can be retained to provide breeding stock for the colony. Male tsetse bite but various methods have been attempted to minimize the risk of disease transmission by the released sterile males. The only recorded attempts to use the auto-sterilization principle has been carried out in tsetse (Hargrove and Langley 1990; Oloo et al. 2000), i.e. attraction of wild flies to a bait which will sterilize them and then allowing them to return to the wild population and mate.

There are strong disagreements about whether the eradication of tsetse in Zanzibar should be taken as a precedent for major efforts to eradicate tsetse from large parts of continental Africa or whether such an attempt would be a hopeless task and a serious diversion of resources from less ambitious but more hopeful anti-trypanosomiasis measures.

Mosquitoes

In mosquitoes, irradiation of late pupae with a male-sterilizing dose causes severe reduction in competitiveness of the emerging adults. This was shown for *Culex* mosquitoes 35 years ago (Smittle et al. 1968) and confirmed for *Anopheles* recently (Andreasen and Curtis in press). Irradiation of adults is apparently not harmful but irradiation of millions of adult mosquitoes would be very inconvenient. It was found in the 1970s that immersion of pupae in an alkylating agent such as thiotepa or bisazir could cause nearly 100% dominant lethality in sperms with little apparent damage to the emerging adults. The treated pupae were carefully rinsed before emergence and the residues of the alkylating agents, which are mutagens, were very low (LaBrecque

et al. 1972). However, from a so far unreplicated study it was reported that spiders fed a diet of nothing but chemosterilized mosquitoes were themselves sterilized (Bracken and Dondale 1972).

In the 1970s it was considered acceptable to go ahead with releases of chemosterilized *Cx. quinquefasciatus* at the WHO/ICMR Unit on Genetic Control of Mosquitoes in Delhi (Yasuno et al. 1978) and *An. albimanus* in El Salvador (Lofgren et al. 1974; Dame, Lowe and Williamson 1981). There would have been almost no contact of these mosquitoes with humans after release because highly effective sex separation was developed so that virtually all were non-biting males. In culicines the male pupae are markedly smaller than females and a carefully adjusted sieving system (Sharma, Patterson and Ford 1972) routinely achieved 99.8% males among releases of 300,000 per day (Singh et al. 1975; Ansari et al. 1977). Demonstration to villagers near Delhi that the males that were being released did not bite satisfied them that the mosquito release activities were at least doing the villagers no harm. In *Anopheles* the pupal size difference between the sexes is not reliable and instead dominant genes causing insecticide resistance were translocated on to the Y chromosome so that females could be selectively killed early in the egg or larval stage, leaving the resistant males unharmed in the *An. gambiae* complex (Curtis, Akiyama and Davidson 1976; Curtis 1978) and *An. albimanus* (Seawright et al. 1978). In the latter work an inversion was used covering the chromosomal segment between the resistance gene and the translocation breakpoint to minimize recombination in this segment that would otherwise progressively reduce the accuracy of sex separation over successive generations. For unknown reasons great difficulty has been experienced in producing comparable sex separation systems in *An. stephensi* (Robinson 1986; Andreasen 2003). This is unfortunate as the latter species, with its urban distribution and role as a serious malaria vector in India, renders it a suitable target for attempts at eradication of urban 'island' populations by SIT as discussed below.

Studies on mating competitiveness were carried out in India with released chemosterilized *Cx. quinquefasciatus* and *Ae. aegypti* (Grover et al. 1976a; 1976b). In the tests with the culicine mosquitoes, males marked with fluorescent powder were released so that the ratio of released sterile to wild fertile males could be determined. Then similarly marked virgin females of wild origin were released, left long enough to mate and then recaptured and made to lay eggs in the laboratory. The proportion of sterile and fertile egg layings was found to be not much less than the sterile:fertile ratio among the males, indicating quite good competitiveness for these females whose marking indicated that they had mated within the village where the males had been released. Similar good competitiveness was found with chemosterilized *An. albimanus* (Dame, Lowe and Williamson 1981).

When releases were made with chemosterilized *Cx. quinquefasciatus* daily over many weeks the percentage sterility among the egg rafts laid was initially disappointingly low and only slowly rose to 80%, which was still far less than the proportion of sterile males within the release village (Yasuno et al. 1978). Because the hypothesis of poor competitiveness of the males had been ruled out by the above described competitiveness test, it was concluded that many of the fertile rafts had been laid by females which had mated outside the release area and then migrated into it, despite attempts to maintain a 3km-wide barrier zone free of breeding by use of larvicide. Because of the generally monogamous behaviour of mosquito females they had refused to re-mate on arrival in the release village. Thus, far from female monogamy being a requirement for the SIT to succeed, it is actually an unfortunate

fact of life, which in this and several other trials has made non-isolation of the target area a major barrier to success.

In the El Salvador project a target population around one lake was apparently adequately isolated; 100% sterility of the eggs laid was achieved and the normal surge in the wild vector population was prevented when the seasonal rains arrived (Lofgren et al. 1974). However, extension to a larger target area again encountered problems of immigration despite maintenance of a barrier zone (Dame, Lowe and Williamson 1981). Nevertheless, a histogram of the data (Benedict and Robinson 2003) on *An. albimanus* densities in the release and a comparison area emphasize how successful the chemosterilized males were in preventing a normal seasonal rise in vector density. Latterly these males came from the strain described above with genetic sex separation and this allowed production and release of a million males per day.

Tests of systems with the potential to drive genetic factors into populations

Cytoplasmic incompatibility

Sterility was found to exist in crosses between different geographical populations of the *Culex pipiens* complex. In some cases both reciprocal crosses were sterile and in other cases only one of the reciprocal crosses was sterile. By repeated backcrosses in one of the latter cases Laven (1967b) showed that control of crossing type was wholly maternally inherited and the phenomenon was given the name cytoplasmic incompatibility. It was possible to choose strains which yielded 'ready made' sterile males with respect to a given target population. By an appropriate backcrossing programme, sometimes involving an intermediate strain, it was possible to equip a strain for release with the chromosomes of a target population and therefore presumably make it well adapted to its environment, without affecting the sterility of crosses between the strains (Krishnamurthy and Laven 1976).

Laven (1967a) demonstrated eradication of a small village population in Myanmar by release of males only of an incompatible strain. It was realized that 100% sex separation was not possible on a large scale and released females could engage in fertile matings with released males leading to population replacement and not eradication. The idea therefore arose of equipping the released strain with another genetic factor with desirable properties and using bi-directional cytoplasmic incompatibility as a means of driving this factor into the population. This should be achievable on the principle of selection for the majority type, where crosses are less fit than either of the pure matings, as described above with regard to autosomal chromosome translocations. It was hoped eventually to drive in genes for non-susceptibility (refractoriness) to filariae but it was more feasible to produce male-linked translocation complexes causing about 65% sterility (Krishnamurthy and Laven 1976). Because they were male-linked, homozygotes could not be produced so they would behave quite differently to autosomal translocations and could not themselves act as gene-driving systems. The idea was that the bi-directional cytoplasmic incompatibility would be the gene-driving system and would also prevent recombination of the cytoplasmic type with the translocation. In a field cage it was shown that this did indeed happen. As releases of the translocation-incompatible strain proceeded there was a phase when many fully sterile (incompatible) egg rafts were produced but, after several weeks, the frequency of these declined to zero, leaving only egg rafts showing partial sterility due to the translocation in matings between males and females with the same cytoplasm (Curtis 1976).

The mating competitiveness in the field of the males of this translocation-incompatible strain was tested by the same method as described above for chemosterilized males and very much the same results were obtained (Grover et al. 1976a). Several weeks of large-scale field releases into a village followed (Curtis et al. 1982) and, as with the chemosterilized releases, the continued appearance of some egg rafts with full fertility indicated that considerable immigration was occurring despite efforts to prevent it.

While these field studies were going on it was shown that complete bi-directional incompatibility of Indian wild strains and laboratory strains of European origin could not be relied on because there was an effect of male aging on incompatibility (Singh, Curtis and Krishnamurthy 1976) and because there was polymorphism for cytoplasmic types in wild populations (Subbarao et al. 1977). At about this time Yen and Barr (1973) produced strong evidence that the maternally inherited factors that caused cytoplasmic incompatibility were symbiotic *Wolbachia*. These occur naturally in *Culex* and some *Aedes* populations but can be eliminated with antibiotic treatment, resulting in strains with males that are universally compatible and females that are only compatible with males from which *Wolbachia* has been removed. It is deduced that *Wolbachia* in males inactivates sperms but these can be 'rescued' if the female carries *Wolbachia* that are compatible with the strain in her mate. This hypothesis explains the crossing properties of strains from which *Wolbachia* have been removed and of naturally existing incompatibility between different geographical populations. With *Aedes* and *Drosophila* it has been possible to inject back *Wolbachia* into strains without them and the expected incompatibility is then expressed (Sinkins, Curtis and O'Neil 1997). It is therefore hoped that *Wolbachia* could be introduced into *Anopheles* where it does not naturally occur.

Meiotic drive

Hickey and Craig (1966) showed in *Aedes aegypti* that combination of a 'driving' *M*, male-determining, gene with susceptibility of its *m* allele led to distortion of the sex ratio in favour of males. This was not due to selective mortality of female embryos but to excess production of male-determining sperm (i.e. meiotic drive – "breaking of Mendel's first law"). At the unit in Delhi this phenomenon was combined with two translocations. These fortunately enhanced the strength of sex-ratio distortion to 13:1 and the double-translocation heterozygote showed about 65% sterility (Suguna et al. 1977a). This combined system, when tested in the field by the above described method, showed good competitiveness for mates (Grover et al. 1976b). In a field cage trial extending over several generations and with simulated density-dependent regulation of the population, the sex-ratio – translocation system showed as good, but no better, population-suppressing ability as chemosterilized males (Curtis et al. 1976).

Apart from a use for attempted population suppression, meiotic drive has been suggested as a possible means of driving refractoriness genes into populations. However, it was found that in Indian wild *Ae. aegypti* populations there are some resistant *m* alleles (Suguna et al. 1977b) which would be selected for and prevent indefinite spread of the meiotic-drive factor. Furthermore, meiotic drive has not yet been reported in *Anopheles*.

Claims about biological warfare

As indicated above there was evidence for a serious level of immigration of *Culex* between Indian villages. It was noted in the 1970s in India that *Ae. aegypti* was

limited to urban areas where there were water tanks. Because of the importance of this species as a dengue vector it was proposed to attempt eradication of an *Ae. aegypti* population from a whole town by sterile-male release (Reuben et al. 1975). Unfortunately an Indian journalist noted that *Ae. aegypti* is commonly called the 'yellow-fever mosquito', that there is no yellow fever in Asia and that yellow fever had been considered in the 1960s as a biological warfare agent. He jumped to the conclusion that the purpose of the research at the unit in Delhi must have been to obtain data of use for biological warfare (Oh New Delhi, Oh Geneva (editorial) 1975; WHO 1976). Powell and Jayaraman (2002) attempted to revive these claims, but it was pointed out to them that the mass releases were to be with males which, because they do not bite, could not be relevant to biological warfare. This press campaign was adopted by opposition politicians and led to cancellation of the town-wide eradication attempt just two days before it was due to begin. From this experience must be drawn the conclusion that any future mosquito release project must pay close attention to relations with, and information to, journalists, politicians and the general public. The WHO restrained its staff from replying to the slanderous accusations on the grounds that the accusations should be viewed as a political matter internal to the host country, but with hindsight this was a very unwise decision.

Transgenic sterile males

Apart from the biological warfare and other accusations the journalist raised the question of the use of chemical mutagens to induce sterility. Though these were handled with great care and the residues in released males were minute as mentioned above, it seems unlikely that their use would be authorized nowadays. There are also problems with radiation-sterilization as mentioned above. It is therefore notable that a transgenic form of sterile male has now been produced in *Ae. aegypti* (L. Alphey, H. White-Cooper, M. Andreasen and P. Coleman, pers. comm.). This uses the system known as RIDL (Release of Insects carrying a Dominant Lethal, Thomas et al. 2000). By adding tetracycline to the rearing medium, the construct is 'switched off' to allow mass rearing. The effect is genetically dominant so that progeny of released RIDL males mated to wild females will die in the absence of tetracycline in normal breeding sites. A multi-centre trial of mating competitiveness of RIDL males is about to begin in cages. At present the additional refinement of making the lethal action sex-limited so that only females die has not yet been achieved. Yet, when it is, it will provide a replacement or a back-up for conventional sex separation systems somewhat extending the impact in the field, as male progeny will be heterozygous for RIDL and will be 50% sterile.

The most hopeful type of sites for eradication using RIDL releases is urban areas having one species of vector, but where the surrounding rural area has another. Based on earlier work it would seem that examples of such cases are *An. stephensi stephensi* in Indian cities (Ramachandra Rao 1984) and *An. arabiensis* in southern-Nigerian cities surrounded by *An. gambiae* in rural areas (Coluzzi et al. 1979; Kristan et al. 2003). *Ae. aegypti* still transmits dengue in Singapore in a human population which now has little immunity (Ooi et al. 2001) because of the impact of legally enforced larval control. However, this form of control has not been able to eradicate *Ae. aegypti* and an attempt to do so with RIDL males would seem to be appropriate.

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4

Genomics and expected benefits for vector entomology

Christos Louis[#]

Abstract

This paper summarizes the gains expected for vector entomology from the acquisition of the genome sequence of disease-transmitting arthropods. The results of this kind of high-throughput science, especially the direct consequences that could be summarily described as post-genomic activities, may lead to a better understanding of the biology of the vectors, including population studies, interactions with the disease agents and, finally, the direct development of tools, biological or bioinformatics-based, to be used in their control.
Keywords: genome sequencing; genome mining; comparative genomics

The facts and the prospects

Although the etymology of the term genomics is obvious, in contrast, its definition is fairly vague. Interestingly, unless one would encompass in it the notion of 'high-throughput research', Bridges' pioneering work on the cytogenetic mapping using the polytene chromosomes of *Drosophila* (Bridges 1935) would definitely qualify as genomic research, possibly marking the beginning of the discipline. The particularly tedious and time-consuming closing of gaps in the whole genome sequence (WGS) of the same organism, on the other hand, can hardly be described as 'high throughput', thus its inclusion in the category of '-*ics*' science could theoretically merely be done as a typical example of 'post-genomics'. In spite of these philological considerations genomics has not only found its place in biological research, even more so, it represents a constantly expanding field, also due to the continual development of new biotechnological, technological and informatics-based tools.

The first reported completion of the sequence of a large segment of eukaryotic DNA, that of chromosome XI of *Saccharomyces cerevisiae* (Dujon et al. 1994), is now about ten years old. Initiated in the late '80s, the WGS of brewers yeast, with a size of about 12 Mb was completed in a time frame of a little less than ten years (Goffeau et al. 1996). *D. melanogaster*'s genome, approximately 10 times larger, took about 5 years to be 'finished' (Adams et al. 2000), while the first draft of the complete WGS of *Anopheles gambiae*, with a genome size about twice as large as that of the fruit fly, took less than 18 months, counting from the date the programme was officially launched till its publication (Holt et al. 2002). This increase of speed also reflects the method chosen for determining the WGS, which was largely switched from clone-based (e.g. whole cosmids or BACs (Bacterial Artificial Chromosomes)) to a whole-genome shotgun approach during the fruit-fly project. Obviously, what

[#] Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Crete, Greece. E-mail: louis@imbb.forth.gr

genomics specialists call ‘complete’ is also a matter of definition. For example, four years after the publication of the full sequence of *D. melanogaster* (Adams et al. 2000) gaps in the sequence are still present, although these have now been reduced to 23 (see http://www.fruitfly.org/annot/release4.html), while the ‘finished’ mosquito genome is still made out of thousands of contigs and scaffolds, the longest of which is, nevertheless, several Mb long.

There is no question that the completion of the *Drosophila* genome gave a major thrust to genetic research. This is best exemplified by the number of papers that have been published in the three calendar years following the publication of the Adams et al. (2000) report. Searching Pubmed with the keyword “*Drosophila melanogaster*” yields 6,052 entries from this period or, in other words, one fourth of all papers that can be retrieved from the database with the same keyword and no restriction for the time of publication. For direct comparison, it should be stated that the corresponding figures for the three years preceding the ‘complete genome’ are 3,293 papers, or about 14% of the total. The more than 83% increase in scientific output can only be attributed to the availability of the WGS, information that is exploited not only by ‘fly labs’ but by researchers working with different experimental systems as well. It is easy to imagine what this wealth of information means for the understanding of the biology in general and that of the fruit fly in particular.

The publication of the complete *An. gambiae* genome sequence is much more recent and three-year statistics are not yet possible; yet, a similar trend, i.e. a significant increase of published papers dealing with the African malaria mosquito, is already apparent. Whether this increase is fully owed to the *Anopheles* WGS cannot be determined easily since an upward trend was already discernible before the Holt et al. (2002) paper: during the last five years some 600 papers described results dealing with the world’s most important malaria vector; strikingly, this number represents a little less than half of all of the Pubmed entries that are retrievable using *An. gambiae* as the sole search criterion. In other words, malaria entomology is experiencing a small boom that started in the 1990s. A few examples justify this statement. While the development of a genetic map of the fruit fly was initiated more than one hundred years ago, it was only in the previous decade, using genomic tools such as microsatellite markers, that a useful recombination map was worked out for the African malaria mosquito (Zheng et al. 1993; 1996). These microsatellites in turn helped give an impetus to population biology (see, for example, Lehmann et al. 2003; Tripet et al. 2003) since they could be translated into easily scored genetic markers. Furthermore, attempts to understand the molecular interactions between the mosquito vector and malaria parasites were, with a few exceptions, initiated only during the previous decade, 10-15 years after the advent of the recombinant-DNA era. Recently, these have been intensified, especially after the acquisition of the WGS. This becomes more apparent in the case of the study of the immune system (Levashina 2004), a physiological apparatus that could potentially be put in use for the development of antiparasitic strategies in the vector (Hemingway and Craig 2004). Finally, the WGS itself could be seen both as an example of this research boom and as a means of sustaining this increased research effort.

It is naturally very difficult, or perhaps even impossible, to translate the impact of the number of scientific publications and correlating it to the importance of the results obtained. This generally true fact may be even more critical in the case of applied or semi-applied sciences such as entomology in general, and more specifically malaria entomology. If one were to describe in only a few words the benefits that whole-genome sequencing offers to the advantage of biology, initially this could be

summarized as the discovery of genes, something that could ultimately lead to the better understanding of any given organism. In the case of disease vectors it is recognized that gene discovery could ultimately lead to the development of potentially novel insect-based intervention mechanisms that are based on molecular mechanisms elucidated by genomic and, especially, post-genomic dissection. This would also be helped by the comprehension of interactions between the two and potentially even three organisms (i.e. the vector and both the vertebrate and invertebrate hosts), again something that will become easier through the availability of WGS of all 'partners'. Finally, the use of the novel genomics-derived tools in the study of populations and, ultimately, the epidemiology of disease could also contribute greatly towards the development of novel insect control approaches.

What are the concrete effects that one can expect from the availability of the genome sequence of *An. gambiae*? A golden bullet should not be expected as an outcome, but a series of silver ones may be a real possibility. A relatively long list of applications is led by the already mentioned understanding of the molecular interactions between *Plasmodium* parasites and the mosquito in the latter's key tissues, i.e. midgut and salivary gland (Alavi et al. 2003; Siden-Kiamos and Louis 2004). Although their existence is assumed, *bona fide* receptors for the parasites have not yet been identified in either of them. It is clear that their potential recognition and detailed study would help devise molecular interventions in order to stop the transmission of *Plasmodium* by anophelines. More or less along the same path, the better understanding of the mosquito's immune system and the way that this could be enhanced in order to attack invading parasites is also one of the goals of post-genomic research. The list obviously cannot stop here, and conceivably the genome can be mined in order to find metabolic pathways that can be used to stop the development of *Plasmodium* in the insect host. In *Plasmodium*, pathways have already been identified and antimalarial drugs are already being developed based on the genomic information (see for example Wiesner, Borrmann and Jomaa 2003). In analogy, in mosquitoes one could potentially think of either novel targets for insecticidal chemicals or for molecules that could block directly the development of the parasite in the insect (Craig et al. 2003).

A further field in which genomic research can find immediate use is that of population biology and genetics. The microsatellite markers preceded the WGS of *An. gambiae* and, as already mentioned, they have helped substantially increase our understanding of the malaria mosquitoes' evolution, ecology and population structure (see Barker 2002). Through the use of single-nucleotide polymorphisms (SNP markers) that the WGS offers as a 'by-product', the availability of genetic markers is now enhanced manifold (Marth et al. 1999; Brumfield et al. 2003).

The potential ease of the SNP analysis brings into discussion a different aspect, namely that of the expansion of genomic analysis into other disease-vector species. The increased research output cited in the beginning of this paper predominantly concerned *An. gambiae* and, to a lesser degree, the non-malaria vector *Aedes aegypti*. These two mosquitoes were established, in a sense, as the model systems for vector biology although, of course, important findings were also described for other vectors such as, for example, the development of germ-line transformation. This latter technology, by the way, although not to be discussed further here, is to be considered equally important as genomics for the advancement of vector biology (Jacobs-Lorena 2003). In addition to *An. gambiae*, prominent among other African malaria vectors are *An. arabiensis* and *An. funestus*, for which genomic data have started accumulating, even though no genome project in the proper sense of the work has been launched.

Asian and American malaria mosquitoes, in contrast to the ones mentioned, lag behind. Looking beyond malaria, genome projects have been initiated for *Ae. aegypti* and the tsetse fly, while *Culex pipiens* is also being discussed, being a vector of the emerging West Nile Virus infection. It is hoped that this accumulation of data will also help bring forward the scientific knowledge pertaining to the other tropical diseases that cost disabilities worldwide. It should be noted here that the relative speed of data acquisition for these 'new' research objects is expected to be even higher than was the case for *Anopheles*. This is not only because WGS, as stated above, is helped by new technological developments, but also by the fact that the available WGS data for insects have a direct effect on the strategies to analyse new related organisms, making their pertinent study much easier.

A last item dealing directly with the sequencing of whole genomes was not addressed so far. This refers to biological databases in general and genome databases in particular. It is a fact that the large amount of information that is obtained by WGS projects cannot be handled by end-users unless sophisticated databases are put in place. This fact was demonstrated early on by FlyBase (The FlyBase Consortium 2003), a database that, since its inception in the late 1980s, has compiled and stored all information dealing with *Drosophila* (http://flybase.bio.indiana.edu/). By now 'life without FlyBase' is no longer possible for the fruit-fly researcher, since all genetic, cytogenetic and biological facts from as early as the 17th century (Metzel 1684) are included in it, as well as every information that has come out of the finished genome, incorporating the annotation of the so-called release 3.0 (Celniker et al. 2002).

Databases should no longer be viewed as simple storage devices using minimal search facilities. This is true not only for the classical sequence databases such as EMBL and Genbank, but also it is even more so the case for refined databases that directly handle genome data. The *Anopheles* genome, for example, has been 'adopted' by ENSEMBL, a joint project of the Sanger Centre and the European Bioinformatics Institute (EBI). The mosquito database at ENSEMBL, thus, contains all sequences, annotations and additional tools that can be used by the end-user to access these data (Mongin et al. 2004). An additional bonus for the mosquito genome is the fact that, in addition to providing the database, ENSEMBL is also responsible for the automatic annotation and re-annotation of the genome, which happens at regular intervals. Finally, ENSEMBL also handles input of hand annotation by members of the research community, using these data in its own automatic annotation pipeline. Thus, information available at http://www.ensembl.org/Anopheles_gambiae/ is updated frequently.

Having mentioned earlier the fact that additional insect vectors have now entered the genomic era, it should also be stated that the genome and biological databases for these species are now to be combined in a single one that will be called Vectorbase, and which will be initiated soon as a novel project. This database is planned to contain the genome information of at least five arthropod vectors (*An. gambiae*, *Cx. pipiens*, *Ae. aegypti*, *Glossina* spp. and the tick *Ixodes scapularis*), while additional vectors may be added at later stages. In addition to the genome data, the plans call for the inclusion of general biological and genetic data similar to what is already stored in AnoBase (http://www.anobase.org), the *Anopheles* database. Finally, new sections are to be developed that will contain data on population biology and data on post-genomics such as information on cDNAs (EST), images, expression profiles, etc.

Conclusions

This brief report presents the situation in vector genomics and, especially, post-genomics in the summer of 2004. As far as the arthropod disease vectors are concerned, it is obvious that the discipline is now almost monopolized by post-genomic research originating in the publication of the WGS of *An. gambiae*. It is, however, expected that in the near future the genomes of additional vectors will have been sequenced. This wealth of information that can only be managed with up-to-date informatics tools is expected to yield results that may soon be useful for the design of alternative strategies aimed at controlling the diseases that are transmitted through these vectors. Recent advances in molecular-biological techniques, especially the possibility to knock down genes through the RNAi technology (Fjose et al. 2001) have now opened up ways to use surrogate genetics for the manipulation of insects (Wimmer 2003). The efficient and sustained regulation of transcription of effector genes, natural and 'artificial' that will interfere with the transmission of disease agents is theoretically achievable, and most of these advantages are the results of genomic and postgenomic research on the fruit fly and the malaria mosquito. The future, thus, although not foreseeable, should definitely be viewed with an optimistic eye.

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5

Genetic approaches for malaria control

Marcelo Jacobs-Lorena[#]

Abstract

The already unacceptable large burden of malaria continues to increase, indicating that the available means to fight the disease are insufficient. The genetic manipulation of the mosquito vectorial competence is a potential new promising weapon for the control of malaria. Considerable progress has been made in recent years towards this goal. It is now possible to introduce synthetic genes into the mosquito germ line, promoters have been identified that effectively drive gene expression in tissues and at times appropriate to target the parasite, and effector genes that impair parasite development in the mosquito have been identified. With these tools, proof-of-concept experiments have already demonstrated that it is possible to interfere genetically with the vectorial competence of the mosquito. At least some of the transgenic mosquito lines that have been created appear to be as fit as their wild-type counterparts. Presently, the major unresolved challenge is the development of methods to drive effector genes into mosquito populations in the field. While several approaches are under consideration, such as transposable elements, *Wolbachia*, meiotic drive and paratransgenesis, their relative feasibility remains to be demonstrated. Additional challenges are the resolution of safety concerns and satisfactorily addressing social, ethical and political considerations. Hopes remain high that the remaining challenges will be solved and that we shall be able to deploy this new genetic weapon in the foreseeable future.
Keywords: mosquitoes; malaria; transgenesis; effector genes; driving mechanisms; refractoriness

Introduction

Insect-transmitted diseases impose an enormous burden on the world population in terms of loss of life (millions of deaths per year) and morbidity. These diseases impose huge economic losses both in terms of health-care costs and lost productivity, mostly in countries that can least afford it. Three basic approaches have been attempted to contain these diseases: 1) treat infected people with drugs that kill the pathogen; 2) control insect vector populations; and 3) develop vaccines that prevent infection.

Drugs

Drugs have been at the forefront of the fight against many arthropod-transmitted diseases. For decades, chloroquine was successfully used against malaria. However,

[#] Johns Hopkins School of Public Health, Malaria Research Institute, Dept. Molecular Microbiology and Immunology, 615 N. Wolfe St.,Baltimore, MD 21205. E-mail: mlorena@jhsph.edu

with time pathogens are selected for drug resistance, forcing the development of new drugs. Unfortunately, this cycle of drug discovery followed by resistance becomes more difficult to perpetuate with the passage of time. In principle, combination drug therapy should greatly alleviate this problem. In practice, other factors (mostly economic) make the implementation of this strategy difficult. While drugs are extremely useful in containing and treating diseases, they are not sufficient on their own for disease eradication. Clearly, a combination of strategies is needed.

Control of insect populations

On the contrary, reduction of vector insect populations will reduce disease transmission. This can be accomplished in various ways, for instance, with insecticides, by managing the environment (elimination of breeding sites) or by interfering with reproduction (sterile-insect releases). Recent technological advances suggest an alternative approach, namely genetic modification of the competence of the vector arthropod to transmit pathogens (vectorial competence), which is the main subject of this article.

Vaccines

The only successful vaccine in existence against an arthropod-transmitted pathogen is the yellow-fever vaccine. Even in this case, the disease has not yet been eradicated. Decades of intense research aimed at the development of other vaccines, notably for malaria and dengue fever, but this has yet to yield a viable product. At the heart of the problem, at least for malaria, is that during thousands of years of association with humans pathogens have been selected that efficiently evade the host immune system. High genetic diversity, variability of potential target molecules (e.g., *Plasmodium var.* genes), and intracellular sequestration are strategies frequently used by pathogens that allow them to elude immune attack. While the search for effective vaccines should continue, this has been an uphill battle.

Insecticides

Under appropriate circumstances, insecticides are powerful weapons to fight vector-borne diseases. For instance, they have been crucial in the eradication of malaria in Europe and of *An. gambiae* in Brazil, and they are important in controlling disease epidemics (e.g., dengue, West Nile) in urban areas. The introduction of DDT in the mid 1940s heightened the hopes of disease eradication. A case in point is the WHO campaign to eradicate malaria, which was successful at its inception (for instance, malaria was almost eliminated from the entire Indian subcontinent). However, problems such as development of insecticide resistance by mosquitoes, the discovery of the harmful effects of DDT to the environment and to non-target organisms, and the 'letting down of the guard' when disease was almost under control, led to the reversal of most initial successes. While judicious use of insecticides is still a powerful weapon to fight disease, one aspect of its use is frequently overlooked: insecticides usually leave intact the biological niche where the target insects reproduce. Therefore, insect populations rapidly return to pre-treatment levels as soon as application is halted, which is a very serious problem. For instance, one can hardly hope for large-scale mosquito-population reduction in Africa, especially if one considers that management of breeding sites (e.g. widely distributed small pools of water) would be required. While residual spraying of house interiors or use of bednets will lower transmission rates and reduce prevalence and incidence of

infections, the breeding sites will continue to generate mosquitoes and this cycle of breeding and killing is likely to hasten the development of insecticide resistance. Thus, insecticides are useful to bring temporary relief but cannot be considered as solutions by themselves. In future, insecticides are likely to become key weapons when used in combination with other approaches such as vaccines, drugs or when used for population replacement (see below).

Sterile-insect technique (SIT)

Insect populations can be controlled by the release of large numbers of sterile males. Thus, if a female mates with a male that has no sperm or whose sperm was rendered unviable, this female will have fewer or no progeny. When many sterile males are released, the local population tends to decline or become extinct. There are a number of cases of the successful local application of this technique, for example, in the control of the Mediterranean fruit fly in Latin America, the New World screwworm in the Americas and Libya, and for tsetse in Zanzibar, Africa. SIT also has been applied, on a limited scale, to *Culex* in India and *Anopheles albimanus* in El Salvador (see Curtis, Chapter 3).

For population control, the crucial parameter is the ratio of the number of released sterile males to the number of males in the local population, which ideally should be around 10:1. Therefore, sterile-insect control is only effective when the resident population to be controlled is small relative to the number of sterile males that can be mass-produced for release or when it can be reduced to very low levels with conventional control tools before the start of releases. It is highly desirable that only males be released for two reasons: 1) In most cases only females bite and transmit disease while, moreover, sterile females can also transmit; 2) Males would court and mate with the released sterile females (instead of local females), thus reducing the efficacy of the programme (Alphey and Andreasen 2002). Large-scale production in the laboratory of a pure male population by non-genetic means may be problematic. It may rely on sex-specific differences of pupal size (culicine mosquitoes) or adult eclosion times (tsetse), but these protocols rarely yield a 100% male population. Clearly, genetic sexing methods (see below) are far superior. The most commonly used technique for male sterilization is exposure to high doses of radiation, a procedure that damages chromosomes and results in unviable sperm. Sterilization by chemical means also has been employed. Because of the large numbers of insects that need to be released, it is crucial that the effectiveness of the sterilization procedure approaches 100%. However, the large doses of radiation and chemicals needed to achieve this effectiveness may reduce insect fitness, survival and mating competitiveness. These strategies can fail if the laboratory-reared males do not mate as effectively as their field counterparts.

The advent of germ-line transformation for a number of different insects has led to the development of genetic alternatives for production of sterile insects (Heinrich and Scott 2000; Thomas et al. 2000). In one version of this approach (Release of Insects carrying a Dominant Lethal or RIDL, Thomas et al. 2000), a conditional dominant lethal gene is introduced into the target insect genome. This gene has two important properties: 1) it is expressed only in females (or it kills only females); and 2) the gene is effectively repressed by a compound that does not occur normally in nature (e.g. tetracycline). Large insect populations are maintained by rearing them in the presence of tetracycline, which represses the dominant lethal gene and allows the survival of equal numbers of males and females. Prior to release, the insects are reared in the

absence of tetracycline, a condition that allows the expression of the dominant lethal gene and the death of all females. The resulting males can be released without further manipulation or treatment. Males carry two copies (homozygous) of the dominant lethal gene. When these males mate in nature, all female progeny will be killed and only males will be produced. Since these surviving males are heterozygous for the dominant lethal gene, the population-reducing effect is still manifest in the second generation.

It should be emphasized that the effectiveness of the SIT is dependent on population structure and dynamics. Furthermore, this technique leaves intact the biological niche in which the target insect is found. SIT is most likely to succeed in cases where target populations are small, the number of target insects is low, and the target area is sufficiently isolated, thereby reducing the likelihood of re-invasion. It is unlikely to be effective for controlling mosquito populations in highly endemic areas of Africa where the mosquito population consists of several vector species in high densities, where access to breeding sites is difficult and where poorly interbreeding mosquito populations co-exist.

Genetic manipulation of vectorial competence

Germ-line transformation

Drosophila melanogaster was the first multicellular organism to be stably transformed (Spradling and Rubin 1982). The same general principles that were used in this pioneering work are still employed today for all germ-line transformation work in insects (Atkinson and James 2002). Embryos are injected with two DNA constructs. One construct contains a gene encoding a dominant selectable marker (e.g., eye color, a fluorescent protein) and the gene of interest, each driven by a separate promoter, and both sequences are together flanked by the inverted repeats of a transposable element. The second construct encodes a transposase, which is an enzyme that recognizes the inverted repeats and catalyses the insertion of the intervening sequences into the genome of the host insect. It took from 1982 until the mid 1990s to develop two crucial technologies: an appropriate transposable-element system (at first scientists did not realize that the *P* transposable element is not active in non-*Drosophila* organisms) and a suitable transformation marker (e.g., GFP). Since then, germ-line transformation of many insects has been accomplished but mosquitoes (*Aedes*, *Anopheles*, *Culex*) are the only insects of medical importance in this list. Importantly, both *An. stephensi* and *An. gambiae* can be transformed, though the success rate in the latter case is still low. Improvement of the transformation efficiency of *An. gambiae* is a high-priority topic for future research. It would also be desirable to develop germ-line transformation procedures for other medically important insects such as sand flies and black flies. Current technology cannot be applied to germ-line transformation of tsetse because these do not lay eggs (that would need to be injected), only fully formed larvae. However, genetic modification of tsetse vectorial capacity could be achieved via genetic modification of one of its symbionts.

The net result of germ-line transformation is the integration into the genome of the host organism of a relatively large DNA sequence, flanked by inverted repeats of the transposable element. The inserted DNA contains at least two genes, the gene to be investigated and a transformation marker gene (e.g., eye color, GFP) that allows transformed individuals to be identified. The integrated DNA is usually stable and transmitted in a Mendelian manner from one generation to the next. In the following

text, we will consider transformation of insects with genes that affect their ability to transmit pathogens. We will start by considering promoters to be used for driving gene expression and then effector genes capable of interfering with parasite development.

Promoters

Promoters that drive the expression of effector genes in transgenic insects should be strong, that is, they should result in abundant transcription. This is because the effectiveness of the gene products is expected to increase with increased abundance. Two general types of promoters can be considered: ubiquitous and tissue-specific. Ubiquitous promoters are less desirable because general expression of a foreign gene product in all tissues of the insect and at all times is likely to impose a fitness load. Tissue-specific promoters have the advantage of being restricted to a tissue and are often developmentally and/or physiologically regulated (not constitutive). Strong tissue-specific promoters that have been characterized in mosquitoes include gut *carboxypeptidase* (Moreira et al. 2000), fat body *vitellogenin* (Kokoza et al. 2000) and gut *peritrophic matrix protein 1* (*PM1*; Jacobs-Lorena laboratory, manuscript in preparation). The *carboxypeptidase* promoter has the advantage of being induced by blood intake, and thus its activation coincides with parasite arrival in the gut. The carboxypeptidase signal sequence effectively promotes secretion into the midgut lumen, which is the compartment where the parasite initially resides. The *PM1* promoter and the protein's signal sequence direct synthesis and storage of the protein in vesicles of the midgut-epithelial cells prior to a blood meal. The vesicle contents are released into the midgut lumen immediately after blood ingestion. The *vitellogenin* promoter is also induced by the blood meal (expression peaks at ~24 h) and the peptide signal sequence promotes secretion into the mosquito body cavity (haemocoel), where the parasite later develops. This promoter is ideally suited for expression of effector molecules that target the ookinete soon after its crossing of the midgut epithelium and emergence into the haemocoel. Once it reaches the haemocoel, the ookinete transforms into an oocyst that is difficult to target because it is covered by a thick protective layer. Upon maturation (at about 10 days after the infective blood meal), the oocyst releases sporozoites that disperse though the haemocoel until they come in contact with, and invade, the salivary gland. It would be desirable to find promoters that drive synthesis of proteins secreted into the haemolymph at the time of sporozoite release. While sequences that direct secretion into the salivary-gland lumen have been identified, their expression levels of the corresponding promoters are low (Coates et al. 1999). It would be desirable to identify a strong salivary-gland promoter to target pathogens stored in the salivary glands. The pathogen usually is stored in the salivary-gland lumen for extended periods of time, and this increased period of contact may favour parasite inactivation by the effector-protein product. One should keep in mind though, that mosquito saliva (and any effector protein secreted into it) is transferred to its vertebrate (human) host, raising safety and ethical questions.

Effector genes

The term effector gene is used here for genes whose products interfere with the development of a pathogen. At least four classes of effector genes can be identified: 1) Genes whose products interact with insect host tissues crucial for parasite development: Examples of this class are SM1, a peptide that occupies putative salivary-gland and midgut receptors for the malaria parasite (Ghosh, Ribolla and Jacobs-Lorena 2001) and phospholipase A2 (PLA2), which is a protein that interferes

with the malaria ookinete invasion of the midgut (Zieler et al. 2001); 2) Genes whose products interact with the pathogen: Examples of this class are genes encoding single-chain monoclonal antibodies that bind to the parasite's outer surface thus blocking their development (Yoshida et al. 1999; De Lara Capurro et al. 2000); 3) Genes whose products kill the pathogen: Examples are peptides from the insect's innate immune system such as defensins and cecropins, and peptides from other sources that act as selective toxins to parasites but do not affect the host insect, such as magainins, Shiva-1, Shiva-3 and gomesin (Kim et al. 2004). Most published work on effector genes deals with effects on the malaria parasite and little is known about such genes for other pathogens. In particular, it is not clear what class of effector genes would be useful for nematodes (filaria). Since these may be encapsulated in certain mosquito strains, genes that activate encapsulation could be considered as possible effector genes. For viruses, genes of the first class (interference of host-tissue invasion) or genes that interfere with virus replication (Olson et al. 1996) are possible candidates. 4) Another possible strategy is to reduce vector competence by manipulation of its immune genes, for instance by using RNA interference or 'smart sprays' (Christophides, Vlachou and Kafatos 2004).

Another important strategic consideration is the stage of malaria parasite development to target. When a mosquito ingests an infected blood meal, it acquires thousands of gametocytes of which only few (usually less than ten) manage to cross the midgut and form oocysts. Later, each oocyst produces thousands of sporozoites, a significant proportion of which invade the salivary gland. Because the strong bottleneck at the level of midgut invasion, this stage of parasite development constitutes a prime target for intervention. Midgut invasion is also a strong bottleneck in the process of arboviral transmission.

Genetically modified mosquitoes

Successful development of the technology described above (transgenesis, promoter characterization and effector-gene identification), permitted the creation of genetically modified mosquitoes impaired in their ability to transmit the malaria parasite. An early example was the creation of an *Ae. aegypti* expressing defensin in the haemolymph (Kokoza et al. 2000). However, the effect of defensin on malaria parasite development has not been reported. At about the same time, the James laboratory reported that a single-chain monoclonal antibody that recognizes a sporozoite surface protein inhibits invasion of the salivary gland (De Lara Capurro et al. 2000). In this instance, the effector gene was transiently expressed from a viral vector that is not inherited by the mosquito progeny. The Jacobs-Lorena laboratory showed that a stably integrated gene encoding SM1 strongly inhibits parasite development in transgenic mosquitoes (Ito et al. 2002). In another example, transgenic mosquitoes expressing PLA2 also had much reduced vectorial competence (Moreira et al. 2002). Recently, it was demonstrated that the capacity to transmit the malaria parasite is reduced by about 60% in transgenic *An. gambiae* expressing cecropin from a *carboxypeptidase* promoter (Kim et al. 2004). Thus, it is clear that mosquitoes can be genetically modified to reduce their vectorial competence. To date, most reported experiments have been done with non-human malaria parasites. An important next step is the transfer of this technology to human pathogens.

Insect fitness

For the introduction of an effector gene into populations, it is important that it confers the least possible detrimental effect on mosquito survival or reproduction

(fitness load). This parameter can be initially tested in the laboratory by use of population cages. For instance, *SM1*-transgenic mosquitoes do not seem to have any load, while *PLA2*-transgenic mosquitoes lay significantly fewer eggs and therefore carry a significant fitness load (Moreira et al. 2004). In contrast to the apparent lack of fitness load of *SM1*-transgenic mosquitoes, Cateruccia, Godfray and Crisanti (2003) reported that transgenic mosquitoes expressing GFP from an *actin* promoter may have a fitness disadvantage. It appears however that in these experiments, loss of fitness was mainly due to inbreeding (the experiments were conducted with homozygous transgenic mosquitoes that may have been subject to the 'founder effect') and perhaps to generalized foreign gene expression from a ubiquitous promoter (see above). Moreover, Irvin et al. (2004) have detected a fitness load in transgenic *Ae. aegypti* that express an eGFP marker gene. However, as for the experiments by Cateruccia et al., they used homozygous transgenic mosquitoes to measure fitness and these experiments cannot determine whether the fitness load originates from nearby recessive genes that were homozygosed with the transgene ('hitchhiking effect') or from a true fitness load imposed by the transgene itself.

Another consideration is that the malaria parasite itself reportedly imposes a fitness load on the mosquito (Hogg and Hurd 1997). In agreement with this observation, the Jacobs-Lorena laboratory has preliminary results indicating that in cage experiments, transgenic mosquitoes expressing SM1 out-compete wild-type mosquitoes with the same genetic background when fed on *P. berghei*-infected mice, presumably because the transgenics have a lower parasite load (unpublished observations).

Eventually tests will have to be devised that measure insect fitness in the field (as opposed to laboratory cages) because other factors may come into play. Moreover, laboratory mosquitoes may not compete well with their field counterparts (important for release studies). One possible solution to this issue may be to cross the effector genes into wild-caught local mosquito populations prior to release.

Between now and field release

While genetic modification of mosquitoes to resistance to the malaria parasite is clearly feasible in a laboratory setting, many issues remain to be addressed before implementation of this approach in the field can be envisioned. Examples of unresolved issues follow.

i) Parasite resistance and multiple effector genes

Parasites tend to have a heterogeneous genome that favours selection of individuals able to overcome barriers such as drugs or possibly effector gene products. It will therefore be crucial that transgenic mosquitoes incorporate more than one (ideally several) effector genes, each of which blocks parasite development by a different mechanism.

ii) Driving effector genes into field populations

This is undoubtedly the major unresolved technical issue. Several approaches have been suggested.

Population replacement by inundatory release. A possible scenario would be to start with an isolated area (e.g., an island) where malaria is prevalent, and reduce to the maximum extent possible the mosquito population by use of insecticides. As discussed above, this would leave an empty biological niche. The next step would be the release of transgenic mosquitoes to occupy this niche. New transgenic releases could follow periodically with the expectation that the original mosquito population will eventually be replaced by the transgenic one. While waiting for the development of an effective driving mechanism (see below), comparison of malaria transmission

before and after population replacement should provide valuable data to assess effectiveness of the transgenic approach. It should be noted however that while population replacement is conceivable for research purposes in small physical or ecological islands, its implementation on a large country- or continent-wide scale is not feasible.

Transposable elements. There is an excellent example in *Drosophila* of how an element can spread through wild populations. In a matter of a few decades, the *P* element spread through virtually all *D. melanogaster* in the world. Presumably, this happened because the transposase causes the element to multiply in the genome, resulting in non-Mendelian transmission. Unfortunately, *P* elements are not active in non-drosophilid insects and more importantly, to date no transposable element with similar properties has been identified in mosquitoes. Even when such elements are identified, there will be major issues to be addressed. One relates to 'cargo' size. As mentioned in the preceding text, it will be critical to use at least two different effector genes. In addition, a gene encoding the transformation marker (e.g., GFP) and a gene encoding the 'driver' (transposase) will be needed. Note that it is crucial for cargo and driver to be tightly linked. Thus, a minimum of four genes are needed representing a substantial cargo of 12~16 kb (calculated at 3~4 kb/[gene + regulatory sequences]). In nature, transposable elements are known to become truncated as they hop from one position to another, and the probability of truncation can be expected to increase with the size of the cargo (for comparison, a typical transposable element has about 3 kb). Thus, cargo damage and consequent inactivation of the genes carried by the element is a major concern. Another consideration is that in some instances, insects carrying a transposable element accumulate a repressor of the transposase, precluding introduction of a different gene with the same transposable element and into the same population. In other words, this could be a 'one-shot' proposition: should one discover that the wrong transgenes were used, that resistance developed or that gene inactivation occurred, there would not be a second chance to spread another set of genes with the same element.

Wolbachia. *Wolbachia* are intracellular bacteria that inhabit the germ line of a number of insects and distort reproduction by killing progeny that do not contain it, by a phenomenon known as cytoplasmic incompatibility (CI). Compelling evidence in favour of *Wolbachia* as a drive mechanism comes from *Drosophila*. Turelli and Hoffmann (1991) observed that *Wolbachia* swept through the *D. simulans* population in California at the rate of 100 km per year. In principle, *Wolbachia* could provide a powerful driving mechanism. However, no *Wolbachia* have yet been identified in anopheline mosquitoes (these are the exclusive vectors for human malaria), although they have been observed in culicine mosquitoes. A major limitation of *Wolbachia* is that it inhabits the germ line while the pathogen develops in the soma. Thus, it is difficult to target parasites with genes introduced into *Wolbachia*. A possible solution to this problem is the identification of genes that cause CI. Currently little is known at the molecular level about how CI functions or how many genes are involved. When identified, such gene(s) could conceivably be used to create a driving mechanism via their insertion into the mosquito genome.

Meiotic drive. Population replacement can be driven by certain genes, such as the *Drosophila segregation distorter* gene, that favour its inheritance over individuals not containing the gene. Unfortunately, very little is known about such genes in insects of medical importance. One complication is that at least in model systems (*Drosophila*, mouse), the drive mechanism depends on multiple genes (e.g., distorter and responder) and this could complicate the implementation of this system in

mosquitoes. Moreover, if such genes were to be employed to drive effector genes into populations, all meiotic drive and effector genes would have to be tightly linked to avoid loss of effectiveness due to recombination.

Paratransgenesis. An alternate approach to spread effector genes through mosquito populations is to introduce effector genes into bacteria that inhabit the mosquito gut, rather than introducing them into the mosquitoes themselves. This approach (known as paratransgenesis) has been successfully tested in another vector/parasite system to disrupt the transmission of *Trypanosoma cruzi*, the causative agent of Chagas disease, by the triatomid bug *Rhodnius prolixus*. *R. prolixus* harbours an obligate bacterial gut symbiont that lives in close proximity to the *T. cruzi* parasite. When this symbiotic bacterium was transformed with an effector gene encoding cecropin A, and fed to naive *R. prolixus* nymphs, *T. cruzi*'s ability to survive in the nymphs was significantly reduced (Durvasula et al. 1997). Preliminary experiments from the Jacobs-Lorena laboratory suggest that the same principle can be used for reducing the capacity of mosquitoes to vector malaria parasites. When *E. coli* displaying the inhibitory SM1 peptide (Ghosh, Ribolla and Jacobs-Lorena 2001) or PLA2 (Zieler et al. 2001) on its surface was fed to *An. stephensi* followed by an infectious blood meal, significantly fewer *P. berghei* parasites developed as compared to control mosquitoes fed on wild-type bacteria. Several considerations argue in favour of the paratransgenic approach: 1) Bacteria live in the same compartment where the initial stages of *Plasmodium* development occur; 2) In principle, bacteria should be easier to introduce into mosquito populations than transgenes. One possible scenario is that baits containing the modified bacteria, a source of sugar and mosquito attractant(s) would be placed in strategic locations (e.g. huts) around a village where malaria transmission occurs. Moreover, genetic modification of bacteria is straightforward and efficient. Bacteria can be easily and cheaply grown in large quantities. This facilitates the introduction of multiple effector genes into mosquito populations, each bacterium being transformed with a different transgene. Unlike mosquito transgenes, inactivation of bacterial transgenes after many generations in the field is not a major concern because of the likely easier logistics of introducing freshly transformed bacteria. Moreover, if an effector gene fails to perform as promised, introduction of alternate transgenes is relatively simple. 3) A paratransgenic approach also poses fewer risks compared with a large-scale release of genetically modified vectors. Not only does a large-scale mosquito release cause increased nuisance to the local population, but there may be an increased health risk if the mosquitoes are capable of vectoring other pathogens. However, the paratransgenic approach is not without concerns: 1) It is not known how adult mosquitoes acquire their bacterial flora. Thus, we do not yet know how to implement a plan to control *Plasmodium* transmission with genetically modified bacteria; 2) Introduction of a genetically modified organism of any kind into the field needs to be approached with much caution because of the unknown consequences. In particular, bacteria are known to be able to spread genes by horizontal transfer (especially if present in plasmids). It is not known whether the modified bacteria could populate the guts of non-target organisms. If so, the consequences need to be assessed.

iii) Population structure

It is quite clear that at least for anopheline mosquitoes, population structure is complex. This is because a number of morphologically identical but chromosomally different (cytotypes) mosquito populations can co-exist in any given area. Importantly, these populations may not freely interbreed and this may seriously affect

efforts to spread effector genes through populations. A better understanding of population structure and mosquito ecology should be given a high priority.

iv) Safety concerns

While there is no reason to believe that any of the effector genes identified to date have any effects on non-target organisms, concerns are being raised by the scientific and lay communities regarding the safety of transgenic mosquitoes. For instance, it has been suggested that these mosquitoes might be better vectors for other (non-malaria) pathogens. While there is no evidence to suggest that this is the case, caution should be used and these possibilities should be tested. Another concern is that of horizontal gene transfer from the transgenic mosquitoes to other organisms, even to humans. Again, this possibility is remote. Moreover, the possibility of horizontal transfer of the effector gene to the germ cells of another organism is even more remote. Finally, most (if not all) effector genes being considered should be innocuous to higher organisms.

v) Political, social and ethical considerations

In addition to scientific concerns, it is important to address concerns of public perception. The issues and debates over genetically modified crops and other attempts of insect releases provide a useful precedent from which we should learn. It will be important to inform the public at large about the benefits and risks of the transgenic technology. The time to start is now, because full public awareness of these issues may take an entire generation to accomplish. The targets of such a campaign must include residents of the affected countries and their government officials. The results of safety tests should be openly and broadly divulgated. It is important to emphasize that no approach will be entirely risk-free and that the balance between potential benefit and risk should prevail in deciding about implementation of a new genetic strategy. It is also crucial to emphasize that any single approach is unlikely to be completely effective on its own, and that a final solution will have to incorporate a combination of several weapons, such as drugs, insecticides, bed nets, and hopefully vaccines and genetic modification of vector competence.

Priorities

Following are some current research priorities.

- First and foremost, a method to drive effector genes into field mosquito populations needs to be devised.
- The efficiency of *An. gambiae* transformation needs to be improved.
- Anopheline mosquitoes cannot be stored frozen or desiccated. Establishment of repository centres for transgenic lines is desirable.
- Whenever possible, work should be conducted with the organisms that are most relevant to human disease (*An. gambiae*, *P. falciparum*).

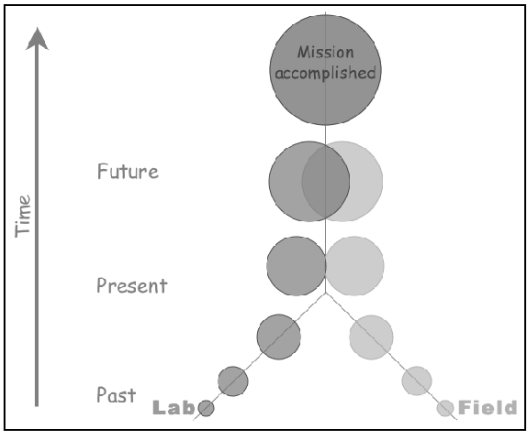

Figure 1. Laboratory and field scientists have mostly worked independently of each other in the past. However, the task of implementing malaria control via genetic manipulation of mosquitoes is so large that only close interactions and collaboration among investigators in the different lab and field disciplines will allow the final goal to be attained.

Prospects

During an historical 1991 meeting sponsored by the MacArthur Foundation and the WHO/TDR in Tucson, Arizona, a consensus was reached that the genetic manipulation of mosquito vectorial competence is an important and attainable goal. Hard work by an emerging community of mosquito molecular biologists delivered the first germ-line transformation in 1998, followed in rapid succession by the development of the necessary tools and by the proof-of-principle demonstration that the approach is feasible. At the same time, field researchers have made fundamental discoveries concerning population structure and vector ecology. Clearly, implementation of the genetic modification of vector competence that was considered in this article will depend on close collaboration among many groups of scientists with a broad range of lab- and field-based expertise (Figure 1). The task ahead of us is so big that it is inconceivable to proceed otherwise. While major lab and field issues remain to be solved, the rapid progress made to date gives one reason to be optimistic that this important new weapon (genetic modification of vector competence) will be ready for field testing within the next decade or so.

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6

Current thoughts about the integration of field and laboratory sciences in genetic control of disease vectors

Thomas W. Scott[#]

Abstract

Realizing the full potential of genetic control of vectors for disease prevention will require development of a research agenda that captures the willingness of people with diverse expertise to work together toward constructive and substantive goals. Below I review the five ecological and population biology topics that are central to contemporary genetic vector-control programmes and present opportunities of collaboration between people engaged in primarily laboratory- versus field-based research activities: (1) spread and stability of introduced genes; (2) evolutionary consequences of mosquito transformation; (3) entomological risk, pathogen transmission and disease severity; (4) quantitative analyses of mosquito biology, disease and genetically modified mosquito (GMM) control; and (5) procedural issues. I point out opportunities for greater, mutually beneficial interaction between laboratory- and field-based scientists. I draw four general conclusions from this analysis. First, an improved understanding of ecological topics associated with GMMs will provide the conceptual and factual foundation for application of genetic-control technology. Second, four topics that should be considered research priorities are male biology, mating behaviour, colonization and mass-production effects, and population biology. Third, in addition to greater collaboration between ecologists and molecular geneticists, genetic-control programmes will require recruitment of expertise from outside the vector-borne disease arena, greater involvement by scientists from disease-endemic countries (DECs), training for young scientists, adequate funding, and a sustained effort. Fourth, collaboration will be a central component of the legacy and success of genetic control for vector-borne disease prevention.
Keywords: genetically modified mosquitoes; genetic control; vector ecology

Introduction

Much of the enthusiasm during the past 15 years for the strategy of reducing mosquito-borne disease with genetic control of vectors was, and continues to be today, based on potential. Evidence of the excitement with which the promise for genetic control has been blessed is the articles that have been published on the topic, both supporting and challenging the concept. Even an incomplete list of published reports restricted to malaria and dengue vectors is impressive in terms of the number in print, the profile of the forums in which these were published, and the reputations of the participants involved (Alphey 2002; Alphey and Andreasen 2002; Aultman et al. 2000; Aultman, Beaty and Walker 2001; Beaty 2000; Benedict and Robinson

[#] Dept. of Entomology, Univ. of California, Davis, CA 95616 USA. E-mail: twscott@ucdavis.edu

2003; Bradbury 2002; 2003; Braig and Yan 2002; Catteruccia, Godfray and Crisanti 2003; Christophides, Vlachou and Kafatos 2004; Coleman and Alphey 2004; Collins 1994; Collins and Paskewitz 1995; Collins et al. 2000; Curtis 1994; 2000; 2002; 2003; Gould and Schliekelman 2004; Irvin et al. 2004; Ito et al. 2002; James 2000; 2003; James et al. 2001; Kiszewski and Spielman 1998; Moreira et al. 2004; Olson et al. 1996; 2002; Reisen 2003; Scott et al. 2002; Scott and Morrison 2003; Spielman 1994; 2003; Spielman, Beier and Kiszewski 2002; Tabachnick 2003; Takken and Scott 2003). In order to successfully complete the next critical steps – i.e., evaluation and application – I submit that the espoused potential will not be realized without more integrated efforts among people engaged in molecular research that is primarily laboratory-based, ecological investigations that are primarily field-based, and population genetics studies that offer a conduit of constructive exchange between the laboratory and field. For this to happen, it is essential that a research agenda be developed that captures the willingness of people with diverse expertise to work together to suppress mosquito vectors, while maintaining their focus on the primary goal of reducing human morbidity and mortality.

Success of genetic-control programmes during the 1960s and 1970s was at least in part due to mutually beneficial interactions among scientists with complementary, but different expertise. Gould and Schliekelman (2004) noted that, "During the era of classical genetic control research there appears to have been incredibly good communication and cooperation between theoretical and empirical researchers. Indeed much of the empirical work was inspired by results of population genetics studies. There has been a tendency for the sophistication of modern science to isolate researchers involved in molecular work from those doing ecological and population genetics studies. We think that more interaction between these scientists at the very early stages of genetic control projects could increase the chances of […] ushering in a new and long-lived, golden era of genetic control". What can be done to address this challenge of elevating cross-disciplinary, collaborative research?

During the 2001 workshop on Genetically Engineered Arthropod Vectors of Human Infectious Diseases at London's Imperial College there appeared to me to be a lack of balance in the contributions of people with laboratory versus field expertise. The geneticists had, in general, given more thought and spent more time engaged in research directed at a contemporary strategy for genetic control than had most vector ecologists. A week later, at the International Congress of the Society for Vector Ecology in Barcelona, Willem Takken and I decided to convene a meeting of vector ecologists for the purpose of defining key ecological and population-biology issues necessary for responsible evaluation and application of genetically modified mosquitoes (GMM) for disease control. The meeting would be largely limited to vector ecologists because we wanted to develop as much as possible a consensus on the topic prior to engaging our laboratory colleagues. This would be the first meeting of vector ecologists to discuss genetic control of mosquitoes to prevent malaria and dengue. That meeting resulted in two publications. One summarized the meeting (Scott et al. 2002) and the other provided detailed thoughts by meeting participants on a list of key challenges that the GMM strategy needs to address in order to be safely and effectively deployed (Takken and Scott 2003). Because at the time the most progress in genetic modification had been made with anopheline vectors of malaria and *Aedes aegypti*, discussion was limited to those taxa. Below I review five topics that are central for a genetic-control programme to prevent malaria and dengue transmission. In the spirit of bridging the gap between laboratory and field research in

disease vector control, when appropriate, I will note opportunities for greater, mutually beneficial interaction between laboratory- and field-based scientists.

Spread and stability of introduced genes

Fundamental to the success for GMMs for disease prevention will be that gene constructs spread and persist in target populations. To achieve this it will be important to understand the genetic structure of mosquito populations, patterns of mosquito reproduction, the size of target populations, how populations are regulated, and requirements for colonization of wild-type mosquitoes and mass rearing of GMMs. Knowledge of target population structure will likely be more important for anophelines than for *Ae. aegypti* because many anopheline populations exhibit restricted gene flow among sympatric sibling species and/or different chromosomal forms (Lanzaro and Tripet 2003). Reproductive barriers between or among different populations could undermine a population replacement strategy if not all competent vectors are eliminated or rendered refractory to parasite transmission.

Because successful mating is a basic component of any genetic-based control strategy and our understanding of mosquito mating is underdeveloped, there is an urgent need for increased research on this topic (see Takken et al., elsewhere in this volume). The application of contemporary molecular tools to dissect the details of mosquito mating offers an opportunity for productive collaboration between laboratory- and field-based scientists. Similarly, an opportunity exists for significant contribution in understanding the ecology of male mosquitoes, which has been largely ignored in preference of females. Females have been the focus of attention because they are responsible for pathogen transmission by bite. But in a genetic-control programme it is likely that releases of GMMs will be limited to males in order not to increase the bites per night suffered by humans living at release sites (Alphey et al. 2002). Therefore, fitness of males – which rarely has been examined for mosquitoes – assumes high status as a critical component of a successful genetic-control programme.

The number of GMMs released will be determined at least in part by the size of the target population. Estimation of effective population size can be complicated by seasonal fluctuations that could, depending on the circumstances, aid or hamper genetic-control efforts. Consequently, there is a need for greater effort with more sophisticated analyses to characterize the size and structure of mosquito populations (Taylor and Manoukis 2003). Likewise, population biologists and ecologists can contribute to the GMM strategy by explaining the processes by which the size of mosquito populations is regulated. Differences in population regulation among genetic subdivisions could lead to an unpredicted advantage for one population over another (Rasgon and Scott 2004).

During the development of GMMs it will be essential that protocols be worked out for colonization of wild-type mosquitoes that will be used for genetic modification and for mass rearing GMMs. Because adaptation to a laboratory setting can reduce fitness compared to the wild-type mosquitoes with which GMMs will be expected to compete (Munstermann 1994; Mukhopadhyay et al. 1997), there is a need to develop requirements or guidelines for minimizing the loss of fitness and altered phenotypic expression due to colonization and mass rearing. This is another area in which people with field- and laboratory-based expertise can work together and make a significant contribution to a GMM control programme.

Evolutionary consequences of mosquito transformation

Understanding and minimizing fitness costs associated with genetic modification, which can be conditional and/or correlated with other life history traits, will be critical in a population replacement scheme for spreading and maintaining stable resistance to pathogen transmission. This is an area that offers multiple opportunities for collaboration between population biologists and molecular biologists who are engineering GMMs. For example, we can ask what are the evolutionary costs of genetic modification to mosquitoes, and how will they shape plans for interfering with pathogen transmission? What effect will imperfect interference have on the evolution of pathogen resistance and how will it be managed in a disease prevention programme? Boëte and Koella (2003) predict that even if a gene for refractoriness was driven to fixation in an anopheline population, if malaria transmission is intense, prevalence of infection in humans will decrease only if transmission interference is close to 100%. If this is true, success of population replacement strategies will depend in large part on the efficacy of the effector gene(s). Thus, Boëte and Koella (2003) provide guidance to the people who are engineering GMMs by predicting the extent to which vector competence will need to be reduced.

What effect will natural environmental conditions and genetic background have on phenotypic expression of resistance? Variation in phenotypic expression can result in some mosquitoes carrying a 'refractory transgene' but not expressing a 'refractory phenotype' (Tabachnick 2003). Even if the gene is driven to fixation, this could lead to less than perfect replacement of competent with refractory mosquitoes and the opportunity for parasites to evolve resistance to GMMs.

What effect will imperfect interference have on the evolution of pathogen resistance and how will it be managed in a disease prevention programme? Although parasite resistance has been discussed in a variety of platforms, there has been relatively little empirical work done to determine to what extent this might be a problem for GMMs. If resistance does evolve, can we predict the virulence characteristics of resistance phenotypes? Every effort should be made to avoid selection of parasites that are more virulent than the ones that preceded release of the GMM.

A question that is frequently asked and will certainly need to be addressed is whether GMMs have enhanced capacity to transmit pathogens other than the one that they are intended to block. Vector competence studies will need to be carried out with co-occurring pathogens demonstrating that GMMs will not transmit unintended pathogens.

Will changes in parasite populations in response to a GMM affect the efficacy of other disease prevention programmes? For example, will vaccines or anti-parasite drugs be compromised? The time is right to begin to provide details for how GMMs will be incorporated into an integrated disease prevention programme. This kind of analysis will require collaboration among people with a diversity of expertise.

Entomological risk, pathogen transmission and disease severity

The conceptual foundation of genetic mosquito control is that reduction in the density of competent vectors, whether directly – population reduction – or indirectly – population replacement – will decrease human infection and disease. For this strategy to be successful we need to know the degree to which mosquito populations must be reduced in order to produce the desired public-health outcome. In other words, we

need to understand the quantitative relationships between density of competent vectors, human infection, and disease (Scott and Morrison 2003). This will include avoiding the so-called 'rebound effect'. That is, if transmission becomes unstable, primary adult infections could result in an unexpected increase in epidemic disease. Short-term reduction in malaria or dengue transmission could, but does not necessarily (Maxwell et al. 2002), create such a situation by increasing the number of people surviving childhood without an infection and the benefit of a protective immunological response. It may also be necessary to partition the relative contributions to parasite transmission by different mosquito species or chromosomal forms if several sympatric mosquito populations sustain transmission. If only one population of mosquitoes is removed from transmission, what will the impact be on the number of new human infections?

The entomological inoculation rate (EIR, i.e. the number of mosquitoes with sporozoites biting a person per unit of time) is a powerful measure of entomological risk for malaria transmission (Smith, Leuenberger and Lengeler 2001). For example, Charlwood et al. (1998) reported that when infections were low, risk of human malaria infection increases with the EIR. When infections were high, an increase in EIR did not raise parasitaemia in infants. Despite its advantages, there are at least two unresolved difficulties associated with application of the EIR for assessing the risk of malaria transmission and disease. First, for ethical reasons it is increasingly difficult to use human bait to collect anophelines that may be infected with parasites. This has resulted in efforts to correlate collections in traps to those from people. In some cases the relationship is good, in others it is not (C. Constantini and D. Fontenille, pers. comm. Mathenge et al. 2004). There is a need to develop a standardized methodology for capturing human-host-seeking anophelines that does not require direct exposure to humans (Mathenge et al. 2002; 2004). Second, establishing the relationship between EIR and malaria-specific mortality centres on the difficulties associated with the quality of cause-of-death data. Smith, Leuenberger and Lengeler (2001) explain the details of this dilemma and highlight the need for continued research efforts at the interface between medical entomology and epidemiology.

Dengue researchers do not have a simple and reliable entomological measure for assessing risk of disease, like the EIR. The rate of *Ae. aegypti* infection with dengue virus is too low and varies too much through time and space to create a dengue risk measure analogous to the EIR. Current measures of entomological risk for dengue transmission are at best weakly correlated with human dengue infection and their relationship to disease is poorly defined. Consequently, predicting and testing the relationships among mosquito density, dengue transmission and disease are among the most important unresolved issues in dengue epidemiology and assessing the application of GMM for dengue prevention.

Quantitative analyses of mosquito biology, disease, and GMM control

Models have made and will continue to make two vital contributions to the development and application of GMM technology (Ribeiro and Kidwell 1994; Kiszewski and Spielman 1998; Turelli and Hoffmann 1999; Focks et al. 2000; Boëte and Koella 2003; Rogers et al. 2002; Rasgon, Styer and Scott 2003; Gould and Schliekelman 2004; Rasgon and Scott 2004). First, they identify knowledge gaps and thus direct new research activities toward high-priority topics. Second, they predict outcomes of scenarios of interest. It is important that in the future modelling efforts in

the GMM arena transcend simulating events retrospectively and predict outcomes of proposed interventions.

This is clearly an area of opportunity for increased interaction among people with different and complementary expertise. Those who create models will seek the most germane and accurate data available from vector ecologists, molecular biologists and epidemiologists. The accuracy and utility of models will need to be refined by interaction among all parties involved. Model output will be invaluable for designing genetic constructs and predicting strategies for deployment and evaluation of GMMs.

Procedural issues

Addressing three procedural issues will provide additional opportunity for interaction between field- and laboratory-based scientists. First, there is a pressing need for development of standardized processes for dealing with the ethical, legal and social issues related to GMM technology (see Touré and Manga elsewhere in this volume). It has been suggested that these kinds of guidelines would be most effective if developed by an international body like the World Health Organization (Scott et al. 2002). During 20-21 September 2004 the Pew Initiative sponsored a conference in Washington, D.C. on science and policy surrounding the release of genetically modified insects. The meeting provided a forum for interaction among people with different backgrounds and the basis for development of guidelines for research and application of GMMs. Second, it is essential that scientists, public-health officials and regulatory personnel in DECs are fully enfranchised in the development and application of GMM programmes. For that to happen, there will need to be greater participation by people and infrastructure development at GMM research field sites. Third, thorough evaluation of GMM technology will require transitional research from laboratories to semi-field facilities – large outdoor cages like those described by (Knols et al. 2003) – followed by release at geographically isolated sites. Challenge 7 in the Grand Challenges in Global Health – i.e., develop a genetic strategy to deplete or incapacitate a disease-transmitting insect population – has already, in the proposal development stage, constituted a unique opportunity for laboratory- and field-based scientists to work side-by-side toward a common and well defined goal. Let us hope grants for this challenge are awarded and exciting crosscutting science will continue.

Conclusions

Four broad conclusions can be drawn regarding the application of genetic control of vectors and the integration of field and laboratory sciences.

First, without an improved understanding of the ecological topics discussed above, application of GMM technology will lack an appropriate conceptual and factual foundation. Understanding and applying ecological processes of a mosquito's role in pathogen transmission will be essential to achieve reduction in disease. When assessing research accomplishment on genetic control of mosquitoes in India during the 1970s, Rao (1974) explained that "Ecology of mosquitoes is the bedrock on which management of genetic control methods have been founded. The impact of the methods applied is largely determined by the behaviour of mosquitoes in nature, their numbers and their resting, feeding, mating, egg laying and dispersal habits which vary from season to season and place to place".

Second, although failures of past efforts have been attributed to factors other than the technology applied, four topics have been problematic in the past and should be

considered research priorities in contemporary programmes. They are male biology, mating behaviour, colonization and mass-production effects, and population biology.

Third, genetic control will require greater collaboration between ecologists and molecular geneticists, recruitment of expertise from outside the vector-borne disease arena, greater involvement by scientists from DECs, training for young scientists, adequate funding and a sustained effort. Genetic control will require a long-term commitment. In this regard it will be important to watch the impact of the Grand Challenges in Global Health on the development of genetic control of vectors.

Fourth, we stand on the threshold of a unique opportunity for all participants in the genetic-control paradigm. Let us hope that in the spirit of constructive and substantive interaction, regardless of participants' expertise or background, we will continue to work together to integrate genetic control into a robust strategy for disease prevention. Collaboration will be a central component of the legacy and success of genetic control for vector-borne disease.

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7

Genetic approaches in *Aedes aegypti* for control of dengue: an overview

Ken E. Olson[1]*, Luke Alphey*[2]*, Jonathan O. Carlson*[3]*, Anthony A. James*[4]

Abstract

The mosquito-borne dengue viruses (DV) cause an estimated 50 million human infections annually. The incidence of severe dengue disease in Southeast Asia and Latin America is increasing at an alarming rate. There are currently no vaccines or anti-viral therapies available to mitigate dengue disease. Current methodologies for controlling the principal vector, *Aedes aegypti*, are inadequate and ineffective. A potential solution to this growing human-health crisis is to develop new genetics-based vector control (GVC) approaches as part of an integrated control strategy. GVC includes both population reduction and population replacement strategies and represents a broad spectrum of genetic mechanisms at various stages in their development for field-testing. To realize the full potentials of these GVC strategies it is critical that we investigate, evaluate and, where appropriate, develop these strategies to the point where they can be deployed at field sites in one or more disease-endemic countries (DECs).
Keywords: dengue; *Aedes aegypti*; genetic-based vector control

Introduction

Dengue fever (DF) and its more serious form, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DHF/DSS) are caused by four closely related but antigenically distinct, single-strand RNA viruses transmitted by mosquitoes to humans. DVs cause more human morbidity and mortality than any other vector-borne viral disease with 2.5-3.0 billion people at risk of infection and 50-100 million DF and 250,000-500,000 DHF/DSS annual cases (Gubler 1996; 1998). All four DV serotypes cause disease and case-fatality rates for untreated DHF/DSS can be 30-40%. The risk of DHF/DSS is highest in areas where two or more DV serotypes are transmitted (Halstead 1988; Monath 1994; Rigau-Perez et al. 1998). At this time, there is no licensed vaccine and no clinical cure for the disease.

[1] Arthropod-borne and Infectious Diseases Laboratory, 3185 Rampart Road, Mail Delivery 1692, Foothills Research Campus, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, USA. E-mail: kolson@colostate.edu
[2] Department of Zoology, Wellcome Trust Center, University of Oxford, Oxford OX1 3PS, UK. E-mail: luke.alphey@zoology.oxford.ac.uk
[3] Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80521-1692, USA. E-mail: jonathan.carlson@colostate.edu
[4] Department of Microbiology & Molecular Genetics, Department of Molecular Biology & Biochemistry, University of California, Irvine CA 92697-3900 USA. E-mail: aajames@uci.edu

Ae. aegypti is by far the most important and efficient vector of DV because of its affinity for humans (Gubler 1998). Dengue control currently depends on reduction or elimination of *Ae. aegypti*. In the 1940-1960s most tropical American countries used integrated programmes of environmental management and insecticides to eliminate mosquitoes (Gubler 1998), but many of these were abandoned in the early 1970s (Reiter and Gubler 1997). *Ae. aegypti* re-infested countries where it had been eliminated and dengue epidemics renewed. In 2004, *Ae. aegypti* is distributed more widely than it was before eradication began, and is now in large urban areas where a greater number of people than in the past are at risk (Gubler 2004). Remarkably, despite the successes of the past, current dengue vector control programmes are often nonexistent or ineffective (Reiter and Gubler 1997). Rather than maintaining integrated programmes that specifically target *Ae. aegypti*, ministries of health merged all mosquito control and relied on outdoor applications of aerosol insecticides to kill adult mosquitoes. This costly approach is ineffective in most cases because the majority of females rest indoors where they avoid insecticide contact (Reiter and Gubler 1997). Furthermore, many insecticides are useless due to the spread of resistance (Hemingway, Field and Vontas 2002).

Several GVC strategies for reducing DV transmission have been identified as potential dengue disease control methods and are designed either to reduce the overall population of DV-transmitting vectors or to replace existing vector populations with populations that cannot transmit the virus. Two vector population reduction approaches are currently being investigated and are in early laboratory cage trials. The first population reduction strategy is the development and use of natural or genetically engineered densoviruses that are pathogenic to *Ae. aegypti* (Carlson, Afanasiev and Suchman 2000). The second population reduction strategy is the development and use of insects carrying dominant lethal mutations (RIDL, see below, Thomas et al. 2000). This approach would require mating of genetically modified vectors (GMV)-RIDL males with local vector populations producing offspring that die prior to becoming adults. Both approaches are designed to reduce transmission of DVs by reducing the vector population. Approaches designed to replace populations of vectors are more long-term, but could have significant consequences for dengue disease control in the future (James 2000). In these approaches, an effector gene, such as an anti-DV gene, is appropriately expressed to block transmission by the vector. GVC approaches require identification of tissue-specific promoters, anti-pathogen effector genes, and genetic drive mechanisms such as synthetic transposable elements (TE) to introgress the effector gene into the population, eliminating vector competence. Successful GVC strategies will require knowledge of vector ecology in DECs and large cage trials in DECs prior to release of biocontrol agents or GMVs.

Current state of the art

Genetic approaches leading to vector population reduction

Mosquito densoviruses as tools for population reduction and transduction

The *Aedes densonucleosis virus* (AeDNV; family *Parvoviridae*) is mosquito-specific and does not infect vertebrates or non-target invertebrates. Larvae are infected in oviposition sites and die in a dose-dependent manner depending on viral titre and stage of infection. AeDNV is maintained through metamorphosis and is transmitted vertically to offspring (Barreau, Jousset and Bergoin 1997). Infected female mosquitoes deliver viruses to multiple breeding sites and viral concentrations

increase as larvae become infected and shed, thus increasing horizontal transmission to other larvae. Survival of infected adult females also decreases significantly in a dose-dependent manner (Kuznetsova and Butchasky 1988, Suchman and Carlson, unpublished). Shortening the female adult lifespan would reduce vectorial capacity since a significant proportion of females would not survive the extrinsic DV incubation period. Recently, a number of other densoviruses have been discovered that also may be adapted as biocontrol and transducing agents (Kittayapong, Baisley and O'Neill 1999).

AeDNV research has the most immediate potential to deliver products for an effective field trial once a field site is selected and more extensive cage experiments completed. Prototype population cage experiments testing the ability of AeDNV to persist, spread and reduce mosquito populations have already been performed and are encouraging: a relatively low inoculum of virus in a larval rearing site replicates to levels that reduce the mosquito population, and female mosquitoes originally from the site inoculate virus into new sites.

Critical laboratory needs and challenges for using densoviruses as biocontrol agents

Optimize densovirus preparations and use of AeDNV in cage experiments

Laboratory-based cage experiments need to be performed to determine 1) if other densoviruses persist and spread more efficiently than AeDNV; 2) if mosquitoes from DEC field sites are susceptible to AeDNV; 3) if different strains of mosquitoes vary in their susceptibility to other mosquito DNVs; 4) if large-scale production and use of the AeDNV bio-control agent is feasible; and 5) whether recombinant viruses expressing anti-vector effectors (such as RNAi interference targeting the expression of critical vector genes) can enhance lethality of the virus for *Aedes aegypti* larvae.

Large-scale cage trials to assess densovirus potential for persistence and spread

Large-scale cages can be used to replicate laboratory experiments with natural populations under field conditions. *Ae. aegypti* from long-term cage experiments need to be compared with the local populations outside the cage at regular intervals by genetic analyses to look for genetic effects of the virus on populations (Gorrochotegui-Escalante et al. 2002; Garcia-Franco et al. 2002; Root et al. 2003). These studies should yield valuable data on the ability of the virus to persist and spread in a wild mosquito population, and to control mosquito populations in the field. These studies will also help refine the experimental design for cage experiments for a number of GVC strategies.

Development and use of Release-of-Insects-carrying-a-Dominant-Lethal (RIDL), a GMV-based development of Sterile-Insect Technique (SIT).

Drosophila melanogaster has been engineered with the basic genetic properties of an RIDL strain (Thomas et al. 2000; Heinrich and Scott 2000), using a repressible gene expression system ('tet-off') based on the tetracycline-repressible transactivator tTA (Baron and Bujard 2000; Gossen and Bujard 1992). Mathematical modelling suggests that for insects with strong density-dependent regulation of population size, a RIDL system imposing lethality at a larval or pupal stage has major advantages over conventional SIT and will provide a simple and effective dengue control method. Preliminary studies in *Ae. aegypti* in which tTA is expressed under tetO/hsp control,

produced >95% lethality in larvae in the absence of tetracycline (Alphey et al., unpublished data). Two transgenic lines are currently being evaluated in cage trials. These lines are being introgressed into the genetic background of local transmitting strains of *Ae. aegypti* to study fitness issues, release parameters, and population-dynamics overtime.

Critical laboratory needs and challenges for using RIDL to reduce vector populations

Increase the penetrance of RIDL-induced lethality

An ideal RIDL system would kill 100% of the individuals supposed to be affected. This is not essential for population suppression or to prevent the spread of the transgene within the target population, and indeed is not provided by current radiation-based SIT programmes for other insects. However, the system can in principle be refined in this regard by using alternative RIDL effectors, such as pro-apoptotic genes (Heinrich and Scott 2000), generating and testing more strains with the current constructs, or combining more than one insertion or construct to give a more highly penetrant and redundant system.

Construct a female-specific RIDL system

Most known female-specific promoters from *Aedes* are induced after the uptake of the blood meal. A RIDL system could potentially be developed around such a promoter to cause females to die soon after biting. Such females would be unable to transmit DV, which have a 10-14 day extrinsic incubation period. Alternatively, it should be possible to identify the sex-specific elements of *Aedes Actin-4*, a gene that expresses an actin in the female pupal developing flight muscles (Muñoz et al. 2004), to drive pre-adult lethality and thereby prevent biting-female development. Such a system would also avoid the need for physical sexing of the release generation and potentially allow the release of any of a wide range of developmental stages.

Determine key parameters for eventual use of RIDL technology in the field

These parameters include the economic and fitness costs of mass rearing of GMV-RIDL strains, the effect of the release ratio (GMV-RIDL/wild-type) of release into cage populations for optimal (most cost-effective) population reduction, and the ability of GMV-RIDL to compete with local mosquitoes for mating and resources. These parameters will feed into a suitable combined epidemiological and entomological model of dengue transmission, the development of which is another key requirement. This will provide a realistic estimate of the cost-effectiveness of RIDL, and a rational method for comparing this to other approaches, applied singly or in combination, in different transmission regimes.

Genetic approaches leading to vector population replacement

Much work has focused on developing GMVs that are refractory for DV transmission by developing germ-line-transformed *Ae. aegypti* that appropriately express an anti-pathogen effector gene. By targeting the pathogen, rather than the vector, expression of the effector gene should have minimal impact on the reproductive fitness of the GMV. The long-term goal is to replace existing transmission-competent vector populations with GMV populations that are no longer permissive for DV transmission. Replacement of *Ae. aegypti* populations to block DV transmission may be a real alternative to current vector control strategies. *Ae. aegypti*

is responsible for most of the severe dengue epidemics, it is relatively easy to manipulate genetically and maintain in the laboratory, and the vectors continuously exchange genes locally and appear to have few gene flow barriers within 150 km (Gorrochotegui-Escalante et al. 2002). At least three genetic-transformation systems have been described and used successfully in *Ae. aegypti* to generate GMVs. These transformation systems are based on the Class II TEs *Mos1* (Mariner), *Hermes* and *piggyBac* (Jasinskiene et al. 1998; Coates et al. 1998; Kokoza et al. 2000). *Mos1* and *piggyBac* are the most commonly used TEs for generating GMVs.

Anti-dengue virus effector genes – RNAi

During the last three years, considerable progress has been made toward identifying effector genes that can profoundly reduce *Ae. aegypti* competence for DV transmission (Adelman et al. 2001; 2002; Olson et al. 2002; Tavanty et al. 2004). The major thrust of research has been to design and express double stranded RNAs (dsRNAs) that make DV-susceptible cells non-permissive for virus replication. This strategy is based on RNA interference (RNAi), an ancient potent, innate immune response in insects and a related response termed post-transcriptional gene silencing in plants (Tijsterman, Ketting and Plasterk 2002).

We now know that *Drosophila melanogaster*, *Caenorhabditis elegans*, humans and plants have the RNAi pathway, which is triggered by the presence of intracellular double-stranded RNA (dsRNA). The presence of dsRNA in cells is an early warning signal of RNA-virus invasion that directs an innate response resulting in destruction of any mRNA having sequence identity with the dsRNA. Many RNA viruses generate dsRNA in infected cells as a byproduct of replication and these replicative intermediates serve as potent recognition patterns for inducing the RNAi intracellular response. If RNA viruses trigger RNAi, why are mosquitoes such efficient vectors of arboviruses? We do not know for sure, but DV may escape the antiviral effects of RNAi in competent mosquitoes either by failing to present the threshold concentration of dsRNA molecules required for triggering the response or by encoding a viral protein that suppresses the RNAi response. Currently, there is no evidence for a DV RNAi suppressor protein. However, Uchil and Satchidanandam (2003) have recently shown that the dsRNA replicative form (RF) of DVs is sequestered in double-membrane structures in the cytoplasm of infected cells which may limit RF exposure to the RNAi pathway.

RNAi is activated by dsRNA and results in a reduced steady-state level of specific RNA molecules with sequence similarity to the dsRNA (Cogoni and Macino 1997; Vaucheret et al. 1998). The mechanism of RNAi has been studied in some detail in *Drosophila melanogaster*. In the fruitfly, the RNase III enzyme Dicer is responsible for digesting dsRNA into 21-23 bp small interfering RNAs (siRNAs). The siRNAs are then unwound into single-stranded siRNAs in an ATP-dependent step and incorporated into an enzyme complex termed the RNA-induced silencing complex (RISC). The single-stranded siRNAs guide RISC to the target mRNA and the complex cleaves the message or inhibits its translation (Schwarz et al. 2002). This strategy has been used in transgenic plants to develop resistance to a number of RNA-virus pathogens. Several groups now have evidence that mosquito species such as *Ae. aegypti*, *Anopheles stephensi* and *An. gambiae* develop an RNAi response very similar to that found in *D. melanogaster.* These vectors are capable of silencing endogenous gene expression or virus replication after introduction of dsRNA targeted to a specific gene (Adelman et al. 2002; Travanty et al. 2004; Brown et al. 2003). Replication of several arboviruses appears to trigger the RNAi response in mosquito cells and we

now have evidence for the genes involved in the *An. gambiae* antiviral response to the arbovirus *O'nyong-nyong alphavirus* (*Togaviridae*) (Sanchez-Vargas et al. 2004; Keene et al. 2004).

RNAi maybe an Achilles heel for replication of RNA viruses and we should be able to induce a robust RNAi response to DVs in the midgut or other relevant tissues of a transgenic mosquito by expressing DV-specific dsRNA. This strategy would sensitize the cells to the presence of the RNA virus leading to the destruction of the virus genome either as the virus uncoats or following virus transcription in the cell. The midgut is a likely target for mounting this line of defence because it is the first tissue the virus encounters in the vector and is the major determinant of vector competence in the mosquito. In addition, oral infection of midguts with high concentrations of virus begin with relatively few foci of infection of epithelial cells that spread throughout the gut over a 5-7 day period prior to dissemination. A virus-specific dsRNA should be able to suppress DV replication during that time frame. Both *Ae. aegypti* midgut and salivary-gland promoters are available to test whether RNAi can be used to promote resistance to DVs in the vector (James et al. 1991; Moreira et al. 2000).

The RNAi approach of developing resistance in *Ae. aegypti* has the following advantages: 1) RNAi does not require expression of a potentially antigenic protein; 2) the strategy utilizes the machinery of a natural innate immune response that is present in the mosquito (Sanchez-Vargas et al. 2004); 3) a number of anti-DV dsRNA effector sequences have already been identified that cause profound resistance in mosquito cell culture and in adult mosquitoes (Adelman et al. 2001); 4) the anti-DV dsRNA effector sequence (500-600 bp) should be less prone to the effects of single-point virus mutations and selection since the active units of RNAi activity are 21-23 bp siRNA blocks formed from the dsRNA trigger (Travanty and Olson, unpublished data, Blair, Adelman and Olson 2000); 5) transgenic lines that express dsRNAs from several non-*Ae. aegypti* promoters have now been generated (Travanty et al. 2004); 6) DV-2 pathogenesis studies of virus in *Ae. aegypti* have been performed to determine the temporal and spatial infection patterns of the virus after oral infection (Sanchez-Vargas and Olson, unpublished data); 7) DV challenge protocols for assessing resistance in transgenic mosquitoes are available (Sanchez-Vargas and Olson, unpublished data).

Critical laboratory short-term needs and challenges for using RNAi-based disease control strategies and other effector gene strategies

Identify Ae. aegypti midgut and salivary gland promoters that can be utilized to deliver anti-DV at the correct time and place in the mosquito tissue.

We are currently evaluating the *Ae. aegypti ferritin heavy chain*, *carboxypeptidase*, *GFAT* and *glutamine synthetase* midgut promoters and the *D7* and *apyrase* salivary-gland promoters for gene-expression potential. To test both RNAi and promoter activity we are developing transgenics that express GAL4 and transgenics with anti-DV dsRNA expression under UAS control (Brand and Perrimon 1993). The two lines can be crossed and offspring evaluated for RNAi efficacy. Identifying suitable promoters is a key to this strategy. It is apparent that the siRNA 23-nucleotide signal is not amplified in insects as it is in plants and *C. elegans* therefore RNAi probably does not spread from cell to cell in mosquitoes (Hoa et al. 2003). This makes it critical that the antiDV dsRNA is expressed in the same vector cells that are critical for DV infection and replication.

Identify the most efficient construct format for delivering the dsRNA

Currently we are designing effector RNAs that comprise 300 bases of DV target sequence in a sense orientation followed by *Ae. aegypti* intron sequence and an exact antisense complement of the sense RNA (Adelman et al. 2002; Travanty et al. 2004). There may be a need to develop new constructs for expression in mosquitoes that form larger dsRNAs in the 500-600 bp range. Does the intron size matter, since it is ultimately cleaved? What untranslated sequences are needed to stabilize expression of the effector gene in target tissues?

Identify the specificity of an effector dsRNA based on DV2 sequence

Will it protect the mosquito from infection with other DV2 genotypes or other DV serotypes? There is indication that it is possible to target multiple serotypes by carefully choosing DV-specific target sequences (Sanchez-Vargas et al. 2004). Will this approach drive selection of DV with altered infection characteristics?

Develop a recombinant/reporter virus to rapidly assess RNAi in transgenic mosquitoes

Researchers have considerable experience developing infectious cDNA clones of flaviviruses and alphaviruses and have developed alphaviruses that express eGFP as a marker of infection (Foy et al. 2004; Keene et al. 2004). The development of a DV-expressing GFP as a marker would greatly facilitate identification and characterization of transgenic lines for virus resistance.

Development of protein-based effector genes

A number of effector-gene strategies will most likely need to be developed to engineer resistance effectively into vector populations. Ito et al. (2002) showed that peptides recognizing mosquito-tissue surface proteins block entry of a malaria sporozoite into the salivary glands of a transgenic mosquito. The challenge here is to identify effector proteins that block DV transmission yet can be effective against a rapidly evolving RNA virus. These peptide-based effectors could take the form of single-chain antibodies (Cappuro et al. 2000??) that bind to and neutralize DV or mimic the envelope glycoprotein domain-III region of DVs (Hung et al. 2004).

Long-term research challenges for GVC-replacement technology

Development of an efficient anti-DV effector gene is only the first step towards the long-term goal of using genetically manipulated insects to control DV. We also need to demonstrate that transposon-mediated systems or other genetic drive systems will successfully invade field populations. The first step in this process is to evaluate transposon-mediated drive of genes through mosquito cage populations. In *D. melanogaster*, studies with autonomous (self-mobilizing constructs that carry a copy of their transposase within the transposon) and non-autonomous (stable constructs mobilized only by externally supplied transposase) TEs carrying marker genes have shown that elements will increase the frequency of the marker gene when introduced into cage population of flies (Carareto et al. 1997). This mobility was characterized by a tight linkage of the transposon with an active marker gene for as many as 40 generations. However, stability of the marker gene varied inversely with the size of the final, 'loaded', autonomous element. Researchers need to conduct cage experiments to evaluate the mobility and stability of loaded autonomous TEs as they spread through cage populations of mosquitoes; maintenance of the integrity of the 'loaded' TE during population replacement and beyond is one of several major

challenges to the development of usable gene drive systems. Obviously serious discussions must take place to identify potential field sites for evaluation of control, especially those strategies involving vector replacement strategies.

Future directions for research and capacity/partnership building

Discussion of other laboratory and field research that will need to be performed to realize GVC approaches fully is found elsewhere in this book. Critical research needs include the development and the characterization of genetic drive mechanisms, the development of a much more complete understanding of the ecology of dengue disease transmission in DECs, and the formation of full and meaningful partnerships with DECs to evaluate GVC approaches. To realize the full potentials of GVC strategies it is critical that we investigate, evaluate and, where appropriate, develop GVC strategies to the point where they can be deployed at field sites in one or more DECs. A number of gaps in knowledge have slowed or prevented the development of genetic control methods. These gaps exist between the state-of-the-art laboratory development of novel anti-DV tools and knowledge of field properties of mosquitoes that will affect their use, and between scientists in the developed world and the DEC scientists who would be responsible for implementing the technology. Further gaps exist among scientists and the agencies that would be responsible for the deployment of any genetic control strategy, and in policies and procedures for evaluating how genetic control methods fit into the overall strategy of existing or planned control programmes; these problems have become acute as the tools have now been developed to allow implementation of some methods. Finally, gaps exist between the enthusiasm of scientists for these genetic methods and the level of awareness of potential end-users of the risks and benefits of using them for controlling dengue transmission.

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8

Malaria and dengue vector biology and control in West and Central Africa

Didier Fontenille[#] *and Pierre Carnevale*[#]

Abstract

In West and Central Africa endemic malaria and epidemic yellow fever are still main causes of morbidity and mortality. From Dakar in Senegal to Kinshasa in the Democratic Republic of Congo the pattern of malaria transmission shows a huge variability, in term of dynamics (rhythm and intensity) of transmission, as well as in terms of the vector species involved. The *Plasmodium* annual entomological inoculation rates (EIR) vary from less than one to more than 1000 infectious bites per person and *P. falciparum* represents more than 90% of malaria infections. In most settings south of the Sahara, several vector species are sympatrically involved in malaria transmission either simultaneously or replacing each other seasonally (Coluzzi 1984; Fontenille and Simard 2004). These vectors differ greatly in terms of density and vector efficiency.

Despite an efficient vaccine, deadly outbreaks of Yellow Fever (YF) virus, which circulates among monkeys and sylvatic vectors, still occur occasionally from Cameroon to Senegal (Mutebi and Barrett 2002). Although *Aedes aegypti*, the local vector, is abundant, and Dengue 2 virus is present in the forest, human dengue remains very rare and localized in Western Africa. But the situation could worsen with the recent introduction and spreading of *Ae. albopictus*, a potential dengue vector, in Central Africa.

Any vector control strategy, whether based on traditional (insecticides and impregnated/treated nets) or genetic control strategies (sterile-male releases or introduction of transgenic mosquitoes), aiming at significantly reducing malaria burden or yellow fever/dengue occurrence in Africa, will have to account for such entomological heterogeneity added to ecological and socio-economic diversities.

This paper provides an update on the bionomics and genetics of the four major African malaria vector systems (the *Anopheles gambiae* complex and the *An. funestus*, *An. nili* and *An. moucheti* species groups) and of the *Ae. aegypti* and *Ae. albopictus* species. It also reviews current vector control measures against malaria and yellow-fever vectors.

Key words: vectors; malaria; yellow fever; dengue; Africa

The vectors

The biology of the main African malaria vectors has been known, in general terms, for more than 50 years. The description and identification of vector species were

[#] LIN, IRD, BP 64501, 911, Avenue Agropolis, 34394 Montpellier cedex 5, France. E-mail: Didier.Fontenille@mpl.ird.fr; pjcarnevale2001@yahoo.fr

traditionally based upon morphological characteristics and sub-divisions (taxa) called sub-species, forms, varieties, races, etc. These have been described not only according to slight morphological differences, but also in terms of distribution, biology, ecology, behaviour, etc. In West and Central Africa 5 different species are considered major malaria vectors: *An. gambiae*, *An. arabiensis*, *An. funestus*, *An. nili* and *An. moucheti.* At least 4 or 5 other species are considered secondary or locally important vectors, such as *An. paludis*, *An. hancocki*, *An. melas*, etc. (Hamon and Mouchet 1961)

***Anopheles gambiae* complex**

Three species of the complex, among seven, are present in West and Central Africa: *An. gambiae sensu stricto*, *An. arabiensis* and *An. melas*. *An. gambiae* is usually predominant in humid environments while *An. arabiensis* is found in drier areas, but they coexist widely over much of their range of distribution. The salt-water species *An. melas* breeds in mangrove swamps along the west coast of Africa south till Namibia (Coetzee, Craig and Le Sueur 2000). Identification of species is based on fixed paracentric inversions or on the recently developed and very convenient PCR-based diagnostic tool detecting species-specific sequence differences in the ribosomal-DNA intergenic spacer (rDNA-IGS) region (Scott, Brogdon and Collins 1993).

Furthermore, extensive studies of karyotype distributions in natural *An. gambiae* populations often revealed strong and persistent deviations from Hardy-Weinberg equilibrium due to a deficit, or even complete absence, of certain heterokaryotypes. These results led to the designation, in West Africa, of five 'chromosomal forms' named under the non-Linnaean nomenclature Forest, Savanna, Mopti, Bamako and Bissau (Coluzzi, Petrarca and Di Deco 1985).

Recently, analysis of the rDNA-IGS region revealed fixed sequence differences between sympatric and synchronous chromosomal forms of Savanna/Bamako and Mopti populations in Mali and Burkina Faso, leading to the designation of two non-panmictic molecular forms named S and M. Both molecular forms are found throughout West and Central Africa (Favia et al. 2001; Della Torre et al. 2001). All Mopti specimens identified so far belong to the M molecular form; however, outside Mali and Burkina Faso, the M form may exhibit chromosomal arrangements typical of the Bissau, Forest or Savanna forms. The S molecular form may also carry standard chromosomes, indicative of the Forest form, or typical Savanna and Bamako karyotypes. While some very rare M/S hybrids have been found in Sierra Leone, Mali and Cameroon, evidence for reproductive isolation between molecular forms has accumulated to the point that incipient speciation is being suggested (Della Torre et al. 2002). For example in South Cameroon, a population-genetic study based on microsatellite DNA markers demonstrated significant genetic differentiation between sympatric M and S populations, both within the standard Forest chromosomal form of *An. gambiae* (Wondji, Simard and Fontenille 2002). The biological and vectorial significance of this genetic subdivision is currently under investigation.

Insecticide resistance has long been recorded in almost all West-African countries. Pyrethroid resistance due to *Kdr* mutation has recently been observed in S, and then also in M forms in every country in which this was investigated (i.e. Senegal, Sierra Leone, Burkina Faso, Mali, Côte d'Ivoire, Ghana, Benin, Cameroon, etc.). One *An. arabiensis* specimen from Burkina Faso was also found to carry the resistance allele (Diabate, pers. comm.). Other resistance mechanisms (resistant AChE, esterases, oxydases, Rdl, GST) have also been recorded in West- and Central-African populations of *An. gambiae* (Weill et al. 2003).

***Anopheles funestus* group**

An. funestus is widespread throughout sub-Saharan West Africa. Since the 1930s this group is known as being composed of several species closely resembling each other that can only be differentiated by very small morphological characters at larval or adult stages (Gillies and De Meillon 1968), or by a recently developed PCR assay (Koekemoer et al. 2002; Coetzee and Fontenille 2004). *An. funestus*, *An. leesoni*, *An. rivulorum* and *An. brucei* have been recorded in West and Central Africa. Their biology and vectorial capacity are very different. With the exception of *An. funestus*, these species are mainly zoophilic and therefore not considered malaria vectors. In 2003, Cohuet et al. have described a new taxon c1osely related to *An. rivulorum*, based on biological, morphological and genetic characteristics. This taxon, provisionally called "*An. rivulorum*-like", is present at least in Burkina Faso and Cameroon, is clearly different from the South African *An. rivulorum*, and does not seem to play any role in malaria transmission.

An. funestus itself is highly polymorphic, both biologically and genetically, showing at least 11 paracentric chromosomal inversions on chromosomes 2 and 3. In populations of Burkina Faso, huge Hardy-Weinberg disequilibrium and linkage disequilibrium between inversions led Costantini et al. (1999) to describe two chromosomal forms called 'Kiribina' and 'Folonzo', based on the presence and association of paracentric inversions, and then to hypothesize incipient speciation within *An. funestus*. In Senegal, 3 chromosomal populations exhibiting different anthropophilic demeanours were recognized, sometime in sympatry (Dia, pers. comm.). In Cameroon a cline of inversion frequencies is observed from the humid forest in the South (with 'Folonzo'-like inverted populations) to the dry savannas in the North (with 'Kiribina' standard populations), with strong heterozygote deficiency in areas where both forms occur. All these data suggest restricted gene flow between chromosomal forms of *An. funestus*. However, on the other hand, several observations from Cameroon (and East Africa) did not detect any evidence for reproductive isolation between 'Folonzo' and 'Kiribina', with heterokaryotypes observed at the expected frequencies within populations. Recent use of microsatellite markers in Senegal and Cameroon showed that gene flow is permitted between chromosomal forms, and showed isolation due to geographic distance between populations. These results strongly suggest that heterozygote deficits at chromosomal loci are mostly locus-specific and reflect environmental selection on the inversions themselves (or the genes they contain) (Cohuet et al. 2005). No pyrethroid resistance has yet been observed in West-African populations of *An. funestus*, in contrast to findings in Mozambique and South Africa, which seriously complicates vector control (see chapter 9).

***Anopheles nili* and *An. moucheti* groups**

Anopheles nili has a wide geographic distribution, spreading across most of West and Central Africa. Larvae of *An. nili* are typically found in vegetation or in dense shade along the edges of streams and large rivers. Extensive morphological, ecological and ethological variations among *An. nili* populations have been reported demonstrating that *An. nili* actually represents a group consisting of at least 4 species: *An. nili s.s.*, *An. somalicus*, and *An. carnevalei* and the recently described new malaria vector *An. ovengensis* (Awono-Ambene et al. 2004). Based on fixed nucleotide differences between ITS2 haplotypes, primers were designed to develop an allele-specific PCR assay for rapid identification of species within the *An. nili* group (Kengne et al. 2003).

An. moucheti is a group of very efficient forest vectors, whose larvae breed in slow-running streams and large rivers of equatorial Africa. Morphological and behavioural variations suggest that at least three taxa may belong to the *An. moucheti* group: *An. moucheti moucheti*, *An. moucheti nigeriensis* and *An. bervoetsi.* However, comparison of DNA sequences of specimens from several populations and countries strongly suggests that possibly only two truly different species, both vectors, exist (Kengne et al., unpublished results). All the populations tested were sensitive to insecticides.

Aedes aegypti* and *Ae. albopictus

Aedes aegypti, the domestic vector of the YF virus, is present in every West-African country, all specimens belonging to the black *formosus* form in both sylvatic and domestic populations. In spite of the fact that West-African populations of *Ae. aegypti formosus* are experimentally able to transmit dengue-2 virus, very few occurrences of dengue have been observed (Burkina Faso, Senegal) (Failloux, Vazeille and Rodhain 2002).

Recently *Aedes albopictus*, an Asian potential vector of dengue, has been discovered in some West-African countries: Nigeria, Cameroon and Equatorial Guinea. In the south of Cameroon this invasive species tends to replace *Ae. aegypti* in many locations (Fontenille and Toto 2001; Toto et al. 2003) and its spreading is a matter of concern.

Vector control

More than 120 years after the discovery of *Plasmodium* by Laveran, malaria remains one of the major public-health problems in Africa south of the Sahara.

From 1955 to 1968 the goal was to achieve global eradication of malaria through Indoor Residual Spraying (IRS) of every house with residual insecticides (DDT, then DLN, HCH, various organophosphates). This programme did not involve Africa south of the Sahara, which remained in the 'pre-eradication stage'. Due to different constraints (lack of funds, technical and operational issues, etc.) this programme was abandoned in 1969 and transformed to 'malaria control' with 4 technical variants dealing primarily with diagnosis and treatment. The 1992 WHO Global Strategy recommended not only case management but also selective and sustainable vector control for malaria prevention. Two main methods are available for such vector control: insecticide-impregnated mosquito bednets (ITNs) and other materials, and IRS, which is still effective and widely used in several countries, mainly in Southern Africa, (Mabaso, Sharp and Lengeler 2004), Burundi, etc. This approach was able to stop malaria epidemics such as the 1987 deadly outbreak in Madagascar and in KwaZulu Natal.

During the African Summit on Roll Back Malaria held in Abuja (25 April 2000) it was agreed to initiate appropriate and sustainable action to strengthen the health systems. Among other things, a decision was reached to ensure that by the year 2005 at least 60% of those at risk of malaria, particularly children under five years of age and pregnant women, will be able to benefit from the most suitable combination of personal and community protective measures such as ITNs and other accessible and affordable interventions to prevent infection and suffering. For a variety of reasons, these goals have not been met (see below).

In West-African countries *Anopheles* control is now mainly based on the large-scale use of ITNs and other impregnated materials supplied by national vector control

programmes or NGOs and private initiatives. Experimental surveys have confirmed efficacy of these methods in terms of reduction of incidence of malarial disease (Carnevale et al. 1991; Lengeler 1998), and overall infant mortality in Ghana (Binka et al. 1996), Kenya (Nevill et al. 1996), Burkina Faso (Habluetzel et al. 1997) and The Gambia (D'Alessandro et al. 1995). Moreover, recent trials showed a mass effect of permethrin-impregnated nets in Ghana (Binka, Indome and Smith 1998), in Kenya (Howard et al. 2000) and with impregnated curtains in Burkina Faso (Diallo et al. 2004) with no rebound mortality even after several years of ITN usage (Binka et al. 2002; Hawley et al. 2003; Maxwell et al. 2003). It was noticed that when some coverage of the population (60 to 80%) was maintained, even people not covered by ITNs can be protected from malaria if they are living inside 'treated' compounds or in their vicinity (less than 300m). These observations open a new field of research and offer hope in terms of public-health outcome of this type of intervention.

These positive results concerning efficacy and effectiveness led to the recommendation and promotion of ITNs for malaria control. Unfortunately, according to the recently (2003) published WHO Africa malaria report, the proportion of children under 5 years sleeping under nets is low – about 15% across 28 countries surveyed. Even fewer children (less than 2%) sleep under ITNs. Only two countries, The Gambia and Sao Tomé and Principe, reported user rates of more than 10% even if the availability of nets has increased noticeably over the last 10 years. However, more and more countries are engaged within the Abuja initiative, and recent unpublished information from different West-African countries (such as Burkina Faso, Ghana and Mali) suggest that coverage rates are increasing. In North Cameroon it was also noticed that mobile teams treating the nets of users directly inside villages were well received and dramatically increased the percentage of ITNs and therefore their actual efficacy (Manga et al., pers. comm.). There are several well-known approaches to increase the affordability of ITNs for people and therefore scaling-up the coverage, and hence the efficiency of ITNs: social marketing, highly subsidized prices, tax-free, as a gift to pregnant women during antenatal clinic visit, given free of charge by companies to their employees and families, 'do-it-yourself kits', 'centre for impregnating mosquito nets', mobile teams doing re-treatment free of charge, ITNs given free of charge during EPI vaccination campaigns, etc. Every method has its advantages and limitations, the crucial point being that it needs to be adapted, tailored, suited to the targeted population in terms of price, size, shape, colour of the nets and cultural behaviour of the population

The main drawbacks in the large-scale use of ITNs are human behaviour (resistance to use), as well as the cost of nets, the need for their regular re-impregnation and their widespread availability and distribution. Recently, prices have decreased; promotion, delivery and affordability have improved with social marketing programmes. Nets are now produced in African countries and are more adapted to human needs in terms of quality, size, shape, colour, opacity, etc. It has been proposed to provide them free of charge (Curtis et al. 2003) for example during the EPI vaccination programme, or as a 'kit for pregnant women' (Guyatt et al. 2002) or a gift for birth through local health systems or NGOs. A technical solution to the re-treatment issue was recently found with the development of 'long-lasting nets' (LLNs) (Guillet et al. 2001), wash-resistant nets such as the Olyset Net® (with permethrin incorporated into the polyethylene fibre) or Permanet2® (with deltamethrin stuck onto the polyester fibre), which sustains their efficacy even after several years of use in the field (N'Guessan et al. 2001).

Another potential drawback in the efficacy of ITNs is pyrethroid resistance of several *An. gambiae* populations recently noticed in several West-African countries (Chandre et al. 1999), attributed to large-scale use of insecticides for agricultural purposes. Resistance to carbamates, organochlorines and organophosphates have also been recorded for a long time in *An. gambiae* populations from several countries of West and Central Africa. Trials of ITNs in experimental huts against pyrethroid-resistant *An. gambiae* showed that they still confer protection to users through a reduction of entry rates, an increase of exit rates, a decrease of man–vector contact and an increase of the mortality rate of resistant specimens (Darriet et al. 2000). Moreover the large-scale use of lambdacyhalothrin-treated nets in the Korhogo area (northern Ivory Coast) where *Kdr* allelic frequencies are > 0.90 among *An. gambiae* populations, induced not only a sharp reduction of entomological parameters (inoculation rate, vectorial capacity, etc.) but also a ~50% reduction of incidence rate of malaria morbidity among children of less than 5 years of age (Carnevale et al. 2001). On the other hand, combination of different classes of insecticides tested in experimental huts in Ivory Coast showed that they might be a potential tool for resistance management. The mixture might also have an advantage in terms of lower cost and toxicity (Hougard et al. 2003). Insecticide resistance of *Culex quinquefasciatus* is also a major drawback for the use of ITNs because 'mosquito control' at household level is mainly directed against nuisance and, therefore, protection conferred by nets must be as comprehensive as possible to gain actual use and participation of the community. The positive 'collateral effect' of ITN (against lice, bugs, ticks, non-vector mosquitoes, etc.) has actually been noticed several times and put forward in their regular use. Mixtures of insecticides could be a solution in such circumstances.

For the time being there is no control of *Aedes aegypti* in West Africa. The management of domestic larval breeding sites was obligatory in most of the West-African countries in the 1960s, but it was gradually stopped and larval indices (Breteau, container, house indices) slowly increased. Currently, control is rarely carried out, by emptying or treating domestic breeding sites, or by ULV pulverization of insecticides only during epidemic episodes.

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9

Malaria and dengue vector biology and control in Southern and Eastern Africa

Maureen Coetzee[#]

Abstract

Malaria vector control has been practiced in the eastern/southern half of the African continent since the beginning of the 20th century, from larval control in the north (Sudan, 1901) to adult control in the south (South Africa, 1931). The major vectors are *Anopheles gambiae, An. funestus* and *An. arabiensis*, with *An. merus*, *An. bwambae* and *An. nili* implicated in transmission in localized areas. Current vector control methods include indoor residual house spraying (± 9 countries out of 19), insecticide-treated bednets for personal protection (± 15 countries out of 19), larviciding under certain circumstances and very limited environmental management. Control programmes are faced with multifaceted problems such as service delivery, species diversity and identification, and insecticide resistance. Population-genetic studies are limited compared with West Africa and this gap in knowledge should be urgently addressed. Current evidence suggests far less polymorphism in all three major vectors, *An. gambiae, An. arabiensis* and *An. funestus*, than is seen in West-African populations.

As far as non-malaria disease vectors are concerned, both *Aedes aegypti aegypti* and *Ae. aegypti formosus* occur in East and Southern Africa. Genetic and disease-transmission studies provide strong evidence for the specific distinctness of these subspecies. Occasional cases of suspected dengue occur in Kenya, presumably transmitted by *Ae. aegypti*. Outbreaks of yellow fever, however, have been caused by other *Aedes* species and not the above two. No targeted control activities are carried out against these species.

Keywords: malaria; dengue; yellow fever; vector control; *Anopheles gambiae*; *Anopheles funestus*; *Anopheles arabiensis*; *Aedes aegypti*; *Aedes formosus*; insecticide resistance; population genetics

Malaria

The dynamics of malaria transmission are not simple, with many factors influencing individual situations. Major problems affecting malaria in Africa include funding and service delivery, political instability, poverty, drug and insecticide resistance, and extremely efficient (i.e. competent and long-lived) vector mosquitoes. Problems specific to vector control include species identification, population

[#] Vector Control Reference Unit, National Institute for Communicable Diseases, Private Bag X4, Sandringham 2131, South Africa; and Division of Clinical Microbiology and Infectious Diseases, School of Pathology of the National Health Laboratory Service and the University of the Witwatersrand, Johannesburg, South Africa. E-mail: maureen.coetzee@nhls.ac.za

diversity, insecticide resistance and choice of control strategy. This paper provides an overview of the vector control situation in Eastern and Southern Africa and highlights issues specific to these regions.

Vector species

Anopheles gambiae complex

Six of the seven recognized species in the *An. gambiae* complex occur in Eastern and Southern Africa (Gillies and Coetzee 1987; Hunt, Coetzee and Fettene 1998). *Anopheles gambiae* and *An. arabiensis* are the major vectors, with *An. arabiensis* occurring throughout the region from South Africa to Sudan and Egypt, while *An. gambiae* is prevalent in the tropical belt only. The saltwater breeder *An. merus* is found in mainly coastal areas of Kenya and Tanzania, but also far inland in Zimbabwe and South Africa (Gillies and Coetzee 1987; Coetzee, Craig and Le Sueur 2000). It is an efficient vector in some areas (Temu et al. 1998, H.T. Masendu, unpubl. data). *Anopheles bwambae* is a minor vector, restricted to the Semliki forest of Western Uganda. The other two species, *An. quadriannulatus sp*. A and B, occur in Southern Africa and Ethiopia respectively. They are mainly cattle feeders and do not play any role in malaria transmission.

The identification of the above species is either by chromosomal inversion polymorphisms (Coluzzi, Petrarca and Di Deco 1985) or rDNA-PCR (Scott, Brogdon and Collins 1993). The occurrence of cattle-feeding species in a given area makes species identification essential so that control measures can target the actual vectors and not look-alike non-vectors. *Anopheles gambiae* is predominantly house frequenting, while *An. arabiensis* will feed on humans and rest indoors as well as feed on cattle and rest outdoors, making this a more difficult mosquito to control by conventional means. The resting and feeding behaviour of these two species, however, may vary considerably depending on locality and availability of hosts (Gillies and Coetzee 1987).

Anopheles funestus group

This group consists of nine morphologically similar species, of which only one, *An. funestus*, is a major vector (Gillies and De Meillon 1968). Species identification of the five most common members of the group is by rDNA-PCR (Koekemoer et al. 2002). *Anopheles funestus* is almost exclusively human biting and preferentially rests indoors, making it very susceptible to control by residual house spraying. It occurs in South Africa and extends northwards to Kenya (Gillies and De Meillon 1968). Early reports of *An. funestus* in Ethiopia have not been confirmed as the only species so far identified by PCR is the non-vector *An. parensis* (Weeto et al. 2004).

Insecticide resistance

Table 1 summarizes the status of insecticide resistance in the three major African malaria vector species (Zahar 1985; Hargreaves et al. 2000; 2003). In most cases the selection pressure has come from agricultural use of the insecticides and can be maintained without selective pressure in many of the mosquito populations through linkage to chromosomal inversion polymorphisms (Brooke et al. 2001).

Table 1. Insecticide resistance in East and Southern African malaria vector mosquitoes (situation in 2004)

Species	Insecticide	Region
An. gambiae	DDT	Zanzibar
	Dieldrin	Kenya and Madagascar
	Pyrethroids	Kenya and Zambia
An. arabiensis	DDT	Sudan, Ethiopia, Zanzibar, South Africa
	Dieldrin	Sudan, Ethiopia, Kenya, Madagascar,
	Organophosphates	Zimbabwe, Swaziland, Sudan
An. funestus	Dieldrin	Kenya
	Pyrethroids	South Africa, Mozambique
	Carbamates	South Africa, Mozambique

Population genetics

Chromosomal studies on both the *An. gambiae* complex and *An. funestus* group in Eastern and Southern Africa show far less inversion polymorphism than in West Africa (Ralisoa Randrianasolo and Coluzzi 1987; Petrarca and Beier 1992; Petrarca et al. 1984; 1986; 1990; 1991; Green and Hunt 1980; Kamau, Hunt and Coetzee 2002; Kamau et al. 2003). Population structure and gene flow studies have been carried out on *An. gambiae, An. arabiensis* and *An. funestus* but these remain few and more are needed (Donnelly et al. 1999; Donnelly, Licht and Lehmann 2001; Garros et al. 2004; Kamau, Hunt and Coetzee 2002; Kamau et al. 2003; Lehmann et al. 1996; 1997; Sinkins et al. 2000; Braginets et al. 2003; Temu, Hunt and Coetzee 2004). Population structuring in *An. funestus* has been demonstrated using microsatellite markers (Temu, Hunt and Coetzee 2004) and restriction-fragment length polymorphisms (RFLP) (Garros et al. 2004), providing evidence of distinct sub-populations. The situation in *An. arabiensis* is less clear with apparently significant gene flow over fairly large areas (Donnelly et al. 1999; Donnelly, Licht and Lehmann 2001).

Vector control

In East and Southern Africa, BHC (cyclodiene) resistance in *An. arabiensis* and pyrethroid resistance in *An. funestus* have had major impacts on control strategies. In the other areas, vector control was implemented in small areas only and discontinued after a few years for various reasons (Zahar 1985). The Zimbabwe and South-African experiences are highlighted here as examples of the problems of insecticide resistance faced by control programmes.

Zimbabwe

In 1974, the Zimbabwe residual house-spraying campaign used benzene hexachloride (BHC), an organochlorine closely allied to dieldrin. A malaria epidemic in the southeastern lowveld indicated problems with the control programme. At the time, the only species identification technique available was the banding pattern of the polytene chromosomes found in half-gravid female anophelines. This meant that only live females could be identified and not dead ones. The following year, an isoenzyme method was developed in Zimbabwe (Mahon, Green and Hunt 1976) that allowed identification of all adults collected. Insecticide susceptibility tests showed that the majority of dead mosquitoes were *An. quadriannulatus* while those surviving exposure to 4% dieldrin were all *An. arabiensis*. The Zimbabwe malaria control

programme changed their policy to DDT spraying and the epidemic was brought under control (Green 1981).

South Africa

In the early 1930s, De Meillon (1934) showed that the malaria vector *An. funestus* fed on humans indoors and rested inside houses until ready to lay eggs. Based on this information, the major epidemic of 1931-33 was brought under control by the use of pyrethrum flit pumps to spray indoors once a week (De Meillon 1936; Park Ross 1936). When DDT became available, South Africa began to implement a house-spraying campaign throughout the malarious areas of the northeast provinces with great success.

Malaria in South Africa is a notifiable disease with records of malaria case incidence kept since 1971. In 1995, a policy decision was taken to move from DDT to the more environmentally friendly pyrethroids. Coinciding with good rains, the number of malaria cases in 1996 almost trebled (Figure 1). Various reasons were postulated, such as cross-border movement of people from Mozambique carrying gametocytes, the weather, and deterioration of the control programme.

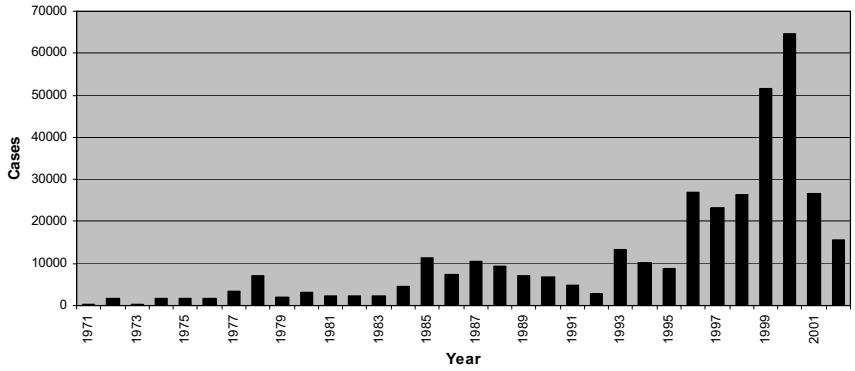

Figure 1. Malaria cases in South Africa, 1971-2001 (source: Department of Health, South Africa)

In 1999, the number of cases doubled again, reaching a peak of over 60,000 cases in 2000. Entomological surveys in December 1999 collected *An. funestus* in window exit traps in pyrethroid-sprayed houses. *Plasmodium falciparum* infection rates were > 5% and susceptibility tests on papers treated with three different pyrethroids confirmed high levels of resistance (Hargreaves et al. 2000). Collections in Maputo, Mozambique, in 2000 showed that carbamate resistance was also present (Brooke et al. 2001). The malaria control programme policy was changed back to the use of DDT for traditional-style housing, with pyrethroids being used in the few western-style houses only. By 2002, the malaria case incidence had decreased by >70%.

A multicentre study in Southern Africa

In 2002 a partnership between five countries in Southern Africa (Namibia, Botswana, Zimbabwe, South Africa and Swaziland) was initiated, supported by the World Health Organization (WHO/AFRO), to obtain baseline information on insecticide resistance. Results showed complete susceptibility of *An. arabiensis* to

DDT and pyrethroids in Namibia, Botswana and Swaziland, with susceptibility to pyrethroids in South Africa and Zimbabwe as well (M. Coetzee et al., unpubl. data). Resistance to DDT was detected in South Africa (Hargreaves et al. 2003) and Zimbabwe (H.T. Masendu, unpubl. data). The impact of this resistance on malaria control is unclear, but at least in South Africa there appears to be no increase in transmission in the areas where the resistance was detected (National Department of Health, unpubl. malaria statistics).

Vector control in East Africa

Early pilot studies in the 1950s and 1960s using dieldrin, DDT and organophosphates for house spraying showed amazing success in many instances (Zahar 1985). Unfortunately, most of these studies were terminated after only a few years, presumably because of financial constraints. Table 2 gives a brief summary of control activities using indoor residual house spraying around the time of the WHO eradication campaign and later (see Zahar (1985) for a comprehensive summary).

Table 2. Vector control in East and Southern Africa using indoor residual spraying

Locality	Dates	Insecticide	Outcome
Sudan, Sennar	1956-59	Dieldrin	Transmission interrupted
Gezira	1973-77	Malathion	Transmission reduced by 74%
Uganda, Kigezi	1959-61	DDT	Transmission interrupted
Kenya, highlands	1954-57	Dieldrin	Prevalence reduced to <2%
Kisumu	1973-75	Fenitrothion	Transmission reduced by 73%
Tanzania, Pare-Taveta	1954-59	Dieldrin	Transmission reduced by >90%
Zanzibar	1958-61	Dieldrin	Transmission interrupted
	1962-68	DDT	Transmission interrupted
Zambia, Copper Belt	1945-70	DDT	Transmission interrupted
Zimbabwe	1948-90	BHC, DDT	Transmission controlled, limited to lowveld areas
South Africa	1945-95	DDT	Transmission controlled, limited to lowveld areas
Swaziland	1960-04	DDT	Transmission controlled
Madagascar	1949-69	DDT	Transmission interrupted on plateau
Mauritius	1949-52	DDT	Eradicated

The 1904-1910 Khartoum, Sudan, malaria control campaign using larviciding only, was successful in reducing malaria transmission within the city limits (Annual Reports of the Gordon Memorial Institute, Khartoum). However, the military precisions with which the campaign was run and the amount of manpower expended on the project were incredible. There was intense coverage of every available larval breeding site within Khartoum, each of them mapped on a monthly basis (the mapping exercise in 1902-04 equalled any modern geographic mapping system using GPS and GIS today!). The problem, however, was that it was impossible to cover all natural water bodies along the Nile rivers, thus enabling sufficient immigration of vector mosquitoes into the city. This, coupled with the constant influx of gametocyte carriers from outside the control area, ensured continued malaria transmission within Khartoum. Andrew Balfour reports (in Ross 1911, p. 530-542) that "... they have met with marked success, and doubtless will continue to do so, provided the work is carried out continually, thoroughly, consistently and with intelligence...", which

highlights the enormous amount of work and commitment that is required to carry out malaria vector control through larviciding even in a very small area. Khartoum today has a human population of over 1 million people compared with 41,000 in 1904.

Current vector control

- Indoor residual house spraying is being carried out to a greater or lesser extent in 9 of the 19 East- and Southern-African countries.
- Insecticide-treated bednets are being distributed or used in pilot studies in 15 countries.
- Larviciding is implemented on an ad-hoc basis where situations allow.
- Only very limited environmental management is undertaken.

Dengue and yellow-fever virus transmission

Both *Aedes aegypti aegypti* and *Ae. aegypti formosus* occur in East and Southern Africa. Genetic and disease-transmission studies provide strong evidence for the specific distinctness of these sub-species (Powell, Tabachnick and Arnold 1980; Failloux, Vazeille and Rodhain 2002). Occasional cases of suspected dengue have occurred in Kenya but these were not confirmed by virus isolation and PCR. Presumably, *Ae. aegypti* transmitted the virus. Outbreaks of yellow fever in East Africa, however, have not been transmitted by *Aedes aegypti*, but by other species such as *Aedes simpsoni* (see Chapter 8 for a more comprehensive summary).

No control activities are currently carried out against *Aedes* larvae, but where indoor residual house spraying is used for malaria vector control, this will presumably affect the adult populations of *Aedes aegypti*.

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Malaria and dengue vector biology and control in Southeast Asia

Pattamaporn Kittayapong[#]

Abstract

This chapter reviews the situation of vector biology and control of both malaria and dengue in the Southeast-Asian region as part of the World Health Organization (WHO/TDR) working-group meeting on strategic planning to bridge laboratory and field research in disease vector control. Many research studies on malaria were related to the survey of malaria vectors and parasites and their spatial and temporal distribution in each country. A few studies demonstrated application of molecular tools to identify sibling species in the vector complexes as well as the genetic structure and gene flow among these complex species. Despite insecticide resistance having been detected in many vector species, insecticide-impregnated bednets are still reported as a cost-effective and efficient way for malaria control. Social-science and socio-economic studies indicate that the level of education and poverty is related to the risk of malaria infection and also emphasize the importance of education as part of successful control programmes. The majority of research on dengue vectors in Southeast Asia involves surveillance for species composition, relative abundance and seasonal distribution of both immature and adult stages. Identification of key breeding containers and patterns of landing/biting of adults are routinely investigated in the study areas. Some aspects of vector ecology and vector biology related to the symbionts of the mosquitoes have been reported. Several studies have pointed out the importance of human transportation as a means for spreading dengue. Recent studies also demonstrated that the disease spread from the larger cities, which serve as the viral reservoirs, to smaller communities in a radial manner. Several socio-economic studies in different countries indicate variations in knowledge and practice related to dengue. Dengue control programmes in Southeast Asia have recently shifted from application of insecticides to integrated vector control strategies using biological control agents, pyrethroid-based insecticides, source reduction and environmental management. However, most of the present vector control measures are not sustainable due to several factors related to both community participation and persistence of public-health vector control programmes. Genetic control using modern molecular technologies may offer novel solutions for future control of vector-borne diseases.
Keywords: *Aedes*; *Anopheles*; dengue; malaria; mosquito; vector; Southeast Asia

[#] Center for Vectors and Vector-Borne Diseases and Department of Biology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand. E-mail: grpkt@mahidol.ac.th

Chapter 10

Malaria

Spatial distribution of malaria vectors and parasites

Malaria remains an important health threat in rural areas of Southeast Asia. All four known human malaria parasites, but predominantly *Plasmodium falciparum* and *P. vivax* are present in the region. Members of three species complexes of *Anopheles* mosquitoes, *An. dirus*, *An. maculatus* and *An. minimus*, are the most important vectors. In Vietnam, malaria is found in mountainous and woody areas as well as in coastal regions. The main vector is *An. dirus*, which is found in stagnant and shaded waters in forested areas, whereas *An. minimus*, another vector species, breeds in running streams in hilly areas. In contrast, *An. sundaicus*, a coastal vector species, adjusts to a variety of habitats. *P. falciparum* and *P. vivax* occur at about the same rate except for the woody regions where *P. falciparum* is more prevalent (75%) (Nguyen 1993).

A preliminary survey of *Anopheles* in 8 provinces in Laos showed that out of 19 species collected, *An. aconitus* is the predominant species, especially in the month of December, and only 3 species, i.e. *An. dirus*, *An. maculatus* and *An. minimus*, are infected with oocysts (Vythilingam et al. 2001). Another malaria survey in one of the southeastern provinces of Laos has reported 28 species collected by both human and animal baits. Sporozoites of both malaria parasites were found in *An. dirus* and *An. minimus* as well as in *An. philippinensis*. Four species, *An. notanandai*, *An. sawadwongporni*, *An. willmori* and *An. Hodgkini*, were recently reported for the first time in this region (Toma et al. 2002). A field survey for malaria prevalence in southeastern Laos by PCR assay has shown that the most common malaria parasite is *P. falciparum*, and mixed infection by 2-4 species of parasites was detected in 23.1% of the samples (Toma et al. 2001). Interestingly, there is a report that *P. falciparum*, but not *P. vivax*, is associated with acute malnutrition among youths in Laos (Takakura et al. 2001).

In Myanmar, *An. dirus* is reported to be one of the primary vectors of *P. falciparum* causing cerebral malaria. The positive rate during 1998-2000 in Bago, Mandalay and Tanintharyi Divisions ranged from 9.9% to 34.3% (Oo, Storch and Becker 2003). A recent entomological survey conducted in Vietnam, Laos and Cambodia showed that *An. dirus* A is still an important malaria vector despite its low density, whereas the role of *An. minimus* A in malaria transmission varies both temporally and spatially. The brackish-water-breeding species, *An. sundaicus* occurs in high density due to the recent changing patterns of land use from rice cultivation to shrimp farming (Trung et al. 2004).

In peninsular Malaysia, *An. maculatus* is the main vector of malaria even though its abundance is only 9.1%, which is about half of the average number of the other two dominant species, *An. aconitus* and *An. barbirostris* (Rahman, Adanan and Abu Hassan 2002). In Indonesia, malaria is common throughout the country and *P. vivax* is the most abundant malaria parasite (Rodhain 2000). The primary vector species, especially in forested hilly areas of Java, are *An. maculatus* and *An. balabacensis* (Barcus et al. 2002).

Analysis of the distribution of malaria-endemic areas in the Indochina peninsula has been conducted using GIS/remote-sensing techniques. These are useful for identification of endemic malaria based on the normalized difference vegetation indices (NDVI) (Nihei et al. 2002). GIS analysis was also used to study distribution of the larval stages of *An. flavirostris*, a principle malaria vector in the Philippines. The study has shown that early larval instars are clustered in shady stream-bank areas,

whereas the late instars are weakly related to shade (Foley, Torres and Mueller 2002). The GIS-based spatial patterns of surface slope and wetness were used to identify the distribution of breeding sites of four major malaria vectors, *An. dirus*, *An. maculatus*, *An. minimus* and *An. sawadwongporni* in Northern Thailand (Sithiprasasna et al. 2003b). Spatial and temporal distribution of *Anopheles* mosquitoes in the same area was reported. A total of 21 species were collected and 86% of species biting humans were *An. minimus*, which was found to be infected with both *P. falciparum* and *P. vivax* (Sithiprasasna et al. 2003a). Konchom et al. (2003) studied malaria incidence during 1991-2001 in 30 highly endemic provinces along the Thai borders. They reported the trend of malaria parasite species shifting from *P. falciparum* to *P. vivax* along the western border to Myanmar and northern border to Laos as well as along the eastern border to Cambodia, while the opposite trend of parasite distribution was found in the southern border to Malaysia. There was also a significant difference in annual parasite incidence between border and non-border especially along the border with Myanmar and Cambodia. A survey on malaria among mobile Cambodians at the Thai-Cambodia border reported an overall infection rate of 2.4% with 93.8% of the infections being due to *P. vivax* (Kitvatanachai et al. 2003).

Population genetics and identification of *Anopheles* species complexes

Understanding population genetics of mosquito vectors is important in planning malaria control. In Thailand, microsatellite markers have been developed for studying genetic variations in natural populations of *An. maculatus* (Rongnoparut et al. 1996). A large number of alleles and high polymorphisms have demonstrated the usefulness of these microsatellite markers in studying gene flow and population-genetic structure of this vector species. High levels of genetic diversity in a small population of *An. maculatus* were detected in the above study. Population structure and population history of *An. dirus*, the main vectors of malaria in Southeast Asia, were studied using sequence analysis of the mitochondrial COI gene (Walton et al. 2000). This study reported that *An. dirus* A extends eastward from Thailand to Laos, Cambodia and Vietnam while *An. dirus* D extends westward through Myanmar. Both species are parapatric but there is little genetic differentiation either within or between species. *Anopheles dirus* C has a patchy distribution along the Thai-Myanmar border and also extends southward into peninsular Thailand. However, no gene flow between populations of *An. dirus* C has been detected. Because of greater genetic diversity in species D, it has been hypothesized that population expansion occurred first in this species and subsequently in species A.

In Southeast Asia, the presence of species complexes makes it more difficult to identify the vector species correctly, which may lead to the wrong target in vector control. In general, members of the sibling species usually exhibit behavioural differences. Two species within the *An. minimus* complex, with differences in resting and biting behaviours, have been discovered in Vietnam using isozyme electrophoresis (Van Bortel et al. 1999). In central Vietnam, the misidentification of *An. minimus* as *An. varuna* was a good example of the importance of the correct identification of vector species in order to implement malaria control effectively. Both *Anopheles* species are different in feeding behaviour, i.e., *An. varuna* is highly zoophilic and is not considered to be a vector, whereas *An. minimus* feeds on both animals and humans and has been confirmed as a vector (Van Bortel et al. 2001). Recently, a multiplex PCR assay was developed to identify the members of the *An. minimus* complex as well as other closely related species, i.e., *An. aconitus*, *An.*

pampanai and *An. varuna* (Phuc et al. 2003). This technique can be applied to all life stages and is simpler, quicker and cheaper than previous assays.

Insecticide resistance and behaviour of malaria vectors

Development of insecticide resistance among vector species in the Southeast-Asian region is an important factor leading to failure in malaria control. Insecticide resistance patterns in mosquito vectors in Thailand have been reported by Chareonviriyaphap, Aum-aung and Ratanatham (1999) and Prapanthadara et al. (2000). Behavioural responses of *An. minimus* to DDT, deltamethrin and lambdacyhalothrin were evaluated using an excito-repellency escape chamber. Both colony-reared and wild populations of *An. minimus* exhibited insecticide-avoidance behaviour, i.e., contact irritancy and non-contact repellency (Chareonviriyaphap et al. 2001). The behavioural avoidance response to insecticides, which was the first sign of insecticide resistance, was detected not only in *An. minimus* but also in three other malaria vectors, i.e. *An. dirus*, *An. maculatus* form B and *An. sawadwongporni*, regardless of insecticide susceptibility, age, nutritional or physiological status (Chareonviriyaphap, Prabaripai and Bangs 2004). Later, the change of feeding behaviour in natural populations of *An. minimus* A and C in responding to DDT spraying was reported. Both species tend to feed on cows rather than humans and there was no preference for indoor, outdoor or forest biting (Rwegoshora et al. 2002). Recently, seasonal abundance and blood-feeding activity of *An. minimus* was studied in Western Thailand (Chareonviriyaphap et al. 2003). Results indicated that this species is more abundant during the wet season and that the human-biting peak in this area is different from other areas suggesting site-specificity in feeding behaviour. This study concluded that site-specific studies were necessary to evaluate vector behaviour accurately as it relates to malaria transmission.

Malaria vector control

Malaria control programmes in Southeast Asia are quite difficult to accomplish due to the presence of vector species complexes and insufficient information on the feeding behaviour of vectors, as well as the resistance of vectors to insecticides. Malaria vector control in this region has recently shifted from routine, residual space-spraying inside houses to the use of pyrethroid-impregnated bednets. In Laos, impregnated bednets have recently been reported to reduce malaria transmission successfully (Kobayashi et al. 2004). Despite a successful reduction of malaria in Thailand, re-emergence of the disease was evident by an increase of the annual parasite indices during 1998 (Chareonviriyaphap, Bangs and Ratanatham 2000). Evaluation of repellency and killing effects of bednets treated with etofenprox, deltamethrin, lambdacyhalothrin and permethrin was carried out in Northern Thailand and results showed that all four insecticides have a high repellence effect. However, the problems of cross-resistance, persistence of chemicals and types of mosquito-net materials should be considered for further evaluation (Prasittisuk et al. 1996).

An evaluation of malaria control in central Vietnam showed that both spraying of insecticides in and around the houses and the use of insecticide-impregnated bednets were efficient (Nguyen et al. 1996). However, spraying with lambdacyhalothrin was more effective than with pyrimiphos and DDT. It was recently reported that malaria in Vietnam, as well as in other Southeast-Asian countries, is related to forest activities so control efforts should target forest workers. Experiences of malaria control in refugee camps on the Pakistan-Afghanistan and Thailand-Myanmar borders had concluded that both government and non-government agencies could play a significant role in

solving issues in malaria control. Moreover, integration of research within implementation programmes may result in innovation and sustainable malaria control (Rowland and Nosten 2001).

Social sciences and socio-economics related to malaria

Social factors related to malaria occurrence have been studied in Eastern Thailand (Butraporn, Sornmani and Hungsapruek 1986). This study demonstrated that poor education and low income as well as long residency and frequent forest association led to a high risk of malaria infection. A study on the behaviour dealing with self-prevention of malaria among mobile populations in Eastern Thailand indicated that the age group of 30-39 years old has the highest risk due to periodic movement into the forested areas. Their moderate knowledge of, and attitude to malaria does not enable them to protect themselves against it (Butraporn et al. 1995). A more recent study in Thailand also indicated the importance of both socio-economic and cultural factors affecting malaria control programmes (Panvisavas 2001; Panvisavas, Dendoung and Dendoung 2001). In Laos, a study on knowledge and behaviour of people regarding prevention of malaria, conducted in 1999-2000, showed that the level of malaria prevention was related to the level of education (Uza et al. 2002). Health education in the target community is, therefore, an important component for the success of vector control programmes.

In Thailand, the use of impregnated bednets to prevent malaria in children is high among mothers who have knowledge about the disease (Sri-aroon et al. 1998). The cost-effectiveness of lambdacyhalothrin-treated bednets was evaluated against the use of DDT spraying and malaria surveillance in Western Thailand. Results showed that the bednet programme was most cost-effective when compared to DDT spraying and malaria surveillance ($1.54 versus $1.87 and $2.50 per case of prevented malaria) (Kamolratanakul et al. 2001). A pilot malaria control programme using DDT-impregnated bednets in Laos during 1995 to 1997 was evaluated. The villages where treatment occurred showed a significant increase of the number of bednets used and a significant decrease of malaria infection when compared to control communities. In addition, this study reported that risk factors were related to occupation, location of the house and use of mosquito nets (Philavong et al. 2000).

Dengue and dengue hemorrhagic fever

Biology and ecology of dengue vectors

In Southeast Asia, both *Aedes aegypti* and *Ae. albopictus* are important vectors of dengue (DF) and dengue hemorrhagic fever (DHF). Several studies on *Aedes* vectors in this region have reported the distribution and abundance of both immature and adult stages. In Sarawak, Indonesia, a survey for larvae of *Ae. aegypti* and *Ae. albopictus* in urban housing indicated that both species shared habitats in houses (9%) and in vacant land (4.5%) (Seng and Jute 1994). In South Sulawesi, Indonesia, *Ae. aegypti* was found mainly in earthen jars indoors while *Ae. albopictus* bred mainly in drum cans in hilly and mountainous areas (Ishak et al. 1997). In Thailand, the biology of both dengue vectors on Samui Island was reported (Thavara et al. 2001). The larval habitats of both species were distinctly separated, i.e., *Ae. aegypti* preferred to breed in earthen jars and concrete water storages while *Ae. albopictus* bred in coconut husks and coconut floral spathes that held rain water. *Aedes aegypti* eggs were not detected in outdoor ovitraps at a distance of 1.5 meters from houses and 75.4% of mosquitoes biting indoors in the daytime were *Ae. aegypti*. The survey for dengue vectors in five

geographical zones of Thailand demonstrated that *Ae. aegypti* predominates in all areas whereas *Ae. albopictus* is restricted to the southern part of the country. As previously reported, water jars are the most important breeding sites of *Ae. aegypti*, while broken cans and plastic containers are the preferred breeding habitats of *Ae. albopictus* (Chareonviriyaphap et al. 2003). An ecological survey of dengue vectors carried out in central Laos during the year 2000 reported that *Ae. aegypti* is dominant among 7 species collected. The key habitats are water jars, cement water tanks, drums and discarded containers, while containers containing *Mesocyclops* do not have *Aedes* larvae (Tsuda et al. 2002).

Longitudinal studies on dengue vectors were conducted in Thailand. Seasonal distribution of *Aedes* larvae in Eastern Thailand reported that even when the larvae are less abundant during the dry season, every part of the studied villages have some of them (Strickman and Kittayapong 2002). This study suggests that vector control in Southeast Asia should concentrate in schools or areas with greatest abundance based on the calculation of larval indices. Another study in Eastern Thailand also showed that breeding containers with high larval nutrients produce large numbers of pupae and large-sized mosquitoes. An estimate of the number of females per house was above the threshold for increasing transmission in all months except from December to February. The number of pupae per house and local temperature were used to calculate transmission risk using Focks' model. Results indicated that the risk is greatest in the months of May and June (Strickman and Kittayapong 2003). Studies on the population dynamics of *Ae. aegypti* in Thailand showed that temperature, but not rainfall, is correlated with female abundance. In addition, high temperature may increase age distribution of young adults and frequent blood feeding due to rapid reduction of energy reserves (Scott et al. 2000b). Blood-feeding behaviour of mosquito vectors is important for the understanding of dengue transmission. Multiple blood feeding and micro-movement to obtain blood sources have been confirmed for *Ae. aegypti* using PCR-based identification of human-blood meals (Chow-Shaffer et al. 2000). In Thailand, it was found that 65% of *Ae. aegypti* feed twice on the same day (Scott et al. 2000a). Mark–release–recapture studies in Thailand indicated that the survival rate of *Ae. aegypti* was age-dependent. Traditional linear regression analysis showed that the survival rate of older females was significantly greater than that of younger ones whereas the more sensitive non-linear regression analysis could not detect differences in the survival rate of both age cohorts in Thailand (Harrington et al. 2001).

Field studies in Thailand concerning the *Wolbachia* endosymbiont of *Ae. albopictus* have been reported. These bacteria are found to infect several species of Southeast-Asian mosquitoes (Kittayapong et al. 2000) and might be used in genetic control through cytoplasmic-incompatibility-induced population replacement. In nature, *Aedes albopictus* is double-infected with these bacteria whereas *Ae. aegypti* has never been reported to be infected. Recently, stable infections of *Wolbachia* in *Ae. aegypti* have been successfully obtained and it was found that these transinfected lines do not exhibit differences in fitness when compared to naturally uninfected populations (Ruang-areerate et al., unpubl. data). Cross-mating between *Wolbachia*-infected and uninfected *Ae. albopictus* has shown that *Wolbachia*-mediated cytoplasmic incompatibility in field-caught and laboratory-reared old-aged *Aedes albopictus* is very strong (Kittayapong et al. 2002). Maternal transmission and field prevalence of 100% in natural populations of *Wolbachia*-double-infected *Ae. albopictus* in Thailand support the potential application of these bacteria as gene-driving mechanism (Kittayapong et al. 2002; Kittayapong, Baimai and O'Neill 2002).

Population genetics, vector competence and dengue transmission

DF and DHF have long been reported as the most common urban diseases in the Southeast-Asian region since the 1950s, before spreading worldwide. All four serotypes of dengue viruses co-circulate in this region (Gubler and Kuno 1997). A study showing the spread of DHF by travellers from East Timor to Townsville, Australia evidenced the importance of humans as vehicles for disease spreading (Hills et al. 2000). There has also been a report on the high risk of DHF in the area where foreigners work for petroleum companies in Indonesia, subsequently returning to their home countries (Mangara et al. 2000). Seroprevalence of dengue virus among German overseas aid workers was found to be 6.4% (43/670), and of these 43, the highest seroprevalence (19.4%) was detected in those returning from Thailand (Eisenhut, Schwarz and Hegenscheid 1999). As reported from the study by Harrington et al. (in press) in Thailand, the human-movement factor may perhaps be more important in the spreading dynamics of the disease than the dispersal and flight range of *Aedes* vectors. This idea is supported by the recent estimates of population-genetic organization and gene flow in *Ae. aegypti* using microsatellite markers (Huber et al. 2004). This study showed that there is less genetic differentiation between mosquito populations from Vietnam (Ho Chi Minh City) and Cambodia (Phnom Penh) than between either of them and Thai populations, suggesting that passive migration through human transportation is the major cause of vector spreading.

Genetic structure of *Ae. aegypti* was studied in Vietnam in relation to vectorial competence and resistance to insecticides (Huber et al. 2003). Estimation of population-genetic organization and gene flow showed that ecological disturbance through urbanization, which had direct impact on sanitation, has a direct impact on the vectorial system. The relationship between genetic differentiation and vector competence for dengue-2 virus has been reported (Huber et al. 2002a). Genetic variations in Ho Chi Minh City and its outskirts were studied using starch-gel electrophoresis and microsatellite markers. Results showed that genetic differentiation is lower in the city when compared to its outskirts, depending on the abundance of breeding sites and human hosts as well as the insecticidal control during dengue outbreaks (Tran et al. 1999; Huber et al. 2002b). Moreover, seasonal and environmental factors also had an effect on the genetic structure of *Aedes* vector populations (Huber et al. 2002c). In Thailand, genetic differentiation was confirmed in *Ae. aegypti* samples collected from different subdistricts. Results may be related to insecticide treatment in these areas (Mousson et al. 2002). In conclusion, further studies on genetic variations of vector populations are required to provide further insights into the understanding of disease epidemiology.

A study on the major mosquito fauna in the forested area undergoing development of an oil palm plantation in Sarawak, Malaysia showed the reduction of the species composition of malaria vectors and the risk of malaria transmission but, on the other hand, an increase of dengue vectors and the risk of dengue transmission (Chang et al. 1997). Evidences for dengue infection in both vector species, i.e., *Ae. aegypti* and *Ae. albopictus*, were reported from Samui Island, Thailand (Thavara et al. 1996). Transovarial transmission of dengue viruses was reported in Singapore. Males of both species were found positive with dengue viruses using a type-specific PCR technique. The serotypes were checked and the results showed a negative correlation between DEN-1 and DEN-4. DEN-1 was higher than DEN-4 in *Ae. aegypti* while the opposite was detected in *Ae. albopictus* (Kow, Koon and Yin 2001).

A study in Selangor, Malaysia revealed a positive correlation between a dengue outbreak and rainfall pattern, which increased the number of breeding habitats of

Aedes vectors (Li et al. 1985). Biological and entomological parameters related to a seasonal pattern of dengue using a mathematical model reported that the strongest influence on the seasonality and pattern of dengue transmission is the duration of infectiousness of the host, vector mortality and biting rates (Bartley, Donnelly and Garnett 2002). Susceptibility to dengue viruses among *Ae. aegypti* mosquitoes collected in different seasons of the year showed no seasonal correlation, even though a seasonal pattern of dengue transmission was observed in Thailand. It was suggested that characteristics of the virus, vector density and frequency of host–vector contact should be considered instead (Thongrungkiat et al. 2003).

Distribution of dengue and Japanese encephalitis among children in rural and sub-rural areas in Thailand has been studied. The results showed that most transmission occurs in residential environments and within a young age group (3-8 years old), which has a significantly higher risk of infection than older children (Strickman et al. 2000). An epidemiological study of DHF in Thailand suggested that vector control activities should concentrate on areas and populations at higher risk (Barbazan, Yoksan and Gonzalez 2002). Integration of geography and pathology for DHF is required to understand the complex epidemiology of the disease, which depends on a variety of factors (Menard 2003). Recently, the spatial and temporal studies of DHF incidences in all 73 provinces of Thailand (1983-1995) could discriminate between seasonal and non-seasonal transmission. The spatial-temporal dynamics of DHF incidence using the data set of 850,000 infections in Thailand from 1983 to 1997 showed that the disease occurred first in Bangkok, the largest city and capital of Thailand, and then moved radially at the speed of 148 km per month. This finding provided a crucial piece of information, namely that the permanent viral reservoir is to be found in large cities; these should therefore be the targets for long-term control of DHF (Cummings et al. 2004).

Dengue vector control

Up until the present, there has been no promising solution for sustainable control of dengue vectors. The trend for dengue vector control in this region has shifted from relying solely on insecticides to biological control, source reduction and environmental management through community participation (Gubler and Kuno 1997). Several countries in the region have recently carried out integration of vector control approaches.

In Vietnam, a survey for *Mesocyclops*, *Micronecta* and fish as biological control agents demonstrated that a large number of breeding containers already contained *Mesocyclops* and the presence of both *Mesocyclops* and *Micronecta* provided some level of control (Nam et al. 2000). A few years later, a successful dengue vector control programme was initiated in three provinces of Northern Vietnam with an application of the biological control agent, *Mesocyclops* spp., and clean-up campaigns through community participation (Kay et al. 2002). Recently, prolonged efficacy in controlling *Aedes* larvae in water containers by a combination of *Bacillus thuringiensis israelensis* (Bti) and copepods was evaluated in Thailand (Kosiyachinda, Bhumiratana and Kittayapong 2003). The enhancement of control activities was observed when rice grains were used as supplementary food for copepods.

From Malaysia, there are few reports on high efficiency of a combination of biological and chemical insecticides (Seleena, Lee and Chiang 2001; Sulaiman et al. 1997; 1999; 2000). The details of dengue vector control and dengue situation in Malaysia were discussed by Poovaneswari (1993), Tham (1993), and Yap et al.

(1994). Singapore has a well-established system for dengue vector surveillance and control. The control strategy integrates case detection, source reduction, health education and law enforcement (Wang 1994). Application of an autocidal ovitrap as a vector control measure against *Ae. aegypti* was developed in Singapore since 1977 by Lok, Kiat and Koh (1977). Recently, a mixture of Bti (Vectobac 12 AS) and pirimiphos-methyl (Actellic 50 EC) applied through thermal fogging at various heights and distances was tested as an efficient approach for simultaneously controlling both larvae and adults of *Ae. aegypti* in Singapore. However, there is a report from Central Java, Indonesia of an unsuccessful vector control trial when *Toxorhynchites* was used as a biological control agent (Annis et al. 1990).

In Thailand, Gratz (1993) pointed out that dengue vector control, as in most other countries, had made little use of the methodologies arising from research. After a long routine application of insecticides as a vector control measure, the trend in Thailand was recently geared toward environmental protection (Chunsuttiwat and Wasakarawa 1994). Several studies were conducted in Thailand regarding the use of repellents, physical and biological control agents. Three types of screen covers were developed for preventing vectors from breeding in water jars, which were the most common and important breeding site of *Ae. aegypti* in Southeast Asia (Kittayapong and Strickman 1993). Thanaka (*Limonia acidissima*) and DEET (di-methyl benzamide) mixture were evaluated for their efficacy as repellents against both *Anopheles* and *Aedes* vectors in Thailand. Almost complete protection up to 6 hours was obtained with a combination of 20% thanaka and 0.5% permethrin. Laboratory bioassays with *Ae. aegypti* indicated that the combination could extend protection from exposure up to 10 hours (Lindsay et al. 1998). The Thai strain of mosquito densoviruses infecting both *Ae. aegypti* and *Ae. albopictus* was reported to be efficient in killing *Aedes* larvae and could be developed as a biological control agent (Kittayapong, Baisley and O'Neill 1999). Evaluation of Bti tablets and temephos ZG were conducted in Thailand. Results showed that at the dosage of 0.37 g per 50 l of water could provide control activities between 90 and112 days while a new formulation of zeolite granules (ZG) of temephos (1%) at the operation rate of 5 g per 50 l of water yielded 100% control for more than 6 months. Both larvicides increased clarity of the water with no unpleasant odour (Mulla et al. 2004).

As synthetic pyrethroids are still used in the public-health system to control vector-borne diseases, the development of insecticide resistance in vectors remains an issue of concern. Some information on vector resistance to pesticides in Thailand was reported by Chareonviriyahpap, Aum-aung and Ratanatham (1999). Brengues et al. (2003) reported the presence of permethrin resistance in a few localities in Southeast-Asian countries, i.e., Indonesia, Thailand and Vietnam. The evidence of both DDT and pyrethroid resistance in Indonesia and Vietnam suggested the presence of a knock-down resistant *kdr*-type mechanism.

Social science and socio-economics related to dengue

It has been reported that health education had an effect on the outcome of a DHF vector control programme in an urban area of Northern Thailand by reducing the Breteau index to about half (from 241 to 126) (Swaddiwudhipong et al. 1992). A recent study on climatic and social risk factors for *Aedes* infestation in rural Thailand reported that factors such as availability of public water wells, existence of transport services and proportion of tin houses, were positively associated with larval indices (Nagao et al. 2003). Socio-economic factors, i.e., per capita gross provincial product (GPPpc) and health-care resources in relation to geographic distribution of malaria

and dengue in Thailand, were also examined. It was recommended from the study that this approach be used for considering resource utilization in integrated control of both diseases (Indaratna et al. 1998). In Northern Thailand, a KAP (Knowledge, Attitudes and Practices) survey reported that 67% of people in the study area had knowledge of dengue, significantly different with respect to age, sex and occupation. Young students had a higher level of knowledge of dengue when compared to older housewives and unemployed persons. In addition, people with knowledge of dengue reported more frequently the use of preventive measures against vectors (Van Benthem et al. 2002). Another KAP survey was carried out among the caretakers of DHF in primary schools in one province in central Thailand. The majority of people in this study area were mothers with primary-school education. Results indicated that they need more understanding of the disease. In general, the caretakers whose children had DHF had higher response in prevention, control and treatment than the group who had healthy children (Kittigul et al. 2003). Both studies demonstrated the importance of educational campaigns to obtain community participation in DHF control. A KAP survey recently reported from Malaysia reflected that most people have a high level of knowledge about dengue and vector control (Hairi et al. 2003). In addition, both were significantly correlated with the attitude towards *Aedes* control. However, there was no correlation between the level of knowledge and the vector control practice, which implied that a high level of knowledge did not necessarily lead to good practice. Therefore, differences in social factors and cultures in each country need to be considered for planning educational programmes.

Conclusions

In conclusion, long-term vector control approaches include source reduction and environmental management, chemical and microbial larvicides, and personal protection using household insecticide products and repellents. Space spray, both thermal fogging and ultra-low volume, is normally used as short-term control measure, especially during disease epidemics. Practical vector control approaches in Southeast Asia rely on persistent efforts by the government sectors as well as communities themselves leading to variations in the success of vector control programmes. Planning and decision-making for resource utilization for malaria and dengue control at both national and regional levels could be more efficient through co-analysis of the disease-epidemic patterns and utilization of health-care resources. Due to the fact that both malaria and dengue vaccines are still under development and the current vector control programmes hardly provide a long-lasting effect, transgenesis-based development of refractory strains of mosquito vectors that are refractory to disease pathogens may offer a new alternative for disease control, which could be successful regardless of the level of community participation. However, genetically modified mosquitoes need to be very efficient in spreading themselves and competing with natural vector populations. In addition, acceptance of the strategy by the community itself will be an important issue. Therefore, the biology and ecology of vectors as well as social and socio-economic factors in different geographic regions need to be investigated in more detail to confirm the possibility of application.

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11

Malaria and dengue vector biology and control in Latin America

Mario H. Rodriguez[#]

Abstract

Malaria and dengue are major public-health problems in Latin America. Malaria is transmitted in 21 countries in the region with over 885,000 cases in 2002. Ninety-five percent of the cases occur in the Amazonian countries, mainly in Brazil. The main malaria vectors in Mexico, Central America and Northern South America are *Anopheles albimanus* and *An. pseudopunctipennis*, and *An. darlingi* and *An. nuñeztovari* in the Amazon and Venezuela. With the exception of *An. darlingi*, these mosquitoes are zoophagic, present low sporozoite indices and have low survival rates. These characteristics make them poor malaria vectors, supporting only seasonal transmission when mosquito abundance peaks. Over 500 million people live at risk of infection with dengue in the region, and all four dengue virus serotypes are in circulation. Over 482,000 clinical cases and 9,893 dengue hemorrhagic fever (DHF) cases were reported in the region in 2003. *Aedes aegypti* was introduced in colonial times and extended to most parts of the continent. After eradication from most part of the continent in the late 1950s and 1960s, re-infestation soon occurred. *Ae. albopictus* was introduced in the region in 1985 and dispersed from the Southern United States into Mexico, Central America and Brazil. The participation of this mosquito in dengue transmission in the area awaits assessment. Several populations with variable vectorial capacities have been identified in Mexico.
Keywords: malaria; dengue; *Anopheles*; *Aedes*; *Plasmodium*; transmission; control; gene flow

Malaria situation in the Americas

The use of DDT spraying for malaria control in the region began in 1941, and by 1948 its efficacy in eliminating transmission in some areas and reducing malaria cases in others, prompted the initiative to eradicate the disease, a strategy adopted until 1955. With DDT indoor residual spraying (IRS) as the spearhead, anti-malarial activities were mainly directed to attack mosquito vectors, but the progressive limitation in success and difficulties to maintain intensive operations were determinant for abandoning eradication for epidemiology-based specific control goals in 1992. In this new strategy, vector control is part of sustained preventive methods selectively applied. This strategy has been maintained to the present, and new elements related to improving health-services decentralization, drug-treatment

[#] Centro de Investigaciones sobre Enfermedades Infecciosas, Instituto Nacional de salud Pública, Av. Universidad 655, Col. Santa María Ahuacatitlan, Cuernavaca, Morelos, México 52508. E-mail: mhenry@correo.insp.mx

surveillance and evaluation, and the integration of resources networks were added in 1998, as part of the worldwide Rollback Malaria initiative advocated by the World Health Organization.

Nowadays, malaria transmission has been interrupted in Canada, the United States and all Caribbean countries except Haiti and the Dominican Republic, but transmission still remains in 21 of the 37 countries of the region, while 915 imported cases were reported in the 18 countries without local transmission. It is estimated that out of 849 million inhabitants of the region, 175 million live in areas with some risk of malaria infection (PAHO 2003).

In 2002, a total of 885,000 malaria cases were reported in a population of 175 million people living in risk areas in the Americas, of which 28.3% were infections with *Plasmodium falciparum* and 71.2% with *P. vivax*. Cases produced by *P. malariae* occurred mainly in Brazil and Surinam. Brazil leads with more than 349,000 of the cases (40%) in the region, followed by Colombia (22%), Ecuador and Peru with 10% each. Along with Guatemala, Guyana, Honduras, Surinam and Venezuela, these countries reported 95% of the regional cases (PAHO 2003).

Malaria control activities

Apart from deploying strategies for better malaria detection and diagnosis, the main vector control activities include environmental sanitation, application of larvicides, IRS (with pyrethroids) and outdoor malathion application. To a lesser extent, biological control using *Bacillus thuringiensis israelensis* and *B. sphaericus* as well as larvivorus fish are also applied in a few countries. Also, a network incorporating the eight Amazonian endemic countries was established in 2001 for drug resistance surveillance.

Particular success was achieved in the control of malaria in Mexico (Chanon et al. 2003). Malaria in this country is unstable with areas of persistent transmission resilient to control interventions. Since the implementation of the global eradication campaign in the fifties, control activities were carried out using mostly IRS (with DDT), and relaxation of these activities was frequently followed by outbreaks that originated from persistent foci. New control strategies with the participation of the endemic communities, based on elimination of filamentous algae from *An. pseudopunctipennis* larval breeding sites, the elimination of the parasite reservoir (*P. vivax*) by repeated treatment of patients in order to eliminate relapses, and the application of low-volume pyrethroid insecticides, have controlled malaria to historically low levels without the use of DDT during the last four years. New insecticide-spraying techniques use less insecticide, act faster and are cheaper. This strategy is now in the process of being tested in other parts of Central America.

Malaria vectors

The main malaria vectors in Mexico and Central America are *Anopheles pseudopunctipennis* and *An. albimanus*, while *An. darlingi*, *An. aquasalis and An. nuñeztovari* are the principal vectors in the Amazonian countries (Zimmerman 1992). Malaria transmission by *An. albimanus* and *An. pseudopunctipennis* occurs in South America with other anophelines like *An. vestitipennis* and *An. punctimacula* as secondary vectors in Mesoamerica, while *An. aquasalis* plays a secondary role in transmission in South America. Only the main vectors are reviewed here.

Anopheles albimanus is widely distributed at low altitudes in the tropics and subtropical regions of the Americas, from the South of the Southern United States to Northern Colombia, Ecuador, Peru and Venezuela, including the Caribbean Islands

(Faran 1980). Their larvae generally prefer exposed areas in the sun, such as shores of lakes, lagoons and small streams, and rain pools, but also swamps or brackish waters (Breeland 1972; Savage et al. 1990).

Anopheles albimanus is highly zoophilic (Arredondo-Jiménez et al. 1992) with human blood indices (HBI) ranging from 0.4 to 0.21 (Garrett-Jones 1964; Garrett-Jones, Boreham and Pant 1980; Loyola et al. 1993). However, host availability and ecological conditions could explain differences in the HBI observed among mosquito populations (Loyola et al. 1993) that readily feed on humans when big mammals are scarce. This anopheline is mainly exophagic, but indoor resting after outdoor feeding has been documented (Loyola et al. 1990; 1993).

Differences in ribosomal DNA structure (Beach, Mills and Collins 1989), egg morphology (Rodriguez et al. 1992b) and allozyme pattern (Narang, Seawright and Suarez 1991) have been documented among *An. albimanus* populations, but polytene chromosomes (Keppler Jr., Kitzmiller and Rabbani 1973; Narang, Seawright and Suarez 1991) and cross-hybridization experiments (Narang, Seawright and Suarez 1991) have failed to demonstrate subgroups within the species.

Estimation of gene flow among *An. albimanus* populations in Mexico using RAPD-PCR analysis indicated low variability (F_{st}= 0.169), distributed in two groups, one in the North and the other in the Southeast, including Guatemala. Nationwide, a migration rate of *Nm* = 1.2 was calculated, but in Southern Mexico, populations collected from five neighbouring different agro-ecological areas had *Nm* values of 1.9 to 2.4, with a mean F_{st} of 0.117 (Villareal-Treviño 2001).

An. albimanus is the main vector of *P. vivax* on the coastal plains of Mexico, Central America and Colombia; it has also been found infected with *P. falciparum* (Rodriguez and Loyola 1990; Herrera et al. 1987). Parasite indices are usually low (0.01-0.1%, Ramsey et al. 1994), and it is estimated that only 2% of mosquitoes live long enough to transmit malaria (Rodriguez et al. 1992a). These characteristics make this species a poor malaria vector. Yet, it supports seasonal transmission during the rainy season, when the abundance of breeding sites produces high mosquito densities.

Anopheles pseudopunctipennis is the most widely distributed anopheline in the Neotropical region (Bruce-Chwatt 1985), covering from Southern United States to Northern Argentina, including the Andean countries and extending to the lesser Antilles (Aitken 1945; Manguin et al. 1995). Throughout its geographical range, *An. pseudopunctipennis* habitats occur at altitudes over 200 m above sea level, where the rugged terrain determines the formation of larval breeding sites in pools, ponds and lagoons developing in the margins of rivers and streams when the rainy season ends (Aitken 1945; Fernandez-Salas et al. 1994).

An. pseudopunctipennis is traditionally considered an anthropophilic mosquito (Vargas 1938; Davis and Shannon 1928). However, although high HBI (between 29.5 and 54.7%) were documented in Southern Mexico, host preference estimates through forage-ratio analysis indicate that the HBI reflects host availability, and that this mosquito has a bias towards horses and dogs (Fernandez-Salas et al. 1993), as documented by higher mosquito collections in horses than in humans (Fernandez-Salas et al. 1993).

At least five subspecies and one variant of *An. pseudopunctipennis* were morphologically described in South America (Knight and Stone 1977). Later, using rDNA analysis and cross-mating experiments, a complex of two allopatric species was identified in Central Mexico and Peru and Bolivia, respectively (Estrada-Franco et al. 1993a; 1993b). Finally, Manguin et al. (1995), by using isozyme analysis, classified the entire population in three clusters, one located in the Antilles, one

extending from the Southern United States though Mexico and Guatemala and the third from South America through Central America to Belize.

An. pseudopunctipennis is frequently the only malaria vector found at altitudes higher than 600 m above sea level (Aitken 1945; Vargas, Casis and Earle 1941). Malaria sporozoite rates range from 2.6% in Peru (Hayes et al. 1987) to 3.16% in Mexico (Loyola et al. 1991). Mark-recapture studies indicate a daily survival rate of 0.875, and that about 60% of nulliparous females require a second blood meal to complete their gonotrophic cycle (Fernandez-Salas, Rodriguez and Roberts 1994). These data may explain why *An. pseudopunctipennis* supports malaria transmission with low population densities.

Anopheles darlingi is distributed in the South of Mexico, Guatemala, Honduras, Belize, Colombia, Venezuela, Bolivia, Peru, Ecuador, Paraguay, Brazil and Argentina (Forattini 1962). This mosquito breeds in shaded clean rainwater pools that are often man-made after forest clearing (Klein, Lima and Toda Tang 1992; Charlwood 1996). Furthermore, the species is frequently clustered in relatively small areas at the end of the rainy season (Charlwood 1980), while there is low mosquito abundance during most parts of the year. Although *An. darlingi* has a longer survival rate than other local anophelines in Rondônia, Brazil (Charlwood and Alecrim 1989), low mosquito densities have hindered extensive studies on survival rates.

Several studies indicate the preference of this mosquito for human blood: offered a choice of hosts *An. darlingi* preferred humans (Oliveira-Ferreira et al. 1992), and 59% of specimens collected in the Brazilian Amazon region were trying to feed on humans or in nearby human dwellings (Tadei et al. 1998). Although this mosquito readily enters houses to feed, it remains inside only for short times.

So far, there is no clear evidence for the existence of subspecies or subpopulations in the *An. darlingi* taxon. Genetic variation is supported by isozyme analysis (Narang et al. 1979) and chromosomal polymorphism (Schreiber and Guedes 1961; Kreutzer, Kitzmiller and Ferreira 1972). However, although differences in biting behaviour were documented in three Brazilian populations, calculated genetic distances (the highest ≤ 0.049) using isozyme and hydrocarbon cuticular analysis only support intra specific variation (Rosa-Freitas, Deane and Momen 1990).

An. darlingi is the main *P. falciparum* and *P. vivax* malaria vector in the endemic areas of Amazonian countries (De Arruda et al. 1986; Klein et al. 1991; Zimmerman 1992; Tadei et al. 1998; Roper et al. 2000). This mosquito is present in the Lacandon forest in Southern Mexico and in restricted areas of Central America, but its participation in malaria transmission in these areas was only inferred in Guatemala because no other anopheline could be incriminated as a vector.

The geographic range of *Anopheles nuñeztovari* covers the regions from Panama to Northern South America, including Colombia, Venezuela, Guyana, French Guyana, Bolivia, Brazil, Ecuador and Peru (Forattini 1962). Larval habitats have been described in flooded areas left by subsided rivers or in flooded pastures. The nature of the breeding sites results in a peak of *An. nuñeztovari* densities during the dry season in Surinam (Zimmerman 1992) and in the rainy season in Venezuela (Rubio 1991).

Anopheles nuñeztovari readily feeds on humans, with HBI between 18.2 and 38.9 in outdoor collections from Venezuela (Rubio 1991). This mosquito is more exophagic than endophagic. Along with *An. aquasalis* it is the main vector of *P. vivax* in Western Venezuela (Rubio 1991) and Colombia (Herrera et al. 1987) and a secondary vector of *P. vivax* and *P. falciparum* in Brazil (De Arruda et al. 1986; Tadei et al. 1998). An isozyme analysis of four Brazilian and two Colombian *An. nuñeztovari* populations provided evidence for some degree of reproductive isolation

between the two groups (Scarpassa, Tadei and Suarez 1996), but no indication of subspeciation.

Anopheles vestitipennis is presented here as a special case, as this is the only malaria vector for which evidence for the existence of subpopulations has accumulated. *An. vestitipennis* ranges from central Mexico to Northern South America and the Great and Lesser Antilles (Wilkerson, Strickman and Litwak 1990). This mosquito has been incriminated as a vector of *P. vivax* in the Lacandon Forest (Loyola et al. 1991) and it probably also transmits malaria in Guatemala (Padilla et al. 1992).

Isozyme analysis of *An. vestitipennis* from Southern Mexico indicated differences between mosquito populations collected on humans and animal baits (D = 0.07) (Arredondo-Jiménez et al. 1996). These findings were later supported by differences in RAPD markers of specimens collected with animal and human baits (D = 0.25) (Murillo-Sánchez 2001). Further mark-recapture experiments confirmed the existence of subpopulations with different host preferences in the field (Ulloa et al. 2002). Interestingly these populations can be separated by differences in the ornamentation of their eggs (Rodriguez et al. 1999).

Special aspects of malaria transmission in Southern Mexico

The main malaria vectors on the Pacific Ocean coast of the State of Chiapas, Mexico are *An. albimanus* on the coastal plains and *An. pseudopunctipennis* in the foothills (Rodriguez and Loyola 1990). *Plasmodium vivax* is responsible for over 95% of malaria cases in the area. The two phenotypes of the circumsporozoite protein (CSP) identified in this parasite, VK210 and VK247 (Rosenberg et al. 1989; Arnot et al. 1985) occur in the area, but so far only VK210 has been identified in patients living on the plains, while both phenotypes, with a predominance of VK247, occur at higher altitudes. This parasite prevalence distribution follows the geographic distribution of the vector mosquitoes (Rodriguez et al. 2000).

Experiments using *P. vivax*-infected patient blood to infect *An. albimanus* and *An. pseudopunctipennis* demonstrated that the former was more susceptible to the phenotype VK210, while the latter was more susceptible to VK247 (Gonzalez-Ceron et al. 1999). Further studies indicated that *P. vivax* ookinetes VK247 either could not invade the midgut epithelium of *An. albimanus* or were killed during crossing it (Gonzalez-Ceron et al. 2001). On the other hand, VK210 parasites were unable to exit the blood-meal bolus and were destroyed when fed to *An. pseudopunctipennis* (Gonzalez-Ceron et al. in prep.).

This situation may not occur in other malarious areas of the Americas where *An. albimanus* transmits malaria. Thus, although a higher prevalence of antibodies to VK210 is observed in Colombia compared to those to VK247, more VK247 sporozoites were isolated later in experimental infections of *An. albimanus* (Gonzalez-Ceron et al. 2001). On the other hand, recent experiments indicate a shift in the infectivity of *An. pseudopunctipennis* by some VK 210 parasites from the foothills, but not by those from the coastal plains. (Gonzalez-Ceron et al. in prep.).

Dengue situation in the Americas

The classic dengue fever (DF) together with their severe forms of dengue hemorrhagic fever (DHF) and shock syndrome (DSS) are serious health problems in many parts of the Americas. The fist dengue-like cases in the Americas were recorded in the Caribbean islands of Martinique and Guadeloupe in 1635. On the continent, this

occurred in 1780 in Philadelphia. The first reported epidemic with over 50,000 cases was recorded in Peru in 1818, and since then many outbreaks varying in intensity and distribution have occurred. Several pandemic outbreaks were recorded in many countries in the region: cases were reported from the United States, Cuba, Brazil and Peru during the pandemic of 1845-51; during the pandemic of 1870-73, cases were reported in Alabama and, notably, over 40,000 cases were reported in New Orleans; the 1901-07 pandemic extended to the Southern United States, the Caribbean, Panama and Colombia; the 1912-16 pandemic included cases in Central America, Northern South America and Puerto Rico. In the 1998 pandemic the more affected countries in the region were Venezuela, Peru, Colombia, Ecuador and Brazil with over 500,000 cases reported (Schneider and Droll 2003). Regional epidemics have affected the whole region, from the United States with over half a million cases in Texas in 1922.

Dengue serotype 1 was introduced into Jamaica in 1977 and the first important epidemic outbreaks produced by this serotype occurred in Bolivia, Brazil, Ecuador, Paraguay and Peru in 1982. Dengue serotype 3 was responsible for epidemics in the Caribbean countries in 1963 and, together with the serotype 2, it was presented again in epidemics occurring in these islands and Venezuela in 1968-1968.

In the late 1970s, serotypes 2 and 3 circulated in the Caribbean and in 1981 this serotype produced a major epidemic in Cuba with 344,203 cases, 10,312 hemorrhagic-fever cases and 158 deaths, (Guzmán et al. 1988). Serotype 4 arrived in the region in 1981 and since then it has been responsible for outbreaks in the Great and Lesser Antilles, Mexico, Central America and Northern South America, generating over 7,000 cases in Brazil in 1982. Serotype 4, in conjunction with serotypes 1 and 2, led to over 10,000 cases in Colombia between 1992 and 1995. Also, between 1980-1994 serotypes 1, 2 and 4 were responsible of all dengue activity in the region. Serotype 3 (Sri Lanka/India genotype) reappeared in Nicaragua and rapidly extended into Central America and Mexico.

Currently, over 500 million people live at risk of infection with dengue viruses and all four serotypes are in circulation in the Americas. Over 482,000 clinical cases (only 16,966 confirmed by laboratory diagnosis) and 9,893 DHF cases were reported in the region in 2003 (PAHO 2004).

The present increase in DF/DHF in the Americas follows the same pattern as seen in Southeast Asia and the occidental Pacific forty years ago, indicating the possibility of ever more important outbreaks with severe illness and higher mortality. The documentation of neutralizing antibodies to dengue virus serotypes 1 and 2 in 22% of a sample of fruit bats in Costa Rica, and to serotypes 2 and 3 in 30% in Ecuador (Platt et al. 2000) raises the possibility of the existence of feral reservoirs.

Dengue vectors

Both main worldwide recognized vectors of dengue, *Aedes aegypti* and *Ae. albopictus,* are present in the region. *Aedes aegypti* was introduced during colonial times (Christophides, Vlachou and Kafatos 2004; Tabachnick 1991) and invaded the whole continent from the United States, the Caribbean, Central and South America, down to Chile.

In 1947, a hemisphere-wide initiative was initiated to combat yellow fever through the eradication of *Ae. aegypti*. This succeeded in eliminating this mosquito in most territories from 1952 (Colombia) to 1963 (Mexico). However, re-infestation soon occurred after 1967, probably from areas where eradication had not been achieved. In spite of additional efforts to re-eliminate this mosquito in Panama, Brazil, Peru and Belize, re-infestation occurred again. Nowadays, *Ae. aegypti* is present in all

American countries except Bermuda, Canada, Chile and Uruguay (Gubler and Trent 1993). It is distributed in altitudes ranging from sea level up to 2,200 m above sea level in Colombia and up to 1,700 m in Mexico.

Aedes albopictus was introduced to the American continent, through the United States where it was fist detected in 1985, but it appeared almost simultaneously in Brazil in 1986. In the United States, it was initially reported in the State of Texas where it dispersed northwards, and by 1995 it was present in 23 states. In 1993, it was recognized in Santo Domingo. *Aedes albopictus* was recently identified in the Southern-Mexican state of Chiapas (Casas-Martinez and Torres-Estrada 2003) and in Guatemala, where, as in other areas (Hobbs, Hughes and Eichold II 1991), it is replacing *Ae. aegypti*. The importance of this vector in dengue transmission remains to be assessed.

Vector population studies

Population genetic analysis of ten *Ae. aegypti* collections from Northern Mexico, using mitochondrial DNA as markers, identified seven haplotypes in two clades. Polymorphic DNA (RAPD) markers indicated that populations are isolated by distance, but free gene flow occurs within a 90-250-km ratio (Gorrochotegui-Escalante et al. 2000). These studies were extended to 32 populations from the Gulf of Mexico to the Pacific Ocean. Twenty-five haplotypes were detected using single-strand conformation polymorphism analysis of the *Nicotinamide Adenine Dinucleotide Dehydrogenase subunit 4* mitochondrial gene. Three genetically different mosquito populations were identified, namely in Northeastern Mexico, the Yucatan Peninsula and the Pacific Ocean coast. These populations were isolated by distance but, again, free gene flow occurred within populations (Gorrochotegui-Escalante et al. 2002).

Further studies, including 22 collections from the same areas in Mexico and two collections from the Southern United States, were conducted to investigate their susceptibility to a dengue 2 virus strain. These studies evidenced differences in the vector competence among mosquito populations with a range between 24 and 83%, the Yucatecan mosquitoes being the most competent ones (Bennett et al. 2002).

Dengue control activities

Eradication efforts have been abandoned long ago in the region, and control is directed mainly to the abatement of mosquito populations. More and more control activities are directed towards the control of domestic breeding sites, and insecticides in the form of fogs are used outdoors during outbreaks. Although these activities are conducted in the face of epidemics, their effect on controlling transmission is uncertain. An excessive dependence on ultra-low-volume application instead of larval breeding control (Gubler and Clark 1995), and untimely and faulty application of insecticides (Gratz 1994) contribute to the inadequacy of the present interventions.

Most countries have limited success in deploying routine control interventions aimed at maintaining *Ae. aegypti* populations at low densities. The main efforts in these interventions seek to diminish larval breeding by treating domiciliary water containers. The application of temephos by public-health personnel often fails because of poor training of field technicians. Biological control using turtles (Borjas et al. 1993) and copepods (Marten et al. 1994), as well as larvicidal measures such as washing containers with chlorine bleach and detergent (Fernández et al. 1998), have the limitation of relying on community participation, even though the perception and attitudes of local people are not frequently taken into consideration.

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12

Transition from the laboratory to the field and effective interplay

Christos Louis[#]

Abstract

Effective interplay between laboratory and field research is dependent upon giving up scientific isolationism, and demands for leadership. An effective, integrated approach has to include all entomologists, and particularly those working in disease-endemic countries (DECs).
Keywords: North-South collaboration; capacity building

State of the art

Historically, biologists working with insects can be subdivided into three main groups. The first one consists of people with a mind mostly oriented towards the whole organism and/or populations. The actual 'field workers' have been, almost uniquely, recruited from this pool of entomologists who, as a rule, only want to understand and, thus, only talk to their peers within the same group. Group two is made up of researchers looking at cells and molecules. They know the general shape of the organism they're working with, as well as some related anecdotes, and again their partners in discussion are only to be found among their equals. Finally, the third cluster consists of the drosophilists. History, though, does unexpectedly choose different roads and this guild-like state of affairs, finally, has slowly but gradually started moving towards a possible merge! Entomologists from all three groups have begun understanding one another better, and this is already exemplified by the lists of co-authors of several papers, who often 'belong' to more than one of the three castes (for example, see Lanzaro et al. 1995). Assuming that this trend will continue, the crucial question is whether it can be accelerated. Ideally, at the end of the day, one would like entomologists to be in position to switch their research from the field to the laboratory or vice versa *ad libitum*, depending on the actual questions that are to be answered. Alternatively, an intensification of *bona fide* collaborations between experts in the respective fields, laboratory and field, is a *conditio sine qua non*, if modern biology is to contribute significantly to novel strategies for the control of vector-borne diseases.

The development of genetic markers based on genomic or 'quasi-genomic' approaches – e.g., microsatellites (Zheng et al. 1993; 1996), RAPDs (Randomly amplified polymorphic DNA) (Kambhampati, Black IV and Rai 1992) etc. – certainly was the major force behind the small boom in population biology of disease vectors that took place during the past decade. The ability to assess genotypes of large

[#] Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Crete, Greece. E-mail: louis@imbb.forth.gr

numbers of individuals in a relatively fast way, using molecular techniques, led to a speed of analysis that could not be obtained with the previously available methodology such as cytological observations (Coluzzi et al. 1979) or allozyme/isozyme-based analysis (Beebe and Cooper 2000). The recent studies performed obviously not only dealt with population biology in its 'conventional' meaning, but also encompassed evolution (Lehmann, Hawley and Collins 1996) and indirectly, to some extent, epidemiology (see Della Torre et al. 2002). Moreover, the possibility of, at last, being able to link phenotypes to actual genetic loci (see for example Zheng et al. 1997), opened up the way for the actual genomic science that culminated in the publication of the complete genomic sequence of *Anopheles gambiae* (Holt et al. 2002).

Recently, a technical, somewhat 'turn-around' trend is also being observed, at least still on the level of planning and not through publications. It is no longer often anonymous genetic markers that are used in population studies, but genes that have actually been implemented in specific interactions with disease agents, such as genes encoding proteins of the immune system. This line of research obviously also brings together scientists from both laboratory and field.

Issues and challenges

The respective research communities can best determine the individual questions that can be resolved by an effective interplay between laboratory and field research. For these collaborations to be successful, the most important condition is to keep a non-biased attitude, always allowing for input from the 'other side'. Needless to say that molecular biology, in its wide sense, is not a scientific branch whose reason of being lies in the provision of better markers. Similarly, the notion that 'bench science' by itself will supply all answers, without recognizing that in real life insects don't live in temperature- and humidity-controlled rooms comes very close to becoming a paradigm for arrogance.

There is no doubt that the big question is how to achieve a better and more efficient interaction between the scientists in the two (and why not, all three) groups mentioned in the beginning. What are, though, the practical steps that have to be taken to achieve an integration of vector biology at a practical level? The first one that comes to mind is relatively simple: if the categorization of scientists in two or three groups is also defined through the lack of cross-communication, then bringing the members of the groups together frequently should help. Holding common meetings and workshops is, perhaps, the only way to overcome the segregation of the two communities.

The second level at which this integration can be addressed is that of education and training. Although it is a fact that University curricula now tend towards an early specialization, there are still two levels at which an integration can be attained, postgraduate and postdoctoral training. This may even be the ideal level at which the new scientists should learn that cooperation between scientific areas may ease the ways leading to the desired goals. Specialized courses, therefore, could try to include an integrated curriculum, and similarly, young scientists from one field should be urged to attend courses and meetings that have an emphasis on the other. I should mention here that the highly successful, annually recurring course on the Biology of Disease Vectors (BDV) might represent the example *par excellence* of such an integrated postgraduate educational event. Though historically focused on molecular biology, notion of the importance of field-based sciences (ecology, behaviour etc.)

may gradually change the curriculum of this course over time to become more 'balanced'.

Capacity and partnership building

Is there an issue that relates to capacity building in DECs that differentiates integration of laboratory and field research from the other topics to be discussed? I don't believe that this is necessarily the case. Of course, having suggested two ways to achieve a better integration of the two branches of entomology, in general, I can only strongly suggest the inclusion of DEC scientists in both integrated meetings/workshops and training courses, perhaps even thinking of an affirmative-action policy.

Finally an issue that is very often addressed and that could perhaps be mentioned here, since it does indeed address crucial questions of both field and laboratory research, is that of sample collections. Several research projects include the collection of insects from the field, which are to be 'processed' in laboratories, often 'molecular' ones, located in the North. Although the scientists in DECs are usually mentioned as co-PIs, the fact remains that the role they play in these projects is often restricted to the actual specimen collection. I am absolutely convinced that laboratories in DECs can actually play a much more important role in scientific research that addresses crucial issues of their own. Equitable partnerships and two-way identification of research needs and priorities will be essential to forward the advances of novel vector control strategies to actual field implementation (Mshinda et al. 2004). Although it is a fact that financial constraints may sometimes make collaboration one-sided, care should be taken to try to integrate DEC laboratories as much as possible and have them play a more central role in entomological research.

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13

Evaluation of drive mechanisms (including transgenes and drivers) in different environmental conditions and genetic backgrounds

Anthony A. James[1], *Mark Q. Benedict*[2,3], *George K. Christophides*[4], *Marcelo Jacobs-Lorena*[5] *and Kenneth E. Olson*[6]

Abstract

Three major objectives, develop viable gene drive mechanisms, identify the epidemiologically significant vectors of pathogens in specific transmission zones, and introgress effector genes into specific populations, must be met in order to move to the field laboratory advances in genetic control strategies.
Keywords: genetic control; mosquitoes; gene drive; population replacement

Current state of the art

A long-term plan was put forward some years ago to research the potential of genetically engineered mosquitoes for the control of disease transmission (Meredith and James 1990; WHO 1991; James 1992). The hypothesis for this research is that increasing the frequency of a gene (or allele) in a population of mosquitoes that interferes with the development or propagation of a pathogen will result in the reduction or elimination of transmission of that pathogen to humans. Furthermore, it is expected that a reduction in transmission will result in less disease. This hypothesis derives from the concept of population replacement in which vectors competent to transmit a specific pathogen are replaced or displaced by ones that cannot transmit (Curtis and Graves 1988). This hypothesis is to be tested by using molecular-biological tools to synthesize genes that result in non-vector phenotypes when incorporated into the genome of mosquitoes. These genes are to be coupled to a drive mechanism such that release of the genetically engineered mosquitoes results in the spread of the anti-pathogen gene through a target vector population. Following

[1] Department of Microbiology and Molecular Genetics, Department of Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, CA 92697-3900, USA. E-mail: aajames@uci.edu
[2] Centers for Disease Control and Prevention, National Centers for Infectious Diseases, Division of Parasitic Diseases, Chamblee, GA 30341, USA. E-mail: mqb0@cdc.gov
[3] Entomology Unit, Agency's Laboratories Seibersdorf, International Atomic Energy Agency, A-2444 Seibersdorf, Austria
[4] European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany. E-mail: Giorgos.Christophides@embl-heidelberg.de
[5] Johns Hopkins School of Public Health, Department of Molecular Microbiology and Immunology, Malaria Research Institute, Baltimore, MD 21205, USA. E-mail: mlorena@jhsph.edu
[6] Department of Microbiology, Immunology, and Pathology, Arthropod-borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO 80521-1692, USA. E-mail: kolson@colostate.edu

implementation of this strategy, there should be measurable decreases in transmission and disease.

Research in three areas was identified as being important to test this genetic-control hypothesis (James et al. 1999). First, in laboratory-based work, it must be shown that it is possible to engineer mosquitoes that are refractory to the transmission of parasites. Second, methods must be identified for moving genes made in the laboratory into wild mosquito populations. Strategies that rely on Mendelian genetics to increase gene frequencies in populations require fitness advantages tightly linked to the anti-pathogen gene and are likely to be too protracted in time to sustain support and interest (Collins and James 1996; Braig and Yan 2002). However, concepts have been developed of genes spreading in an infection-like wave through mosquito populations, and there is enthusiasm for using transposable elements or other mobile nucleic-acid vectors (Kidwell and Ribeiro 1992) and bacterial symbionts (Curtis and Sinkins 1998) to drive genes. The third research area provides the information about target vector populations and disease transmission dynamics that is needed to model and predict how anti-pathogen genes will affect the epidemiology of a disease in a specific endemic region. This is important for both the introduction of genes and for establishing the parameters by which the success of introductions will be measured. The genetic structure, population migration indices, gene flow and other population-genetic factors of the vectors will influence the outcome of a genetic strategy, and the effects of these factors on introduced genes in the target population must be anticipated and integrated. Threshold values for entomological inoculation rates, the fraction of the vector population that must be made pathogen-resistant, and the effect of immune states of people living in disease-endemic areas also must be considered. As a final measure, the most significant outcome of research efforts in all three of these areas will be a reduction or elimination of human disease as a result of interrupting transmission of the target pathogen.

The demonstration of the function of isolated promoters (Coates et al. 1999; Kokoza et al. 2000; Moreira et al. 2000), the development of transgenesis technologies (Jasinskiene et al. 1998; Coates et al. 1998; Catteruccia et al. 2000; Allen et al. 2001; Grossman et al. 2001; Kokoza et al. 2001; Lobo et al. 2002; Perera, Harrell II and Handler 2002) and the identification and synthesis of potential anti-pathogen effector genes (Olson et al. 1996; De Lara Capurro et al. 2000; Ghosh, Ribolla and Jacobs-Lorena 2001; Moreira et al. 2002; Ito et al. 2002; Osta, Christophides and Kafatos 2004; Blandin et al. 2004) complete the proof-of-principle for the laboratory-based research area. What is required next for this area is to transfer the methodology and approaches developed in animal models of disease to mosquito–pathogen interactions relevant to humans, and this work is ongoing. Important for the context of this book, the challenges of the second research area must be engaged by designing safe (low risk-to-benefit ratio) and efficient mechanisms for driving genes through vector populations.

Issues and challenges

Movement of laboratory-developed genes into wild populations requires addressing three major issues that need innovation and research. The first is to develop viable gene drive mechanisms that can be used to move genes into target populations. Strategies based on competitive displacement, reduced heterozygote fitness and meiotic drive have been analysed and arguments favouring some approaches over others have been made (Braig and Yan 2002). Properties of an ideal

gene-driving system are being identified (Braig and Yan 2002; James 2005) and these have generated robust debate. For example, whether or not a transgene inherently confers a fitness load on the mosquito is controversial. While Catteruccia, Godfray and Crisanti (2003) and Irvin et al. (2004) detected fitness load in mosquitoes *homozygous* for a transgene, Moreira et al. (2004) detected no significant fitness load in *heterozygous* transgenic mosquitoes expressing a tetramer of the SM1 dodecapeptide. The experiments in the former two reports could not distinguish whether the fitness load was imposed by homozygosity of nearby deleterious recessive genes (cf. 'founder effect', 'hitchhiking effect') or by the transgene itself. This is not an issue in the Moreira et al. (2004) experiments because transgenic mosquitoes were backcrossed at each generation, with wild-type mosquitoes. Thus, transgenic and wild-type controls had similar genetic backgrounds. Specific options for gene drive mechanisms include extracellular and intracellular symbionts, viruses and transposons. Extracellular bacterial symbionts ingested during coprophagy have proven remarkably efficient in spreading exogenous genes through reduviid bugs in an effort to control Chagas' disease (Beard, Cordon-Rosales and Durvasula 2002). In principle, a similar approach could be used to deliver effector genes to the mosquito midgut. In preliminary experiments, the Jacobs-Lorena laboratory constructed recombinant *E. coli* expressing an SM1-Omp (Outer membrane protein) fusion protein and showed that mosquitoes that had ingested these bacteria were impaired in transmission of the malaria parasite *P. berghei*. Advantages of such 'paratransgenesis' approach include: 1) logistics are simpler (bacteria can be genetically manipulated with ease, they can be cheaply grown in large quantities); 2) the approach is compatible with the concomitant use of insecticides in the target area; 3) many effector genes can be delivered simultaneously using a mixture of transgenic bacteria; and 4) the nature of the effector genes can be changed at any time during the control programme. A major unresolved issue is that no one knows how adult mosquitoes acquire their bacterial flora and therefore, a strategy to deliver the genetically modified bacteria to the mosquitoes in the field has yet to be developed. Intracellular symbionts, such as the *Wolbachia* species (Curtis and Sinkins 1998), and the endosymbionts of tsetse (Aksoy 2000), hold some promise, but a current lack of efficient *ex vivo* manipulation presents challenges to their further development for mosquitoes (Noda, Miyoshi and Koizumi 2002; O'Neill et al. 1997). However, the extent to which these agents cause cytoplasmic incompatibility may make them useful in population reduction strategies of genetic control (Curtis and Sinkins 1998). A number of viruses have been identified that may provide the basis of a gene drive system. Mosquito densoviruses appear particularly attractive because of their apparent inability to infect non-target organisms (Afanasiev and Carlson 2000). Many of these viruses are lethal to insect larvae and seem more appropriate to develop as a population reduction tool to be used in conjunction with other anti-vector strategies for local eradication of a target vector population. Class-II transposable elements (those that transpose via a DNA intermediate (Finnegan 1985)) also are attractive as the basis for a gene drive mechanism because of their ability to excise and insert (mobilize) in DNA. Transposons are spread through populations by replicative transposition in the germ line and this process circumvents standard Mendelian inheritance (Kidwell 1983; Collins and James 1996). Thus, we have a number of requirements and candidate mechanisms available for gene drive mechanisms.

The second major issue is to recognize that not all mosquitoes are the same, and that this has a significant influence on the transmission dynamics of specific pathogens. For example, some of the most efficient vectors of malaria, *An. gambiae*

and *An. funestus,* each consist of a group of closely related, highly anthropophilic sibling species that adapted genetically to different ecological niches (Coluzzi et al. 2002). These mosquitoes have different larval habitats with the former exploiting transient water sources created by seasonal rains or agricultural irrigation, and the latter favouring permanent sites found in pools in stream beds, in small ponds, marshes and long-term flooded rice fields. The complementary life cycles of these two mosquitoes are sufficient to maintain malaria transmission throughout the year in many areas. *Aedes aegypti* is by far the most important and efficient vector of dengue viruses also because of its affinity for humans (Gubler 1998). Within a specific transmission environment, little intraspecific variation is observed. Immature mosquitoes develop primarily in man-made containers near human dwellings and adult females rest indoors where they feed frequently and preferentially on human blood. Extended flight can be limited because food, mates and oviposition sites are readily available within or near human habitations (Edman et al. 1998; Harrington et al. 2001). This makes possible explosive dengue epidemics even when mosquito population densities and DV entomological thresholds are low (Kuno 1995; 1997; Focks et al. 2000; Scott et al. 2000). Thus, before it is possible to expect a positive outcome of a genetic intervention strategy it is necessary to define for a specific locale the exact mosquitoes that are targets of the intervention. If malaria transmission is observed in different environmental conditions, it is likely that different species or subspecies (genetically isolated sympatric populations) may be the primary vectors. Furthermore, different vectors are important at different times of the year and, therefore, a comprehensive approach for utilizing population replacement strategies must target all the important vectors within a given area.

The third major issue is that it is likely that laboratory-derived mosquitoes will differ significantly from the target insects in the field. Fitness factors that affect fecundity, fertility and mate choice are likely to drift as they adapt to selection pressures in the laboratory. Therefore, it is preferable to develop procedures that introgress the important genes into strains of mosquitoes that are as similar as possible to the targets in the field. Following up on the discussion above, particular attention must be paid to the time of year that the release will take place so that the release mosquitoes match as much as possible the correct seasonal target. Thus the major challenges are to be able to develop a viable gene drive mechanism, define as much as possible what are the important vectors of a the target disease and determine how the drive mechanisms and effector molecules are going to be introduced into those specific populations.

Research and control opportunities

The research opportunities follow directly from the challenges listed above. The research of drive mechanisms will follow a path of studying natural or synthetic genetic phenomena that alter segregation ratios to spread specific genes or alleles through populations. From these phenomena, specific drive systems must be designed that incorporate features that mitigate concerns about the release of genetically modified organisms. Such drive systems must be demonstrated first to work in laboratory experiments and progress to large cage trials in the field. The systems then must be introgressed into field-derived mosquito strains and evaluated for their reproductive and competitive success. This can only be done based on a description of the target mosquitoes in the proposed release area.

Future directions for research and capacity/partnership building

The future direction of research in this area overlaps those described in Chapter 12. All future work should emphasize the enlistment and involvement of DEC personnel to foster the level of awareness required for workable guidelines for the evaluation and application of genetic control programmes. Furthermore, a research agenda that requires a DEC component should include experiments to demonstrate that drive sstems can spread an effector through caged populations of mosquitoes, introgress laboratory-derived gene drive systems into target populations recently derived from the wild, demonstrate drive systems in field cage settings, determine the effects on life-history traits of drive systems in field cages, and determine the possibility of movement in field cages of drive mechanisms among closely related species.

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14

Tools for monitoring the genetic structure and stability of mosquito populations

Gregory C. Lanzaro[#], Sergey Nuzhdin[##] and Frederic Tripet[#]

Abstract

Interest in mosquito population genetics has risen dramatically over the past decade, driven mainly by renewed interest in vector control as a means of controlling malaria and dengue fever. Earlier work in mosquito population genetics focused on resolving taxonomic issues, especially in distinguishing and defining the geographic distributions of cryptic taxa that are common in mosquitoes, especially in the genus *Anopheles*. The lessons learned from this early work include the realization that our concept of vector species is often incorrect and that even at the within-species level substantial genetic divergence among local populations exists. The explosion of research into the molecular genetics of mosquito vectors has dramatically altered the direction of, and interest in, mosquito population genetics. The most obvious difference is in the nature of the genetic markers that lie at the heart of studies aimed at describing the genetics of mosquito populations. Perhaps even more significantly, the study of mosquito molecular biology has led to changes in the questions being asked and in our ability to provide answers. The tools (e.g. markers) and questions are intimately related because the availability of new methodologies has allowed us to seek the answers to questions that seemed intractable in the past.

In this paper we discuss several tools that are currently available, but not yet widely used in population-genetics studies. We do not, however, provide a comprehensive review of those tools that are currently used in mosquito population genetics (with the exception of microsatellite DNA), as these have been reviewed elsewhere (Norris 2002).

Keywords: population genetics; microsatellites; single-nucleotide polymorphisms; sequence-specific amplification polymorphisms; microarrays

The raw material: Procuring specimens from natural populations

Unquestionably the most important tools for anyone interested in studying the biology of mosquito populations remain a pair of sturdy boots, a passport and the willingness to travel and spend time in the field. The point here is that the development of a sampling strategy that satisfies the question(s) being asked is the most important component of work aimed at understanding the genetics of natural populations. The sampling plan must include temporal and/or spatial components and

[#] Department of Entomology, University of California, One Shields Avenue, Davis, CA 95616, USA. E-mail: gclanzaro@ucdavis.edu and ftripet@ucdavis.edu

[##]Section of Evolution and Ecology, University of California, Davis, CA USA. E-mail: svnuzhdin@ucdavis.edu

provide for the procurement of an adequate sample size from each population under study, regardless of scale, which may vary from a single household within a village, to broad ecological areas spanning multiple national boundaries. The best way to obtain the necessary samples is to participate directly in collecting expeditions, which also provides the important benefit of learning something of the natural history of the species under study. Thus, an important role to be filled by the population geneticist is bridging the gap between the molecular genetics and field ecology.

Although there is little doubt that obtaining material for population studies by collecting it oneself is the best approach, doing so is expensive, both in time and in money, and often the questions being asked involve populations that may have already been extensively sampled. Therefore it is reasonable that organized specimen archives be created and maintained. Informal 'specimen sharing' among mosquito population geneticists is part of our tradition, however in the past, studies based on chromosome or isozyme markers usually led to the destruction of samples, so that only samples in excess of what was needed for a study were available. Most of the markers being used today are PCR-based, requiring only a small portion of the total amount of single mosquito-genomic DNA, so single specimens should be available for a relatively large number of assays. In addition, a method known as Multiple Displacement Amplification exists which allows a 100–400-fold amplification of whole mosquito genomes (Gorrochotegui-Escalante and Black IV 2003). This essentially makes individual mosquito DNA samples available for a very large number of studies. A network of collaborating investigators could be established and their contact information and description of material available posted on an appropriate website available to the community at large. Beyond this, arrangements can be made by individual investigators with respect to sharing material.

Microsatellite DNA

The discovery of hyper-variable microsatellite-DNA sequences undoubtedly revolutionized the fields of population genetics and ecology (Zhang and Hewitt 2003). For population geneticists, having at hand a marker evolving much faster than mitochondrial genes or genes coding for isozymes, equated to being able to resolve the structure of populations at a much finer geographical and evolutionary scale. For behavioural ecologists it translated into being able to establish kin relationships using DNA from smaller and smaller organisms, samples from live organisms, or even their gametes. Because microsatellites provide higher resolution for estimating genetic differentiation between populations within taxa, they allowed population biologists to make better inferences about population structure, and in some cases, about the movement of individuals between populations.

Not surprisingly, the number of studies taking advantage of their versatility has grown exponentially and the advances made possible by microsatellites render them indispensable in many fields (Zhang and Hewitt 2003). Today, an equally important body of literature points out the limitations of microsatellites for some applications (e.g. Chambers and MacAvoy 2000; Balloux and Lugon-Moulin 2002; Zhang and Hewitt 2003). The stepwise mutation process that adds or subtracts repeats to existing alleles (Armour et al. 1999; Eisen 1999) results in alleles of identical size having different mutational histories, a phenomenon known as allele size homoplasy (Estoup and Cornuet 1999; Estoup, Jarne and Cornuet 2002). Homoplasy is made more likely if the range of possible allele sizes itself is constrained (Garza, Slatkin and Freimer 1995; Lehmann, Hawley and Collins 1996; Estoup, Jarne and Cornuet 2002). There is

evidence that some microsatellite tracts located in promoter regions may affect gene expression and protein binding (Kashi and Soller 1999; Rothenburg et al. 2001). These loci are clearly not neutral because selection may favour an optimal size range of repeat tracts (Estoup, Jarne and Cornuet 2002; Zhang and Hewitt 2003). Generally speaking, microsatellite loci that are near genes that are themselves under selection will be subject to hitchhiking and background selection and this may be another source of deviation from neutrality (Charlesworth, Nordborg and Charlesworth 1997; Barton 2000). Homoplasy is a concern when estimating genetic divergence between taxa (Garza, Slatkin and Freimer 1995; Estoup, Jarne and Cornuet 2002). Genetic distances such as Wright's F_{ST} estimate (1931), Weir and Cockerham's θ F_{ST} estimate (1984), or Nei's *Ds* and *Da* distances (Nei 1972; Takezaki and Nei 1996) are based on the assumption that mutations generate only new alleles (infinite-allele model). Homoplasy will therefore result in an underestimation of genetic distance. As a result a number of new genetic distance statistics that assume stepwise or mixed stepwise and non-stepwise mutational models have been proposed. These include R_{ST} (Slatkin 1995), $(\delta\mu)^2$ (Goldstein et al. 1995) and D_{SW} (Shriver et al. 1995). Simulations presented along with these distances show that they out-perform classic distances for phylogenetic inferences (Slatkin 1995; Goldstein et al. 1995; Shriver et al. 1995). However, assessing which is better suited for use on real data remains difficult and depends on the evolutionary scale and the organisms considered. For population-genetic studies, F_{ST}'s (Wright 1931; Weir and Cockerham 1984) are still widely used and it is generally accepted that they perform better in studies of populations that exchange migrants – e.g. subdivided populations or hybrid zones (Rousset 1996; Estoup, Jarne and Cornuet 2002).

Another major concern for using microsatellites was the practice of directly translating F_{ST}'s into *Nm*, the number of migrants per generation, using the simple relationship $F_{ST} \approx 1/(4Nm+1)$ (Slatkin 1985; 1987). As emphasized by Whitlock and McCauley (1999), the temptation of translating F_{ST} estimates into units that make immediate ecological sense is understandable but treacherous. This is because few populations meet the assumptions required for translating F_{ST}'s into *Nm*'s, and F_{ST}'s may be biased by homoplasy (Bossart and Prowell 1998; Whitlock and McCauley 1999). Here again, it is generally recognized that *Nm* estimates have to be interpreted with caution. As a result, they are best suited for qualitative comparisons except when their reliability has been assessed using direct measures of dispersal or by comparing them to estimates of hybridization rates (Taylor et al. 2001; Tripet, Dolo and Lanzaro 2005).

Sequence-specific amplification polymorphisms

Sometimes, it is necessary to develop a set of markers private for one genotype and absent in the remaining individuals within a population. One of the promising and inexpensive ways is Sequence-specific amplification polymorphism (SSAP) (Waugh et al. 1997). SSAP analysis has been designed to resolve genetic distances of very closely related crop-plant varieties. It relies on the presence of multiple transposable elements – that are similar to retroviruses – frequently inserting into new genomic positions. SSAP is similar to AFLP except that only those bands are visualized that are tagged into highly polymorphic transposable element sites. Specifically, one ligates ~20bp adapter to the ends of restricted DNA (usually with a four-cutter enzyme), and PCR-amplified DNA using a labelled primer homologous to the transposable element sequence and an unlabelled primer homologous to the adapter.

Bands of different size correspond to transposable elements from different occupation positions. An advantage of these markers is that they are as polymorphic as microsatellites. As the mosquito genome is sequenced and its transposable element population becomes known, thousands of SSAP markers can be developed within a week. But unlike microsatellite markers, SSAP bands are frequently population- and individual-specific (Yang and Nuzhdin 2003).

Single-nucleotide polymorphisms

As the amount of sequence data available from many organisms increases and entire genomes are being assembled, more and more researchers have the possibility to use yet another type of marker. Single-nucleotide polymorphisms (SNPs) have much lower mutation rates than microsatellites and provide an alternative tool for pedigree analyses (Blouin et al. 1996; Glaubitz, Rhodes and Dewoody 2003). Their increasing popularity is driven in large part by advances in biomedicine where genomic studies have linked SNPs with phenotypic characteristics of diseases and hosts, and increasingly powerful methods are used for screening variation at multiple loci (Vignal et al. 2002; Hirschhorn et al. 2002). Here we discuss their potential use as an alternative to microsatellites for population-genetic studies as proposed elsewhere (Brumfield et al. 2003; Morin et al. 2004). We are currently involved in studies of the population structure of *Anopheles gambiae* Giles, the main vector of malaria in Africa. Since the entire genome has been sequenced (Holt et al. 2002), SNPs are a real alternative to microsatellites in this organism.

Microsatellites versus SNPs

There are a number of aspects that need to be taken into account when comparing the two types of markers. One of the big advantages of SNPs, when whole genome sequences are available, is their abundance. Users can decide the polymorphism they prefer (transition, transversion or both) and pick loci away from coding regions to insure that they are not influenced by selection on nearby genes. In theory, when genomes are available, microsatellite loci could also be selected away from genes but their lower density may prevent that luxury in many study organisms. With regard to mutational processes, SNPs with their low mutational rate, i.e. $\sim 10^{-9}$ compared to $\sim 10^{-4}$ to 10^{-6} for microsatellites, are expected to feature few alleles per locus (Hancock 1999; Zhang and Hewitt 2003). In fact, in most cases, SNPs will be equivalent to di-allelic markers (Vignal et al. 2002; Morin et al. 2004). It is expected that multi-allelic microsatellites should have higher power – per locus – over di-allelic SNPs for estimating genetic divergence or gene flow using *F*-statistics or assignment tests (Vignal et al. 2002; Brumfield et al. 2003; Morin et al. 2004). Mariette et al. (2002) estimated that four to ten times more di-allelic markers (dominant markers were simulated in this study) were necessary for reliably estimating genome-wide levels of variation. Studies by Blouin et al. (1996) and Glaubitz, Rhodes and Dewoody (2003) suggest that measures of pair-wise genetic relationships using SNPs would require analysis of more than 5 times more loci.

Comparing the resolution of SNPs to that of the faster evolving microsatellites is critical to assess their potential for population genetics. Despite extensive discussion of the potential for SNPs in population-genetic studies (Brumfield et al. 2003; Morin et al. 2004), there are, as yet, no qualitative comparisons of the two types of markers available.

Practical implications for population studies

Assessing what is the optimal marker for the organism and populations under study is a fundamental step in designing and planning population genetic studies. In many instances, however, time and cost optimization is just as important for the success of a project. The costs and technical aspects relating to the use of either marker have been adequately evaluated and discussed elsewhere (reviewed in Kwok 2001; Chen and Sullivan 2003; Zhang and Hewitt 2003). Despite important developments in methods of SNP allele discrimination and detection, all techniques rely on PCR amplification. Given the much higher number of SNP loci required, the costs in reagents and manpower will be multiplied 6-10-fold. Choosing SNPs over microsatellites also involves considerable investment in equipment that often has higher operating costs than the sequencers used for typing microsatellites. Nowadays, microsatellite libraries can be ordered from companies at a reasonable cost. More importantly they can be ordered with pre-evaluated primer pairs thus significantly cutting down the costs of manpower required for these steps.

In conclusion, SNPs should theoretically generate better estimates than microsatellites when the populations under study are fully isolated either reproductively or geographically. Switching to SNPs does not, however, prevent biases due to co-ancestry and it should also be noted that the impact of ascertainment biases or problems of null alleles remains to be adequately evaluated. In animal systems or populations with either known ongoing gene flow or low microsatellite mutation rates, e.g. *Drosophila* (Zhang and Hewitt 2003), the benefit of using SNPs is questionable. For the reasons discussed above and because advantages and potential flaws of microsatellites are so well documented, we predict that they will remain essential tools in population genetics. The popularity of SNPs will strongly depend on the number of whole genomes available, the development of simpler protocols for their design in other organisms, and the availability of affordable automated PCR procedures for processing large number of loci.

Microarrays for population genetics

In addition to PCR-based techniques, SNP-scoring techniques relying on ligation and hybridization are being developed and evaluated. Because of the large sample sizes required for studies of population-genetic structure, ligation-based techniques are currently the only real options for typing large numbers of SNPs at an affordable cost. For ligation-based approaches, two primers are designed to ligate if they perfectly hybridize to a PCR-amplified genomic template. The specificity of the ligation reaction is much higher than that of polymerization, thus the typing error is much smaller. The use of a bar-code system of ligation detection greatly reduces labour. It enables typing at the cost of about US $0.05 per SNP. Millions of SNPs are rapidly and reliably typed with this approach (Genissel et al. 2004). However, current applications focus on scoring large numbers of SNPs within a few large amplicons. For population-genetic studies, the reverse should be achieved, namely scoring fewer loci but from a large number of amplicons spread out across the genome, but here again the number of PCR reactions required can be very large. Assuming that a genome is available and money would not be a limiting factor, SNP typing can be made at a much larger scale with microarrays. Currently available Affymetrix CustomSeqTM re-sequencing arrays enable the analysis of up to 30,000 bases of double-stranded sequence. These arrays carry in excess of 240,000 features, each

feature being 20x25 micrometer of glass surface covered by millions of copies of a 25-mer oligonucleotide. To identify the nucleotide at a given position, the Affymetrix platform compares the levels of template hybridization to four oligonucleotides that match the reference genome sequence and are identical except at the position that is being analysed. This position (the exact middle of the oligo) contains either A, T, C or G. The strongest hybridization indicates which of the four oligonucleotides represents a perfect match, as opposed to a mismatch. This inference is confirmed if the sequencing of the two opposite strands produces concordant results. To identify the next nucleotide, the analysis is repeated with all oligos shifted by one base. Affymetrix arrays are intended for hybridizations with templates amplified by LPCR as described above. This technology provides base calls at >99.99% accuracy and 90% calling rate. This compares favourably with the accuracy and calling rate achievable with direct ABI sequencing. Using this platform also generates data on small indels.

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15

What are relevant assays for refractoriness?

George K. Christophides[1], Louis C. Gouagna[2], Marcelo Jacobs-Lorena[3], Anthony A. James[4] and Kenneth E. Olson[5]

Abstract

The engineering and testing of genes that result in refractory phenotypes are important components of the continuing effort towards the use of population replacement strategies for vector control. Both 'endogenous' and 'synthetic' refractory phenotypes are being considered. Additional research is required to determine the prevalence of such phenotypes in the various vector–pathogen combinations, and the threshold levels of activity of genes conferring transmission blocking, as well as to develop efficient methods for the evaluation of their entire spectrum of biological effects.
Keywords: refractoriness; immunity; melanization; RNA interference; SM1

Current state of the art

Refractoriness phenotypes could be divided into 'endogenous' and 'synthetic'. To date, a number of endogenous refractory phenotypes have been recognized, including melanotic encapsulation and lytic destruction of malaria parasites in the mosquito midgut (Christophides, Vlachou and Kafatos 2004) and RNA-interference (RNAi)-mediated suppression of viruses (Sanchez-Vargas et al. 2004). A small number of additional refractory phenotypes have been recorded; however, their specifics are, to a large extent, unknown and could be attributed to differences in vector physiology (e.g. lack of vector factors).

Melanization is an insect immune reaction that has been reported for many mosquito–parasite combinations. In a genetically selected *Plasmodium*-refractory strain of *Anopheles gambiae,* parasite melanization occurs immediately after the ookinete has traversed the midgut epithelium, between the epithelial cells and the basal lamina (Collins et al. 1986; Paskewitz et al. 1988). Although these refractory mosquitoes block development of the primate malaria parasite, *P. cynomolgi*, the rodent parasite, *P. berghei*, and allopatric strains of the human parasite *P. falciparum*, they fail to

[1] European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany. E-mail: christop@embl.de
[2] International Centre of Insect Physiology and Ecology, PO Box 30772, Nairobi, Kenya. E-mail: lgouagna@mbita.mimcom.net
[3] Johns Hopkins School of Public Health, Department of Molecular Microbiology and Immunology, Malaria Research Institute, Baltimore, MD 21205, USA. E-mail: mlorena@jhsph.edu
[4] Department of Microbiology and Molecular Genetics, Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697-3900, USA. E-mail: aajames@uci.edu
[5] Department of Microbiology, Immunology, and Pathology, Arthropod-borne and Infectious Diseases Lab., Colorado State University, Fort Collins, CO 80521-1692, USA. E-mail: kolson@colostate.edu

melanize sympatric *P. falciparum* populations (Collins et al. 1986), suggesting that the reaction is based on specific recognition and is most probably regulated by parasite-induced immune evasion. Recent data showing that silencing of specific immunity genes unrelated to the final steps of the melanization reaction suppress the melanization phenotype allowing parasite development and support a hypothesis that specific parasite recognition and killing may precede melanization (Blandin et al. 2004; Osta et al. 2004). Three quantitative-trait loci (*Pen1*, *Pen2* and *Pen3*) have been found to be responsible for the melanization phenotype, with one (*Pen1*) having the most important contribution (Zheng et al. 1997). However, the specific genes responsible for the quantitative traits have yet to be identified. Furthermore, a multidisciplinary morphological, biochemical and genomic approach has demonstrated broad physiological differences between the refractory and susceptible mosquitoes, implicating the level of reactive oxygen species as a factor contributing to the observed phenotype (Kumar et al. 2003).

A similar refractoriness phenotype has been described in a genetically selected line of *An. dirus* that is fully refractory to the rodent parasite, *P. yoelii* (Somboon, Prapanthadara and Suwonkerd 1999). Melanization starts early after midgut invasion and ranges from small melanized parasites to fully encapsulated oocysts. However, these mosquitoes are susceptible to natural infections with the human parasites *P. falciparum* and *P. vivax*, again emphasizing the specificity of the mosquito–parasite interaction.

The second refractoriness mechanism, lytic destruction of parasites, was reported first in a genetically selected strain of *An. gambiae* that kills *P. gallinaceum* ookinetes inside the midgut epithelial cells (Vernick et al. 1995). The ookinetes appear to be initially vacuolated and subsequently lysed while still in the cytoplasm of the midgut cells. A single dominant gene is responsible for this phenotype. Two recent studies have shown that mosquito innate immunity plays a central role in lysis of *P. berghei* ookinetes in the midgut epithelium (Blandin et al. 2004; Osta et al. 2004). RNAi-mediated gene silencing of two immunity genes, *TEP1*, a complement-like protein, and *LRIM1*, a leucine-rich repeat immune protein, results in an average fourfold increase in parasite numbers. However, two other mosquito genes encoding C-type lectins, CTL4 and CTLMA2, protect the parasites from immune reaction, since their absence leads to parasite killing and melanization (Osta et al. 2004). Specifically, the two parasite agonists protect the ookinetes from the immune action of LRIM1. Whether the protective function of the C-type lectins is the result of evolutionary adaptation remains to be examined (Osta, Christophides and Kafatos 2004).

A proteomic approach in a strain of *An. stephensi* showing reduced susceptibility to infection with *P. falciparum* (Feldmann and Ponnudurai 1989) revealed many differences in the midgut expression profile following a blood meal, when compared to a susceptible strain (Prevot et al. 1998). A factor responsible for the death of *Plasmodium* in the midgut of the mosquitoes is the production of nitric oxide (NO) and nitrite/nitrate radicals, all products of NO synthase (NOS) activity. NOS is induced in the *Anopheles* midgut upon parasite infection, resulting in elevated levels of these highly reactive species, and inhibition of this enzyme promotes parasite development (Luckhart et al. 1998).

A field-based study (Southern Tanzania) has revealed that *Plasmodium* melanization also is detected in natural mosquito populations (Schwartz and Koella 2002). However, the same study provided evidence that melanization of Sephadex beads, which is commonly used to measure the mosquito melanization capacity, does not accurately model *An. gambiae* susceptibility to *P. falciparum* in field conditions.

Strong evidence for genetic variability of *An. gambiae* in terms of susceptibility to natural malaria populations, manifested as reduced *P. falciparum* oocyst numbers detected in the mosquito midgut, has also been revealed (Niare et al. 2002). This phenotype is attributed to segregating alleles of two chromosomal loci (*Pfin1* and *Pfin2*). The apparently high natural frequency of resistance alleles suggests that natural mosquito populations exhibit significant variation in permissiveness for parasite development, and supports the hypothesis that malaria parasites exert a significant selective pressure on vector populations.

Synthetic refractoriness phenotypes are the result of genetic engineering of the vectors through the introduction of genes that determine anti-pathogen activities. These effector molecules could be exogenous or endogenous and act as pathogen anti-ligands (e.g. single-chain antibodies against parasite surface proteins), host mosquito anti-receptors (e.g. peptides blocking mosquito midgut invasion by the parasite), immune factors (e.g. antimicrobial peptides; AMPs) and toxins, or molecules acting through RNAi interference specific for pathogen or vector genes (Nirmala and James 2003).

Along these lines, a short-chain peptide, SM1 that binds to the *An. gambiae* midgut and salivary glands has been transformed into *An. stephensi* generating mosquitoes that express the peptide in the midgut lumen (Ito et al. 2002). These mosquitoes exhibit strongly reduced capacity to support *P. berghei* transmission. Similarly, a *P. berghei*-refractory *An. stephensi* has been generated by midgut-specific expression of a bee-venom phospholipase (Moreira et al. 2002). The mode of action of the two effectors remains unknown. Transgenic overexpresion of a gene encoding the AMP Cecropin A (*CEC1*) in the midgut of *A. gambiae* reduced the number of developing *P. berghei* oocysts by 60% (Kim et al. 2004). Similarly, in the yellow-fever mosquito *Aedes aegypti*, transgenic overexpression of the endogenous AMPs Defensin A and Cecropin A under the control of the *vitellogenin* gene promoter leads to robust inhibition of *P. gallinaceum* development (Shin, Kokoza and Raikhel 2003). Furthermore, *Ae. aegypti* resistant to *P. gallinaceum* have been developed via a different approach (De Lara Capurro et al. 2000): single-chain antibodies, engineered from a cDNA encoding an anti-*Plasmodium* monoclonal antibody, bind to *P. gallinaceum* sporozoites and largely prevent infection of the salivary glands when expressed by Sindbis virus.

Issues and challenges

Much work remains to be done on the identification and full characterization of effector traits as well as the construction and analysis of penetration and expression of transgenes targeting human malaria parasites. In addition, issues such as phenotypic variation depending on environmental conditions, development of resistance or even increased virulence of the pathogen, must also be addressed. Assuming that a strategy is chosen that does not rely on a complete replacement of populations, the relative frequency (prevalence) of the transgenes will be important and the threshold levels of refractoriness needed to interrupt transmission will need to be determined. Field tests of specific genes and outcome evaluation parameters also are necessary, as are assays, possibly based on membrane-feeding of gametocytemic blood, to evaluate refractoriness to endogenous malaria pathogens.

Research and control opportunities

Vector-borne diseases are fast increasing in most parts of the developing world, and there is an immediate need to develop novel strategies for disease control. Recent advances toward development of methods for vector transgenesis and identification and engineering of anti-pathogen effectors make the concept of vector population replacement with innocuous vector populations particularly attractive. However, many technical issues remain to be resolved, including the development of ideal effector traits that confer full resistance against human pathogens in natural transmission conditions. To date, efforts to characterize endogenous or synthetic effector genes have focused on model vector–pathogen combinations. However, a limited number of studies have suggested considerable differences among the various vector–pathogen combinations, and therefore, experimental approaches focusing on the main mosquito vectors and human pathogens should be undertaken in the future.

Furthermore, experiments will have to be performed to determine the genetic characteristics (dominance relations, penetration and expression) of mosquito genes and effects of both endogenous and synthetic refractoriness genes on the selection of resistance or increased virulence of the target pathogen. In addition, research on combinations of effector mechanisms to thwart the emergence of new pathogen phenotypes is needed. Additional studies of natural refractoriness and its potential to adapt as a 'death-on-infection' effector mechanism also are needed. Field-based work is necessary to assay the potential for phenotypic variation in refractory gene function in response to different environmental conditions, and protocols for field tests and outcome evaluation are required. Work with both laboratory- and field-based components includes modelling and experiments to establish the needed frequency of genes with a refractoriness phenotype in mosquito populations, in order to achieve the threshold levels needed for interrupting transmission.

Finally, research is needed to identify key qualitative and quantitative endpoints for the efficacy of effector genes. This includes routine and reliable methods for transmission-blocking assays that do not pose threats to humans. For this, protocols for human subjects must be standardized.

Future directions for research and capacity/partnership building

The future direction of research in this area that will bridge the gap between laboratory and field work will likely include the further analysis of endogenous, field-derived refractoriness mechanisms, the development of cage-trial protocols that allow the evaluation of engineered genes under (semi)natural conditions, the refinement of socially acceptable assays for transmission blocking, and outcome evaluation protocols that measure accurately the impact of a refractoriness gene on transmission dynamics.

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Fitness studies: developing a consensus methodology

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

Abstract

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

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

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

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

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

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

Introduction

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

Definitions

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

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

Complexities of fitness

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

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

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

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

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

Research on GMM fitness

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

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

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

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

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

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

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

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

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

Recommendations for measuring fitness

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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17

Mosquito mating behaviour

Willem Takken[1]*, Carlo Costantini*[2]*, Guimogo Dolo*[3]*, Ahmed Hassanali*[4]*, N'Fale Sagnon*[2] *and Eli Osir*[4]

Abstract

Mating is one aspect of behaviour that has been much ignored in mosquito biology. Yet, the success of a transgenic release strategy depends on normal, competitive mating between introduced and wild individuals. An overview is presented of current knowledge of mating behaviour in *Culicidae*, including timing of mating, means of sperm transfer, refractory behaviour and multiple mating. Most lacunae were found in mate finding: it is known that some species use swarming while other mate on or near the vertebrate host. At short range males locate females by acoustic signals, but there is no knowledge how the sexes locate each other from a distance. It is argued that mass rearing of mosquitoes for sterile-insect release or transgenic release should include steps to safeguard male fitness. A series of challenges for future studies are discussed, including cues that control swarming behaviour, mate-finding behaviour and identification of genes that control mating behaviour.
Keywords: *Culicidae*; fitness; swarming; multiple mating; sperm; gene

Introduction

Of the critical behaviours that characterize the mosquito life strategy, mating is probably the least understood and most understudied. Yet, as mosquitoes depend on sexual reproduction for species maintenance, this aspect of mosquito biology should receive the highest attention when seeking new avenues for mosquito control and interventions for mosquito-borne disease. Which behavioural steps need to be considered when mating is concerned? As a rule, newly emerged male mosquitoes are unfit for coupling with a female, as the external genitalia require a morphological change. This is accomplished by inversion of the terminalia within the first 24 hr following emergence. In many species, male accessory glands mature during the first few days of adult life, and this is needed before sperm can be successfully transferred (Clements 1999). Thus, males of many mosquito species require several days to mature before a first successful mating can take place. In *Anopheles gambiae* Giles *sensu stricto* and *An. arabiensis* Patton optimal mating occurs with 5–7-day-old males

[1] Laboratory of Entomology, Wageningen University and Research Centre, PO Box 8031, 6700 EH Wageningen, The Netherlands. E-mail: Willem.Takken@wur.nl
[2] Centre National de Recherche et Formation sur le Paludisme (CNRFP), 01 B.P. 2208, Ouagadougou, Burkina Faso. E-mail: costantini.cnlp@fasonet.bf
[3] Malaria Research and Training Center, Faculté de Médecine de Pharmacie et d'Odonto-stomatologie, B.P. 1805, Bamako, Mali. E-mail: dolo@mrtcbko.org
[4] International Centre of Insect Physiology and Ecology (ICIPE), PO Box 30772, Nairobi, Kenya. E-mail: ahassanali@icipe.org ; eosir@icipe.org

(Reisen 2003; Verhoek and Takken 1994). In other species, males may mate at an earlier age, but sperm may then not be successfully transferred due to immaturity. Females, by contrast, are ready to mate almost as soon as they emerge from the pupal cases. As an extreme example, in some species females are inseminated immediately following emergence by males who sit and wait next to the emergence site to pounce on any female as they are unfolding their wings, or they even grab the female pupa shortly before emergence (Provost and Haeger 1967). In most species, though, there is a 24–48-hour time lag between emergence and mating. Mating is not needed for egg development and maturation, but in most species eggs can only be deposited when insemination has occurred (Clements 1999). As a rule, female mosquitoes mate before taking a first blood meal, but in several anophelines a large proportion of virgins may blood-feed prior to mating. Such a blood meal is essential for the development of a metabolic energy reservoir (Gillies 1954; Lyimo and Takken 1993; Takken, Klowden and Chambers 1998). Many females may imbibe nectar or other carbohydrate sources prior to mating, presumably again to acquire an energy reservoir for flying and mate-finding (Foster 1995; Foster and Takken 2004). In *Aedes aegypti* L. mating is accompanied by a change in behaviour, caused by the transfer of 'matrone', a male hormone, which makes the female refractory to successive matings and induces host-seeking behaviour (Craig Jr 1967). A similar hormonal effect was also reported from *Culex tarsalis* Coquillet. Such behavioural physiology does not occur in *An. gambiae s.s*., where male accessory-gland substances do not induce a change in female behaviour (Klowden 2001). The success of male mating is determined by fitness, and this may have consequences for the number of times a male can mate. Obviously, this is determined by male size and feeding behaviour, and the efficiency of finding nectar sources. Aspects governing male fitness are poorly understood and appear to be difficult to estimate, in particular in the field (Charlwood 2003).

One of the most critical issues in mosquito mating is our lack of understanding of mate-finding. Many culicine species, characteristically, mate in swarms, when males aggregate in sometimes large numbers, forming nearly-cylindrical swarms of several metres height. This has been observed most notably in the genera *Anopheles*, *Culex* and *Ochlerotatus*, but species of other genera may also exhibit swarming (Clements 1999). Such swarms are often found in characteristic sites, presumably guided by a visual marker (Marchand 1984; Charlwood et al. 2002b; Yuval and Bouskila 1993; Charlwood, Thompson and Madsen 2003; Yuval, Wekesa and Washino 1993). It is unknown how males aggregate or what factors influence the sustenance of swarms. Even more intriguing is the fact that we do not know how females locate male swarms. Single females fly into the swarm and are detected by their lower wing-beat frequency (Belton 1994; Clements 1999). Several males may arrive near the female, which departs with one of them from the swarm *in copula*. Larger males were reported more successful in mating than smaller ones (Yuval and Bouskila 1993; Yuval, Wekesa and Washino 1993) although Charlwood et al. (2002a) showed that in *An. gambiae* Giles *s.s.* there was no effect of male body size on mating success. Intriguingly female body size has also an advantage in mate selection, larger females of *An. gambiae s.s*. being preferentially selected for mating (Okanda et al. 2002). It has been suggested that female swarm finding is directed by olfactory cues (Takken 1999; Takken and Knols 1999), perhaps, in addition, aided by the same visual cues that guide males to swarming sites. Many culicines mate near the vertebrate host, males of *Mansonia* spp. being attracted to host odours (McIver, Wilkes and Gillies 1980) and therefore being able to locate females in search of a blood meal. In

conclusion, other than proof of acoustic communication between the sexes, the behavioural process governing mating in mosquitoes remains a black box.

Whereas hybridization between closely related species has frequently been observed in the laboratory (Davidson 1964), such encounters are relatively rare in the wild (White 1971; Tripet et al. 2001). Apparently mating barriers exist, which serve to prevent coupling between related species and, hence, waste of resources. Nevertheless, Tripet et al. (2001) reported 1.2% cross-mating between two molecular forms of *An. gambiae s.s.* in Mali, demonstrating that cross-form hybridizations are not entirely excluded. As a rule female mosquitoes become refractory to male encounters following insemination (see above), but from laboratory studies it is well-known that female *An. gambiae* can mate several times (Charlwood and Jones 1979; Gomulski 1990). Field studies concerning this aspect are rare, but molecular techniques using genetic fingerprinting have now been developed that allow detailed study of this phenomenon. For instance, it was reported that in *An. gambiae s.s*. up to 2.5% of field-collected females had been inseminated by at least 2 different males, of which two-thirds had mated with males of the same chromosomal form (Tripet et al. 2003). As studies on genetic exchange between mosquito populations are important with regard to population genetics and behaviour, the extent of multiple matings needs to be considered as well.

Genetic control and mating behaviour

Past efforts for the genetic control of mosquitoes using the sterile-insect technique (SIT) have been less successful than expected, partially because of low degree of competitiveness between sterile and wild males (Lounibos 2003; Reisen 2003). Many mosquito species can be cultured in large numbers under controlled conditions, but due to genetic selection and loss of natural traits, such insects may behave differently from their wild siblings. Newly developed tools for genetic manipulation of mosquitoes rendering them refractory to human pathogens or altering host preference appear promising, in theory, as effective solutions for disease control (Ito et al. 2002; Besansky, Hill and Costantini 2004). However, the required establishment of laboratory cultures and subsequent genetic transformation of target mosquito species may result in insects with widely different mating behaviours compared to their wild siblings. Unless competitive ability and mating behaviour are adequately understood, the release of transgenic or sterilized mosquitoes may result in failures akin to those observed in several former SIT studies.

Challenges for future research

Mating in mosquitoes remains a poorly understood process. Yet, successful mating is critical for the success of proposed strategies for vector-borne-disease control using SIT or genetically modified mosquitoes (GMM). Some progress with studies on mating behaviour under field conditions has recently been reported with anophelines in São Tomé and Mozambique (Chambers and MacAvoy 2000; Charlwood et al. 2002a; 2002b; Charlwood, Thompson and Madsen 2003). However, such studies are few, and do not answer the question of how mating is accomplished and by which factors it is regulated. As insemination of wild female mosquitoes by released transgenic or sterile males is obviously a requirement for any genetic-control

programme, it is proposed that research focuses on the following aspects of mating behaviour:

- Cues that control male swarming
- Male feeding behaviour and fitness
- Female mate-location behaviour
- Pre- and post-mating behaviour
- Frequency of multiple-species swarming
- Genes that affect and/or regulate mating behaviour
- Factors that prevent hybridization of closely related species
- Factors that control multiple mating.

These aspects appear critical for a proper understanding of mosquito population biology and genetics. For instance, in population modelling of the behaviour of gene transfer between GMM and wild populations, the frequency of wild versus GMM matings should be well understood in order to predict the number of released individuals required for effective results. Also, SIT programmes require a constant monitoring of wild versus sterile matings to adjust the release rate over time. Finally, any driving mechanism of foreign DNA into wild populations requires a normal mating behaviour, and can only be evaluated once this behaviour is properly understood.

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18

Pathogen evolution issues in genetically modified mosquito vector strategies

Mario H. Rodriguez[#]

Abstract

In this paper, pathogen evolution will not be considered in the extended concept of adaptation through mutability, heredity and long-term adaptation through speciation. In contrast, the main issues deal with aspects related to polymorphism and population diversity that arise by selection processes and how these may influence vector–pathogen–human relationships. Three aspects related to these relationships will be presented in order to discuss the possibility that a reduction of the vector capability to transmit malaria could result in the selection of parasites. First, aspects of population size and diversity of *Plasmodium* (with emphasis on *P. falciparum*) relevant to epidemiology and host interactions will be presented. Next, *Plasmodium*–vector molecular interactions, determinant of infectivity, will be reviewed; these could determine the efficacy of a trait introduced by the genetic modification of the mosquito and/or could result in selection of parasites resistant to the trait. Finally, the possibility of virulence shifts in pathogens as a result of the genetically introduced traits in mosquitoes will be discussed.
Keywords: evolution; *Plasmodium*; population; mosquito vectors; molecular interactions; virulence shifts

Plasmodium population size and diversity

Genetic variation in malarial parasites has practical significance for control strategies based on the elimination of parasite transmission. Highly polymorphic molecular targets in the parasites could limit the efficacy of the mechanism introduced for their elimination. On the other hand, selective pressures imposed by the intervention could generate parasite mutants in highly plastic genome populations much easier than in reduced genetic diversity ones. There is an extensive contradictory literature on the variability of *Plasmodium falciparum*. The almost complete absence of silent nucleotide substitutions in coding sequences of nine genes (including the circumsporozoite protein (CSP)) and 25 introns of eight independent parasite isolates was interpreted to reflect a recent origin of the world's parasite populations (Rich, Hudson and Ayala 1997; Volkman et al. 2001); this would be inconsistent with extant polymorphisms in genes of ancient proteins (MSA-1, AMA-1) (Verra and Hughes 2000) as well as CSP, which originated before *P. falciparum*

[#] Centro de Investigaciones sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Av. Universidad 655, Col. Santa María Ahuacatitlan, Cuernavaca, Morelos, México 52508. E-mail: mhenry@correo.insp.mx

split from *P. reichenowi* (Hughes 1992; Dubbeld, Kocken and Thomas 1998; Okenu, Thomas and Conway 2000).

Others have identified over 400 polymorphic sites including 238 single-nucleotide polymorphisms (SNPs) and 165 microsatellites (Mu et al. 2002), and an effective population size of (N_e) 10^5 for the past 300,000-400,000 years was calculated through the analysis of the DNA sequence of 23 nuclear protein-coding loci on the basis that these were not subject to selection (Hughes and Verra 2001). This estimation is consistent with that obtained using microsatellite data ($N_e = 10^2$-10^4) (Anderson et al. 2000), indicating a large population with extensive possibilities for variation.

Several possible explanations point towards unique characteristics of the *Plasmodium* biology and gene protein structure to conciliate an extensive polymorphism in genes with specific functions and low rates on SNPs in non-coding regions. Compared to homologues in other organisms, *P. falciparum* proteins are longer because they contain low-complexity regions (Pizzi and Frontali 2001), these regions usually form tandem repeats located at non-globular domains of the open reading frames. *P. falciparum* genes have high A-T contents, a feature more pronounced in the repeat regions imposing limitations to the structure of transfer RNA, thus limiting the amount of nucleotide substitution that could maintain the viability of the organism (Forsdyke 2002).

On the other hand, analysis of polymorphism in non-repeat regions in the CSP protein indicated that these are not randomly distributed, but restricted to B- and T-cell epitopes (Rich, Hudson and Ayala 1997) indicating that these result from strong natural selection. Interestingly, haplotypes in the two non-repeat regions of the protein correlate with one another, but not with the intervening repeat region, which may indicate the clonality of the parasite population structure.

Beside an extensive effective population size, the plasticity of the *Plasmodium* genome and the effect of selection were documented by analysing 342 highly polymorphic microsatellite markers (Wootton et al. 2002) in chloroquine-resistant parasites. This analysis indicated at least four geographically different founding events, and extensive linkage disequilibrium surrounding the *ofcrt* (resistance-encoding gene) that occurred over only 20-89 parasite sexual generations (~6-30 years).

Thus, for the purpose of this discussion we can state that the *P. falciparum* parasite origin is ancient with a large effective population size. Although the parasite has stringent constraints with respect to mutation, its plasticity confers this pathogen the possibility to adapt to a variety of conditions in its vertebrate and insect hosts. This plasticity is reflected in the range of mosquito genera that could transmit *Plasmodium* and the wide range of anophelines that do transmit malaria to humans (Bruce-Chwatt 1985); moreover, it indicates that during evolution accumulated mutations in the parasites have enabled them to explore and exploit new niches with different conditions requiring molecular modifications for the interactions with the complex histological structure and defence responses of mosquitoes.

Plasmodium-vector molecular interactions as determinants of infectivity

Much information on antigenic polymorphism and antigenic variation exists for parasite stages present in the vertebrate host, but little is known about possible adaptations to modulate the parasite interactions with their mosquito vectors. Understanding the biology of ookinete and sporozoite invasion and survival is

necessary to design the topological and temporal expression of introduced resistance genes. However this will not be examined at length here, yet some simplifications will be necessary to present some aspects of parasite development for the sake of a concise discussion.

Malaria parasites develop in a complex, compartmentalized milieu in the mosquito (Shahabuddin and Costero 2001). Survival in the blood meal bolus imposes a bottleneck in the ingested population. Invasion of the midgut epithelium and salivary glands requires molecules specialized for motility, epithelium recognition and survival. Thus the main characteristics of molecules required for the interaction of parasites with their host relevant for our discussion are: a) the variability of those molecules involved in cell recognition for invasion, and b) their possible variations to avoid the immune response.

Around 10-15 parasite molecules have been identified on the surface of ookinetes, but two proteins, P25 and P28, predominate and are very similar in rodent, bird and human malaria parasites (Kumar and Carter 1985; Kaslow et al. 1989; Paton et al. 1993; Tsuboi et al. 1998). These proteins, containing epidermal growth-factor domains, protect ookinetes within the midgut contents (Grotendorst and Carter 1987), and participate in interactions with the peritrophic matrix (Sieber et al. 1991) and the basal lamina (Vlachou et al. 2001). The most interesting characteristic of these proteins relevant for our discussion is that they have partially redundant functions and the exclusion of only one of them does not abrogate invasion (Tomas et al. 2001). Molecule receptors on the midgut surface could be sialic-acid-like carbohydrates (Zieler, Nawrocki and Shahabuddin 1999), but other glycoproteins with N-acetyl-glucosamine residues have been implicated (Ramasamy et al. 1997). Taken together these indicate that, as in the case of merozoites, parasite mosquito stages have developed mechanisms to better exploit variable conditions in potential mosquito vectors.

Non-synonymous nucleotide substitutions were documented in two proteins participating in the interaction of sporozoites with mosquito salivary glands and hepatocytes: thrombospondin-related anonymous protein (TRAP) and CSP (Hughes 1991; Hughes and Hughes 1995) might be the result of immune pressure in the vertebrate host. However, regions of genetic polymorphism have also been identified in both P25 and P28 molecules (Tsuboi et al. 1998), indicating that although these proteins are not under the immune pressure of the vertebrate host, genetic variation occurs; whether or not this has an effect on protein function and parasite selection resulting from unidentified mosquito factors remains unknown.

Except for sporozoites that remain for relatively prolonged periods in salivary-gland ducts, developing *Plasmodium* in mosquitoes stay for a short while inside the individual mosquito compartments. These parasites are not under the immune pressure faced by blood stages (Bull 1994). These stages have developed antigenic variation mechanisms (Beeson and Brown 2002; Recker et al. 2004; Craig and Scherf 2001; Blythe, Surentheran and Preiser 2004). Nevertheless, parasites encounter diverse protective mechanisms in mosquitoes, some of which render most anopheline species resistant to malaria infection (Bruce-Chwatt 1985); among those susceptible species, very few mosquitoes are found infected in the field (Haji et al. 1996). Even more, those that are reputedly good vectors block more than 99.9% of ingested parasites (Vaughan, Noden and Beier 1992).

As mosquito defence mechanisms are multiple, and their induction and regulation have only recently begun to be deciphered, these mechanisms will only be mentioned here to highlight the possibilities of parasite evasion mechanisms. Nitric-oxide

induction during ookinete migration through the epithelium induces destruction of the invaded cell (Han et al. 2000) and probably reduces infection, but surviving parasites may have evolved detoxification mechanisms, as it occurs with superoxide dismutase, that block oxygen species (Bécuwe et al. 1993). Interestingly, two C-type lectins induced in mosquitoes during parasite invasion prevent ookinetes from developing into oocysts by inhibiting parasite melanization (Osta, Christophides and Kafatos 2004). Concomitantly, immune responses (Richman et al. 1997; Dimopoulos et al. 1998; Gorman, Andreeva and Paskewitz 2000; Vizioli et al. 2001) are induced during malaria parasite development in mosquitoes, the *LRIM1* gene is activated during ookinete invasion and this is probably responsible for the activation of the innate immune response via TOLL-like receptors (Osta, Christophides and Kafatos 2004). Theoretically, surviving parasites may avert these defence mechanisms by lacking surface protein determinants that stimulate immune responses, and are therefore not recognized by the immune system, or secrete molecules that interfere with the immune response (Beerntsen, James and Christensen 2000).

Should we worry about mutation in the pathogens that could enable them to escape the control mechanisms introduced in the mosquito vector? There are very few empirical data that address the question of "what parasite traits are being selected in the process of natural selection?" or "which phenotypes or genes are targets of selection?". A few theoretical models address the mechanisms (including mutation or survival strategy, recombination or reproductive strategy) by which variations are generated and persist in *Plasmodium* populations. It is evident that the possibility of avoiding the newly introduced resistant traits in mosquito vectors will depend on the nature of these traits. The capabilities of parasites to avert the new weapons should be inferred by the extensive variability in mosquito susceptibility to malaria parasites, indicative of the wide possibility of strategies these ancient parasites posses.

Virulence shifts in pathogens as a result of the GM mosquitoes

Virulence may be defined as the severity of the effect of infection on host mortality. In the case of *Plasmodium*–vector interactions, the pathogen virulence will have an effect on lifetime reproductive success (survival and fecundity) of the invaded mosquitoes. The general theory on the evolution of virulence assumes that selection tends to increase the parasites' basic reproductive rate (R_o). If the rate of transmission is linked to virulence, selection may result in intermediate levels of virulence, but ever-increasing virulence may also result (Anderson and May 1979). Individual selection within parasite populations will favour whatever level of virulence maximizes their R_o (May and Anderson 1983a). When competitive exclusion occurs among populations, only the strain with maximum R_o can survive under general conditions (Bremermann et al. 1989), and selection will always favour the most virulent strain (Bremermann and Pickering 1983). On the other hand, factors determining the effect of the infection on the host, also determine the final outcome on the evolution of virulence.

Conventional wisdom (May and Anderson 1983b), supported by empirical observations (Levin and Eden 1990), states that virulence evolves towards less host-harmful parasites. But trade-off between transmission and parasite-induced host mortality leads towards an intermediate level of virulence (the enlighten theory). This is also predicted by simple models (Lenski and May 1994), and stable virulence will occur when the increased transmission becomes increasingly costly in terms of increased virulence. These theoretical considerations should be taken into account in

assessing the possibilities of shifts in virulence in natural infections and in GM vectors.

Plasmodium infections in mosquitoes are present as a mixture of parasite genotypes (Taylor, Walliker and Read 1997). When clones share resources the population dynamics of individual clones are affected by the presence of the others (Read and Taylor 2001). Competition can affect transmission success (fitness) of individual clones, thus shaping the evolution of virulence. This competition will also determine the fate of new mutants arising in the course of infection. Very little is known about parasite clonal competition in mosquitoes, but some insights could derive from observations in infections in humans. The number of clones present in older children and adults has no effect on parasite titres (Smith et al. 1999) indicating that clonal densities within hosts are not regulated independently, but density-dependent clonal diversity also occurs (Arnot 1998). Also, fewer clones are present in symptomatic patients than in asymptomatic infections, indicating competitive suppression (Mercereau-Puijalon 1996). Finally low parasite turnover in areas of low malaria transmission indicates that competitive exclusion also occurs (Daubersies et al. 1996). Thus, it is likely that clonal competition will also occur among parasites developing in mosquitoes (Taylor, Walliker and Read 1997).

In infections with related parasite strains, evolutionary stable virulence occurs if there is a trade-off between virulence and infectivity (Frank 1992). The final outcome of altering the parasite's genotype composition as a result of diminishing the force of infection (of some clones) of parasites in genetically modified mosquitoes will depend on whether virulent parasite species/strains will adaptively adjust their fitness in response to the change in mosquito genetic makeup, in the same way as they do in natural vector populations, and whether the selected parasites clones and the underlying clonal competition will increase or decrease transmission. More virulent strains may have intra-host competitive advantages, but may kill the host, while less virulent strains may have an inter-host advantage, because they are less harmful to the host and are transmitted for longer periods (Nowak and May 1994). We know little about fitness differences among *Plasmodium* populations across different endemic zones or even in the host. The same occurs on the ability of clones to produce gametocytes (that reflects parasite fitness) and to infect humans.

In nature, the hosts typically live in partially structured populations with transmission events occurring locally. The epidemiology of malaria could be considered a complex case of metapopulation ecology (Anderson 1991). Ecological patches are represented by areas of disease transmission, and each vertebrate and mosquito hosts as subsections of these patches. This puts a limit on the transmission rates of pathogens even without a trade-off with virulence (Rand, Wilson and McGlade 1994; Haraguchi and Sasaki 2000). A further clustering of resistant hosts (introduced by transgenesis) may have important implications for the invasion of recently evolved pathogen strains and their relative fitness. A pathogen that is transmitted very quickly may be more aggressive not only to non-resistant individuals but probably also to those with the resistant trait (Boots, Hudson and Sasaki 2004).

On the other hand, inhibition of transmission by the introduction of GM resistance traits in mosquitoes results in destruction of some but not all the ecological patches. Ecological models of competition indicate that patch destruction could lead to an increase in the number of patches, occupied by inferior parasite competitor (reviewed by Read and Taylor 2001). The outcome could be an increase in disease prevalence. But all this will depend on the established clonal competitiveness resulting from the intervention.

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Models to investigate some issues regarding the feasibility of driving refractoriness genes into mosquito vector populations

Christopher F. Curtis[#]

Abstract

A genetic driving system extremely closely genetically linked to a refractoriness gene is needed if such genes are to be of any use in the control of vector-borne disease. *Wolbachia* cytoplasmic symbionts and/or appropriate factors from *Wolbachia* incorporated into the mosquito genome may be usable as driving factors. Maximal fitness of refractoriness factors is needed, otherwise any genetic recombination between the refractoriness factor and the driver can be shown, by simple models, to lead ultimately to fixation of the driver no longer linked to the refractoriness factor. Models can also show the serious impact of non-isolation of the target wild population and incompleteness of the refractoriness.
Keywords: driving systems; refractoriness genes; linkage; *Wolbachia*; fitness; immigration

The need for a driving system

It is likely that *Anopheles* strains that have been genetically engineered to be non-susceptible (refractory) to *Plasmodium falciparum* will soon be available. It is sometimes implied that the production of such strains will instantly provide a new method for controlling malaria. However, in fact the production of the genetic constructs giving refractoriness is only a beginning, and the much larger problems will still have to be solved of how to drive these constructs into very large wild vector populations and establish them there stably in such a way that they actually have a worthwhile impact on prevalence of malaria infection in humans and on incidence of morbidity and mortality.

Theoretically one could introduce a high frequency of refractoriness genes by prolonged mass release of males carrying these genes. However, as with release of sterile males, this method would require mass rearing facilities and would be very vulnerable to reversal by the effects of immigration of wild-type females, already mated to wild-type males outside the release area and unwilling to re-mate on arrival in the release area. Use of a mass rearing facility for sterile males would actually be more efficient than for a strain only carrying a refractoriness factor because – as pointed out by Knipling (1955) – if one begins to succeed with sterile males, the target population would decline and the ratio of released males to the residual wild population would become more and more favourable. It is likely that the problem of

[#] London School of Hygiene and Tropical Medicine, London, UK. E-mail: chris.curtis@lshtm.ac.uk

immigration could be minimized by targeting urban vector populations on geographical islands or ecological 'islands' where the same vector species does not exist in the surrounding area. If there is a serious level of disease transmission in such an urban area this could be a very worthwhile approach, as a release programme covering a given urban area would protect far more people than a similar effort to cover a similar sized rural area.

Africa's malaria problem is mainly a rural one and to hope to deal effectively with this vast problem by introduction of refractoriness genes will require that these genes are tightly linked to driving factors which will lead to increase in gene frequency and spreading from small 'seeding' releases as well as 'resistance' to the effects of immigration. Appropriate types of transposons or *Wolbachia* symbionts are the currently favoured forms of driving factor.

The mechanism of action of *Wolbachia*

Wolbachia is maternally transmitted and has evolved the capacity to inactivate sperm of many host male insects and also to 'rescue' sperm activity after the sperms are introduced into females. Thus, matings within uninfected and within infected stains are fertile and self-reproducing and matings of infected females to uninfected males are fertile and yield infected progeny. However, matings of *Wolbachia*-infected males to uninfected females are sterile because the sperms are inactivated by the females and not 'rescued' by the female. Thus the infected state tends to be selected for and has indeed been observed to spread in *Drosophila* (Turelli and Hoffmann 1995). Figure 1 shows a method of calculating the outcome of random mating between infected and uninfected strains and including the effect of immigration of already mated uninfected females from outside the area (Curtis and Sinkins 1998).

		Male parents		Uninf. migrants	Total
		Uninf. (q)	Inf. (p)		
Female parents	Uninf. (q)	q^2	sterile	m	$q^2 + m$
	Inf. (p)	pq	p^2	–	$p^2 + pq$
				TOTAL	$1 - pq + m$
q at following generation = $(q^2+m) / (1 - pq + m)$					

Figure 1. Method of calculating whether the driving effect of *Wolbachia* infection can overcome the effect of immigration of uninfected mated females (Curtis and Sinkins 1998)

This model was applied to simulate successive generations after a single release. If there is no immigration, the frequency of uninfected insects is driven to zero. If there is an immigration rate per generation of 5% or 20% of the population, selection against the uninfected type can still be achieved by making initial releases of sufficient size. If immigration continues steadily, the driving effect of the *Wolbachia* comes to equilibrium with the immigration. Releases of only 10-40% of the wild population size, made once, were sufficient to start the process of selection for the released type and the 'resistance' to immigration should be contrasted with the massive and

prolonged releases and fatal effects of immigration with sterile-male releases aimed at eradication.

Genetic linkage of refractoriness factors to *Wolbachia*

If driving of *Wolbachia* into a population is to do anything useful it is necessary that a gene or transgenic construct for refractoriness to *Plasmodium* is inherited maternally, as *Wolbachia* is. Possibly the construct could be introduced into *Wolbachia* itself or into some other maternally inherited entity such as mitochondria. However, experience shows that maternal inheritance of such entities is not 100% reliable. If occasionally the construct is not inherited maternally there would be the possibility of production of 'recombinant' individuals with the driving *Wolbachia* but without the construct. It seems probable that a transgenic construct would usually entail some degree of fitness cost and the recombinants, with *Wolbachia* but without the construct, would therefore be the fittest type. Simulations by D.Campbell-Lendrum and P.Coleman (Curtis et al. in press) showed that a limited release could lead to almost 100% replacement by the *Wolbachia*-infected *Plasmodium* refractory type, despite the fact that the refractoriness causes a 20% fitness cost. However, they assumed that once in a million there is a failure of maternal inheritance of the refractoriness construct and, because of the relief from the burden of its fitness cost, the end result is fixation of the *Wolbachia*-infected *Plasmodium*-susceptible type, i.e. no sustained impact on malaria transmission. It would be impossible to carry out laboratory studies on a sufficient scale to exclude the possibility of one in a million failures of maternal inheritance. Some would argue that the assumption of a 20% fitness cost is too pessimistic, especially if it is arranged that the refractoriness construct is only expressed when *Plasmodium* are present. *Plasmodium* cause some damage to mosquitoes and refractoriness might contribute some advantage by preventing this damage (Boëte and Koella 2002). However, infection rates are low in wild populations and it can be supposed that if refractoriness contributed a net selective advantage it would already be the wild type in *Anopheles,* since surely the necessary mutations would have occurred in evolutionary history. The model of Campbell-Lendrum and Coleman is available to test the effects of more common occurrence of failures of maternal inheritance and of lower or zero fitness cost of refractoriness.

If refractoriness was successfully introduced into a vector population there would be intense selection on the *Plasmodium* population to evolve an evasion mechanism (as commonly occurs when new plant varieties are introduced conferring resistance to a plant pathogen). This might be made less likely by incorporating two or more independent refractoriness factors into the release strain. However, it should be recognized that this would multiply the problems of ensuring reliable linkage to the driving factor.

Sinkins and Godfray (2004) have pointed out that the technology for inserting transgenic constructs into *Wolbachia* is not yet available and that it might be more feasible to insert the *Wolbachia* factor which 'rescues' inactivated sperm into a mosquito chromosome (a 'nuclear rescue construct' or NRC). The refractoriness construct would then be closely linked to the NRC. They assume that *Wolbachia* could be injected into, and propagated in, *Anopheles* and that a release would lead to fixation of this strain in a wild population. Then it is assumed that a strain carrying an NRC linked to refractoriness is released resulting in fixation of the NRC (Sinkins and Godfray 2004). With their assumptions *Wolbachia* is selectively eliminated from the

population because the fixation of the NRC cancels the selective advantage of *Wolbachia* and the authors expect some failures of maternal transmission of this symbiont.

Reliability of refractoriness

Boëte and Koella (2002) investigated the conditions under which a transposon-refractoriness construct would go to fixation depending on the transposition frequency (i.e. conversions from heterozygosity to homozygosity for the transposon), the fitness cost of the transposon and of refractoriness and on whether the latter is conditional on the mosquito being *Plasmodium* infected.

Assuming that fixation of the transposon-refractoriness construct occurs, Boëte and Koella (2002) studied the extent to which prevalence of human malaria is, or is not, reduced, depending on intensity of transmission of malaria and efficacy of refractoriness. The conclusion is that there will be little or no benefit in terms of reducing malaria prevalence if transmission is very intense, and unless refractoriness is close to 100% effective in mosquitoes that carry this construct.

Conclusion

In the coming years the engineering of refractory *Anopheles* with driving factors will certainly be a rich source of NPS (*Nature* paper synthetase). However, the sobering conclusion emerges from the models that there are many things that could go wrong and prevent any real benefit for populations suffering from malaria. The main requirements for success are nearly isolated target populations, extremely reliable refractoriness factors, extremely reliable driving factors, and extremely close linkage between them. Calling for 100% reliability for any of these is probably unrealistic but, as data emerge on % reliability for each of these aspects, the above types of models, and other more sophisticated ones, should help judgments to be made about whether a given construct has sufficient promise to be worthwhile to scale it up for field trials.

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Identification and characterization of field sites for genetic control of disease vectors

Bart G.J. Knols[#] *and Hervé Bossin*[#]

Abstract

Genetic control of disease vectors consists of a large variety of approaches. Each of these bring along an often-substantial number of prerequisites in order to stand a chance of being successful. Of the large number of trials documented, only few have taken the scale and scope of operation to sustain long-term area-wide benefits. Broadly speaking, two categories of issues will be faced by any genetic control programme when moving from the laboratory to the field: those that relate to *biological* and/or *environmental* factors and those that relate to *stakeholder* power. The ongoing debate on genetically engineered crops has heightened the interest of many (often antagonistic) stakeholders in the possible impact that biotechnological advances may have on society and the environment, which necessitates a much larger impetus towards stakeholder management. Identification of suitable field sites for anticipated genetic-control trials therefore requires a stepwise approach, which is outlined in this chapter. A coordinating entity, consisting of stakeholders from disease-endemic countries (DECs) and (inter)national experts, is urgently needed to provide support to governments in their decision-making process regarding genetic-control trials. Such entity should also serve a mediating function between researchers, end-users and influential stakeholders (e.g. press) in order to further the potential of biotechnological developments. Failure to steer this process efficiently may result in substantial opposition and stalling of progress, similar to hindrances experienced with the introduction of transgenic crops in the European Union.
Keywords: genetic control; transgenesis; field site selection; biological variables; environmental variables; stakeholder management

Introduction

The ongoing development of genetically modified disease vectors (or their symbionts) for application in genetic-control programmes against major tropical diseases such as malaria (Ito et al. 2002; Alphey et al. 2002), dengue (Olson et al. 1996), and Chaga disease (Beard et al. 2002), is gradually advancing to a stage where scientists involved are planning field trials or, more in the short term, semi-field evaluations in contained near-natural environments. This gradual transition of research efforts from the laboratory to field environments raises a number of

[#] International Atomic Energy Agency (IAEA), Agency's Laboratories Seibersdorf, A-2444 Seibersdorf, Austria, and Laboratory of Entomology, Wageningen University and Research Centre, PO Box 8031, 6700 EH Wageningen, The Netherlands. E-mail: B.Knols@IAEA.org; H.Bossin@IAEA.org

important issues like ELSI (ethical, legal and social issues; see Macer (2003) and Touré and Knols (in press, Chapter 23), and also the selection of appropriate field sites. Both site identification and characterization are discussed in this paper.

A priori, it can be noted that previous genetic-control trials (e.g. employing cytoplasmic incompatibility (CI) (Laven 1967), chromosomal translocations (Curtis 1968; Laven 1972; Lounibos 2003) and the sterile-insect technique (SIT) (Knipling 1955; Klassen and Curtis in press), etc.), amongst other approaches, have addressed many analogous problems related to site selection and characterization, albeit without the genetic-engineering component that adds new and unique concerns. The following sections focus on GM mosquitoes, though most concepts apply similarly to other disease vectors.

Biological and environmental prerequisites

Laven's CI trials (1967) in Myanmar were conducted in a small village surrounded by rice fields, where the target pest (*Culex pipiens* fatigans) did not occur. In Kenya, genetic-control trials against *Ae. aegypti* in the 1970s focused on villages and a small area surrounding them (Lounibos 2003). The necessity for applying genetic control against isolated populations remains valid today. It has been proposed to target *Anopheles arabiensis* populations in urban areas surrounded by *An. gambiae s.s.* or urban *An. stephensi* populations surrounded by *An. culicifacies* (Curtis 2003; Kristan et al. 2003). Others have suggested going beyond 'ecological islands' described above, and move to physical islands. Genetic-control trials have delivered dramatic successes through eradication of target pests, such as the eradication of *Glossina austeni* from the island of Zanzibar (Msangi et al. 2000).

With regard to the application of GM approaches for disease-vector control, further containment (in terms of selecting isolated populations) is needed to overcome potential adverse effects of the introduction of GM insects. The choice for physical islands, far from mainland vector populations, seems the best option in that regard (G. Lanzaro, pers. comm.).

Beyond (1) geographic isolation, there are several more key factors affecting the selection of a field site and the target species, such as: (2) occurrence in a narrow geographic range and (3) appropriately sized area (small enough to be manageable, large enough to be convincing); presence of (4) panmictic populations (i.e. all individuals within the population are potential recombination partners) of (5) one vector species (although another closely related non-transmitting species may be useful as a 'control'); the target species occurs in relatively (6) low density and can be suppressed with existing vector control tools; and (7) disease transmission, in order to measure the public-health impact resulting from the intervention.

These biological prerequisites should serve as the first criteria when selecting a field site. Secondary criteria include (8) site accessibility and availability of research infrastructure, and the availability of (9) detailed entomological and epidemiological information.

Additionally, the release of genetically altered mosquitoes necessitates further precautionary measures as outlined by Spielman, Beier and Kiszewski (2002). In their view, releases should not be permitted unless: (1) the nuisance caused by released organisms remains lower than that caused by ambient vector organisms; (2) the release results in no increase in abundance of haematophagous arthropods; (3) the release requires no reduction in ongoing health-promoting activities; (4) the risk of transmission of microbes other than the target pathogen would not increase; (5) the

release does not compromise future interventions against the target disease; and (6) any improved state of health of people living in the release site is sustainable. In addition, a release must pose no environmental threat due to horizontal transfer of transgenes to predators or other organisms in the release site.

Clearly, both of the above lists result in only a limited number of potential field sites that may be suitable for proof-of-principle experimentation beyond the confines of the laboratory. Arguably, biological and environmental prerequisites should be given priority when identifying potential sites, as additional requirements (e.g. research infrastructure, local capacity and opportunities for collaboration) can be obtained and/or developed. Nevertheless, it should be clear from the onset that research efforts relating to the former are futile if the latter are insufficiently addressed. A perfect field site may be useless if consent from major stakeholders cannot be obtained. Ideally, therefore, both processes should be undertaken simultaneously.

Stakeholder management

Public outreach

Given the controversy that has already been associated with projects involving genetic engineering, it is reasonable to expect that future research efforts will draw the attention and concern of people who are not directly involved with the field studies (Gaskell et al. 2002). Studies on risks associated with large-scale technological and scientific activity are capable of attracting significant public opposition (Beck 1992), and it is becoming clear that involving broad sectors of the public in discussion, planning and even conduct of these activities may be the only effective way to cope with this challenge (National Research Council NRC 1996).

Community consent is essential to this, as to any attempt to release genetically modified organisms requires (Knols and Scott 2003). Given the absence of any existing mechanisms for assisting with this process (Pew Initiative on Food and Biotechnology 2005), investigators currently rely on approaches used elsewhere, adapt them to local culture and introduce them through national counterparts and authorities. The ethical component of forthcoming projects relates to both potential risks and public perception and opinion of the project and its research components. Regretfully, the ongoing process of identifying potential field sites currently lacks a policy framework that adequately protects against possible public-health and environmental risks. Bearing in mind the negative outcomes of certain (anticipated) massive releases of mosquitoes in the past (Desowitz 1993; Oh New Delhi, Oh Geneva (editorial) 1975; WHO 1976), a stepwise approach toward the residents of potential study sites needs urgent development.

Oversight mechanism

Considering the fact that various research groups are actively seeking collaboration in potential field sites, and given the fact that research activities in preparation for possible releases are underway (see for instance Chen et al. 2004), absence of oversight of these activities may harm the entire endeavour if inadequately conducted. A clear outcome of the Nairobi meeting (see Chapter 1) was the recognized need to oversee and coordinate field-related research. It was proposed to set up a steering committee (Figure 1) that will consist of GMO experts, public-health entomologists, representatives of ministries, social scientists and ELSI experts, WHO regional and Geneva representatives, etc.

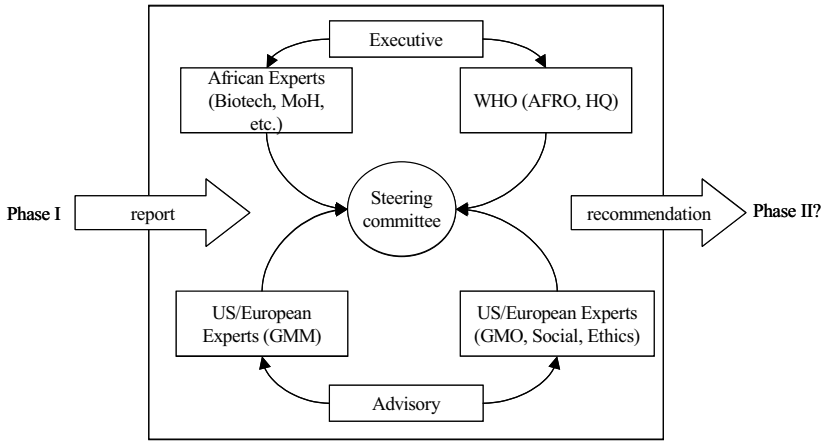

Figure 1. A proposed mechanism to guide the transitional phases for implementation of genetic-control trials from laboratory to field settings

The group, which should remain impartial and without conflict of interest, could have its members assigned to either the executive or advisory part of the committee. Larger representation of disease-endemic country representatives with executive power should be foreseen as a means to transfer 'ownership'. This expert panel should review project progress at defined intervals and have the penultimate decision-making power to endorse phase advancement (Phase I (laboratory cage) to II to III trials (semi-field systems in tropics) based on written documents (for more details of this process, see Chapter 16). The committee, in turn, would justify its decisions by reporting to the governments of the countries involved, funding agencies, and other relevant stakeholders they identify. Further justification for this approach has recently been published (Mshinda et al. 2004; Pew Initiative on Food and Biotechnology 2005) and has significant analogies with the (former) African Malaria Vaccine Testing Network (now residing under the African Malaria Network, AMANET) that oversees and evaluates all vaccine trials in Africa.

Perhaps the most significant challenge in terms of GM insect development and implementation is the full participation of and genuine collaboration with partner institutions in disease-endemic settings that meet the above criteria. For instance, it is likely that the suitability of certain island settings will be countered by the absence of appropriate institutional frameworks and local competence. In view of this, it seems appropriate to develop more generic guidelines for GM insect implementation irrespective of the field site/country involved. Again, the regulatory framework proposed above seems best suited for this task (Mshinda et al. 2004).

Field site characterization

Specific research topics related to field site selection and characterization should follow an evaluation of the existing human resources and scientific/public-health infrastructure at the chosen sites, and include:

(1)collection of basic ecological and biological data of the target species, including relative population densities, (adult and larval) distribution patterns and an

assessment of the role of the target species in disease transmission (Touré et al. 1998; Scott et al. 2002; Taylor and Manoukis 2003; Billingsley et al. in press);

(2) an assessment of the degree to which the target population is genetically isolated from surrounding populations and a description of the genetic structure of the population (see Chapter 14 for details and methods);

(3) Creation of a geographic information system (GIS) that describes the ecology of each site and is fully integrated with information from the ecology and population-genetics studies.

It is important to note at this juncture, that these activities benefit not only planned genetic vector-control interventions, but any vector-control programme. Essential though, is recognition of the fact that the tools for this kind of field research are available and that field site identification and preparation is therefore a key activity underpinning the transition of research from the bench to the field.

Conclusions

Given the long list of prerequisites that determine selection of appropriate field sites for trials to evaluate GM insects in Phase II and III settings (Chapter 16), it is unlikely that many such sites will be identified. Currently this selection process is not monitored and in the hands of individual scientists. Given the intricacies of this process and the difficulties associated with stakeholder management, both general guidelines and an oversight mechanism are urgently needed. Previous investment in the development of GM insects warrants careful progression towards moving technology and capacity to DEC settings. Failure to do so may result in significant delays or even blockage to evaluate the full potential of these approaches to curb some of the world's most debilitating diseases.

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Application of genetically modified mosquitoes in national vector control programmes: thoughts on integrated control

Abraham Mnzava[1]*, Chris F. Curtis*[2]*, Lucien Manga*[3] *and Yeya T. Touré*[4]

Abstract

Advances in transgenic technology have allowed the development of genetically transformed insects that have reduced ability to support the development of disease pathogens. The integration of this new method within national vector control programmes is indeed the biggest challenge, notwithstanding the current weak health systems in most disease-endemic countries (DECs) to efficiently apply vector control interventions. Moreover, where integration is considered, it is essential that reliable data are available on the multiple effects of the interventions. This should be done in parallel to the general strengthening of both human, technical, financial and physical resources at all levels of the national health system.
Keywords: genetically modified mosquitoes; integrated vector management; transgenesis; vector control programmes

Introduction

The long-term goal of research on genetically modified mosquitoes is to develop strains that are unable to support the development of human pathogens and then introduce and drive such a trait into natural insect vector populations. Progress has been achieved in two important disease vectors – *Anopheles stephensi* (vector species of malaria in South Asia) and *Aedes aegypti* (vector of dengue and yellow fever) (Moreira et al. 2002). In *An. gambiae*, on the other hand, germline transformation has been accomplished but not yet with any potential useful effects.

Knowledge from the work on *An. stephensi* and *Ae. aegypti* has allowed the construction of refractory mosquitoes with the potential to be extended to other vector species and to other important biological and behavioural traits that are relevant in vector disease transmission. For example, the development of a 'genetically transformed' mosquito that no longer has a preference for human blood would be a breakthrough (Coluzzi and Costantini 2002). The biggest challenge, however, is how and when such a tool can be integrated into existing national vector control programmes.

[1] Vector Control, Eastern Mediterranean Region of WHO, Abdul Razzak Al Sanhouri Street, Cairo 11371, Egypt. E-mail: Mnzavaa@emro.who.int
[2] London School of Hygiene and Tropical Medicine, London, UK. E-mail: Chris.curtis@lshtm.ac.uk
[3] Vector Biology and Control, African Regional Office of WHO, Harare, Zimbabwe. E-mail: Mangal@who.int
[4] Molecular Entomology, WHO-TDR, Geneva, Switzerland. E-mail: Tourey@who.int

A global trend – from disease-specific to integrated control

From a programmatic point of view, the current global trend is to move away from vertical or disease-specific programmes. One such approach in vector-borne disease control is Integrated Vector Management (IVM). This is an approach that emphasizes the promotion of inter-sectoral action for health as well as the synergistic impact of various interventions targeting more than one vector-borne disease where this is feasible (WHO 2004a). Whereas a number of countries in the African, Asian and the Eastern-Mediterranean regions of WHO are now adapting this strategy, there is still a lot to be done to ensure an effective integration of vector-borne disease programmes in each country (WHO 2001; 2004b).

Generally speaking, the integration of genetically modified mosquitoes into current vector control programmes will be faced with problems that are inherent to any conventional control tool, be it indoor residual spraying (IRS) or the use of insecticide-treated bednets (ITNs) (De Savigny 2004). As hinted above, the problem does not relate to the tools but, rather, the availability of a vector control system already in place to deliver the control tools in an alternative and sustainable manner. This includes strengthening of institutional, human and technical capacity at country level.

Specific challenges with respect to genetically modified mosquitoes

Before the integration of genetically modified mosquitoes into control programmes can be considered, there are still some important challenges and risks that need to be addressed. These include the unanticipated phenotypic changes resulting from transgenic alteration by effector genes and drive systems in a wild population and the unanticipated epidemiological impact/effects of control programmes (Alphey et al. 2002). There is indeed no proven technique to drive a 'refractory construct' into a field population, and this area requires further investigation. Moreover, both epidemiological and entomological risks must be assessed separately before releases can be performed (Benedict and Robinson 2003).

For early testing of mating competitiveness of transgenics, radiation sterilization may be used as a precaution against any unforeseen harmful effects of transgenic mosquito releases (Krafsur 1998). If and when questions about competitiveness and harmful effects have been resolved it will be necessary to proceed to non-sterilized mosquito releases to initiate the spread of transgenes in wild populations.

The introduction of transgenics to areas where other control measures such as ITNs and IRS are in place, might be thought to pose a problem. There is evidence from the field that male anophelines enter houses and would be vulnerable to killing by ITNs or IRS (Marchand 1985). However, this could only compromise the impact of the release technique where integration is proposed if, for some reason, the insecticidal method had proportionately more effect in killing the released males than the wild males. Such an effect does not seem very likely but should be considered.

Whereas current global initiatives are being explored on how best to apply laboratory advances in the field, conventional vector control programmes have many of the appropriate skills and facilities to participate in a vector control programme using genetically modified mosquitoes. The requirement for successful application of this method is indeed the ability to deliver to the field, over large areas, large numbers of sexually active genetically sterile males. Which control programme has such capacity?

If transgenics are to be integrated with conventional control it is essential that reliable data are available on the effects of the conventional control methods so that the additional effects of the transgenics can be accurately assessed. It may be noted that in earlier trials of genetic control, larvicides were used to attempt to create barrier zones around release areas in order to minimize the effects of immigration. Such procedures may also be necessary with trials of transgenics unless these focus on distinct and geographically or ecologically isolated populations.

Strengthening the capacity of control programmes

Vector control programmes intending to include genetically modified mosquitoes as one of the control options need to ensure the following:

1. A national vector control focal point with a broad understanding of vector control tools and basic entomology and ecology.
2. The vector control focal point must be supported by a core group of experts, either available within the control programme, or available within national research and academic institutions. This group will be responsible for the implementation of this method and for monitoring and evaluation of impact and potential risks.
3. In mosquito-borne disease-endemic areas earmarked for control using genetically modified mosquitoes, there must be facilities for the large-scale rearing of mosquitoes (insectaries and field laboratories).
4. An accurate assessment of the availability of reliable data on the effects of the conventional interventions and the additional effects of the transgenics is needed.
5. The integration of these new advances to currently weak health systems will require coordination and support from both national and international entities.

Conclusions

Most national vector control programmes are still struggling to effectively deliver conventional control tools/interventions such as the indoor residual spraying of houses with insecticides or the use of insecticide-treated bednets. Integrating the application of genetically modified insects (mosquitoes) with the current control programmes would require the general strengthening of the health system through IVM. To achieve this would need, among other things, additional resources – human, technical, financial and physical infrastructures.

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Entomological correlates of epidemiological impacts: how do we know it is working?

Andrew K. Githeko[#]

Abstract

Assuming that transgenesis of malaria vector populations is feasible, it will be necessary to carry out entomological, morbidity and mortality trials to measure the degree of efficacy of this new intervention strategy. Taking into consideration the necessity to maintain reproductive fitness of a transgenic mosquito, the best strategy would be to target the sporogonic cycle and/or reduce the anthropophilic feeding behaviour of the vectors. The former would impact on the entomological inoculation rate (EIR) whereas the latter would impact on vectorial capacity. A three-phase trial would be carried out to test safety, efficacy and impact on morbidity and mortality. After confirmation of safety and efficacy, phases two and three would involve large-scale multiple site trials.
Keywords: transgenic; *Anopheles gambiae*; entomological inoculation rate; vectorial capacity; efficacy

Introduction

Assuming that it will be possible to produce a *Plasmodium*-refractory variety of *An. gambiae* through transgenesis, a number of options are available to reduce the vectorial efficiency and transmission competence. Currently, barriers to genetic transformation include, among others, gene-driving mechanisms, maintenance of gene frequency and stability of the effector constructs (Ghosh, Moreira and Jacobs-Lorena 2002).

An important property of a transgenic mosquito is its reproductive fitness compared to that of wild-type conspecifics (Moreira et al. 2004). It must be noted that natural selection has resulted in the survival of very fit varieties (inversion karyotypes) of *An. gambiae* with reference to vector habitats and bioclimatic zones.

In assessing the entomological correlates of the epidemiological impacts of transgenic forms of *An. gambiae* we must recognize that only reproductively fit transgenic individuals will have a chance to compete with the wild counterparts. Thus, while it may be possible to construct mosquito that have genes for low fecundity, this variety would be unable to compete with the wild types (Irvin et al. 2004; Moreira et al. 2004).

The current genetic modification strategies that probably have the least effect on fitness are those that focus on blocking the penetration of the midgut and salivary glands by sporozoites. The result is females that cannot support the sporogonic cycle

[#] Kenya Medical Research Institute, Centre for Vector Biology and Control, Climate and Human Health Research Unit, P.O. Box 1578, Kisumu 40100, Kenya. E-mail: AGitheko@kisian.mimcom.net

(Ito et al. 2002; Moreira et al. 2000). In terms of transmission this could result in a great reduction in the entomological inoculation rates and, consequently, of malaria transmission.

A second strategy would be to change the blood-feeding preference of *An. gambiae* from man to animals. Some of the sibling species of the *An. gambiae* complex such as *An. arabiensis* and *An. quadriannulatus* can be highly zoophilic. But this would assume that animals and predominantly bovids would always be available (Pates et al. 2001).

Entomological correlates

Assuming the two strategies indicated above can be achieved, a number of impacts can be expected on the vectorial capacity and entomological inoculation rates. Vectorial capacity is the future daily sporozoite inoculation rate arising from a currently infective human case, on the assumption that all female mosquitoes biting that person become infected. It is the product of the vector density in relation to man, the proportion that bite man twice, and the expectation of the infective life span of the vector (MacDonald 1957; Garrett-Jones and Shidrawi 1969).

Vectorial capacity is mathematically expressed as:

$$VC = \frac{Ma^2 p^x}{-\ln p}$$

where
M = man-biting rate or vector density in relation to man
a = the daily man-biting rate
p = daily survival rate
x = duration of the sporogonic cycle.

Expectation of the life span of a vector:

$$\frac{1}{-\log p}$$

Expectation of the infective life span:

$$\frac{p^x}{-\log p}$$

Entomological inoculation rate (EIR):
EIR = Mas

where M and a are as defined above and s is the sporozoite rate.

Effects of reducing sporozoite rates on vectorial capacity and EIR

Sporozoite infection rate terms are not included in the vectorial capacity equation, thus a reduction in sporozoite infection rates would not be expected to have any impact on vectorial capacity. It should be noted that the vectorial capacity assumes that all female mosquitoes biting an infective human case will become infected. This assumption would be invalid for transgenic mosquitoes that are resistant to *Plasmodium* infection. A decrease in sporozoite rate would however, have a

substantial impact on the EIR. Nevertheless in areas of holoendemic malaria transmission this may not lead to a proportional decrease in disease prevalence because the level of transmission is several-fold greater than what is required to maintain prevalence at a certain (high) level. For example, a study in Tanzania showed that where the mean annual EIR was 34 infective bites per person, mean annual parasite prevalence ranged from 33% to 76%, whereas in an area where the mean EIR was 405 the prevalence ranged from 80% to 84% (Ellman et al. 1998). Consequently a 91.6% reduction in EIR led to an 8-51% reduction in malaria prevalence. In a vector control study in Kenya using permethrin-impregnated sisal curtains a 72% reduction in EIR reduced malaria prevalence by only 10% (Oloo et al. 1996).

Effects of changes in human blood-feeding behaviour and daily man-biting rates

The human-feeding rate term occurs twice in the vectorial capacity equation, thus changes in this parameter would be expected to have an impact on the ability of the vector to transmit diseases. A good example is *Anopheles arabiensis*, which, in certain areas, has a high preference for feeding on animals. This leads to a low mean annual sporozoite rate of about 1-2% compared to *An. gambiae* which preferentially feeds on humans and consequently shows a mean annual sporozoite rate of 6% (Githeko et al. 1993; 1994; Taylor et al. 1990). A reduction in human blood-feeding and the daily man-biting rate would have a substantial impact on EIR because the reduction would concurrently lead to lower sporozoite rates.

Survival rates

Classical vector control programmes using residual insecticides have two aims: killing vectors and reducing their longevity. Survival rates are a useful measure of the impacts of an insecticide on malaria transmission. However, low survival rates would be associated with a poor reproductive capacity of the transgenic mosquitoes.

Epidemiological impacts

The epidemiological impacts expected from any entomological intervention are a reduction in parasite prevalence, incidence, morbidity and mortality. Most of the published studies refer to interventions that reduce man-biting rates as a result of the application of residual insecticides or impregnated bed nets. The impact of a reduction in man-biting rates or sporozoites may be best observed by comparing transmission in areas that have similar densities of *An. arabiensis* and *An. gambiae.* Unfortunately *An. funestus* occurs frequently in habitats occupied by the two former vectors. It is therefore difficult to determine the epidemiological impact of an inefficient vector such as *An. arabiensis* versus that of *An. gambiae*, a much more efficient one.

The rate of epidemiological impact would depend upon the rate of gene drive in the wild populations. Ideally the gene would be driven to fixation. The big question, therefore, is how to know whether or not this intervention is working. It is assumed that the trial would take a similar design to malaria vaccine trials, which normally have three phases. There are two possible end points for phase-I and -II trial, namely a

significant reduction in man-biting and human blood-feeding rates and a significant reduction in EIR.

Phase I would entail a safety and efficacy trial in the laboratory to demonstrate that significant and preferably full transmission blocking occurs. In these trials it should be demonstrated that there is no increased chance of enhanced transmission of other (vector-borne) diseases, including arboviral infections, by the transgenic mosquitoes. This phase should also include life-table studies to evaluate mosquito fitness and might include competition experiments.

Phase II would be a limited field trial, possibly in a greenhouse and/or in an isolated small island where natural vector populations exist. It would be necessary to demonstrate that gene flow is occurring at an acceptable rate and stability is maximized. Phase-II studies should also evaluate the potential for horizontal gene transfer.

Phase III would involve a full field-scale trial where, after success in phase I and II, the efficacy of the intervention tool would be assessed at vector population level and subsequently in terms of human disease outcome. Ideally this would be a multiple-site trial where all climatic and other environmental parameters would be taken into account. The end point of this trial would be a reduction in parasite prevalence and disease incidence. This stage would be challenged by many ethical and political issues.

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Ethical, legal and social issues in the use of genetically modified vectors for disease control

Yeya T. Touré[#] *and Lucien Manga*[##]

Abstract

Genetic control of vectors may have an important role to play in the interruption of vector-borne disease transmission if the main biotechnological and implementation challenges are adequately addressed. Following the demonstration, in the laboratory, of the technical feasibility to develop transgenic mosquitoes unable to transmit malaria and dengue pathogens, the following actions will need to be taken in order to make this approach a control method applicable for public-health purposes: establish a proof of efficacy and safety to be approved by authorized biosafety and regulatory bodies before any experimental release; ensure the public and the media that this goal is desirable, feasible and can be accomplished safely; develop a plan to gather all the information necessary for legal and regulatory approvals; design a monitoring system for early detection and evaluation of adverse outcomes and plan strategies to remedy their effects; develop mechanisms for dissemination of information; enhance capacity in disease-endemic countries, promote research partnership and create an international consortium for genetic control of disease vectors to coordinate research activities and suggest future directions.
Keywords: vector-borne diseases; genetic control; efficacy; Biosafety assessment; Ethical, legal, social issues; Interruption of transmission

Current state of the art

The technical feasibility of the development of transgenic mosquitoes unable to transmit malaria and dengue pathogens has been demonstrated in the laboratory. *Anopheles stephensi* was made refractory to *Plasmodium berghei* growth and transmission (Ito et al. 2002) and *Aedes aegypti* was made resistant to DEN-2 virus replication and transmission (Olson et al. 1996). However, key issues remain to be addressed in order to make this approach a control method applicable for public-health purposes. They include biotechnological challenges dealing with the development of the tool, such as devising suitable gene driver systems and developing and evaluating appropriate effector gene constructs. They also consist of implementation challenges for the use of the tool in the field. The requirements to be considered before genetic control methods, in general, can be used in the field and refractory transgenic insect vectors, in particular, can be released into the wild, comprise the need to demonstrate a proof of efficacy and safety for humans and the

[#] Special Programme for Research and Training in Tropical Diseases (TDR), World Health Organization, 20 Avenue Appia, CH-1211 Geneva 27, Switzerland. E-mail: tourey@who.int
[##] Regional Advisor, VBC, WHO/AFRO, Harare, Zimbabwe. E-mail: mangal@who.int

environment, and to address Ethical, Legal and Social Issues (ELSI) (Alphey et al. 2002; Macer 2003). These matters represent important considerations to address in order to ensure the public about the efficacy and safety of the control strategy and develop an evidence base for policy decision-taking. Consequently, lessons are to be learned from the genetically modified food debate and previous experiments about genetic control of vectors in El Salvador and India (see Curtis, this volume, Chapter 3).

Issues and challenges

The main issues to consider include:

- *To provide a proof of efficacy and safety of the use of genetically modified vectors (GMVs) for disease control*

  Laboratory experiments and contained semi-field tests would need to be undertaken under different conditions and during a sufficient period of time to provide the evidence that the tool is efficacious and safe.

- *Biosafety assessment and management to minimize potential risks for humans and the environment*

  Proper safety assessment and management is an important basis for policy decision. It needs a strong scientific base such as the identification of scientific principles and practices for conducting safe laboratory experiments and field trials with GMVs, following Good Developmental Practices (GDP) (Touré et al. 2003; Touré, Oduola and Morel 2004). It also requires the setting up of procedures during the research and development process to minimize the potential adverse human and environmental consequences by anticipating detrimental effects that might follow the release of GMVs during experimentation. In addition, it can be achieved through the provision of guidance on the design and performance of minimum-risk field research, the development of criteria and test methods for environmental monitoring, the provision of the basis for collection of data addressing safety in the field and, finally, the development of guidelines for dispersal, contingency measures and site rehabilitation (Macer 2003). Moreover, it would need the design of monitoring systems for early detection and evaluation of adverse outcomes, and of the planning of interventions strategies, so that new information can be gathered and interpreted to avert and, if necessary, remedy adverse health or environmental effects (Edmonds Institute 1998).

- *Site selection and preparation*

  There should be prior environmental and health studies for site selection, and based on these data the most appropriate sites should be chosen. In this regard, ecological studies are needed to improve understanding of gene flow in vector populations (mating patterns, behaviour, male biology, population size and structure, mechanisms of population regulation, fitness and phenotypic effects of colonization and mass production). They will help identifying suitable isolated field sites, characterize vector populations in terms of genetic and ecological make-up, determine epidemiological patterns (transmission, disease), and develop appropriate contained semi-field systems to improve understanding of the biology of (transgenic) vectors (Scott et al. 2002). Furthermore, models can be used to enhance understanding of biological processes, spatial and temporal variations, selection of ‘suitable’ areas, prediction of effects of transgene introduction and public-health outcome.

A proof of efficacy and safety to be approved by authorized biosafety and regulatory bodies before any experimental release should be properly established (Hoy 2000; Macer 2003).

- *To address the Ethical, Legal and Social Issues (ELSI) of the potential use of GMVs*

ELSI of the potential use of GMVs need to be properly addressed through the following actions:

– Integrating with the scientific studies those ELSI factors that are relevant to the use of GMVs and ensuring that the biosafety information reaches the public, the communities and the decision-making bodies.

– Ensuring that all stakeholders (i.e. parties with legitimate concerns) have mechanisms for including their input into the proposed control programmes.

– Translating risk assessment procedures into language(s) that is (are) easily understood by the communities concerned.

– Collaborating with end-users on rationale and practical bases for the choice of sites and planning for deployment, in clear and legally appropriate concepts of informed consent. Consent should be obtained from the communities involved. The mechanisms to obtain individual and group consent need to be specifically developed for public-health interventions.

– The data should be openly provided to all as broadly as possible in a two-way process, so that they can benefit from global expertise and develop an international consensus.

– Building public awareness and confidence about the benefits and risks in order to develop implementation strategies that involve the end-user communities and decision-making bodies and to provide means to the public (including the media) to be sufficiently knowledgeable to understand the real measures of success of the programmes and to make informed decisions about the merits of deploying these programmes in their communities.

– Provide adequate means for information dissemination and communication.

– Promote South-South and South-North research cooperation, develop partnerships and enhance capacity in disease-endemic countries (DECs) for the understanding and the potential use of the control tool.

- *Gather all the information necessary for legal and regulatory approvals*

The efficacy and biosafety data gathered along with the actions taken with the public, the media and the communities will be used to provide a complete documentation for biosafety review, ethical review and approval by national and local authorities. A global endorsement (international level) would be most desirable to allow multi-country implementation of the strategy.

Research and control opportunities

Despite the international commitment to control vector-borne diseases such as malaria and dengue, the disease burden still remains high. Under such circumstances, the ultimate goal for vector-borne disease control remains the interruption of transmission. Genetic control of vectors would have an important role to play in the interruption of transmission if the challenges indicated above are adequately addressed.

The ongoing research activities with support from WHO/TDR, NIAID/NIH, IAEA, Wageningen University and the forthcoming Gates Foundation-supported activities

provide a basis for concerted efforts to address the challenges. A coordination of the research activities and the organization of a network would strongly enhance our ability to address the challenges. The creation of a consortium to coordinate the network activities from the different sources of support would be needed. The activities of the consortium may involve an annual meeting to review the progress achieved and suggest future direction.

Future directions for research and capacity/partnership building

The main goal would be to undertake the actions necessary to provide proof of efficacy and safety and address public concerns as bases for policy decision. The actions would include:

- *To provide a proof of efficacy and safety of the use of GMVs for disease control*

– Conduct studies on efficacy, biosafety and risk/benefit evaluation through long-term efforts to clarify the scientific uncertainties under different experimental conditions and with the involvement of investigators in DECs.

– Provide the basis for collection of data on vector biology, ecology, behaviour and genetics addressing efficacy and safety in the field.

– Develop guidelines and principles on the design and performance of efficacy and minimum-risk field research.

– Develop criteria and test methods for environmental monitoring.

– Develop criteria to identify and prepare the sites.

– Design monitoring systems for early detection and evaluation of adverse outcomes, and plan interventions strategies, so that new information can be gathered and interpreted to avert and, if necessary, remedy adverse health or environmental effects.

– Develop guidelines for dispersal, contingency measures and site rehabilitation.

- *To ensure the public that this goal is desirable, feasible and can be accomplished safely*

– Develop a strategy to make the information available to the public and the media such as to raise their awareness and address their concerns about possible environmental and human-health risks.

– Bring all parties together on common ground that can lead to objective, scientific, legal, ethical and social-based decisions by policymakers bearing in mind that most people may not trust the scientific risk analyses.

- *To develop a plan to gather all the information necessary for legal and regulatory approvals*

– Documentation for biosafety review.

– Documentation for ethical review.

– Documentation and mechanisms for national and local authorities' approval and international endorsement.

- *To enhance capacity in DECs* for biosafety assessment, risk/benefit evaluation, environmental monitoring, and research on vector biology, ecology, genetics and behaviour.
- *To promote South-South and North-South research collaboration* based on well defined ethical and scientific standards.
- *To develop mechanisms for dissemination of information* to researchers, decision makers, the communities, the public and the media.
- *To create an international consortium for genetic control of disease vectors* to coordinate the research activities and suggest future directions.

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List of participants

Akogbeto, Martin	Centre de Recherche Entomologique de Cotonou, Cotonou, Bénin.
Awono-Ambene, Herman	Institute of Medical Research and Studies of Medicinal Plants (IMMP), Medical Research Centre, Yaoundé, Cameroon
Bossin, Hervé	IAEA, Seibersdorf, Austria
Christofidis, Giorgos	EMBL, Heidelberg, Germany
Coetzee, Maureen	National Institute for Communicable Diseases, Johannesburg, South Africa
Costantini, Carlo	Centre National de Recherche et Formation sur le Paludisme, Ouagadougou, Burkina Faso
Curtis, Chris	London School of Hygiene and Tropical Medicine, London, UK
Dolo, Guimogo	Malaria Research and Training Center, Bamako, Mali
Fontenille, Didier	IRD Centre de Montpellier, Montpellier Cedex, France
Githeko, Andrew	Kenya Medical Research Institute, Kisumu, Kenya
Githure, John	ICIPE, Nairobi, Kenya
Gouagna, Louis	ICIPE, Nairobi, Kenya
Hassanali, Ahmed	ICIPE, Nairobi, Kenya
Jacobs-Lorena, Marcelo	Johns Hopkins School of Public Health, Baltimore, MD, USA
James, Anthony	University of California, Irvine, CA, USA
Kittayapong, Pattamaporn	Mahidol University, Bangkok, Thailand
Kamau, Luna	Kenya Medical Research Institute, Kilifi, Kenya
Knols, Bart	IAEA, Seibersdorf, Austria, and Wageningen University, Wageningen, The Netherlands
Lanzaro, Gregory	University of California, Davis, USA
Louis, Christos	Institute of Molecular Biology and Biotechnology, Heraklion, Crete, Greece
Manga, Lucien	World Health Organization, Harare, Zimbabwe
Mbogo, Charles	Kenya Medical Research Institute, Kilifi, Kenya
Mukabana, Richard	University of Nairobi, Nairobi, Kenya
Ole-Moiyoi, Onesmo	ICIPE, Nairobi, Kenya
Olson, Ken	Colorado State University, Fort Collins, CO, USA

Osir, Eli	ICIPE, Nairobi, Kenya
Sagnon, N'fale	Centre National de Recherche et de Formation sur le Paludisme, Ouagadougou, Burkina Faso
Sang, Rosemary	Kenya Medical Research Institute, Nairobi, Kenya
Scott, Thomas	University of California, Davis, CA, USA
Takken, Willem	Wageningen University, Wageningen, The Netherlands
Touré, Yeya	World Health Organization, Geneva, Switzerland
Yan, Guiyun	State University of New York at Buffalo, Buffalo, NY, USA

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