Co-ordinated Research Programme on

*Explore genetic molecular, mechanical and behavioral methods of sex separation in mosquitoes*

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To explore irradiation and classical genetic approaches for sex separation in mosquitoes - development of GSS based on irradiation and classical genetics

To explore molecular approaches for sex separation in mosquitoes - Development of GSS based on molecular genetics

To explore mechanical, behavioural, developmental and symbiont-based approaches for sex separation in mosquitoes

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LIST OF PARTICIPANTS
Title: Exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes.

Budget Cycle: 2013-2018

Project: Project 2.1.4.4 (Development of the SIT for the control of disease transmitting mosquitoes) as part of Sub-programme 2.1.4 (Sustainable Control of Major Insect Pests).

Division: NAFA

Responsible Project Officer: Jeremie Gilles

Alternate Project Officer: Kostas Bourtzis

Summary: Requests from Member States for exploration of the potential of applying the Sterile Insect Technique (SIT) against mosquitoes in area-wide integrated vector management (AW-IVM) programmes continue to increase. However, because female mosquitoes, unlike male mosquitoes, can transmit disease, means to eliminate them from the mass production process are a critical pre-requisite. In addition, not releasing sterile females would increase SIT programme efficiency due to the fact that sterile males can then focus only on achieving matings with wild females. Thus mosquito SIT programme efficiency and safety would be considerably enhanced by the development of improved strains for mass-rearing and release. These include strains that: (a) produce only male insects for release and (b) carry easily identifiable markers to monitor released males in the field. Although also assessing mechanical, behavioural and developmental approaches, this CRP will primarily explore classical genetic and modern biotechnology techniques to accomplish female elimination in major mosquito vectors of disease. Whenever possible, these methods will be created with a view to application to a wide spectrum of mosquito species. Major beneficiaries will be operational AW-IVM programmes in Member States that plan to apply the SIT (classical, transgenic, and/or symbiont-based approaches) against mosquitoes. By the end of the CRP, methods for developing sexing strains will have advanced and some strains will be available for evaluation.

The development and evaluation of such methods through this CRP will have the following tangible benefits for SIT mosquito control programmes:

1.) Safety of SIT mosquito programmes will be enhanced. Mosquito SIT implementation cannot include the release of even sexually-sterile biting females. Therefore mosquito SIT requires the exclusive release of sterile males, which is impossible on a large scale without sex separation methods.
2.) Male-only releases are several-fold more efficient than releases of both sexes. Consequently, when genetic sexing technology is available SIT programmes are significantly more efficient and therefore more cost-effective.

3.) As only the males are needed for the SIT, the production, handling and release costs can be reduced significantly if sexing strains are used and females are eliminated early in development.

**Background Situation Analysis:**

Among the major vectors of human diseases, mosquitoes are the most devastating ones. Urbanisation, globalisation and climate change have further accelerated the spread and outbreaks of new mosquito-borne diseases. In view of the problems associated with conventional mosquito control, such as resistance and health effects, major efforts are required to develop new or complementary control techniques, including the SIT, for major mosquito species.

The Sterile Insect Technique (SIT) is an increasingly important component of area-wide integrated vector management (AW-IVM) programmes for key insect vectors such as mosquitoes. With the increase in vector-borne diseases and their toll on human health and mortality, there have been recurring requests from Member States to develop tools and techniques for mosquito SIT, including the development of sexing strains (GC(56)/RES/19) to be able to apply the SIT to control mosquito vector populations (Resolution GC(52)/RES/13). The SIT has the ability to suppress or in special situations to eradicate existing vector populations and to prevent the establishment of new outbreaks.

Operational use of the SIT in insect agricultural pest species continues to reveal areas where new technologies could further improve efficiency and thus lead to more efficacious programmes. There are many aspects of the mosquito SIT package that require increased efficiency to be able to reach the operational level, e.g. improved mass rearing, release technology, quality control, field monitoring, etc. However, one critical area where important advances need to be made before any SIT application is possible concerns the development of genetic sexing strains (GSS). Unlike agricultural pests where the release of both sexes is primarily of economic concern, in mosquitoes, it is an essential prerequisite to release only males since females are blood feeders and transmit disease. **Without male-only releases, SIT applications against mosquitoes are not possible.**

In respect to symbiont-based control methods, the Incompatible Insect Technique (IIT) relies on the sustained, inundative releases of *Wolbachia*-infected incompatible males to sterilize the targeted female population. Several *Wolbachia*-related (semi)field trials (IIT alone or combined SIT/IIT) of various scope and size are underway in different countries and against different mosquito vector species. Field trials include interventions against *Ae. aegypti* in Thailand and the USA, *Ae. albopictus* in China and the USA, and *Ae. polynesiensis* in French Polynesia. The success of IIT could be affected by the accidental release of fertile females.
infected with the incompatible *Wolbachia* type. As the population is suppressed, there is the risk that accidental female release would permit the establishment of the incompatible *Wolbachia*, leading to population replacement instead of population elimination. To overcome this issue the combination of SIT with IIT has been proposed which eliminates the risk associated with accidental release of fertile females. Furthermore, the use of transmission blocking *Wolbachia* provides protection against pathogens like DENV, CHIKV and ZIKV. Altogether, the combined SIT/IIT approach represents a safe and secure genetic approach for the control of mosquito vector populations.

Currently, AW integrated pest management (IPM) programmes with an SIT component have been successfully implemented for several very important fruit fly and lepidopteran species where the development of improved strains, especially GSS (in the case of fruit flies), led to major increases in applicability and efficiency of the SIT component. The experience gained from these programmes would prime the new CRP for the development of GSS in mosquitoes.

There are several mosquito species that are major vectors of different pathogens in different countries. Of these, based on the severity of the disease, requests from Member States and the availability of scientific tools and information, the consultants recommended that the initial efforts to develop mosquito GSS should focus on the following species: *Anopheles arabiensis* (Aar), *Anopheles stephensi* (ASl), and *Anopheles gambiae* (AAg) (vectors of malaria), and *Aedes albopictus* (AAl) and *Ae. aegypti* (AAe) (vectors of dengue, chikungunya, zika and other viruses).

Mosquito GSS development can be achieved using different approaches, but they all rely on some form of stable genetic change introduced and maintained in the developed strain. Genetic change can be introduced using irradiation and classical genetics (as in the case of the Mediterranean fruit fly GSS) or modern biotechnology, specifically genetic transformation or gene editing approaches. Both approaches have advantages and disadvantages relating to transferability of systems between species, stability in mass-rearing, regulatory approval, etc. In addition, existing alternative sex separation methods based on mechanical, behavioural developmental and symbiont-based approaches should also be considered. However, it should be noted that these alternative methods are based on species-specific traits and are likely more appropriate only for small scale operations, highlighting the need to develop state of the art sexing strategies based on genetic and molecular approaches for large scale SIT and non-SIT operations.

Developing sex separation methods for mosquitoes is a prerequisite for the use of SIT in an AW-IVM approach. Four specific objectives should be addressed:

1. To explore classical genetic approaches including irradiation for sex separation in mosquitoes
2. To explore molecular approaches for sex separation in mosquitoes
3. To explore mechanical, behavioral, developmental and symbiont-based approaches for sex separation in mosquitoes

4. To continue encouraging and attracting participants to the CRP in the field of classical genetics

To explore irradiation and classical genetic approaches for sex separation in mosquitoes - development of GSS based on irradiation and classical genetics

One example of how strain improvement can significantly enhance SIT applicability and efficiency has been the use of GSS in the Mediterranean fruit fly, *Ceratitis capitata* AW-IPM programmes, a technology developed through the Agency’s CRP programme with support from the FAO/IAEA Agriculture and Biotechnology Laboratories in Seibersdorf. Using irradiation and classical genetic approaches, a series of genetic sexing strains were developed for the Mediterranean fruit fly. Several of them were evaluated and are currently being used in all mass-rearing facilities of this pest for large scale SIT and AW-IPM programmes, including the VIENNA 8 strains. These achievements in the Mediterranean fruit fly field later resulted in the adoption of irradiation and classical genetic approaches for the development of GSS in other major tephritid species like the Mexican fruit fly (*Anastrepha ludens*), the melon fly (*Bactrocera cucurbitae*) and the Oriental fruit fly (*Bactrocera dorsalis*).

To develop efficient sex separation methods, including genetic sexing strains for mosquito SIT programmes, basic genetic and molecular tools are required as well as any available mechanical and behavioural methods. This section provides an overview of what genetic and molecular tools are currently available in the toolbox and illustrates that despite recent advances in this field, there is an urgent need for further improvement.

The sequence of the genomes of some 20 species of mosquitoes is currently available and is an invaluable resource for the development of GSS using transgenic and genome modification technologies.

The first mosquito GSS were developed in ‘70s, using classical genetic approaches. However, these strains did not prove to be very useful for various reasons including instability of the genotypes produced in the lab and essential determinants of the effectiveness of the GSS. While none of the original strains are currently available, one of the original mosquito GSS was recreated based on dieldrin resistance in *An. arabiensis*. The use of insecticide resistance as a selectable marker is unlikely to be acceptable for large scale applications for a number of reasons including: (a) protection of the environment and human health; (b) accuracy and (c) potential contamination of the mass-rearing colony.

In view of the lack of highly efficient GSS there is a major demand for the development of new GSS for target mosquito species, which would be based on the successful strategy followed for Mediterranean fruit fly and other fruit fly species. An ideal GSS must have two features: (a) the ability to maintain desirable genotypes using selectable or morphological markers (b) a mechanism to ensure sex-specific selection. In addition, the development of GSS allowing early elimination of females in the mass production process (ideally at the
embryonic stage), should be preferred to improve cost efficiency. Thus, the development of a mosquito GSS using conventional genetic technologies would demand the following:

(a) isolation and mapping of genetic markers (preferably morphological markers) from natural populations and/or laboratory populations following mutagenesis; most of these markers will be useful for genetic studies, some of which may meet the criteria for a selectable marker;

(b) irradiation-induced chromosomal rearrangements (i.e. inversions, translocations) and their characterization;

(c) isolation of conditional early lethal mutations (for example tsl mutation) and

(d) combination of the above components into a stable GSS. Making the decision about the most suitable selectable marker is crucial and will ultimately result in the construction of a robust GSS.

At this point, it should be emphasized that the isolation of genetic markers from natural populations or random mutagenesis is a rather challenging and labour-demanding serendipitous process. The achievement of this goal will require the recruitment of a dedicated and fully committed expert, as well as sufficient technical support, to join the mosquito group at the IPCL, Seibersdorf. Despite the fact that the principles of the classical genetic approach are easily applicable to most species, the actual tools that will be developed for a given species cannot be transferred to another mosquito species. On the other hand, the benefit of the classical genetic approach is that no legal restrictions or other regulations apply and strains can be directly used for SIT application.

*Expected outputs*

1. Genetic markers from different sources including natural populations and/or mutagen-based screens. These markers will facilitate genetic studies as well as the isolation of suitable selectable markers for the development of mosquito GSS.

2. Chromosomal rearrangements in the target mosquito species. These chromosomal rearrangements will facilitate genetic studies as well as the development of stable mosquito GSS.

3. (Cyto) genetic data and analyses in support of sex separation. Cytogenetic analysis will facilitate genetic studies and the evaluation of the most suitable GSS.

4. Morphological and selectable markers. These markers will allow the construction of GSS.

5. A classical GSS. The GSS will consist of at least: (a) a selectable marker; (b) an inversion and (c) a sex chromosome/autosome translocation.

6. Lab and semi-field evaluation of a classical GSS. This evaluation will provide data on the actual stability and the overall fitness of the developed GSS.
To explore molecular approaches for sex separation in mosquitoes - Development of GSS based on molecular genetics

Enhancing SIT through the development of molecular-based genetic sexing strains has been proven successful for fruit flies, including the tephritid species *C. capitata* and *A. suspensa*, for which 100% conditional/repressible female lethality has been achieved through a well-understood apoptotic pathway combined with sex-specific splicing. The application of transgenic systems has the potential advantage of a faster development time and the ability to be transferred to other species. In addition, an approach to generate a male-only population by sex conversion of females to males has been tested in *C. capitata* with very encouraging results. Some of the molecular-based GSS have been successfully evaluated under semi-mass rearing and field cage conditions with support from the FAO/IAEA Agriculture and Biotechnology Laboratories in Seibersdorf. These achievements will help to explore different options for molecular-based GSS in mosquito species.

To generate molecular-based GSS in mosquitoes there are a number of options that are currently being considered which include female-specific lethality, sex ratio distortion, sex-conversion or sex-specific markers to be used with sorting. In all these systems, transgenic constructs enable genetic sexing using effector transgenes of different classes, and if needed combining them with sex-specific expression using validated sex-specific promoters. Alternatively, sex-specific expression of effector transgenes can be ensured through linkage to male-specific chromosomes or male-specific loci (e.g. *M* locus). Finally, to be suitable for implementation GSS will have to integrate conditional expression of the genetic sexing phenotype to permit mass rearing.

Transgene effectors using all transgenic based approaches to GSS have now been demonstrated in mosquito vectors. A female-killing transgene was recently generated in *Anopheles stephensi* by inserting an additional copy of the Y-chromosome linked male-determining factor *Guy1* onto an autosome, which lead to dominant embryonic lethality of females but did not affect males. Sex-ratio distorters using both naturally occurring endonucleases and more recently using CRISPR/Cas9 (see below) and expressed during spermatogenesis have been developed in *Anopheles gambiae* showing high degrees of male bias (~95%) in the offspring of transgenic males. Sex-conversion in the form of masculinization of genetic females has been shown in *Aedes aegypti* by transiently mis-expressing the primary sex-determiner *Nix* in females. Finally, cassettes consisting of fluorescent markers, under the control of male-specific promoters or inserted directly on the Y chromosome, have been developed for automated sorting and selection of transgenic male using a FACS-based technology. Significant progress in the study of mosquito Y chromosome biology has been made in several *Anopheles* and *Aedes* species. This has led to the identification and functional characterization of several primary male determining genes in mosquitoes, which have in turn acted as the effector transgenes in some of the above strategies.

Conditional or repressible expression systems will be required to consider the use of such GSS in a mass-rearing setting. In this context, the Q-system has now been successfully transferred to *Anopheles gambiae* and its transfer to additional mosquito species is now being
anticipated. In addition, the UAS-Gal4 and the tet-system system are already available in a number of mosquito species. The suitability of these conditional systems will largely be dictated by the different sexing strategy and/or effector system used. To date, only the tet-system has been evaluated in the mass-rearing environment with some success, but reports regarding leaky expression and insertion site-specific variation suggest that alternative systems should be explored.

Creating genetically modified mosquitoes for biological control will require predictable and highly regulated gene expression and to achieve this kind of site-specific integration systems will be important. These systems enable the integration of transgenes into known sites within the genome, thereby eliminating the uncertainties associated with transposable element integrations and the variable gene expression that can arise due to ‘position effects’. A number of site-specific recombination systems are available and the phiC31/attP and Cre/LoxP systems have been introduced into *Aedes aegypti*, *Anopheles stephensi*, *Anopheles gambiae* and *Anopheles arabiensis*. These systems have been shown to not only enable the integration of transgenes but in some cases for the exchange of transgenes by a method called Recombination Mediated Cassette Exchange (RMCE). The number of well characterized recombination sites in genomic locations known to result in minimal or no ‘position effects’ is currently quite small and the continued creation of mosquito lines with useful site specific recombination ‘docking sites’ is essential.

RNA-guided endonucleases such as Cas9 from the CRISPR/Cas9 system have been widely adopted by insect biologists and there are now many published examples of its use in insects as both a somatic and germline mutagen. It is clear from these published data that the creation of null alleles can be quite efficient if the necessary guide RNAs are well designed and the Cas9 endonuclease can be effectively delivered to germ cells or presumptive germ cells. The use of these systems to insert transgenes by homology directed repair mechanisms has also been demonstrated in mosquitoes. The development of ‘gene editing’ systems is particularly significant because they provide insect biologists with a new powerful set of tools and capabilities that will facilitate the creation of genetic sexing strains.

The use of RNAi to control gene expression in insects has progressed with the discovery and development of new modes of dsRNA delivery to insect tissues and cells. It has been shown that feeding mosquito larvae bacteria or yeast expressing dsRNA specific to mosquito genes can be an effective way of regulating the expression of some mosquito genes. In addition, feeding mosquito larvae only dsRNA or dsRNA associated with inert carriers such as chitosan can also result in altered mosquito gene expression. When the dsRNA-targeted genes were involved in sex determination, sex ratio distortion was observed suggesting that the use of dsRNA may has some potential as a means for genetically sexing mosquito larvae.

**Expected outputs**

7. Construction and characterization of transgenic strains with site-specific landing sites and known performance characteristics. *Some progress has been made in both the generation and assessment of transgenic strains harbouring*
site-specific landing sites (AGa, AAe), but additional sites within species in which progress has been made are needed and some important species continue to lack well characterized landing sites (AAr, AAI).

8. Methods, protocols and procedures for using RNA-guided endonuclease-based (‘gene editing’) technologies in mosquitoes. Progress in establishing these systems in mosquitoes has been made. (AGa, AAe, AST) TALENs are no longer being used and have been substituted by CRISPR-based gene editing.

9. Data on the structure, organization and patterns of gene expression of the sex chromosomes of mosquitoes. Successful strategies have been developed and applied to a number of vector species that have provided data into the biology of sex chromosomes and sex determining genes (AGa, AAr, AST, AAe, AAI). These strategies are applicable to other mosquito species.

10. Gene expression regulatory elements that confer sex-specificity. Regulatory sequences with male and female specific expression have been identified and characterized in transgenic strains in a few mosquito species (AGa, AAr, AST, AAe).

11. Effector molecules that can confer predictable phenotypes when expressed in mosquitoes. Effectors falling in the classes of female specific lethals (AST), sex ratio distorts (AGa), sex-converters (AAe) and fluorescent markers (AGa, AAr, AST, AAe) have been identified, characterized and used in a number of species.

12. Strains of genetically modified mosquitoes created from elements generated from outputs 8, 10 and 11 that have the potential to be adapted to permit conditional genetic sexing. Combinations of regulatory elements and effector molecules can be used to generate female-specific effects. Conditional sexing would be an ideal output.

13. Strains developed from output 12 assessed for performance characteristics in lab and/or semi-field conditions. The results of these assessments will contribute to the development of standard operating procedures if there is sufficient time.

To explore mechanical, behavioural, developmental and symbiont-based approaches for sex separation in mosquitoes

Mechanical, behavioural, and developmental tools for sexing need to be developed or existing ones to be refined to further improving the elimination of females at different developmental stages (ideally at the embryonic stage). The accessibility and integration of less efficient systems can provide a stop-gap measure that allows rapid start up with a minimum of investment, but are currently not considered long-term substitutes for true sexing.
systems. The following are being used and could be made more specific and efficient, or have not been explored in detail, but might be investigated by CRP participants. More than one method could be combined in order to attain better separation.

**Mechanical Tools.** Sex separation is possible at the pupal stage for some *Aedes* and *Culex* species due to size dimorphism between male and female pupae: females are usually larger. In other mosquito species, these differences are not significant enough to make sexing by size feasible. Many trials have been made in the past to assess pupal body size at different larval densities by measuring the dorsal width of the cephalothorax. While cephalothorax width was shown to be density dependent in *Ae. albopictus*, the results indicate that the size difference between males and females remains fairly constant across densities. Methods that have exploited this difference include: (i) sieving methods (e.g. standard sieves) for *Ae. albopictus, Ae. aegypti,* and *Cx. quinquefasciatus; (ii)* use of the Fay-Morlan glass plate sex separation system (later modified by Focks) for numerous species; and (iii) use of the McCray adjustable opening separation system for *Aedes sp.*

Both female and male pupae are positively buoyant in pure water, but they may not have the same absolute density. Differences in buoyancy between male and female pupae could be explored using cold water (in which different rates of ascent might exist) or aqueous solutions whose density has been modified using e.g. salt or sugar solutions. If such differences are found, they could be exploited in mechanized sex-separation systems, so their utility likely overlaps that of pupa size methods.

Female adult mosquitoes are heavier and larger than males. These characteristics may be of value for separation of adults using a system similar to that which has been developed for *Musca domestica* in which a regulated air stream was used to separate anesthetized (CO$_2$) adults. Such methods will be most efficient for species in which size difference between sexes is greatest.

Sexual dimorphism can be also used for automatic identification by artificial vision algorithms. Once both sexes have been identified, mechanical separation/removal of females controlled by computer can be used. One possible method to eliminate female pupae is to use high speed positioning and accuracy precision laser systems driven by a dual axis galvanometer optical scanner. These systems are widely used in industrial applications (ie. stereolithography rapid prototyping systems).

**Behavioural Tools.** For all blood-feeding mosquitoes, sex separation could occur at the adult stage by spiking blood with insecticides (malathion, dieldrin) or other mosquito toxins (ivermectin, Spinosad). Recent studies have shown that all female *An. arabiensis* could be eliminated by spiking blood meals with 7.5 ppm ivermectin within 4 days. For *An. albimanus*, using 0.5 % malathion in citrated bovine blood gave 95% effective elimination of females but resulted in significant (25%) male death due to inadvertent exposure. Improvements could involve toxicants with no vapour or contact toxicity.

Host-seeking behaviour by females could be exploited even if they do not actually consume any blood. By creating a mechanical system for capturing females that are drawn to host cues
(heat, CO₂, odours), females could be partitioned into separate chambers or killed upon resting on a metal screen (electrocution). If the electrocuting screen operated intermittently, females might rest on it more readily.

Males of some species (Culex and Anopheles particularly) begin swarming as young adults. Swarms contain very few females when they form in nature. This behaviour might be exploited for small-scale studies in which males could be collected from the swarms.

**Developmental Tools.** On average, male mosquitoes develop more rapidly than females (protandry). Mosquito larvae must achieve a critical weight to be able to pupate and this critical weight is higher for females leading to a longer developmental time. This feature has been exploited for Aedes particularly, but could be considered for other species where better sex-separation methods are not available. Protandry is known to be affected by several factors including rearing temperature, larval density and diet. Renewed efforts have been developed to standardize this process for at least three Aedes species, namely Ae. aegypti, Ae. albopictus, and Ae. polynesiensis.

*Ae. albopictus* and *Ae. polynesiensis* harbour endosymbiotic microrganisms like Wolbachia which influence larval survival and adult fitness. Very little work has been done on the effect of this bacterium on pre-imaginal development comparing lines harbouring different Wolbachia strains (natural or artificially established) with their aposymbiotic counterparts. Exploring the role of bacterial endosymbionts (e.g. Wolbachia) in influencing the duration of pre-imaginal development, protandry, sex ratio and pupal dimorphism may allow the development of more effective sex separation methods using appropriate mosquito strains and mass rearing conditions. Recent evidence supports the need for further investigation of the Wolbachia properties on mosquito developmental biology. The dynamics of endosymbiont density in their host is also being investigated in view of its possible importance in SIT and/or IIT applications.

**Expected outputs**

14. Data that allows the use of Wolbachia symbiosis for sex separation. *Recent results suggest that Wolbachia symbiosis can enhance protandry in at least one mosquito species and could thus improve existing sex separation methods.*

15. A set of mechanical (e.g. size dimorphism), behavioural (e.g. phototaxis) and developmental (e.g. protandry) properties for developing sex separation tools. *Mechanical and developmental parameters have been used to conduct limited scale (semi-)field trials for several vector species thus facilitating studies as well as the development of classical sex separation tools.*

16. Novel sex separation tools exploiting tested mechanical, behavioural and developmental properties. *At least one novel sex separation tool has been developed which is being evaluated.*
Data on the efficacy and reproducibility of mechanical, behavioural, and developmental tools under mid to large production scales using standard quality control parameters. Mid to large scale productions were undertaken that provided data on the actual efficiency, reproducibility, and applicability of these tools.

Selected References


Curtis CF (1979) Genetic sexing techniques based on translocation of insecticide resistance to the Y chromosome. *Bull OILB/SROP*, 2:


Other Insect Pests, Joint Proceedings of the International Conference on Area-wide Control of


NAPPO: *Guidelines for the Importation and Confined Field Release of Transgenic Arthropods in NAPPO Member Countries* 2007


**Nuclear Component:**

All the activities in the CRP relate to the development and evaluation of mosquito strains for use in SIT programmes. The SIT relies on the use of ionizing radiation to sterilize large numbers of insects and currently there is no alternative that could replace radiation. However, there are developments taking place which intend to use molecular methods for generating lethality in field populations. These approaches are not included in this new CRP as their non-confined use would create significant concerns relating to biosafety and long-term effectiveness. Radiation-induced sterility provides a very high level of biosafety and can be used in combination with improved strains developed in this CRP. As radiation induces random dominant mutations, there is no possibility of resistance developing to this physical process, a possibility which cannot be excluded with molecular approaches that involve genomic insertions.

**Explanation / Justification:**

1. Publication of results. Activities and findings of the CRP will be published in a special issue of a peer-reviewed journal.

**Participation of Agency's laboratories (Yes/No)**

As few institutions are applying irradiation and classical genetics for the development of GSS in mosquitoes, the CRP needs therefore to be supported through adaptive research and development carried out at the IPCL, FAO/IAEA Agriculture and Biotechnology Laboratories, Seibersdorf as part of Project 2.1.4.4. This R&D will focus on the development of molecular markers using classical genetics, and the evaluation of marker strains and genetic sexing strains developed using modern biotechnology.

**Assumptions:**

Member States will continue to suffer major losses due to dengue, chikungunya, zika, and malaria outbreaks.

That IAEA Member States continue requesting the development of the SIT package for mosquitoes, and that enough scientists and institutions are willing to participate in the CRP and can be motivated to apply classical genetic approaches to develop sex separation systems in mosquitoes.

The demand for area-wide integrated insect pest management approaches, including SIT and augmentative biological control as non-polluting suppression/eradication components, continues to increase, mandating expansion and improvement in cost-effectiveness of these environment-friendly, sustainable approaches.
Related TC Projects

MEX5031: Using the Sterile Insect Technique to Control Dengue Vectors.

PHI5033: Building Capacity in Using the Sterile Insect Technique against Dengue and Chikungunya Vectors.

SAF5014: Assessing the Sterile Insect Technique for Malaria Mosquitos in a South African Setting, Phase II.

SRL5047: Establishing a National Centre for Research, Training and Services in Medical and Molecular Entomology for Vector-borne Disease Control.

SUD5038: Implementing the Sterile Insect Technique for Integrated Control of Anopheles arabiensis, Phase II.

RAS5066: Promoting the Sharing of Expertise and Infrastructure for Dengue Vector Surveillance towards Integration of the Sterile Insect Technique with Conventional Control Methods among South and South East Asian Countries.

RER5022: Establishing Genetic Control Programmes for Aedes Invasive Mosquitoes.

RLA5074: Strengthening Regional Capacity in Latin America and the Caribbean for Integrated Vector Management Approaches with a Sterile Insect Technique Component, to Control Aedes Mosquitoes as Vectors of Human Pathogens, particularly Zika Virus.

INT5155: Sharing Knowledge on the Sterile Insect and Related Techniques for the Integrated Area-Wide Management of Insect Pests and Human Disease Vectors.
# LOGICAL FRAMEWORK:

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<td><strong>Overall Objective</strong></td>
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<td>Requests by Member States in the area of mosquito control using the SIT are increasing. To make this nuclear technology available to Member States for several mosquito species, the development of male-only releases is either a requirement to reduce programme costs or an essential precondition. Given the large number of different target species and the lack of any efficient tools for mosquito control, strategies based on genetic, molecular, mechanical and behavioural techniques are amenable. Biological material is available. An expert on classical genetics and a technician in the mosquito group in Seibersdorf are recruited.</td>
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## Specific Objectives

1. To explore irradiation and classical genetic approaches for sex-separation in mosquitoes

2. To explore molecular approaches for sex separation in mosquitoes

3. To explore mechanical, behavioural, developmental and symbiont-based approaches for sex separation in mosquitoes

4. To continue encouraging and attracting participants to the CRP in the field of classical genetics

## Outcomes

1. Classical genetic approaches for sex separation in mosquitoes assessed

2. Molecular approaches for sex separation in mosquitoes developed

3. Mechanical, behavioural, developmental and symbiont-based approaches for sex separation in mosquitoes improved and validated

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<th>Geneticists pursuing a classical approach attracted to the CRP.</th>
<th>Irradiation and classical genetics can be applied for the construction of mosquito GSS.</th>
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<td>1. Genetic markers from different sources including natural populations and/or mutagen-based screens.</td>
<td>Genetic markers isolated or developed.</td>
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<td>2. Chromosomal rearrangements in the target mosquito species.</td>
<td>Chromosomal rearrangements selected.</td>
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<td>3. (Cyto)genetic data and analyses in support of sex separation.</td>
<td>(Cyto)genetic markers and chromosomal rearrangements described.</td>
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<td>5. A classical GSS.</td>
<td>Classical GSS constructed and characterized</td>
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<td>6. Lab and semi-field evaluation of a classical GSS.</td>
<td>Classical GSS in laboratory and semi-field testing evaluated</td>
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<td>7. Construction and characterization of transgenic strains with site-specific landing sites and known performance characteristics.</td>
<td>Strains with landing sites constructed and performance characteristics assessed</td>
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<td>8. Methods, protocols and procedures for using RNA-guided endonuclease-based (‘genome editing’) technologies in mosquitoes.</td>
<td>Accurate genome editing in target loci and species achieved</td>
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<td>9. Data on the structure, organization and biological material and methods available.</td>
<td>Sex determination pathways and sex</td>
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Biological material and methods available.  
Scientists working on classical genetics and methods are available.  
(Cyto)genetic methods, chromosome maps and experts are in place.  
Morphological and selectable markers are identifiable.  
Suitable genetic markers, irradiation sources, chromosomal rearrangements are available.  
Laboratory and semi-field testing is feasible.  
Landing sites and performance characteristics are available.  
Genome editing technologies, genomic data are available and endogenous repair mechanisms are functional.  
Tools and omics data for the analysis of complex sex determination pathways and
1. Patterns of gene expression of the sex chromosomes of mosquitoes.

2. Specific expression in mosquitoes better understood. Content of sex chromosomes studied.

3. Regulatory elements tested

4. Reports and or published papers

5. Wolbachia influence on sex separation exploited

6. Laboratory and semi-field testing of GSS is feasible.

7. Wolbachia infection influences developmental parameters in a sex-specific manner

8. Tools for characterization are available.

9. Factors influencing the efficiency of sex separation tools characterized

10. Reports and or published papers

11. Effector molecules tested

12. Effector molecules are available and can be tested.

13. Molecular-based sexing strains developed

14. Components for the application of molecular-based technologies are available

15. Molecular-based GSS performance characteristics evaluated in lab or semi-field scale.

16. Laboratory and semi-field testing of GSS is feasible.

17. Novel sex separation tools tested

18. Reports and or published papers

19. Efficacy and performance characteristics evaluated in lab or semi-field scale.
and reproducibility of mechanical, behavioural, and developmental tools for lab and/or semi-field testing.

18. Publication of results in a peer reviewed journal.

| Activities                                                                 | reproducibility of tools assessed | papers                               | place.                  |
|                                                                           | Papers drafted and submitted.     | Journal issue with published papers | Data for publication available. |

| Proposals evaluated and 6 Research Contracts, 11 Research Agreements and 2 Technical Contracts awarded. | Signed contracts and agreements. | Suitable proposals submitted, funding available and approval of Contracts and Agreements by CCRA-NA committee. |
| 1st RCM held 2013.                                                          | Participants’ activities and logical framework revised. | Contracts and Agreements signed by counterpart organisations. |

| 2nd RCM held 2015.                                                          | Participants and RCM Progress Reports. | Progress satisfactory. |
| 3rd RCM held 2016.                                                          | Participants and RCM Progress Reports. | Progress satisfactory and mid-CRP evaluation approved by CCRA-NA committee. |

| 4th RCM held 2018.                                                          | Participants and RCM Final Reports | Final reports are submitted to the Agency. |
| Scientific publication.                                                     |                                       | Consensus can be found on appropriate international journal and acceptance by journal obtained. |
FUTURE ACTIVITIES

**Contract 17939:** Margareth Capurro, University of São Paulo, Brazil (Collaborators: Jake Tu, Chun-Hong Chen)

**Construction and characterization of GSS (Aedes aegypti and Aedes albopictus)**

The goal of this project is to generate transgenic *Aedes aegypti* mosquitoes that will produce male-only progeny. The hypothesis is to knockout the *dxx* female form (exon 5b) resulting in female masculinization, deformities in feminine organs or death of female mosquitoes. We will use CRISPR-Cas9-system to knockdown exon 5b from *dxx* gene to create male only progeny.

**Aims for the next 18 months:**

- Finish the constructs CRISP-EX5b-HR-AePUb-eGFP;
- Obtain transgenic lines with visible mutation;
- Design the recover-female gene to produce homozygous lines.

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**Contract 17958:** Flaminia Catteruccia, Harvard T. H. Chan School of Public Health, Boston, USA (Collaborators: Eric Marois)

**Mosquito sex separation via TALEN-mediated female-specific lethality**

Our initial proposal suggested a system to generate a sex separation scheme in which a pair of TAL-endonucleases transgenically inserted on opposing X-chromosomes could be used to induce lethality in females leaving males as the sole survivors. The scientific developments of the past three years have revealed TAL endonucleases to be an outdated technology, eclipsed by the high-efficiency and straightforward implementation of CRISPR technologies. Therefore our research focus has shifted to develop CRISPR technologies for our original purposes.

**Optimization of CRISPR inducibly sterile knockouts**

We have developed a system for induced tissue-specific sterility male *Anopheles gambiae* using the CRISPR/Cas9 system. For this system we have generated two transgenic lines: one expressing germline Cas9 under the Vasa2 promoter (Papathanos et al. 2009), and a second encoding three gRNAs targeting *zpg* (AGAP006241), an innexin required for male and female germ cell development (Thailayil et al. 2011). Each of these transgenes is inserted onto the docking line, X1, located on chromosomal arm 2L (Volohonski et al. 2015). Upon crossing these transgenic lines, simultaneous co-expression of Cas9 from the Vasa2 promoter, and gRNAs targeting *zpg* causes severe atrophy of germline tissues in the F1 generation. Because the Vasa2 promoter is also characterized by maternal deposition of Cas9 protein into the developing oocyte, progeny deriving from a Cas9 mother result in a more
penetrant germ-cell-less phenotype caused by early embryonic Cas9 expression. From this cross 95% of sons are characterized by complete atrophy of germ cell tissues, no visible sperm, and almost no detectable fluorescence from a Vasa-YFP germline reporter. Mating these males to wild type females renders 95% of females sterile and refractory to further mating, concordant with male testicular phenotype. Males not completely sterilized by this system either contain a CRISPR mutation that maintains fertility or escape detectable mutagenesis completely.

Sterility resulting from Cas9 and gRNA-ZPG mosaic mutagenesis is currently 95% efficient. We aim to optimize this system to achieve >98% spermless phenotype, which is more acceptable for field releases. Following optimization, this system could be modified to include a female-killing strategy.

Aims for the next 18 months:
- Optimize CRISPR for mosaic mutagenesis
- Select for natural variance in our Cas9-expressing transgenic we aim to optimize this system to achieve >98% spermless phenotype
- Test mating competitiveness of genetically sterilized *Anopheles gambiae* males
- Mating capture experiments against non GM control line

**Contract 17882**: Andrea Crisanti, Imperial College of London (Collaborators: Federica Bernardini, Philippos Papathanos, Tony Nolan, Nikolai Windbichler).

**Transferring a synthetic sex ratio distortion system between members of the Anopheles complex**

We have previously characterized an *Anopheles gambiae* transgenic strain, named T4, with a transgenic construct inserted onto the Y chromosome. Expression of fluorescent maker genes in T4 males suggested that the transgene construct had inserted within a region of chromatin that allows transcriptional activity. The fact that the T4 locus was transcriptionally active made it an exciting target for genetic engineering. Using meganuclease-induced homologous repair a site-specific recombination signal, AttP, was introduced onto the Y chromosome and the resulting docking line, named YAttP, was proven to allow secondary integration. This transgenic strain allows automated sex separation and generation of male-exclusive genetic traits, therefore it represents a valuable tool for the improvement of vector control strategies already in use, such as sterile insect technique, and encourage the development of new approaches such as those based on endonuclease genes (Bernardini et al., 2014). *A. arabiensis* is another important vector of human malaria. The generation of a transgenic line in this species carrying a Y-linked AttP site is of great interest. Since active insertions onto the Y chromosome are extremely rare, we used a scheme based on crossing and selection to overcome F1 hybrid male sterility, predicted by Haldane’s rule, and we introgressed the engineered *A. gambiae* Y chromosome, YAttP, in the *A. arabiensis* genetic background. Whole genome sequencing showed that the Y retained its original sequence content. We find that the effect of the presence of a heterospecific Y-chromosome on the expression of *A.
*arabiensis* genes is minute and can be explained almost exclusively as a direct consequence of transcripts arising from sequence elements present on the *A. gambiae* Y-chromosome itself. We find that Y hybrids show no obvious fertility defects and no substantial reduction in male competitiveness. Our results demonstrate that, despite their radically different structure, Y chromosomes of two species of the gambiae complex that diverged ~1.85Myr ago function interchangeably thus indicating that Y degeneration may be complete. The Y hybrid strain, similarly to the *A. gambiae* YAttP allows automated sex separation and generation of male-exclusive genetic traits. Furthermore, our finding suggests that malaria control interventions based on endonuclease genes, such as sex-distorting Y drive, would be transferable, whether intentionally or contingent, between the major Malaria vector species.

Sex distortion technique has been previously validated in *A. gambiae*. This technology is based on the activity of I-PpoI, a homing endonuclease that has high specificity for a conserved sequence within the ribosomal rDNA repeats located in a single cluster on the *A. gambiae* X chromosome. If this endonuclease is expressed during spermatogenesis in transgenic mosquitoes the paternal X chromosome is expected to be cut and therefore only Y chromosome bearing sperms are produced. In *A. gambiae* >95% male offspring has been achieved as result of the I-PpoI activity and it has been proven that distorter male mosquitoes can efficiently suppress caged wild-type mosquito populations. The *A. gambiae* distorted strain harbours the endonuclease I-PpoI on an autosomal chromosome (Galizi et al., 2014). For the efficacy of a vector control strategy based on sex distortion technique, the possibility to insert the endonuclease directly on the Y chromosome would represent a powerful tool which in theory could lead to the elimination of the target population even after releasing a very low number of transgenic males. The AttP and Y hybrid strains, carrying a docking site on the Y chromosome, represent a valuable tool for the improvement of this technology. However, expression of Y-linked genes driven by germline promoters is not straightforward, this is likely due to meiotic sex chromosomes inactivation (MSCI) a mechanism responsible for the silencing of chromosomes that fail to pair with their homologues partners during meiosis (Vibranovski, 2014). While efforts are made in order to overcome MSCI and achieve expression from the Y chromosome, we wanted to test the validity of sex distortion technique when applied to *A. arabiensis* mosquitoes. We introgressed the autosomal transgene carrying the I-PpoI distorter, originally integrated in the *A. gambiae* genome, into the *A. arabiensis* genetic background. Our preliminary data show that the *A. arabiensis* X chromosome is susceptible to the I-PpoI activity resulting in production of 99% male progeny. This finding suggests that sex distortion technique can be successfully applied to target *A. arabiensis* populations. During the next 18 months we will validate the value, in the context of vector control, of the *A. arabiensis* distorted strain.

**Aims for the next 18 months:**

- Establish, by further backcrosses and inbreeding, the *A. arabiensis* distorted strain.
- Assess the genetic composition of this strain by deep DNA sequence analysis.
- Assess the reproductive fitness of *A. arabiensis* distorted males (fertility, fecundity, mating competitiveness).
Creation of conditional female embryonic lethal strains of *An. arabiensis*

We have previously identified in *Anopheles gambiae* a Y chromosome gene, which after ectopic delivery causes death of female embryos in both *An. gambiae* and *An. arabiensis*. During the last 18 months we have extensively characterized the gene in a number of *in vivo* tests. We demonstrated that the gene (named *Yob*) represents a primary sex determination signal conferring maleness. With the onset of expression between 2.0 and 2.5 h after oviposition, it is among the earliest expressed zygotic genes. Transfection of the female cell line with the *Yob* mRNA led to a shift in splicing pattern of *doublesex* gene (*dsx*), the final element of the sex determination cascade, toward male transcript isoform. The shift in *dsx* splicing has been also observed in transgenic females that ectopically express *Yob* (see below), confirming *Yob*’s involvement in the sex determination pathway. In addition to conferring maleness, *Yob* controls activation of dosage compensation, the process that drives overexpression of the X-linked genes in males to the levels of expression from the two X chromosomes in females. Apparently, female embryo death caused by *Yob* expression results from an inappropriate activation of dosage compensation and a toxic hyper-expression of the X chromosome genes during early female development.

We have created a piggyBac-based transgenic construct with the *Yob* cDNA under the control of the *An. gambiae* vasa promoter (with supposedly female germline-specific activity). Using the construct, we have generated 10 transgenic *A. gambiae* lines with non-inducible ectopic expression of *Yob*. Our expectation was that all females in these lines would be killed as embryos (which would require maintaining the lines by backcrossing transgenic males to the wild type females). However, that was not the case. A range of phenotypes caused by position effect was observed, and the extent of female killing, if any, was correlated with the expression level of the dsRED marker tag. One line exhibited no sex bias, which coincided with very weak expression of dsRED. In the lines most strongly expressing dsRED there was a strong female killing effect (~75% males). Importantly, the surviving females from the latter lines were masculinized to a considerable degree, they never attempted to feed on blood, and were dying shortly after emergence, most likely due to dosage compensation issues. We determined that the *vasa* promoter activity was “leaky” in these lines and *Yob* was expressed in various female tissues; however, not at levels sufficiently high to kill all the females at the embryo stage.

To set the stage for generation of strains with a fully penetrant female-lethal phenotype, we have been working on the identification of highly active early zygotic promoters. Our attempts to isolate the endogenous *Yob* promoter, which may fulfil this requirement, has been yet unsuccessful, possibly because the regulatory elements of *Yob* may have a peculiar structure associated with location on the entirely heterochromatic Y
chromosome. Therefore, we focused on isolation of promoters from other early zygotic genes. To date we have confirmed that the An. gambiae orthologs of the Drosophila even skipped, hunchback and Phk3 genes are highly expressed starting at the maternal-to-zygotic transition. We isolated by PCR putative promoters of these three genes, and cloned the obtained fragments into a reporter plasmid. These putative promoters await functional characterization in An. gambiae embryos. We have also generated Gal4-UAS plasmids for functional analysis of the promoters in a bipartite expression system.

Aims for the next 18 months:

● Continue testing the activity of the putative early promoters of selected genes in An. gambiae.
● Once strong early zygotic promoters are identified, generate inducible female-lethal An. gambiae strains ectopically expressing Yob.
● If the above goals are achieved and time permits, generate inducible female-lethal strains of An. arabiensis.

Publications:


**Contract 17981:** David O’Brochta, University of Maryland, College Park, USA (Collaborators: Jake Tu)

**CRISPR/Cas9 mutagenesis of An. arabiensis and the creation of ΦC31 attP landing site lines**

During the previous 18 months an extensive enhancer trap screen was conducted in Anopheles stephensi in which 130,000 larvae were screened for tissue-specific gene expression indicating the identification of tissue specific enhancers. While many tissue specific enhancers were identified, including approximately 20 for hemocytes, no enhancers were identified that were expressed in the gonads of larvae or adults. The enhancer-trap lines with hemocyte-specific expression were used to regulate the expression of various transgenes the directly or indirectly impact immunity gene expression. The failure to detect and recover
gonad-specific enhancers was unexpected because gonad specific gene expression is known to occur, eg. beta-tubulin, yet none of these loci were detected in these screens.

CRISPR/Cas9 and its use as a site specific mutagenesis tool were explored in *Anopheles gambiae*. Protocols were developed for the creation, detection and maintenance of cryptic mutations. These protocols were used to create a knockout mutation of the *saglin* gene in *An. gambiae*. The protocols developed here will be applicable to *An. arabiensis*. Although planned, the application of CRISPR/Cas9 technology to *An. arabiensis* during the previous 18 months was not completed because our development of efficient protocols and procedures took longer than expected in *An. gambiae*.

The *piggyBac* transposon was tested for its ability to be remobilized in *An. gambiae* by the expression of the germline-specific expression of *piggyBac* transposase. Efficient remobilization of *piggyBac* would enable gene-trap and enhancer-trap technologies to be developed for this species and perhaps *An. arabiensis* as well. During the last 18 months *An. gambiae* lines were created with a stable transgene expressing piggyBac transposase in the germline. This line was used to remobilize a small *piggyBac* element containing a marker gene. Remobilizations were readily detected in approximately 2% of the progeny, a frequency comparable to that seen in *An. stephensi* in which functional enhancer-trap and gene trap systems have been developed.

The QF/QUAS system, a binary transcription system that is similar to the Gal4/UAS system with some added capabilities for small molecule modulation was introduced into *An. gambiae* and shown to be functional. This transcriptional regulatory system provides an additional system for constructing regulated transgene expression systems. The Q-system has the added capability of being regulated with the small molecule, quinic acid.


Aims for the next 18 months:
- Use CRISPR/Cas9 to create visible mutations.
- Use CRISPR/Cas9 to create a reciprocal translocation involving a visible marker.
- Create transgenic *An. arabiensis* lines that carry either 1) a stably integrated source of *piggyBac* transposase expressed in the germ-line (using promoter from *vasa*). 2) a single copy of a *piggyBac* elements with an ΦC31attP site and a reporter gene sensitive to ‘position effects’.
• Remobilize PB-attP in *An. arabiensis* to create a collection of lines with ΦC31attP landing sites on different chromosomes that are free from ‘position effects’.

**Contract 17952**: Philippos Papathanos, University of Perugia, Italy (CRP collaborators: Andrea Crisanti, Jake Tu, Jaroslaw Krzywinski, Francesca Scolari; External collaborators: N. Windbichler, N. Besanszy, I. Sharakov)

Over the last 18 months we have seen the completion of two large scale genomics projects in the lab, whose results will inform on the development of genetic sexing systems. First, we completed the first annotation of the *An. gambiae* Y chromosome, resolving the content of the Y, its structure and evolution within the *An. gambiae* complex. A significant outcome of this work was the confirmation of YG2 as the likely male determining gene, based on its unique conservation on the Y in all members of the complex (this was subsequently confirmed by J. Krzywinski and the gene was called Yob). The second project focused on the study of sex-biased gene expression in 4 species of the *Anopheles* genus, their conservation and evolution in the genus. The results have characterized genes that are expressed uniquely in males from which regulatory elements can be extracted in the future for generating genetic sexing strains, and whose function can be anticipated to cross species boundaries, should that be required. We have been unable to establish the RNAi based approach using feeding of larvae with *E. coli* lines expressing dsRNAs as outlined in the previous report for a number of reasons, including IP. However, we have been heavily involved in the development and testing of CRISPR/Cas9 based approaches for gene-knockout and phenotype testing, a system that obviously offers a number of advantages compared to RNAi. We are currently assessing the role of a Y chromosome gene that is expressed during male spermatogenesis using site directed knockin of a transgene, which will permit the generation of either male-sterile strains (assuming that this gene is essential for male sterility as similar genes in humans or *Drosophila*) or for overcoming meiotic sex chromosome inactivation:

In the next 18 months our activities will continue to address the:

• Development of sex-ratio distorters based on X-chromosome shredding for *Anopheles* mosquitoes but also now additional agricultural pests using either

  1. repressible expression of X shredders (as autosomal non-invasive approaches) or
  2. Y-linked invasive sex ratio distorters

• Assembly of the Y chromosome of *Anopheles* species. While our latest report documents the content and structure of the Y chromosome, we were unable to achieve a full assembly of the Y chromosome. We are continuing to test novel technologies in genome sequencing that may help resolve this problem.

• The current state of the genome assembly of *Aedes albopictus* is still in a highly fragmented state, as a result of the technologies that were used in combination with the highly repetitive nature of this genome. We are leading a new international consortium to address this issue and are implementing new technologies including
PacBio, Bio-nano and Hi-Seq to overcome assembly problems. This new assembly will be made available to the community pre-publication.

**Contract 17896:** Francesca Scolari, University of Pavia, Italy (Collaborators: Jake Tu, Romeo Bellini, Jaroslaw Krzywinski, Philippos Papathanos, Margareth Capurro)

**Development of sex-specific markers in the tiger mosquito Ae. albopictus**

The mitotic chromosome protocol that allows the sex-discrimination of 4th instar larvae of Ae. albopictus has been validated in the Rimini strain (CAA) by the correspondence between the male larvae expressing the Nix gene and the chromosome 1 banding pattern. Nix sequence has been derived from the annotated Fellini genome and showed 100% identity to the sequence already reported in Science (Hall et al. 2015). Nix is transcribed principally in the head and antennae of adult males, and therefore it might be involved in the regulation of male behavioural processes. Nix may be used as targets for PCR assays for molecular sexing. Two annotated genomes of Ae albopictus are now available, one derived from the strain Foshan (from the putative source area of the species; China-USA Consortium; genome sizes 2.0 Gb; Chen et al. 2016) and the other from the strain Fellini (from a derived population; European Consortium; genome sizes 0.94 Gb; Drisou et al. 2015). Differences in genome size among populations have been already reported, as well as size variation in chromosomes. Genetic variation analysis of populations newly introduced in Congo, having had a Chikungunya outbreak, doesn’t select nor transmit the CHIK E1-226V mutation causing the Chikungunya epidemic in La Reunion (Vazeille et al. 2016). Therefore it was proved that genetically differentiated populations of Ae. albopictus have impact on vector competence. Transcriptomes from the antennae of Ae. albopictus adults from four wild populations have been developed and 44 Odorant Binding Proteins (OBPs) and 78 Odorant Receptors OR genes were identified. Some of these sequences display transcriptional and/or SNP variations among the populations and sex-biased expresional variation is currently analyzed. Three OBP genes showing sex/tissue specificity have been expressed in heterologous systems and the resulting proteins are subjected to binding assays using known odour ligands. In addition RNA-seq libraries derived from males and females separately in relation with different physiological stages have been developed in order to provide a reservoir of genes important for sexual behaviour and sex separation.

Citations:


2016 - Importance of mosquito "quasispecies" in selecting an epidemic arthropod-borne virus. Sci Rep 6: 29564


Aims for the next 18 months:

· The mitotic karyotype of Ae albopictus obtained from the imaginal discs of sexed fourth instar larvae will be evaluated using banding patterns and fluorescence in situ hybridization (FISH) in the Rimini strain. Potential variations in karyotype will be assessed in other strains and wild populations, as well as in Nix sequences, in different strains and wild populations (collaboration with Jake Tu).
· Genome size variation among lab strains and wild populations will be estimated by flow cytometry method with the aim to see if the genome size might be related to different vector competence.
· The set of OBP and OR genes identified in Ae. albopictus antennal transcriptome separately, derived from male and female adult mosquitoes in different physiological stages, will be screened to identify any potential sex-specific expression profile. Three heterologous OBP proteins showing sex/tissue specificity will be subjected to binding assays using known odour ligands, in order to analyze sex-specific behavior.

List of Publications


Previously, we had created landing site lines for $\text{loxN-lox2272}$ Cre-Recombinase-mediated cassette exchange experiments to site-specifically target the $\text{Ae. aegypti}$ genome, and performed RMCE experiments in 3 of the 4 lines. With these lines, we didn’t obtain a cassette exchange, but instead we obtained donor plasmid integration either at the $\text{loxN}$ or the $\text{lox2272}$ site, showing that $\text{lox}$-integration is functional in $\text{Ae. aegypti}$.

In the past 18 months we constructed landing site lines for $\text{loxN-loxP}$ and performed cassette exchange experiments to see if use of the wildtype $\text{loxP}$ site in combination with a mutated $\text{lox}$ site is more efficient in cassette exchange than the two mutated sites $\text{loxN}$ and $\text{lox2272}$, but we didn’t obtain any site-specific events. However, the site-specific integration via single recombination of either $\text{loxN}$ or $\text{lox2272}$ sites in the previous experiments produced integration lines containing two pairs of homospecific $\text{lox}$ sites. These homospecific sites should readily recombine to either yield reversion of the integration reaction or completion of RMCE. Indeed, injection of a $\text{loxN}$ integration line with Cre recombinase successfully resulted in completed RMCE events, showing that Cre-RMCE is functional in $\text{Ae. aegypti}$. A prerequisite for creation of useful transgenic strains is the fitness of the transgenic insects and a good expression level of the transgene construct. Therefore, we performed fitness tests with the landing site lines and one integration line to assess the fitness cost of transgene insertion at different genomic positions. Fitness parameters tested were female fecundity, fertility, larval development time and adult survival, in addition to male mating competitiveness and determined the most fit line for downstream modification.

**Aims for the next 18 months:**

PhiC31-RMCE is another versatile RMCE method for site-specific targeting of the $\text{Ae. aegypti}$ genome. Landing site lines for phiC31-RMCE are already available in the lab, but one of them has 2 integrations, the other one has fitness costs upon inbreeding, therefore both are not suitable for RMCE applications.

- Characterize the landing site of the line with the fitness cost to understand the underlying reason
- Create more landing site lines for phiC31-RMCE
- Characterize genomic insertion sites new landing site lines
- Prove feasibility of RMCE with these lines
- Provide lines for community

**References:**


**Contract 17945**: Carlos Tur Lahiguera, TRAGSA, Gustavo Salvador Herranz, Universidad CEU Cardenal Herrera, Spain (Collaborators: Romeo Bellini, A. Puggioli, H. Bossin, P. Tortosa)

*Aedes albopictus* female removal based on pupal dimorphism using artificial vision algorithms and computer controlled laser beamer

During the last 18 months a first fully functional hardware and software prototype have been developed and tested in different rearing conditions. Device parameters as light intensity, laser shot frequency and image acquisitions have been adjusted in order to increase the efficiency of the sex sorter. At this moment more than 80% of males can be recovered with a sex purity of 99.66% of female removal. Finally, a system for administration and removal of pupae from the device has been developed.

**Aims for the next 18 months:**

- Confirm the obtained results in terms of male recovering and female removal with *Ae. albopictus* pupae from Valencia strain.
- Continue improving the system in order to minimize residual presence of females and to increase male productivity.
- Test the feeding system, in order to drain and distribute pupae uniformly over the conveyor wheel, minimizing pupae physical contact.
- Evaluate the influence of rearing conditions in sexual dimorphism using the pupae measurements obtained in the image analysis.
● Collaborate with CAA and CRVOI to analyze the frequency size distribution curves of pupae from different *Ae. albopictus* strains (including La Réunion, Rimini, Valencia and CAA protandry strains following CAA rearing SOP).
● Determine frequency size distribution curves of *Ae. polynesiensis* and *Ae. aegypti* to assess the potential for laser-based sex separation in collaboration with Hervé Bossin (Institut Louis Malardé, French Polynesia).

**Contract 17956:** Zhijian Jake Tu, Virginia Tech/Biochemistry, USA (Collaborators: Jeremie Gilles, Kostas Bourtzis, Tony James, Marc Schetelig, Igor Sharakhov, Antony James)

**Characterization and application of male-only transgenic lines in *Anopheles* mosquitoes**

Report on work related to this CRP (the past 18 months):

1) In collaboration with Drs. Zach Adelman, Igor Sharakohov, Maria Sharakhova and Xiaoguang Chen, we have discovered a male determining factor (Nix) in *Aedes aegypti* and demonstrated its ability to masculinize genetic females in transient assays (Hall et al., 2015).

2) In addition to demonstrating that the Guy1 gene confers stably-inherited and complete female lethality when integrated in the autosomes of *Anopheles stephensi* (Criscione, Qi and Tu, 2016), we have also shown that a related Y chromosome gene sYG2 also confers stably-inherited and complete female lethality of *Anopheles stephensi* (Tu et al., unpublished). We have also shown that Guy1 specifically binds to the promoter region of sYG2, possibly activates its expression. Because our hypothesis is that Guy1 and sYG2 confer female lethality through mis-regulation of dosage compensation, we confirmed that complete dosage compensation is operating in *Anopheles stephensi* (Jiang et al., 2015).

3) We discovered a small number of genes on the *Anopheles gambiae* Y chromosome including gYG2 which is the earliest transcribed Y chromosome gene (Hall et al., 2013). As part of efforts of a Y consortium (members include labs of Nora Besansky, Philipppos Papathanos, Igor Sharakhov and Zhijian Tu) to investigate the evolution of the Y chromosome in the *Anopheles gambiae* species complex, it was also shown that gYG2 is the only known Y chromosome gene that is shared among species of the *Anopheles gambiae* complex, suggesting that it is likely the male determining factor in *Anopheles gambiae* (Hall et al., 2016).

Plan for the next 18 months

1. We will make transgenic lines that ectopically express Nix in *Aedes aegypti*. We will test the effect of Nix expression in genetic females under the conditions of stable Nix integration. We will also generate transgenic lines in which Nix transgene expression is under the control of a tet-off conditional system. This will allow us to produce homozygous transgenic lines that will potentially produce all male progeny outside the laboratories when the tetracycline cue is not provided.
2. In *Anopheles stephensi*, we will further test the fitness of Guy1 and sYG2 transgenic males by focusing specifically on mating competitiveness. We will continue our
efforts to generate transgenic lines in which Guy1 or sYG2 transgene expression is under the control of a tet-off conditional system. This will allow us to produce homozygous transgenic lines that will potentially produce all male progeny outside the laboratories when the tetracycline cue is not provided.

Peer-reviewed publications related to this CRP


Book chapters on topics relevant to this CRP


**Contract 17959:** Wimaladharma Abeyewickreme, Menaka Hapugoda. Nilmini Gunawardena Nayana Gunatilake of NRC - Dengue Mega Project, Faculty of Medicine, Univ. of Kelaniya, Sri Lanka (Collaborators: Jeremie Gilles; Romeo Bellini).

**Mechanical sex separation in Aedes species**

Our research group intended to explore mechanical and behavioral approaches for sex separation of *Ae. albopictus*. During the first 18 months we have been mainly working on establishment of laboratory colonies of *Ae. aegypti* and *Ae. albopictus* in two separate laboratories at the Anti Malaria Campaign and the Molecular Medicine Unit of the University of Kelaniya. Since it was important to standardize colonization following IAEA protocols to obtain maximum production with synchronization of development at different stages attention was given to have healthy colonies in both laboratories by exchanging ideas and sharing material between these two laboratories.

During the second 18 months we have made some experimental studies on separation of larvae/pupae separating *Ae. albopictus/Aedes ae.agypti* males from females using the following three mechanical and behavioral methods;

2. Fay and Morlan Glass Plate separation and
3. Testing Behavioural Methods by spiking blood meals with toxicants.
Results of standard metal sieves method revealed that a mesh with 1.2 mm pore size gave the maximum separation of male pupae of both species which was 73%.

In Fay and Morlan glass plate separation method experiments it was revealed that when larvae were reared under enhanced colony conditions the glass plate could separate 100% of the male pupae of both species along with 1.15% female pupal contamination. In this method separation resulted a mortality of 4-5%.

Efficacy of Ivermectin (Ivotec, 1% w/v) and Spinosad (Spinosin, 12% w/v) were separately and together spiked in cattle blood to feed both *Aedes aegypti* and *Aedes albopictus* were tested separately. Spiking blood meals with Ivermectin 8 ppm and Spinosad 8 ppm separately killed 100% of the *Aedes* females within 24-48 hours. In experiments with combined spiking blood of both ivermectin and Spinosad, percentage of females fed was decreased with the increase of the toxicity (concentration). However it could be observed that when females of both species were fed on a combination of Ivermectin 4 ppm and Spinosad 4ppm could fed fed females in two blood meals given 24 hrs apart could eliminate 100% of the females exposed. Results also revealed that there was no significant difference in male mortality in these experiments in males in experimental cages compared to test cages.

Overall results of these experiments done in under laboratory conditions in small scale reveal that mechanical separation of Sri Lankan strains of *Aedes* mosquitoes using Fly and Morlan separator followed by spiking blood meals with Ivermectin/Spinosad in combination has a very high potential of sex separation of *Aedes* mosquitoes as an efficient method to be used for 100% male releases for SIT and or IIT approaches for dengue vector control.

Aims for the next 18 months:

- Further improve these methods with successful results obtained during the past 18 months by standardising the protocols followed and taking into account the natural protandry of *Aedes* species and the collection of the male pupae only during the first 24hr after the first set of pupae emerge.

- Optimise methods for behavioral sex separation at the adult stage by feeding female mosquitoes with blood spiked with other insecticides (malathion, dieldrin etc.) and/or other potential mosquito toxins (other than Ivermectin and Spinosad) which may have quicker female knock down effects.

- Explore seeking behaviour of males/females to create a mechanical separation system for capturing males/females that are drawn to host cues (heat, CO₂, odours and other male attractants such as nectar, plant extracts, etc.). Partitioning of sexes into separate chambers and/or killing upon resting on different surfaces (eg. metal and electrocution) will also be investigated.

- Identification of markers for future collaborative molecular studies will be carried out during the larval rearing process. Attempt will be made to establish collaborative links
with CRP partners particularly working on molecular aspects of *Ae. albopictus* for SIT and/or IIT.

Publications:

Sex separation of Aedes mosquitoes for Sterile Male Technique (SIT) and Incompatible Insect Technique (IIT) using Mechanical and Behavioural Methods. -Manuscript in preparation for publication.

**Contract 17950:** Hervé Bossin, Institut Louis Malardé, French Polynesia (Collaborators: Romeo Bellini, Maurizio Calvitti, Carlos Tur Lahiguera, Gustavo Salvador Herranz, Pablo Tortosa, IAEA IPCL)

**Developmental and mechanical sex separation in *Aedes polynesiensis***

The efficacy and sustainability of the *Wolbachia* incompatible insect technique (IIT) to control the disease vector *Ae. polynesiensis* is being evaluated through open release trials in French Polynesia. A proof of concept trial was conducted on the island of Raiatea and a ranging trial was later performed on the private atoll of Tetiaroa, which supported the pursuit of field evaluation. Since the last RCM, the ILM mosquito team initiated an IIT suppression trial of significant size (75 ha) to control and possibly eliminate the *Ae. polynesiensis* mosquito population present on an isolated islet of this atoll.

**Progress to date and main achievements:**

Over the past 18 months, entomological and environmental baseline data were collected at the field site to characterize and monitor the dynamics of the *Ae. polynesiensis* mosquito before and during the IIT intervention. Weekly releases of *Wolbachia* incompatible males initiated in Sept. 2015 continued until the end of Sept. 2016 (57 weeks of release in total). Over 3 million incompatible males were successfully produced, transferred and released at the Tetiaroa site during that time frame, with an average of 56,000 incompatible males released each week (750 males/ha). These releases resulted in the drastic suppression and possibly elimination of the targeted *Aedes polynesiensis* population as determined by adult trap data and ovitrap indices compared to adjacent no-release control islets. *Wolbachia*-induced sterility was detectable within a few weeks of treatment, and reduction of the adult female population became manifest after only 4 months of intervention.

Our efforts to use the open IAEA rearing pans for male production were not successful. This is likely due to relatively fluctuating environmental conditions observed in the ILM insectary particularly during the dry (relatively cooler) season, which resulted in suboptimal synchrony of development across trays. More consistent results were obtained with standard ILM trays (closed with lids). Tests involving IAEA rearing trays will be resumed once climate control in the ILM insectary gets improved.

In the absence of a genetic sexing strain for *Ae. polynesiensis*, male/female sorting relied upon both developmental (protandry) and mechanical sex separation techniques (Focks mechanical sorter). To assess the quality of sex separation and minimize the risk of accidental female release, over 1,500 pupae were randomly sampled each week following mechanical
separation and their sex was verified individually under the microscope. Under ILM standard laboratory rearing conditions, the sexing accuracy averaged 99.99% male purity (0.01% ± 0.0003 females, mean ± SD) with rare female contamination events detected during intervention. This confirms the relative stringency and reproducibility of *Aedes polynesiensis* mechanical sexing under mid-scale production conditions (≤ 100,000 males/week).

While the limited number of females accidentally released did not result in population replacement, these rare events highlight both the critical need to develop more efficient sexing systems for *Ae. polynesiensis* and other mosquito vectors and the added safety that the combination of IIT with SIT can bring (irradiation of male batches at low doses that specifically sterilize contaminating females) (Bourtzis et al., 2014). Furthermore, these observations warrant the need to strengthen available models of inadvertent *Wolbachia* release to develop contingency plans at scales appropriate to management actions.

Aims for the next 18 months:
- Explore ways on how to further synchronize egg hatching for the purpose of male mosquito mass production and measure the impact on the stringency and reproducibility of sex separation. This work will be conducted at ILM following CAA and University of São Paulo proposed experimental protocols.
- Determine frequency size distribution curves of *Ae. polynesiensis* and *Ae. aegypti* pupae to assess the potential for laser-based sex separation (collaboration with Carlos Tur Lahiguera, TRAGSA, and Gustavo Salvador Herranz, Univ. Cardinal Herrera, and IAEA IPCL).
- Compare the influence of *Wolbachia* strains on developmental parameters (e.g. protandry, time to pupation) of *Ae. polynesiensis* wild type (*WolbA*) and incompatible (*WolbB*) colonies toward their exploitation to improve sex separation.
- Monitor the dynamics of *Wolbachia* in *Ae. polynesiensis* wild type and incompatible strains and study the effects of rearing and sex-separation standard procedures on *Wolbachia* (collaboration with Maurizio Calvitti, ENEA and Pablo Tortosa, PIMIT).
- ILM trial data will be provided to feed mathematical models designed to predict the outcome of accidental *Wolbachia* female release (collaboration with ENEA).


In 2014, in the framework of our participation to this CRP, we have uncovered unexpected biological traits that contribute to make the ARwP mosquito strain (single infected with *wPip* Wolbachia) potential enhancer of conventional SIT or IIT for suppressing *Ae. albopictus* populations.

In 2014 a colony of this mosquito strain was established at the CAA “G. Nicoli” (former Italian IAEA Collaborating Center), in order to study how this strain responses to Standard Operating Procedures developed at CAA for mass rearing and sex separations of the wild type Rimini strain (RN).
We evaluated the following functional parameters: i) male and female pupation dynamics; ii) efficiency of mechanical sexing; iii) male mating competitiveness in comparison with irradiated and wild-type males.

In the first series of experiments, carried out in 2013-2015, this mosquito strain showed a higher rate of production of male pupae in the 24 hours after pupation onset and, for similar level of male productivity, a lower percentage of residual contaminant females when applying mechanical sexing procedures (0.22%). Furthermore, ARwP were more efficient, than irradiated RN males, in competing with wild-type males for virgin females (wild-type) in large enclosures, thus inducing a level of sterility significantly higher than that expected for an equal mating competitiveness (CIS=3).

During the last year, a second series of experiments have been done to confirm the differences found in pupation dynamics and efficiency of mechanical sex separation between ARwP and RN when intensively reared (3 cycles for ARwP; 61 cycles for RN). Furthermore, we wanted to understand if the differences were specifically linked to the presence of the wPip strain or to a different genetic background (or ecological adaptation) probably arisen from a different number of SOP cycles to which RN and ARwP have been exposed over generations.

To provide these answers, in the laboratory of ENEA we set up a parallel experiment, to compare the pupation dynamics and the efficiency of mechanical sex separation of five mosquito strains of which we knew the geographical origin and the number of generations from the laboratory colonization:
ARwP-F85 (wPip infected), AR-F85 (same genetic background but Wolbachia free), RN-F65, SR-F5, HeF5 (all naturally superinfected but recently laboratory colonized).

Results

Pupation onset
The wPip Wolbachia, studied in ARwP, significantly shortens the time needed by first instar larvae to reach pupal stage (4.9 d), when compared to the aposymbiotic line (same genetic background) (5.25 d) and other naturally-infected (range 5.14-5.33 d). Genetic background effects seem to have a secondary role on the pupation onset confirmed in triple infected HC strain (Zhang et al., 2016).

Pupal production
ARwP shows a significantly higher percentage of developed pupae by 24 h after the pupation onset (32% of the initial number of L1 larvae), in comparison with the aposymbiotic ARF85 (19.7%) and the wild-type RNF65 strain (22.7%) while newly colonized Ae. albopictus seem to not significantly differ from ARwP.

Sieving efficiency and mosquito size
Although ARwP showed the lowest rate of pupae passing through the meshes of metal sieve, at 24hs from the pupation onset, differences between the Ae. albopictus strains were not significant.

However differences in wing size of males has been measured comparing ARwPF85 with long term colonized superinfected RNF65, and LRF30. In both cases wing size of ARwP males were larger.
Female residual contamination

When we compare ARwPF85 and RNF65 and HeF5, ARwP strain showed a reduced percentage of residual females among pupae expected to be males (0.36 vs 1.47 and 1.03). This difference were statistically significant. Although the improvement due to the wPip strain in Ae.albopictus on the efficiency of mechanical sex separation is relatively low, it should be considered an interesting result because easy to integrate with more advanced methods (i.e methods using artificial vision and controlled laser beamer).

Future workplan in order of priority

In the next 18 months we’ll continue, in collaboration with CAA, to test the effect of more intensive rearing protocols on ARwP strain for the field application of effective suppression protocols. Larvae will be reared in larger trays containing 4000 larvae (2 larvae/ml) at higher temperature (30°C) to enhance the production system.

We will monitor eventual effects on the following colony parameters:
- Male productivity-quality (numbers and quality in terms of male mating competitiveness in field conditions (gardens, backyards made available by volunteers) by release and recapture at different times and distances from the release points.
- Pupal size, sexual dimorphism and sex separation efficiency. So far, we have observed that the ARwP pupae are slightly bigger compared to tested wild type strains (RN_{f60} and LR_{30}). But now we need to understand what wPip implies in terms of sexual dimorphism. We wonder if female and male pupae have been affected to the same extent. In case of ascertained increased sexual dimorphism, it would be worth setting up a cooperation with Tragsa to combine to apply the laser beam technology to the ARwP strain.

As comparison, we will analyze the response of He and SR, naturally infected strains, to a higher number of cycles of intensive rearing in terms of male productivity-quality, sex separation efficiency, so as to compare ARwP with naturally infected strains exposed to an equivalent number of mass rearing generations.

- Outcome of accidental female co-release. This CRP has for primary objective to develop methods that can fully prevent the unwanted release of females. Until such methods become available, understanding and predicting the consequences of accidentally female release is critical, in particular when using Wolbachia transinfected/incompatible strains. Regulators need feedback from scientists to take appropriate decisions.

Laboratory cage tests already started, will be carried out to validate mathematical models (Moretti et al., in prep, Dobson et al., 2002) predicting the outcome of simultaneously accidentally released Wolbachia transfected females (fertile and after radiation substerilization) in the framework of a bidirectionally incompatible pattern (ARwP vs naturally infected mosquitoes) exploited in combination with low irradiation dosage treatment (SIT+IIT). Pressure of biting females (generation after generation) will be monitored as the frequency of different Wolbachia infection types (PCR).

- Irradiation on Wolbachia density. Since we properly consider SIT+IIT an effective methods to neutralize the potential negative effects of releasing females, we need to evaluate the effect of irradiation (the lowest dose enough to sterilize females), combined with intensive rearing, on Wolbachia density in ARwP and in naturally infected strains (Rimini). This because an
eventual significant depletion of the endosymbiont load might alter the vectorial capacity of females.

Objective of this experimentation will be the set up of sterile male release strategies ensuring the best possible compromise between efficacy, safety and sustainability.

Publications


**Contract 17925:** R. Bellini, A. Puggioli, M. Carrieri, Centro Agricoltura Ambiente “G. Nicoli”, Italy (Collaborators: Maurizio Calvitti, Jeremie Gilles, Herve Bossin, Carlos Tur Lahiguera, Gustavo Salvador Herranz, Pablo Tortosa)

**Exploiting sex dimorphism and protandry for *Aedes albopictus* sex separation**

During the second 18 months period of this CRP, we concentrated our work on the possibility to increase the male productivity in mass rearing by enhancing the protandry of *Aedes albopictus*, while we stop working on the sexual dimorphism because we realized during the previous CRP period that the sexual dimorphism was not an easily selectable character. While it seems that the protandry has more useful genetic pattern to be possibly exploited for sex separation in *Ae. albopictus*. After nine generations of lab selection by crossing earlier developing males with slower developing females we observed that the sex ratio in the pupae collected at 24 h from the pupation onset, is significantly shifted in favor of males compared to the control strain (we get about 98% of males in the selected strain against 87% males in the control), while the productivity (calculated as the number of pupae produced at 24 h on the number of starting larvae) resulted not different between the two strains. The difference in the sex ratio between the two strains seems mostly due to an induced delay in the development of the females then to an induced precociousness in the development of the males.
We also realized that by using our standard egg hatching protocol we may lose some advantage in protandry. This may results from the fact that eggs are kept in the hatching glass for about 14-16 hours and therefore the hatching time may be distributed in a large time window.

We therefore conducted experiments to see if it might be possible to start the larval rearing from more synchronous larvae by reducing the time window of hatching. From this study it seems possible to reduce to 4 hours the hatching time by conditioning the hatching container with the standard nutrient broth before introducing the eggs, without any reduction in the hatching rate.

This new hatching method has been therefore adopted in the procedure to select the enhanced protandry strain.

We also conducted specific experiments on the effect of shaking the egg hatching container, and of using boiled water and changing the pH range. Shaking the egg hatching container and using boiled water did not induce any egg hatching. Operating the egg hatching in the pH range 1.5 – 13.5 produced very low hatching rate at pH below 5.0 and above 8.0, with no clear effect on sex ratio.

We continued the collaboration with ENEA on the evaluation of parameters of the Ae. albopictus ARwP strain. The data are presented in the ENEA report.

Aims for the next 18 months:

● We plan to continue the work on egg hatching by refining the procedure to improve synchronicity in egg hatching and larval development, by testing hatching times shorter than 4 hours. In this context, we will test the possibility to use nutrient broth conditioned hatching solution as well as boiled water after conditioning the eggs by wetting them shortly.

● We will continue to select the enhanced protandry strain using the improved egg hatching procedure. The outcome will be regularly evaluated during the selection process by checking the following parameters: male and female pupae size, male pupae productivity, sex ratio. The comparator will be the dataset obtained at the beginning of the selection process.

● We will analyze more precisely the effect of pH in the range 5.0 – 8.0 on egg hatching and sex ratio in Ae. albopictus.

● We will continue the cooperation with ENEA and PIMIT on the evaluation of the ARwP strain, and with TRAGSA on the evaluation of the performance of the prototype sexing laser machine.


**Contract 17926**: Gulzamin Khan, Alam Zeb, Inamullah Khan, Nuclear Institute for Food and Agriculture (NIFA), Pakistan (Collaborators: C. Tur, R. Bellini, A. Puggioli, W. Abeyewickreme, P. Tortosa)

Exploring mechanical, behavioural and nutritional methods of sex separation in mosquitoes.

Dengue is a deadly disease with alarming spreading potential in Pakistan thus need proper attention. As no vaccine for the disease is available globally, therefore, vector control is the only option in the present scenario. The use of insecticides for vector control have environmental constraints, health hazards and resistance development in mosquitoes, thus, environment friendly vector control strategies with main emphasis on Sterile Insect Technique (SIT) are needed. Efforts are being made under the umbrella of IAEA CRP in this regard. Besides our research efforts towards SIT, we regularly carry out the entomological surveillance of Aedes species and seeking environment friendly conventional methods for vector control. The efforts on our part for the sex separation of *Aedes* and *Culex* mosquitoes using nutritional and mechanical methods are in progress for successful launching of SIT programs.

Mechanical, behavioural, nutritional and natural protandry for sexing are needed when efficient systems for eliminating females in the embryo stage have not been developed or are not yet available. This project was proposed to develop a specific and efficient system for male female separation. Sex separation is currently possible at the pupal stage for both *Aedes* albopictus and *Culex* species due to size dimorphism between male and female pupae: females resulted by different combinations of nutrition that resulted in distinct dimorphism, exploiting the natural protandry and behaviours that favoured the different means of mechanical methods. The trials on large scale are needed for the confirmations and effectiveness of the results. Work on devising an effective blood spiking methods for eliminating/knocking down the left over female were also needed and its standardization/optimization is going on.

By testing the different sources of protein and carbohydrates under repeated trials, it was found that these ingredients in different ratios and combinations have significant effect on the dimorphic development of mosquitoes both on their developmental time and on their size. The combined effect of both sources favor the sexual dimorphism due to significant difference in size. The pupal size (cephalothorax) was significantly different (0.9mm for male
and up to 1.3mm for female Aedes albopictus). This variation effect due to different formulation of diets was exploited successfully in mechanical sex separation. It was concluded that Stevia 75%+ Bovine Liver20%+ Yeast5% diet in combination showed the significant effect (47% over control) on Sexual dimorphism in male/female pupae of Aedes albopictus. However, the effect of this diet combinations did not favor the sexual dimorphism in Culex species. From the results on mechanical sex separations, it was concluded that the mesh size of 1.25 separated both the sexes effectively with a mean accuracy range (97-100%). It is therefore, recommended for the mechanical separation of the male/female having sexual dimorphism resulted due to nutritional effect. During behavioral studies, the protective sheath formation (32% variation in size) was observed at 5 degree Celcius in both species of mosquitoes pupae (Culex and Aedes). This low treatment of temperature for 24-48 hours of exposure time facilitated the sex separation of the female from mix culture. However, the protocol need to be standardized and need further studies for minimizing the mortality effect. In the trials of spiking blood meals with different toxicants, the NIFA bio-larvicide has shown high potential (97-100% mortality) as a viable treatment to eliminate female Aedes from mix culture colonies comprising of the both the female and male adults and needs to be tested in further study by adding some palatable ingredients. The devised diet formulation will further be used on mass rearing scale and the effect of larval density plus male mating capacity will also be tested for this particular diet formulation. The fabrication of the mass rearing cages on IAEA design is also in progress and will be streamlined in the upgrading mass production for the subsequent SIT program.

Aims for the next 18 months:

- Fabrication of mass rearing cages as per IAEA design (our Technical services Division is working on this)
- Simulation of mass rearing program on the larval diet that lead to distinct sexual dimorphism
- Diet quality (the diet that resulted in distinct dimorphism will be subjected to quality tests on large scale); Effect of larval density and food concentration on larval development etc.
- Effect of different larval densities on sexual dimorphism.
- To standardize the behavioral effect (buoyancy between male and female pupae) due to light stimuli on the sex separation.
- To work out the correlation of hatching period in the fashion of pupal stage on sex determination/sex ratio production.
- To work out the optimum period in the first day pupae production on percent male/female production (Natural protandry).
- To determine the optimum time period for the effective sex separation through sieving devices.
- Comparative efficacy of sieving apparatus and John Hack larvae/pupae separator for sex separation. (Fabrication of locally designed mesh for comparatively large scale is in progress based on the cephalothorax size of Aedes albopictus that resulted due to the diet effect).
- To standardize the effective concentration (NIFA-Bio-products) that favored palatability and the subsequent elimination of the female in the blood spiking methods
● Development of nutritional based strain on this specific diet (Seeking of any possibility).
● Trial on sexual dimorphism on Aedes aegypti will also be carried out as this species has been recorded and was found as dominant over Aedes albopictus during our surveillance activities in some epidemic area of dengue disease (e.g. Swat Valley).

Recent Publications related to the Project:


Booklet/pamphlet (Local language):


Products:

● Developed an effective plant based environment friendly mosquitoes repellant "Dengue Guard" for protection against mosquitoes species.


Institute: Centre for Opportunistic, Tropical and Hospital Infections, National Institute for Communicable Diseases, Private Bag X4, Sandringham, Johannesburg 2131, South Africa and Wits Research Institute for Malaria, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

Collaborators: Cyrill Ndo, Pablo Tortosa and Kostas Bourtzis

Development of sex separation tools to eliminate female Anopheles arabiensis during mass production.

Introduction:
In the context of eliminating malaria transmission by 2018 the South African government is investigating use of the sterile insect technique (SIT) as an additional tool for vector control intervention. Over the past years South Africa has optimised various technical aspects of the sterile insect technique for control of the malaria vector Anopheles arabiensis. A sexing system utilising dieldrin resistance as a selectable marker to separate males from females has been developed. The resultant strain has been characterised for its potential as mass release strain for pending pilot SIT releases in South Africa. However, use of this genetic sexing strain (GSS)- denoted GMK depends on overcoming a number of challenges namely (a) dieldrin is a banned insecticide because of its effects on the environment and human health and (b) the GMK strain has inherent low fertility levels which has a major impact on cost and efficiency during mass rearing (our investigations indicated that 6 -10% males survive into adulthood after dieldrin treatments. In view of this and current lack of an alternative and reliable sex-separation strategy for An. arabiensis our group is participating in a Coordinated Research Programme to explore classical genetic and modern biotechnology techniques to accomplish female elimination in major mosquito vectors of disease. Within this CRP our institute is involved in the development of alternative sex-sorting systems based on (i) exploiting the exclusive blood feeding behaviour of adult females, and (ii) exploring use of temperature sensitivity lethality as a tool for sex separation in An. arabiensis. Additionally since dieldrin is a banned insecticide, we intend to alleviate regulatory concerns by evaluating the effectiveness/efficacy of other environmentally-acceptable insecticides targeting GABA receptors on the developed GSS An. arabiensis GMK strain.
Summary of progress over the past 18 months.

Over the past 18 months we have done tests to check on the stability of the insecticide resistance marker used to develop the GSS_GMK strain. Results showed that the marker is fairly stable and the strain begun losing the marker in a small proportion (0.3%) of the males in the 8th generation however, the marker did not migrate into females. Dose optimization of the four alternative GABA receptor antagonists (picrotoxin; alpha-endosulfan; lindane and isoxazole) resulted in LC90 of 0.25ppm, 4.8ppm, 0.38ppm and 3.2ppm respectively after exposing 3rd to 4th instar larvae of an An. arabiensis susceptible control strain. Exposure of the GSS strain larvae to double the LC90 and an intermediate of the double the LC90 and LC90 showed that the intermediate dose selected most of the heterozygotes and killed all susceptibles while the LC90 killed susceptible and most of the heterozygous resistant males. However this data is based on one replicate and experiments are in progress.

In the context of developing a sexing strain based on tsl we carried out experiments to determining the minimum (permissible) and maximum (restrictive) temperature at which an An. arabiensis laboratory strain targeted for induction of tsl mutation tolerates. Three developmental stages of the strain (eggs, larvae and pupae) were exposed in different batches to seven different temperatures (25, 30, 35, 37, 40, 42 and 44 oC) at 5 different temperatures (1, 2, 3, 4 and 6hrs). Results indicated that An. arabiensis is not restricted by any of these temperatures irrespective of time exposure. Although there was difference in lethality between each temperature and time of exposure none of the temperatures at the times tested can be used to screen for a tsl lethality in this An. arabiensis strain. Further experiments will be done by increasing the time of exposure for each of the temperature for up 24hrs. Meanwhile experiments on induction of conditional temperature sensitivity mutations using ethyl methane sulphonate (EMS) showed that a dose of 0.01M is the optimal dose which can induce conditional mutagenesis in male An. arabiensis without affecting their reproductive fitness.

Experiments on exploring the specific blood feeding behaviour females for sex separation in An. arabiensis were carried out. This started by optimising concentration of the toxicant (ivermectin) to be used for blood spiking. A concentration of 7.5ppm was found to be optimal and it killed all females in mixed population of males and females within 10days with a median survival of the females being only 2 days. The remaining males did not show any loss of both physiological and reproductive fitness.

Activities planned for the next 18 months.

In the next 18 months we propose to:
• Continue on tests on the optimal concentration of each of the four alternative GABA receptor insecticides which will select for heterozygous males in the newly developed GSS strain.
• Carry out genetic analysis of a morphological marker (green pupae) screened from, a laboratory An arabiensis strain.
• Finalise the maximum (restrictive) temperature at which An. arabiensis laboratory colony can survives.
• Inducing conditional mutations in *An. arabiensis* male mosquitoes using EMS and screening for iso-families with the potential to contain tsl alleles.

**Publications:**


**Contract 17953:** Cyrille Ndo (OCEAC, Cameroon), Yacouba Poumachu (OCEAC, Cameroon), Parfait Awono-Ambene (OCEAC, Cameroon), Igor Sharakhov (University of Virginia Tech USA) (Collaborators: IPCL, Jeremie Gilles, Kostas Bourtzis)

**Developing a genetic method for accurate sex separation and female elimination in *An. arabiensis***

The general aim of our project is to develop an *An. arabiensis* genetic sexing strain in which females are homozygous for a recessive temperature-sensitive lethal (TSL) leading to lethality under non permissive conditions (i.e. 40-41°C), thus allowing selection of heterozygous resistant males. *Anopheles arabiensis* colony from North Cameroon has been established in our insectary and as first screened for temperature sensitive phenotype. After that, we performed mutagenesis of *An. arabiensis* male mosquitoes using 3 distinct EMS doses and screened isofamilies for the *tsl* phenotype as well as for the presence of morphological markers. We have identified 2 strains named ANA_S1 and ANA_S2 together with 3 other families expressing temperature sensitivity. During the last 18 months, we definitely confirmed the presence of the *tsl* in these strains and selected a temperature sensitive strain which is now maintained in the insectary of OCEAC at 25±1°C. Additionally, the inheritance pattern of the *tsl* has been determined by standard crossing followed by phenotypic screening. So far, screening of both natural and laboratory reared populations in order to isolate morphological marker remain unsuccessful, while the presence of the *kdr* mutation in our *An. arabiensis* colony has been confirmed.

**Aims for the next 18 months:**

- Continue screening both natural and laboratory reared populations in order to isolate morphological markers;
- Create a Genetic Sexing Strain by irradiating *wt* males with the aim of translocating *tsl*+ on the Y chromosome, and assess it fitness;
- Characterize irradiation-induced chromosomal rearrangements and assess the stability of the *An. arabiensis* GSS

**Contract 17933:** Pablo Tortosa, Célestin Michelle Atyame, Louis-Clément Gouagna Centre de Recherche et de Veille sur les Maladies Emergentes dans l'Océan Indien, La Réunion, France (Collaborators: G. Munhenga and M. Calvitti)

**Construction of an RdlR Genetic Sexing Strain in *Aedes albopictus***.
Our laboratory is currently developing 2 innovative vector control strategies, namely the Sterile Insect Technique and the Incompatible Insect Technique, targeting *Aedes albopictus*, a vector species of global medical concern. Although both strategies are showing promising results, they both require the availability of an efficient sexing strain allowing the mass production of sterile or incompatible males.

*The main goal achieved*
We have generated an *Aedes albopictus* Genetic Sexing Strain (GSS) using translocation of *rdl*R allele conferring dieldrin resistance. The strain named GSS-1 has been now successfully amplified and maintained in our facilities.

**Perspectives**
- Investigating the molecular basis of the linkage between *rdl* translocation and M locus (*nix* gene) in GSS-1 line (collaboration with TU Zhijian, SCOLARI Francesca).
- Construct isogenic lines and test fipronil instead of dieldrin
- Analyze the life history traits of GSS-1.
- Monitoring of *Wolbachia* density and tissue localisation in comparison with other strains (collaboration with BOSSIN Hervé, CALVITTI Maurizio, BELLINI Romeo).
- Analyze the frequency size distribution curves of pupae from our *Ae. Albopictus* strains with other strains through CAA rearing SOP (collaboration with LAHIGUERA Carlos Tur).

List of publications
THIRD FAO/IAEA RESEARCH COORDINATION MEETING ON
“Exploring genetic molecular, mechanical and behavioural methods of sex separation in mosquitoes”

10-14 October 2016

Centro Regional de Investigación en Salud Pública (CRISP)
Tapachula, Chiapas, Mexico

AGENDA

MONDAY, 10 OCTOBER 2016

08:45 – 09:00  **Kostas Bourtzis:** Welcome statement, administrative issues and goals of the meeting.

09:00 – 09:30  **Kostas Bourtzis:** Combined SIT/IIT approach as a tool for the population control of *Aedes* mosquito species.

09:30 – 10:00  **I. Fernandez-Salas, G. Bond, C. F. Marina, A. Dor, P. Liedo:** Pilot test to assess the effectiveness of the combined SIT/IIT approach to suppress *Aedes aegypti* populations in Chiapas, Mexico.

10:00 – 10:30  **Hervé C. Bossin, Hereiti Petit, Michel A. Cheong Sang, Jérôme Marie:** *Wolbachia* trial on Tetiaroa, French Polynesia: toward a possible elimination of the mosquito vector *Ae. polynesiensis*.

10:30 – 11:00  COFFEE BREAK

11:00 – 11:30  **Maurizio Calvitti, Arianna Puggioli, Elena Lampazzi, Riccardo Moretti:** Under intensive rearing, the ARwP *Aedes albopictus* strain enhances efficiency of mechanical sexing.

11:30 – 12:00  **Pablo Tortosa, Cyrille Lebon, Aude Benlali, Patrick Mavingui:** Towards the construction of an *Aedes albopictus* genetic sexing strain: almost there.

12:00 – 12:30  **Cyrille Ndo, Yacouba Poumachu, Parfait Awono-Ambene and Jeremie Gilles:** Development and evaluation of the fitness of an *Anopheles arabiensis* temperature sensitive strain using ethylmethanesulfonate.

12:30 – 14:00  LUNCH
14:00 – 14:30  **Givemore Munhenga, Thabo Mashatola, Leonard C Dandalo, Oliver R Wood, Leanne N Lobb, Basil D Brooke, Maria Kaiser and Lizette L Koekemoer:** Development of sex separation tools to eliminate female *Anopheles arabiensis* during mass production.

14:30 – 15:00  **Romeo Bellini, Arianna Puggioli and Marco Carrieri:** Exploiting sex dimorphism and protandry for *Aedes albopictus* sex separation.

15:00 – 15:30  **Carlos Tur Lahiguera, Gustavo Salvador, Ignacio Plá Mora:** *Aedes* female removal based on pupal dimorphism using artificial vision algorithms and computer controlled laser beamer.

15:30 – 16:00  **COFFEE BREAK**

16:00 – 16:30  **W. Abeyewickreme, Tharaka Ranathunga, Nayana Gunatilake, Asha Wijeguanwardena and Menaka Hapugoda:** Sex separation of *Aedes* mosquitoes for sterile insect technique (SIT) and incompatible insect technique (IIT) using mechanical and behavioural methods.

16:30 – 17:00  **Gul Zamin Khan, Inamullah Khan and Alamzeb:** Exploring mechanical and nutritional methods of sex separation in *Aedes* and *Culex* species of mosquitoes.

17:00 – 17:30  **Open discussion on the presentations.**

19:00  **Group Dinner.**

**TUESDAY, 11 OCTOBER 2016**

09:00 – 09:30  **F. Bernardini, A. Kriezis, R. Galizi, T. Nolan and A. Crisanti:** Transferring a synthetic sex ratio distortion system between members of the *Anopheles* complex.

09:30 – 10:00  **Philippos A Papathanos:** Engineering invasive Y chromosomes for insect control.

10:00 – 10:30  **Elzbieta Krzywinska, Nathan Dennison, Gareth Lycett and Jaroslaw Krzywinski:** Analysis of sex determination pathway in the African malaria mosquito, *Anopheles*.

10:30 – 11:00  **COFFEE BREAK**

11:00 – 11:30  **David A. O’Brochta:** Genetic technologies and the exploration of mosquito/plasmodium interactions.
11:30 – 12:00  **A. Smidler and Flaminia Catteruccia:** CRISPR-generated spermless Anopheles gambiae.

12:00 – 12:30  **Irina Haecker and Marc Schetelig:** Site-specific landing site systems to improve transgenic mosquitoes for their use in SIT programs.

12:30 – 14:00  **LUNCH**

14:00 – 14:30  Brantley Hall, Frank Criscione, Sanjay Basu, Yumin Qi, Jim Biedler, Xiaofang Jiang, Michelle Anderson, Vladmir Timoshevskiy, Xiaoguang Chen, Maria Sharakhova, Igor Sharakhov, Zach Adelman and Zhijian Jake Tu: Manipulating genes in the mosquito sex-determination pathway for sex separation and population suppression.

14:30 – 15:00  **Margareth Lara Capurro and Helena Rocha Corrêa de Araújo:** Construction and characterization of genetic sexing strain (*Aedes aegypti* and *Ae. albopictus*).

15:00 – 15:30  Francesca Scolari, Grazia Savini, Ludvik M. Gomulski, Anna R. Malacrida and Giuliano Gasperi: Exploring genomic and transcriptomic resources to find sex-linked markers in *Aedes albopictus*.

15:30 – 16:00  **COFFEE BREAK**

16:00 – 16:30  Open discussion on the presentations.

16:30 – 17:30  Working Group Discussions.

**WEDNESDAY, 12 OCTOBER 2016**

05:30 – 06:00  Transportation to Medfly emergence and release facility

06:00 – 08:30  Post-irradiation operation for sterile insect releases

08:30 – 10:30  Transportation and breakfast at Baos restaurant

10:30 – 11:00  Transportation to Moscafrut mass-rearing facility in Metapa

11:00 – 13:00  Tour mass-rearing and sterilization facility

13:00 – 14:30  Transportation to Argovia, an eco-touristic coffee finca

14:30 – 16:00  Lunch at Argovia restaurant
16:00 – 18:00  Tour through coffee plantation, process and flowers production
18:00  Transportation to hotel.

THURSDAY, 13 OCTOBER 2016

08:30 – 10:30  Working Group Discussions.
10:30 – 11:00  COFFEE BREAK
11:00 – 12:30  Working Group Discussions.
12:30 – 14:00  LUNCH
14:00 – 15:30  Drafting Report.
15:30 – 16:00  COFFEE BREAK
16:00 – 17:30  Drafting Report.
19:00  Reception at El Navengate restaurant

FRIDAY, 14 OCTOBER 2016

08:30 – 10:30  Reports of Working Groups and Revision of Logical Framework.
10:30 – 11:00  COFFEE BREAK
11:00 – 12:30  Drafting of Final Report.
12:30 – 14:00  LUNCH
14:00 – 15:30  Drafting of Final Report.
15:30 – 16:00  COFFEE BREAK
16:00 – 17:30  Presentation and approval of the Final Report - General discussion.
17:30  Closing.
Abstracts
THIRD RESEARCH COORDINATION MEETING

On “Exploring Genetic, Molecular, Mechanical and Behavioural Methods of Sex Separation in Mosquitoes”

Tapachula, Mexico

10 - 14 October 2016

TITLE OF WORKING PAPER: Pilot test to assess the effectiveness of SIT+IIT to suppress Aedes aegypti populations in Chiapas, Mexico.

AUTHOR (S): I. Fernandez-Salas, G. Bond, C. F. Marina, A. Dor, P. Liedo

ORGANIZATION: Centro Regional de Investigación en Salud Pública (CRISP), INSP, Tapachula, Chiapas, Mexico. El Colegio de la Frontera Sur (ECOSUR), Tapachula, Chiapas, Mexico

SHORT SUMMARY OF PAPER

**Abstract:**

Our aim in this project is to assess the effectiveness of the Sterile Insect Technique (SIT) plus the Incompatible Insect Technique (IIT) for the suppression of Aedes aegypti populations in Chiapas, Mexico.

Two sites of about 30 hectares each with similar environmental conditions have been selected near Tapachula city. Populations of Aedes mosquitoes have been monitored since October 2015 using ovitraps inside and outside of 15 houses for each locality. BG sentinel traps and GAP traps will also be used for monitoring of adult populations. Mark-release-recapture method will be use to evaluate population size and dispersal of adults Ae aegypti. Five transects around each community have been set in the surrounding vegetation zones. Traps are located at 0, 50 and 100 m) from the village perimeter. These are to evaluate spatial distribution of Ae. albopictus and Ae. aegypti. After 18-24 months monitoring, one site will be selected for sterile male mosquito releases, and the other site will be used as a control. After one year, the sites will be switched, with the purpose of have one year releases in each site.

A genetically diverse colony was established at the laboratory, collecting wild mosquitoes from 12 sites in the coast of Chiapas. Egg samples of this colony were sent to the IPCS laboratory at Seibendorff, Austria to produce a Wolbachia infected strain. Our goal is to characterize this new strain, to evaluate its performance under field cage conditions and to mass-rear it for field releases.
The rapid emergence and expansion of Zika (Musso & Gubler, 2016) and other arboviruses (e.g., dengue, chikungunya) in the last decade have become major public health issues. The situation is particularly critical in Latin America, in several U.S. and European overseas territories and in small island developing states (SIDS) in the Caribbean and the Pacific. *Aedes aegypti* and, to a lesser extent *Ae. albopictus* have been linked with most known arbovirus outbreaks but the ecology of transmission in the Pacific may prove uniquely diverse, as other species within the *Stegomyia* subgenus also play a role in transmission to humans. For example, the native species *Ae. hensilli* and *Ae. polynesiensis* have been implicated in Zika outbreaks in Yap (Duffy et al., 2009) and French Polynesia (Cao-Lormeau et al., 2014) respectively. *Ae. polynesiensis* is widely distributed across the Pacific (incl. French Polynesia, Wallis and Futuna, Fiji, Samoa, American Samoa, Tuvalu, Tokelau, Cook Islands, Kiribati, and Pitcairn) where it is the primary vector of lymphatic filariasis and of zoonotic diseases, such as the dog heartworm *Dirofilaria immitis*. Overall, no less than 12 potential dengue vectors have been identified in the Pacific Islands. With no vaccine currently available, nor targeted viral therapeutics, vector control plays a critical role in protecting individuals against infection by Zika and other existing (dengue, chikungunya) or future mosquito-borne diseases. Yet, control or elimination of *Ae. aegypti*, and especially its sister species, *Ae. albopictus* and *Ae. polynesiensis* remains difficult due to their day-biting activity as well as the heterogeneity and cryptic nature of the containers they use. New approaches are urgently needed. For *Ae. polynesiensis*, a significant suppression trial (*AeLIMIN+*) conducted by Institut Louis Malardé (ILM) is being implemented on an isolated islet (75 ha) of the atoll of Tetiaroa, French Polynesia. This integrated control approach has relied for the most part on the release of *Wolbachia* incompatible males mosquitoes that seek, court and mate with wild females, thereby reducing their reproductive capacity. Entomological and environmental data were collected at the field site to characterize and monitor the dynamics of the *Ae. polynesiensis* mosquito before and during the IIT.
intervention. Initial control measures consisted mostly in the removal of domestic breeding containers and the deployment of gravid *Aedes* traps (GAT) in the built environment. *Wolbachia* male releases were then initiated for population suppression and possibly elimination. Ca. 55,000 incompatible males have been released once weekly since Sept. 2015 (54 weeks of release to date). In total, over 2.9 millions incompatible males have been successfully produced, transferred and released to date at the Tetiaroa treatment site. The sustained, inundative releases resulted in the suppression of the targeted *Aedes polynesiensis* population as determined by adult trap data and ovitrack indices compared to adjacent no-release control islets. *Wolbachia*-induced sterility was detectable within a few weeks of treatment, and reduction of the adult female population became manifest after only 4 months of intervention. The last eclosion from eggs collected at the treatment site was recorded in May, 2016, the last egg was collected in July and, for the first time since the start of the intervention no adult females were captured in early September 2016. Taken together, these results suggest that elimination of this isolated mosquito vector population on the atoll of Tetiaroa might be within reach. This successful trial supports the evaluation of novel genetic control strategies in larger islands of French Polynesia which provide an ideal context for studying ecological questions (Cressey, 2016, Davies et al. 2016) and to evaluate novel mosquito control approaches that would otherwise be more difficult to investigate in continental systems.

In the absence of a genetic sexing strain for *Ae. polynesiensis*, male/female sorting relied upon both developmental (protandry) and mechanical sex separation techniques. QC activities (rearing/sorting parameters, male mating competitiveness, lab and field *Wolbachia* molecular monitoring) were undertaken throughout the project. To assess the level of sex separation and minimize the risk of accidental female release, over 1,500 pupae were randomly sampled each week following mechanical separation and their sex was verified individually under the microscope. Under ILM standard laboratory rearing conditions, the sexing accuracy averaged 99.99% male purity (0.01% ± 0.0003 females, mean ± SD) with rare female contamination events detected during intervention. Although these limited female releases did not result in population replacement, they highlight the critical need to develop more efficient sexing systems for *Ae. polynesiensis* and other mosquito vectors and the safety benefit that combined IIT-SIT (irradiation of male batches at doses that specifically sterilize contaminating females) can bring (Bourtzis et al., 2014).

**Cited references**


Eliminating, or reducing as far as possible, the co-release of females during the application of SIT, against invasive *Aedes* mosquitoes, is still a major challenge. The absence of an efficient method for sex separation, as a genetic sexing strain, has promoted the research of other solutions (genetic, mechanical and behavioral methods). The *ARwP* mosquito line was developed thanks to the replacement of the natural *Wolbachia* superinfection (*w*Alb A+ *w*Alb B) with a heterologous *Wolbachia* strain (*w*Pip), resulting in a bidirectional incompatibility pattern with wild-type *Ae. albopictus*.

In last two years, in the framework of our participation to this CRP, we have uncovered unexpected biological traits that contribute to make the *ARwP* strain a potential enhancer of conventional SIT for suppressing *Ae. albopictus* populations.

In 2014 a colony of this mosquito strain was established at the CAA “G. Nicoli” (former Italian IAEA Collaborating Center), in order to study how this strain responds to Standard Operating Procedures developed at CAA for mass rearing and sex separations of the wild type Rimini strain (RN).

We evaluated the following functional parameters: i) male and female pupation dynamics; ii) efficiency of mechanical sexing; iii) male mating competitiveness in comparison with irradiated and wild-type males.

In the first series of experiments, carried out in 2013-2015, *ARwP* males showed a higher rate of production of male pupae in the 24 hours after pupation onset and, for similar level of male productivity, a lower percentage of residual contaminant females when applying mechanical sexing procedures. Furthermore, *ARwP* were more efficient, than irradiated RN males, in
competing with wild-type males for virgin females (wild-type) in large enclosures, thus inducing a level of sterility significantly higher than that expected for an equal mating competitiveness.

During the last year, a second series of experiments have been done to confirm the differences found in pupation dynamics and efficiency of mechanical sex separation between AR\textsubscript{w}P and RN when intensively reared (3 cycles for AR\textsubscript{w}P; 61 cycles for RN).

Furthermore, we wanted to understand if the differences were specifically linked to the presence of the \textit{w}Pip strain or to a different genetic background (or ecological adaptation) probably arisen from a different number of SOP cycles to which RN and AR\textsubscript{w}P have been exposed over generations.

To provide these answers, in the laboratory of ENEA we set up a parallel experiment, to compare the pupation dynamics and the efficiency of mechanical sex separation of three mosquito strains: AR\textsubscript{w}P, the \textit{Wolbachia} free strain (AR) that was used as receiver strain to generate AR\textsubscript{w}P in 2009 and a wild type strain (SR) collected North of Rome. These 3 strains were maintained in laboratory conditions for at least 5 generations.

Results and more details will be presented in Tapachula.
THIRD RESEARCH COORDINATION MEETING
On “Exploring Genetic, Molecular, Mechanical and Behavioural Methods of Sex Separation in Mosquitoes”
Tapachula, Mexico
10 - 14 October 2016

TITLE OF WORKING PAPER: Towards the construction of an Aedes albopictus Genetic Sexing Strain: almost there
AUTHOR (S): Pablo TORTOSA¹, Cyrille LEBON², Aude BENLALI², Patrick Mavingui¹
ORGANIZATIONS:¹Reunion University, CNRS 9192, INSERM 1187, IRD 249, Research Unit “Processus Infectieux en Milieu Insulaire Tropical (PIMIT)”. Technological Platform CYROI - Saint Denis - Reunion Island - France
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SHORT SUMMARY OF PAPER
Abstract:
Following the construction of two homozygous mosquito lines either sensitive or resistant to dieldrin, we carried out two strategies aiming at obtaining a mosquito line with strong sex ratio distortion. Homozygous resistant males were submitted to increasing doses of irradiation in order to induce chromosomal rearrangements. These treated males were then used for the construction of the Genetic Sexing Strain (GSS) line following 2 distinct strategies. We first developed an original selection protocol in which males bearing the insecticide resistance gene in linkage disequilibrium with the M locus have a fitness advantage over non-distorted males. This strategy did not lead to the selection of a GSS line, likely because the fitness advantage was counter selected by a low fecundity rate of the GSS males. We thus implemented a distinct non-selective and previously described protocol by identifying those crosses between irradiated resistant males and susceptible females producing significant sex ratio distortion. This protocol allowed us to identify a first GSS line producing around 95% males following dieldrin treatment. Some life history traits of this line will be presented, as well as the improvements to be carried out in order to obtain a perfect or nearly perfect GSS.
THIRD RESEARCH COORDINATION MEETING

On “Exploring Genetic, Molecular, Mechanical and Behavioural Methods of Sex Separation in Mosquitoes”

Tapachula, Mexico

10 - 14 October 2016

TITLE OF WORKING PAPER: Development and evaluation of the fitness of an Anopheles arabiensis temperature sensitive strain using ethylmethanesulfonate

AUTHOR (S): Cyrille Ndo¹, Yacouba Poumachu¹, Parfait Awono-Ambene¹ and Jeremie Gilles²

ORGANIZATIONS: 1 - Organisation de Coordination pour la Lutte contre les Endémies en Afrique Centrale (OCEAC), Yaoundé-Cameroon; 2 - Insect Pest Control Laboratory, International Atomic Energy Agency

SHORT SUMMARY OF PAPER

Abstract:
The Sterile Insect Technique (SIT) is among new tools being investigated to combat malaria in the context of expansion of drug and insecticide resistance in Plasmodium parasites and Anopheles vectors respectively. The development of this technique in Anopheles vectors require among other, a good sexing method allowing to completely remove hematophagous females in baths of mosquitoes to be released.

Using Ethylmethane sulfonate, a mutagen agent known to induced temperature sensitivity in insect, an An. arabiensis strain containing a putative temperature-sensitive lethals (TSL) was selected from wild mosquitoes and was established at the insectary at OCEAC (Cameroon). Temperature sensitivity pattern of this strain has been assessed at various temperatures and time duration using early larval stages or eggs. The fitness, in term of female fecundity, eggs fertility and adults longevity, was assessed over generations and was compared against the normal strain. Moreover, the inheritance pattern of the putative TSL was investigated using crossing experiments.

The results obtained confirm the presence of a TSL in the An. arabiensis strain selected and suggest that mutagenesis didn't significantly affect its fitness. Crossing experiments are still underway to assess the inheritance pattern of the TSL prior irradiation of wild males to induced the translocation of the TSL locus on the Y chromosome.
TITLE OF WORKING PAPER: Development of sex separation tools to eliminate female Anopheles arabiensis during mass production.


ORGANIZATION: WITS Research Institute for Malaria and National Institute for Communicable Diseases, Vector Control Reference Laboratory, South Africa

SHORT SUMMARY OF PAPER

Abstract:

Over the past five years South Africa has optimised various technical aspects of the sterile insect technique (SIT) for control of the malaria vector Anopheles arabiensis including the development of a sexing system. A sexing system utilising dieldrin resistance as a selectable marker to separate An. arabiensis males from females has been developed and characterised for its potential use during SIT pilot releases. However, use of this genetic sexing strain (GSS) - denoted GMK - depends on overcoming a number of challenges namely (a) dieldrin is a banned insecticide because of its effects on the environment and human health and (b) the GMK strain has low fertility levels which has a major impact on cost and efficiency during mass rearing (approximately 6-10% males survive into adulthood after dieldrin treatment). In view of this and because of a lack of an alternative and reliable sex separation strategy for An. arabiensis, we are developing alternative sex-sorting systems based on (i) blood feeding behaviour of adult females, and (ii) sex linkage using a selectable temperature sensitivity gene in which temperature conditions favour the production of males only. We are also evaluating the effectiveness/efficacy of other environmentally-acceptable insecticides targeting GABA receptors using the GMK An. arabiensis strain.

During the meeting results on the minimum (permissive) and maximum (restrictive) temperatures which do not affect egg, larval or pupal viability in An. arabiensis will be presented as part of the work currently underway to develop the use of temperature-sensitive lethality as a selectable marker for sex separation. In addition, progress in evaluating the effectiveness of five GABA antagonists (lindane, fipronil, isoxazole, alpha-endosulfan and picrotoxin) as alternatives to the use of dieldrin in separating males from females in the newly developed GMK sexing strain will be presented. We will also provide results on experiments carried out to determine the practicability and potential of using blood spiked with ivermectin to eliminate females during mass-rearing. Additionally, data on the stability of the newly developed GMK GSS will be discussed.
In order to release sterile male only, without any residual presence of females, we must develop an efficient and reliable sexing method. The current methods for sexing *Aedes albopictus* are mainly based on the sex dimorphism of pupae but the male pupae productivity with these methods is in the range of 22-28% (calculated on the total number of reared males), with a residual presence of females in the range 0.3-1.0%. In case we need to keep the presence of residual females close to 0% the productivity of the male decreases considerably.

These productivity rates are not acceptable in case of large scale mass rearing facilities and better performing system are under development. We are exploring the possibility to increase the male productivity by enhancing the time window separation between males and females.

**Protandry**

*Ae. albopictus*, as well as other species, shows natural protandry which may be exploited to increase sex separation efficiency. We investigated the possibility to enhance protandry by the artificial selection of a strain obtained by crossing the males that pupate earlier with the females that pupate later on. At each generation, 250 precocious males and 250 late females were collected and placed into a cage (40x40x40 cm) for mating and egg production.

After nine generations of selection we observed that the sex ratio, within 24 h from pupation onset, was significantly different compared to our control strain, with results suggesting a delay in the development of the females.

In our standard mass rearing procedure, the *Ae. albopictus* egg hatch is stimulated with a nutrient broth culture overnight, therefore the newly hatched larvae collected in the morning may have an age in the range 1-12 hours, which may be critical when coetaneous larvae are needed.
Therefore, in order to produce a protandry strain starting from more coetaneous larvae, we tested the effect of boiled water, shaking movement and combination of these methods including the use of nutrient broth, on the egg hatching in comparison with our standard procedure.

We also analyzed the development of larvae obtained from eggs hatched in time intervals of 4 hours, using two different methods to prepare the hatching solution.

**Effect of pH on Ae. albopictus sex determination**

The effect of pH on *Ae. albopictus* sex determination was evaluated by analyzing the sex ratio of larvae developed in hatching water with different pH levels.

**Cooperation with ENEA**

In collaboration with ENEA we evaluated the effect of a newly developed *Ae. albopictus* strain obtained through a *Wolbachia* insertion (wPip strain), on the pre-imaginal development and sex separation.

We established a permanent ARwP colony at the CAA mass rearing facility in order to monitor *Wolbachia* density, pre-imaginal development, efficiency of sex separation, and CI level, after 3 generations of CAA rearing SOP. Results regarding this activity will be presented by ENEA.
THIRD RESEARCH COORDINATION MEETING

On “Exploring Genetic, Molecular, Mechanical and Behavioural Methods of Sex Separation in Mosquitoes”

Tapachula, Mexico

10 - 14 October 2016

TITLE OF WORKING PAPER: Aedes female removal based on pupal dimorphism using artificial vision algorithms and computer controlled laser beamer

AUTHOR (S): Carlos Tur Lahiguera, Gustavo Salvador, Ignacio Plá Mora

ORGANIZATION: Centro de control biológico de Plagas (TRAGSA), Universidad CEU Cardenal Herrera.

SHORT SUMMARY OF PAPER

Abstract:

The purpose of this work is to develop mechanical tools for sexing Aedes sp pupae. This subject is included in the frame of the Co-ordinated Research Programme on “Explore mechanical, molecular, behavioral or genetic methods of sex separation in mosquitoes”.

The proposed method is based on identification of both sexes by artificial vision algorithms due to the existing pupae sexual dimorphism. Previous data analysis have shown substantial difference in size between male and female pupae when analysis is performed within the same cohort. This enables an artificial vision system to separate up to 70 percent of males with a negligible risk of female presence. First, Watershed algorithm is used to separate pupae that are in contact and measure their individual size. Then, the frequency curve of pupae size is obtained with two differentiated peaks. Each peak represents the highest range of size for both sexes. Size threshold between males and females is obtained using an Expectation-Magnification algorithm. Both the frequency curve and the threshold are updated in real time with any new pupae analysis, which allows the continuous analysis of large amounts of pupae in a very short time.

Once the threshold size has been calculated, the female centroid coordinates are obtained and a laser beam is directed using a system driven by a dual axis galvanometer optical scanner. The laser beam will kill all female pupae and only male pupae will survive.

During the last 18 months both hardware and software have been tested in different rearing conditions. Device parameters as light intensity, laser shoot frequency and image acquisition have been adjusted in order to increase the efficiency of the sex sorter. The influence of rearing conditions in sexual dimorphism is being analyzed using pupae measurements obtained in the image analysis. Finally, a system for administration and removal of pupae from the device has been developed.
THIRD RESEARCH COORDINATION MEETING
On “Exploring Genetic, Molecular, Mechanical and Behavioural Methods of Sex Separation in Mosquitoes”

Tapachula, Mexico

10 - 14 October 2016

TITLE OF WORKING PAPER: Sex separation of Aedes mosquitoes for Sterile Insect Technique (SIT) and Incompatible Insect Techniques (IIT) using Mechanical and Behavioural Methods.

AUTHOR (S): W. Abeyewickreme1&2, Tharaka Ranathunga2, Nayana Gunatilake2, Asha Wijeguanwardena2, Menaka Hapugoda2.

ORGANIZATION: 1. Dept. of Parasitology 2. Molecular Medicine Unit Faculty of Medicine, University of Kelaniya, Sri Lanka.

SHORT SUMMARY OF PAPER

Abstract:

Background
Dengue fever is a rapidly emerging arthropod-borne viral disease that has threatened approximately one-third of the world’s population. Due to the absence of an effective drug or vaccine for dengue, prime focus of health sectors lies on the biological controlling of vector densities via innovative approaches such as Sterile Insect Techniques (SIT) and Incompatible Insect Techniques (IIT) etc. Both these approaches require mass rearing and releasing of vectors in to the environment. As female mosquitoes are capable of transmitting the disease, elimination of females from mass releasing remains to be critical. Furthermore, such eliminations promote the mating of released males with wild females, increasing the efficiency of the techniques. A few behavioural and mechanical sex separation methods were recently tested at the Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya, Sri Lanka.

1. Mechanical methods.
A. Fay and Morlan glass plate separation
Methodology: Batches of 500 Ae. aegypti larvae were reared under normal and enhanced colony conditions. The emerged pupae from each colony were screened by devising the Fay and Morlan glass plate separator. The separated sets of pupae from each colony were placed in separate cages and reared up to adults for morphological separation to two sexes.
Results: Using the separator, 98.69% (n=227) of the males were separated in the first band along with 15.93% (n=43) of females. The second band included the rest of the males (1.30%, n=3) and females (84.07%, n=227). Meanwhile, out of 500 Ae. aegypti pupae reared under enhanced rearing conditions, 100% (n=240) of the males were separated as the first
band along with 1.15% (n=3) of females. Second band included the rest 98.85% (n=257) of the females. According to the Paired Chi-Square statistics, the percentage of males and females separated at each band differed significantly (p < 0.05) at 95% level of confidence. In this method, these separations resulted a mortality of 4% and 5%, respectively.

**Conclusion:** Fay & Morlan glass plate separator was able to yield a 100% separation of males together with 1.15% females under enhanced culture conditions, exhibiting its potential to be used as a separation method of Ae. aegypti mosquitoes with colony enhancements.

B. **Standard metal sieves separation.**

**Methodology:** Pupae (n=300) were poured through standard sieves with pore sizes of sieves - 1.12 mm, 1.25 mm, 1.40 mm and 1.60 mm. Test was replicated four times.

**Results:** The majority of males (73%) were separated by 1.12 mm pore size. Only 63% of the females were separated by 1.25 mm while only 28% of the females were separated by 1.12 mm pore size. The percentage mortalities of 4% and 3% were recorded at pore sizes of 1.12 and 1.25 mm respectively.

**Conclusion:** The majority of males were separated by 1.12 mm pore size. Therefore, using sieves with this pore size can be used for sex separation.

2. **Testing behavioral methods- Spiking blood meals with toxicants**

**Methodology:** Efficacy of two veterinary preparations (Ivermectin [Ivotec, 1% w/v] and Spinosad [Spinosyn, 12% w/v] as a behavioral sex separation tool for adult Aedes aegypti and Ae. albopictus mosquitoes was investigated. A batch containing 300 mosquitoes of Ae. albopictus with 1:1 male to female ratio was allowed to feed on spiked blood of cattle origin with different concentrations (2, 4, 6, 8 and 10 ppm) of Ivermectin and Spinosad separately. After 24 hours all remaining females and males were aspirated out and transferred in to new cages separately. An additional blood meal with the initially fed concentrations of ivermectin and spinosad was provided after 24 hours, followed by a 48 hour observation of mortality. This experiment was repeated by feeding cattle blood containing a mixture of ivermectin and spinosad in 1:1 ratio with concentrations ranging 2, 4, 6, 8 and 10 ppm. The whole procedure was followed for Ae. aegypti mosquitoes as well.

**Results:** Spiking blood with 8 ppm ivermectin and spinosad killed all the fed females of Ae aegypti and Ae. abopictus within 24 to 48 hours. It was observed that the number of fed females gradually increased, when decreasing the toxicity in combination of ivermectin and spinosad (1:1) in blood for both species. Male mortality did not show any significant difference during the study.

**Conclusion:** Double feeding of blood with 8 ppm ivermectin and 8 ppm spinosad separately have shown the potential of using both ivermectin and spinosad as a viable treatment to eliminate female Ae aegypti and Ae. abopictus from laboratory colonies.

**Overall Conclusion:** Mechanical separation of Aedes mosquitoes using Fly and Morlan separator followed by spiking blood meal with Ivermectin/Spinosad has the potential of total elimination of females before males are released to the environment for SIT/IIT.
Dengue is a deadly disease with alarming spreading potential and need proper attention. As no vaccine for the disease is available globally, therefore, vector control is the only option in the present scenario. The use of insecticides for vector control have environmental constraints, health hazards and resistance development in mosquitoes, thus, environment friendly vector control strategies with main emphasis on Sterile Insect Technique (SIT) are needed. Efforts are being made under the umbrella of IAEA CRP in this regard. Besides our research efforts towards SIT, we regularly carry out the entomological surveillance of Aedes species and seeking environment friendly conventional methods for vector control. The efforts on our part for the sex separation of Aedes and Culex mosquitoes using nutritional and mechanical methods are in progress for successful launching of SIT programs.

By testing the different sources of protein and carbohydrates under repeated trials, it was found that these ingredients in different ratios and combinations have significant effect on the dimorphic development of mosquitoes both on their developmental time and on their size. The combined effect of both sources favor the sexual dimorphism due to significant difference in size. The pupal size was significantly different (0.9mm for male and upto 1.3mm for female Aedes albopictus). This variation effect due to different formulation of diets was exploited successfully in mechanical sex separation. It was concluded that Stevia 75%+ Bovine Liver20%+ Yeast5% diet in combination showed the significant effect (47% over control) on Sexual dimorphism in male/female pupae of Aedes albopictus. However, the effect of this diet combinations did not favor the sexual dimorphism in Culex species. From the results on mechanical sex separations, it was concluded that the mesh size of 1.25 separated both the sexes effectively with a mean accuracy range (97-100%). It is therefore, recommended for the mechanical separation of the male/female having sexual dimorphism resulted due to nutritional effect. During behavioral studies, the
protective sheath formation (32% variation in size) was observed at 5 degree celcius in both species of mosquitoes pupae (Culex and Aedes). This low treatment of temperature for 24-48 hours of exposure time facilitated the sex separation of the female from mix culture. However, the protocol need to be standerdized and need further studies for minimizing the mortality effect. In the trials of spiking blood meals with different toxicants, the NIFA bio-larvicide has shown high potential (97-100% mortality) as a viable treatment to eliminate female Aedes from mix culture colonies comprising of the both the female and male adults and needs to be tested in further study by adding some palatable ingredients. The devised diet formulation will further be used on mass rearing scale and the effect of larval density plus male mating capacity will also be tested for this particular diet formulation. The fabrication of the mass rearing cages on IAEA design is also in progress and will be streamlined in the upgrading mass production for the subsequent SIT program.
TRANSFERRING A SYNTHETIC SEX RATIO DISTORTION SYSTEM BETWEEN MEMBERS OF THE ANOPHELES COMPLEX

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Abstract:
I-PpoI is a homing endonuclease that has high specificity for a conserved sequence within the ribosomal rDNA repeats located in a single cluster on the Anopheles gambiae X chromosome. If this endonuclease is expressed during spermatogenesis in transgenic mosquitoes the paternal X chromosome is expected to be cut and therefore only Y chromosome bearing sperms are produced. This system has been validated in Anopheles gambiae with a distortion to >95% male offspring as a result of the I-PpoI activity. Distorter male mosquitoes can efficiently suppress caged wild-type mosquito populations, providing a powerful tool for vector control strategies. Given that malaria mosquito vectors belong to a complex comprising at least two major vectors, a technology that can be transferred to sibling vector species would be of great interest. According to Haldane’s rule, F1 hybrid male sterility is known to occur in all intercrosses in the A. gambiae complex. A scheme based on crossing and selection was used to overcome the F1 hybrid male sterility and to introgress the transgene carrying the I-PpoI distorter, originally integrated in the Anopheles gambiae genome, into the Anopheles arabiensis genetic background. Our data suggest that this sex distortion technique can be successfully applied to target Anopheles arabiensis mosquitoes.
Selfish sex chromosomes have been identified in a number of species, since their existence within a population - manifesting as extraordinary sex ratios in the offspring of individuals carrying them - is striking. Sex chromosomes harboring alleles that favorably bias their transmission to the next generation act to selectively eliminate gametes bearing the opposite sex chromosome. As a result, both the allele (sex distorter) and the sex chromosome that harbors it increase in frequency in a population over time until the population either becomes extinct or until resistance alleles arise that counteract the activities of the sex distorter. Beyond their captivating significance for biology and evolution, the adverse effect that such selfish sex chromosomes could have on population fitness has been considered for its application in the control of pest species, particularly insects. Synthetic genetic constructs can now be engineered to behave similarly to naturally occurring sex distorters, be as impervious to the development of resistance alleles as possible and to be transferable between species. Towards this end, we are building invasive Y chromosomes in malarial mosquitoes that could spread within natural populations, dramatically reducing the frequency of biting females and thereby diminishing the reproductive capacity of the population, leading eventually to collapse. We will present the progress on the engineering of such strains and insights our exploration is revealing on the genome and reproductive biology of Anopheles mosquitoes.
THIRD RESEARCH COORDINATION MEETING
On “Exploring Genetic, Molecular, Mechanical and Behavioural Methods of Sex Separation in Mosquitoes”
Tapachula, Mexico
10 - 14 October 2016

TITLE OF WORKING PAPER: Analysis of sex determination pathway in the African malaria mosquito, Anopheles
AUTHOR (S): Elzbieta Krzywinska, Nathan Dennison, Gareth Lycett, Jaroslaw Krzywinski
ORGANIZATION: The Pirbright Institute, UK

SHORT SUMMARY OF PAPER

Abstract:
We have recently identified a primary sex determination signal gene (called Yob) in the African malaria mosquito Anopheles gambiae mosquitoes by comparing transcriptomes of male and female early embryos. The gene is encoded on the Y chromosome and is involved in sex-specific splicing of doublesex, the final gene within the sex determination pathway, as demonstrated by the experiments in the cell line and in transgenic mosquitoes. Ectopic expression of Yob kills female embryos of A. gambiae and A. arabiensis, but does not affect male development. Conversely, silencing of the gene’s expression is lethal to males. These opposite lethal effects suggest that Yob regulates dosage compensation (DC), the process, which in the Anopheles males, similar to Drosophila, drives overexpression of the X chromosome to the levels of expression from the two X chromosomes in females. We have been using the female killing property of Yob to create transgenic male-only strains of A. gambiae. The strains generated thus far do not have fully penetrant female-lethal phenotype. However, the surviving females are strongly masculinized, and do not feed on blood. These results give good prospects for the generation of male-only strains for Anopheles control.
Title of Working Paper: Genetic technologies and the exploration of mosquito/plasmodium interactions.

Author(S): David A. O’Brochta

Organization: University of Maryland College Park, Institute for Bioscience and Biotechnology Research and the Department of Entomology

Short Summary of Paper

Abstract:

Transgenic and RNA-guided nuclease-based technologies are being used to investigate Plasmodium sporozoite interactions with salivary glands of Anopheles mosquitoes. Saglin is a salivary gland-specific protein implicated in the recognition/invasion of salivary glands by sporozoites. Our recent findings challenge the current model of the role of this protein in the salivary gland recognition/invasion process by sporozoites. The distribution of saglin transcripts and protein in the salivary gland does not match the salivary gland invasion patterns of sporozoites with saglin being found only in the medial lobe of the salivary gland. Misexpression of saglin in the lateral lobes of the salivary glands of Anopheles stephensi using transgenic technologies did not alter the intensity of infection of the salivary glands by sporozoites. Anopheles gambiae homozygous for a RNA-guided nuclease-generated null allele of the saglin gene have significantly reduced intensities of infection of the midgut as reflected in the reduced number of oocysts, although neither saglin protein nor saglin transcripts are detectable in the midgut epithelium. Saglin-mutation homozygotes also had reduced infection of their salivary glands but it is unclear how much of this reduction was due to the pleiotropic effects on the midgut stage of the infection process. While creating null alleles is the most popular and easiest application of contemporary RNA-guided nuclease-based technology, it is not the only application. A brief synopsis of the scope of applications of RNA-guided nuclease technologies will also be provided, including their use in creating chromosome rearrangements, to enable full consideration of the technological options available for creating or enhancing sex separation technologies for mosquitoes.
Abstract:

Malaria kills 450,000 annually and controlling the vector, the anopheline mosquito, is the most effective way to slow the spread of disease. Alarmingly, *Anopheles* mosquitoes are evolving resistance to insecticides, rendering existing control measures potentially ineffective. Developing CRISPR in mosquitoes not only enhances our ability to study the basic biology of this important vector, but it also promises to enable novel vector control strategies. Here we use CRISPR transgenic system in *Anopheles gambiae* to generate inducible and specific germline knockouts for biological study and as candidates for release in Sterile Insect Technique (SIT). This system is composed of two transgenic lines: one expressing germline Cas9, and a second encoding gRNAs targeting ZPG, a gene required for germ cell development. Upon crossing these transgenic lines, progeny fail to develop normal germlines. The majority of males generated in this system are characterized by the absence of the male germline, and contain no observable sperm. Mating these spermless males to wild type females renders females refractory to further mating and sterilizes the female in 95% of matings. This suggests that such a system, with optimization, could be used to generate genetically sterilized males for use in SIT. Our work supports the utility of CRISPR as a high-efficiency tool to answer outstanding questions in *Anopheles* basic biology and enable new vector control strategies.
A successful strategy to control pest insect populations is based on the Sterile Insect Technique (SIT), which uses the release of mass-reared, radiation-sterilized male insects to cause infertile matings and thus reduce the pest population level. Genetic modifications offer new possibilities in pest insect control, e.g. by developing conditional genetic sexing or sterility systems needed for SIT.

Genetic modification is often achieved by random genomic insertion of a transgene construct via transposons. This is challenging for GMO strain development due to genomic position effects that could suppress transgene expression or by insertion mutations that negatively affect host fitness and viability. Recombinase-mediated cassette exchange (RMCE) allows for site-specific integration of transgene constructs into a well-characterized integration site of so-called landing site lines. RMCE has been successfully established in Drosophila and in Caribfly, and was recently also achieved in *Aedes aegypti* by us and others using the phiC31 recombinase (phiC31-RMCE). Moreover, the so-called iRMCE was established in *Ae. aegypti*. While both methods allow the site-specific integration, iRMCE, in contrast to phiC31-RMCE is not a true cassette exchange. And both systems are not reversible, i.e. the landing site can only be modified once. In contrast, Cre- and FLP recombinase systems allow for the repeated modification of the landing site due to their reversibility. We therefore wanted to establish the reversible Cre-RMCE system in *Ae. aegypti*.

We first created several landing site lines for Cre-RMCE. These landing site lines contain heterospecific *lox* sites flanking a fluorescent marker. We inbred these lines to homozygosity and injected early embryos with the Cre recombinase and a donor plasmid containing the corresponding *lox* sites flanking a different fluorescent marker. A change of marker color in the offspring (*G*₁) of the transformed individuals (*G*₀) would indicate a successful cassette exchange event. *G*₁ individuals with the new marker could indeed be identified. However, positive individuals still showed the original marker in addition to the new colour, indicating
integration instead of a recombination event, which was confirmed by molecular analysis. We then injected embryos of such an integration line with Cre recombinase to induce a second step recombination that resulted in a completed RMCE event. We thereby established a two-step Cre-RMCE protocol for *Ae. aegypti*. Moreover, we performed extensive fitness tests with the landing site lines to evaluate the fitness costs due to transgene insertion at different genomic positions. We included one of the integration line to evaluate the effect of integration of additional exogenous sequence.
THIRD RESEARCH COORDINATION MEETING

On “Exploring Genetic, Molecular, Mechanical and Behavioural Methods of Sex Separation in Mosquitoes”

Tapachula, Mexico

10 - 14 October 2016

TITLE OF WORKING PAPER: Manipulating genes in the mosquito sex-determination pathway for sex separation and population suppression

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SHORT SUMMARY OF PAPER

Abstract:

In mosquitoes, sex determination is carried out via at least two types of sex chromosome karyotypes: the heteromorphic sex chromosomes (X and Y) in Anopheles and the homomorphic sex chromosomes in Culicinae mosquitoes. Genetic evidence suggests that a dominant male-determining factor (M factor) from either the Y chromosome or a male-determining locus (M-locus) on the homomorphic sex chromosome is the primary signal that initiates male development and controls sex determination. We have recently discovered a male determining factor Nix in Aedes aegypti and a strong M factor candidate Guy1 in Anopheles stephensi mosquitoes. In this presentation, I will discuss evidence that supports Nix and Guy1 as the M factors in these two divergent mosquito species. I will also compare and contrast the outcome of ectopic expression of Nix and Guy1 in Aedes and Anopheles mosquitoes in the context of the need for or lack of dosage compensation. I will also discuss our efforts to produce all males for genetic sexing and for population suppression.
THIRD RESEARCH COORDINATION MEETING
On “Exploring Genetic, Molecular, Mechanical and Behavioural Methods of Sex Separation in Mosquitoes”
Tapachula, Mexico
10 - 14 October 2016

TITLE OF WORKING PAPER: Construction and characterization of genetic sexing strain
(Aedes aegypti and Ae. albopictus)
AUTHOR(S): Margareth Lara Capurro and Helena Rocha Corrêa de Araújo
ORGANIZATION: Universidade de São Paulo

SHORT SUMMARY OF PAPER
Abstract:
The mosquito borne diseases as dengue, zika and chikungunya have achieved the world attention due to the high number of cases and deaths reported annually. The development of new strategies for vector control that aim reducing the mosquito population or preventing the transmission of pathogens is the main goal of the research institutions worldwide. The uses of classical sterile insect technique or genetic manipulation are ones of the approaches to control the mosquito population, which use the mass-reared and the release of sterile-male mosquitoes in the environment. The utilization of technologies to supress wild mosquito populations needs an effective sex-separation method to obtain exclusively male mosquitoes. Seeking to achieve this goal, our project aims to eliminate females produced during mosquito mass reared through generation transgenic line of Aedes aegypti and Ae. albopictus that will produce males-only progeny. These mosquitoes will be valuable to public health programs in charge of controlling arbovirus epidemics. The key components of this strategy are: RNAi that will knock down the doublesex pathway and the 3’ UTR of the transgene will include a SV40 polyadenylation signal to stabilize that transcript. This gene will be regulated by Tet-On Tet-Off systems to produce laboratory colonies. Unfortunately, we did not obtain transgenic larvae to establish the lineage, thus we will use a tool for genome editing called CRISPR-Cas9 that is a highly effective tool for precision genome editing in the mosquito Ae. aegypti.
SHORT SUMMARY OF PAPER

Abstract:
The focus of our work is to characterize Chromosome 1, which, in the tiger mosquito *Aedes albopictus*, carries the sex determining region. We approached this problem both at the cytological and the genomic level. We previously developed a protocol that distinguishes the sex of mature fourth instar larvae by microscopic observation of developing gonads. This protocol permits the unambiguous identification of male and female larvae and thus facilitates the characterization of their karyotypes using mitotic chromosomes obtained from larval imaginal discs. Both DAPI- and Giemsa C-banding staining performed on mitotic preparations from the male larval imaginal discs consistently confirmed the presence of a male-specific banding pattern on Chromosome 1.

We are now applying the stretched chromosome approach to further characterize this region of Chromosome 1.

It is known that on this chromosome a homologue of the *Ae. aegypti* M-locus gene, *Nix*, is present.

We are now characterizing this sequence in different strains and populations, both at the DNA and RNA levels, in order to assess the level of variation, and potentially, its regulation, and to identify sex-specific polymorphisms.
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