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Présentée par Mlle **OLIVA Clelia** :

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Études biologiques et comportementales de deux espèces de moustiques (*Aedes albopictus* et *Anopheles arabiensis*) vectrices de maladies en vue du développement de la Technique de l'Insecte Stérile (TIS) contre ces vecteurs à l'île de la Réunion

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Biological and behavioral studies of two disease-transmitting mosquito species (*Aedes albopictus* and *Anopheles arabiensis*) with the aim of developing the Sterile insect technique (SIT) against these vectors on Reunion Island

Directeur de thèse : Pr. LEMPERIERE Guy (IRD, Réunion)

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RÉSUMÉ

Études biologiques et comportementales de deux espèces de moustiques (*Aedes albopictus* et *Anopheles arabiensis*) vectrices de maladies en vue du développement de la technique de l'insecte stérile contre ces vecteurs à l'île de la Réunion.

Les femelles moustiques peuvent être vectrices de nombreux agents infectieux (virus, protozoaires, helminthes) pour l'Homme, qui peuvent être la cause de maladies graves comme le paludisme et la dengue. Ces maladies menacent respectivement 50 et 40% de la population mondiale; le paludisme étant responsable de près d'un million de décès par an. Les méthodes de lutte anti-vectorielle destinées à limiter les populations vectrices et stopper la transmission de maladies, se heurtent au développement incessant de résistances de la part des moustiques et des agents infectieux vis-à-vis des traitements employés. Bien que certaines régions du monde aient réussi à stopper efficacement la transmission de certaines de ces maladies, une grande partie des régions tropicales reste menacée. De plus l'expansion rapide de certaines espèces vectrices, telles qu'*Aedes albopictus*, accroît les risques sanitaires dans de nouvelles régions du globe. La technique de l'insecte stérile (TIS), qui a permis l'éradication ou la suppression des populations de nombreux insectes nuisibles aux cultures et à l'Homme, représente un moyen de lutte prometteur contre les moustiques. Cette technique s'appuie sur le lâcher en masse de mâles stérilisés par rayonnements ionisants qui, en transférant un sperme stérile aux femelles sauvages, vont permettre une diminution progressive de la population cible. Suite à l'épidémie de chikungunya à l'île de la Réunion en 2005 et face aux menaces permanentes de recrudescence de la dengue et du paludisme, les services de lutte anti-vectorielle réunionnais mettent en place d'importants moyens de lutte contre les populations de moustiques concernées. Toutefois, ces mesures ne permettant pas une diminution durable des densités de vecteurs, une étude de faisabilité est en cours quant à l'utilisation de la TIS pour diminuer et contrôler les populations d'*Ae. albopictus*, vecteur de la dengue et du chikungunya, et d'*Anopheles arabiensis*, vecteur du paludisme. Ce travail de thèse s'inscrit dans le cadre du projet TIS Réunion, dans le but d'étudier la biologie et le comportement des souches destinées aux lâchers de mâles stériles. Dans un premier temps, cette étude s'intéresse à la comparaison entre les souches d'élevage d'*An. arabiensis* et les souches sauvages, ainsi qu'aux modalités de stérilisation des mâles de la souche à sexage génétique. Une seconde partie est consacrée à l'étude de l'effet de l'irradiation sur les mâles d'*Ae. albopictus*, en étudiant plus particulièrement leur stratégie de reproduction, leur capacité d'insémination en laboratoire, ainsi que leur compétitivité sexuelle et longévité face aux mâles sauvages en conditions semi-contrôlées.

Mots clés : Technique de l'Insecte Stérile – Ile de la Réunion – *Aedes albopictus* – *Anopheles arabiensis* – stérilisation – reproduction

ABSTRACT

Biological and behavioral studies of two disease-transmitting mosquito species (*Aedes albopictus* and *Anopheles arabiensis*) with the aim of developing the sterile insect technique against these vectors on Reunion Island.

Mosquito females are potential vectors of numerous pathogens (viruses, protozoa, helminths), which can cause serious diseases such as malaria and dengue in humans. These two infectious diseases are threatening 50 and 40% of the world population respectively. Malaria is responsible for nearly one million deaths per year, and is considered by many experts as the most important insect-transmitted disease. Anti-vectorial control methods, intended to limit the vector populations and to stop the disease transmission have to face many challenges such as the development of mosquitoes' and pathogens' resistance to the treatments employed to control them. Although various regions of the world have succeeded in efficiently stopping the transmission of some diseases, most of the tropical regions remain under threat. In addition, the rapid expansion of some vector species, such as *Aedes albopictus*, increases the risks in previously safe areas of the world. The sterile insect technique (SIT) has allowed the eradication or suppression of various insect pest populations threatening crops, animal, and human health, and could offer a promising control tool against mosquitoes. The classical SIT relies on the mass releases of males sterilized by ionizing radiation; they transfer sterile sperm to wild females, which results in a progressive reduction of the target population. Following the chikungunya outbreak in Reunion Island in 2005 and considering the constant threat of a recrudescence of dengue and malaria, the anti-vectorial services in Reunion Island are deploying important means to control the relevant mosquito populations. However, these measures do not confer a permanent, or long-lasting reduction of vector densities. A feasibility study is ongoing, evaluating the use of the SIT to diminish and control the populations of *Ae. albopictus*, a vector of dengue and chikungunya, and *Anopheles arabiensis*, a vector of malaria. This PhD work was developed in the context of the SIT Reunion project, with the aim of studying the biology and the behaviour of some strains intended for the sterile male releases. Firstly, this study endeavours to compare colonized and wild strains of *An. arabiensis*, and to determine the sterilisation procedures of the genetic sexing strain males. The second part of this work studies the effect of irradiation on male *Ae. albopictus*, and most notably their reproductive strategy, the insemination capacity in laboratory, and finally their sexual competitiveness and longevity against wild males under semi-field conditions.

Key words: Sterile insect technique – Reunion Island – *Aedes albopictus* – *Anopheles arabiensis* – sterilization – reproduction

Laboratoires et organismes d'accueil

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List of publications

Peer-reviewed publications

OLIVA C.F., M.Q BENEDICT, S.M. SOLIBAN, G. LEMPÉRIÈRE, F. BALESTRINO and J. GILLES. 2012. Comparisons of life history characteristics of a genetic sexing strain with laboratory strains of *Anopheles arabiensis* (Culicidae: Diptera) from Northern Sudan. Journal of Medical Entomology 49(5):1045-1051.

OLIVA C.F., M.Q BENEDICT, G. LEMPÉRIÈRE and J. GILLES. 2011. Laboratory selection for an accelerated mosquito sexual development rate. Malaria Journal 10:135.

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YAMADA H., M.Q BENEDICT, C.A. MALCOLM, **C.F OLIVA,** S.M. SOLIBAN, and J. GILLES. 2012. Genetic sex separation of the malaria vector, *Anopheles arabiensis*, by exposing eggs to dieldrin. Malaria Journal 11:208.

BALESTRINO, F., S.M. SOLIBAN, J. GILLES, **C. OLIVA** and M. Q. BENEDICT. 2010. Ovipositional behavior in the context of mass rearing of *Anopheles arabiensis*. Journal of the American Mosquito Control Association. 26(4):365-72.

Submitted publications

Effects of irradiation, presence of females, and immediate sugar supply on the longevity of sterile males *Aedes albopictus* in Reunion Island under semi-field conditions. **C.F. OLIVA**, M.J. MAIER, J. GILLES, M. JACQUET, G. LEMPÉRIÈRE, S. QUILICI, M.J.B. VREYSEN, F. SCHOONEMANN, D. CHADEE, and S. BOYER. Submitted to Acta Tropica.

X-Ray induced sterility in *Aedes albopictus* (Diptera: Culicidae) and male longevity following irradiation. H. YAMADA, A.G. PARKER, **C.F. OLIVA**, F. BALESTRINO, and J.R.L. GILLES. Submitted to Journal of Medical Entomology.

Publications in progress

The malaria struggle and the sterile insect technique: a review. **C.F. OLIVA**, M.J.B VREYSEN, S. DUPÉ, R. LEES, L-C GOUAGNA, J. GILLES, G. LEMPÉRIÈRE, and R CHHEM. For Vector-Borne and Zoonotic Diseases.

Are male *Aedes albopictus* sexually prudent? **C. F. OLIVA**, D. DAMIENS, M.J.B VREYSEN, G. LEMPÉRIÈRE, and J. GILLES. For PLoS Biology.

Contrasting experimental habitat optima for *Anopheles* and *Aedes*. **C.F. OLIVA**, O. MADAKACHERRY, and J. GILLES. For Journal of Medical Entomology.

Oral communications

C.F. OLIVA, D. DAMIENS, M.J.B VREYSEN, LEMPERIERE G., L-C. GOUAGNA, S. BOYER and J. GILLES. Are male *Aedes albopictus* sexually prudent? 18th European Society for Vector Ecology Conference, Montpellier, FRANCE. 8-11 Oct. 2012.

Posters

OLIVA C., BENEDICT M.Q., LEMPERIERE G., and GILLES J. Contrasting experimental habitat optima for *Anopheles* and *Aedes*. 59th Annual Meeting of the American Society of Tropical Medicine and *Hygiene*, Atlanta, GA, USA. 3-7 Nov. 2010 (Abstract).

OLIVA C., BENEDICT M.Q., LEMPERIERE G., and GILLES J. Laboratory-reared *Anopheles arabiensis* males, reduction of the sexual maturation period. International Conference on Emerging Vector-borne Diseases in a Changing European Environment (EDEN), Montpellier, FRANCE, 10-12 May 2010 (Abstract).

Invited presentations at conferences and workshops

Oct. 2011. Mating system of male *Ae. albopictus* and effect of irradiation. IAEA, Special meeting on Male Mosquito Biology. Bologna, Italy.

April 2011. The Sterile Insect Technique and sterilization aspects for mosquito control. Special meeting organized by ARS (Agence de Santé de l'Océan Indien), Reunion Island.

March 2011. Effect of irradiation on male *Ae. albopictus* longevity, mating capacity and competitiveness in semi-field conditions in Reunion Island. IAEA, Special meeting on Mosquito Mass Rearing System. Reunion Island.

Feb. 2011. Sterilization aspects in the Sterile Insect Technique for mosquito control. Special meeting organized by the Mauritian Ministry of Health and Ministry of Agriculture. Mauritius.

Oct. 2010. Laboratory-rearing induced selection of early sexual maturity in males *Anopheles arabiensis*. IAEA, Special meeting on Male Mosquito Biology. Vienna, Austria.

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"*Non, la science n'est pas une illusion. Mais ce serait une illusion de croire que nous puissions trouver ailleurs ce qu'elle ne peut pas nous donner."*

"Science is not illusion. But it would be an illusion to suppose that we could get anywhere else what it cannot give us."

-- Sigmund Freud, 1927

À ma famille,

TABLE DES MATIÈRES

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PRÉAMBULE

Ce manuscrit est structuré en deux parties sous forme d'un enchainement d'articles publiés en tant que premier auteur (Article 2) et coauteur (Article 4), d'articles acceptés pour publication (Articles 3 et 7), et de trois articles aboutis en cours de révision (Articles 1, 5 et 6).

Afin de présenter le cheminement logique de ce travail, chaque partie est précédée d'un résumé en français permettant d'introduire le contexte, les objectifs, la stratégie employée et les résultats principaux obtenus pour chaque article. Leurs implications pour l'application de la TIS et leurs apports aux connaissances actuelles sur la biologie et l'écologie des Culicidés sont discutés à la fin de chaque partie. Enfin, ce travail ayant été réalisé en collaboration avec une institution non francophone, une conclusion générale en langue anglaise résume l'ensemble du travail, et les implications et perspectives soulevées par cette thèse.

PROLOGUE

This thesis is divided in two sections: the first one reporting the study on *Anopheles arabiensis* and the second dealing with the study of *Aedes albopictus*. It consists of a sequence of articles published with myself as first author (Article 2) and as co-author (Article 4), articles accepted for publication (Articles 3 and 7), and three finished articles under review (Articles 1, 5 et 6).

In order to illustrate the logical flow of this work, each part is preceded by a summary in French introducing the context, the objectives, the strategy employed, and the results obtained for each article. The implications for the implementation of the SIT and the contributions to the current knowledge on the biology and ecology of the Culicidae are discussed at the end of each part. Finally, as this work has been realized in collaboration with a non French-speaking institution, a general conclusion in English summarizes the entire work, including the implications and prospects raised by this thesis.

INTRODUCTION GÉNÉRALE

O. Madakacherry

O. Madakacherry

1.1 Les maladies vectorielles transmises par les moustiques

1.1.1 Émergence et réémergence de maladies vectorielles

Les maladies vectorielles émergentes[1] ou réémergentes sont au centre des préoccupations de santé publique depuis les dernières décennies. Sont principalement en cause les mouvements humains et les transports liés au tourisme, au commerce ou aux conflits, ainsi que la déforestation et les projets d'irrigation, l'urbanisation, une hygiène dégradée, mais aussi les changements climatiques qui modifient la distribution des arthropodes, des arboviroses[2] ou des parasites associés, leur efficacité, et/ou leur cycle de développement (Gould & Higgs 2009).

Beaucoup de ces maladies ont pour vecteurs plusieurs espèces de Culicidae, qui appartiennent notamment aux genres *Aedes, Anopheles,* et *Culex* (voir Encadré). Toutes les espèces de moustiques ne sont pas vectrices de maladies. Les parasites protozoaires responsables du paludisme sont spécifiques du genre *Anopheles* ; en revanche, les virus et nématodes parasites (filaires) peuvent être transmis par différentes espèces.

1.1.2 Le paludisme

Le paludisme (voir Article 1) menace la moitié de la population du globe (Figure 1), et est de loin la maladie transmise par des insectes causant la plus grande mortalité (Gilles & Warrell 1993). En 2010 il était responsable du décès de 655 000 personnes, dont 86% étaient des enfants. L'Afrique fut la région la plus touchée avec 91% des décès, suivie de l'Asie du Sud-Est (World Health Organization 2012c). Un parasite protozoaire du genre *Plasmodium* est à l'origine de cette maladie, il est transmis à l'Homme par la piqure de moustiques du genre *Anopheles* (Cox 2010). *Plasmodium falciparum* est responsable de la majorité des cas mortels, les espèces *P. vivax*, *P. malariae,* et *P. ovale* induisant généralement des formes bénignes du paludisme (Trampuz *et al.* 2003; Antinori *et al.* 2012). Après infection par le parasite, les formes sporozoaires se développent dans le foie et infectent les globules rouges. Les cycles d'invasions des globules rouges et leur rupture, qui relâchent des grandes quantités de parasites dans le sang, sont à l'origine des cycles typiques de fièvre, tremblements et transpiration. Dans certains cas, peuvent s'ensuivre anémie, obstruction des capillaires, défaillance du foie et des reins, coma et finalement décès. Les enfants sont particulièrement sujets aux formes sévères du paludisme et peuvent développer anémie, détresse respiratoire ou lésions neurologiques. Des traitements curatifs permettent de stopper le développement des parasites cependant la recherche pharmaceutique doit sans cesse faire face au développement de résistance de la part des parasites (Wellems 2002). Des recherches de vaccins sont menées depuis près d'un siècle mais se heurtent principalement à l'évolution rapide du *Plasmodium* (Desowitz 2000; Girard *et al.* 2007).

L'Europe, l'Amérique du Nord, et le Nord de l'Australie ont éradiqué avec succès le paludisme dans les années 1970 grâce à une élimination rigoureuse des populations de moustiques vectrices (lutte anti-larvaire, assainissements, etc.) et à des traitements curatifs (Feachem *et al.* 2010). Cependant ces mesures n'ont pas permis de contrôler efficacement le vecteur et de réduire la transmission du parasite dans les régions d'Afrique, d'Asie du Sud-Est, d'Amérique du Sud et d'Europe de l'Est. Toutefois le récent séquençage du génome du parasite ainsi que celui du vecteur laissent entrevoir de nouvelles opportunités (Ashburner 2002; Morel *et al.* 2002; Sachs 2002; Wellems 2002; Touré, Oduola, & Morel 2004).

1 **Maladie émergente** : maladie dont l'incidence réelle augmente de manière significative, pour une population donnée, dans une région donnée, par rapport à la situation habituelle de cette maladie.

2 **Arbovirose** : zoonoses (infection naturellement transmissible de l'animal à l'homme et vice-versa) causée par un virus transporté et transmis par des arthropodes piqueurs (Arthropod-borne virus). L'arthropode (moustique, tique ou phlébotome) s'infecte mais ne développe pas de maladie; il transmet le virus par la salive lors du repas sanguin.

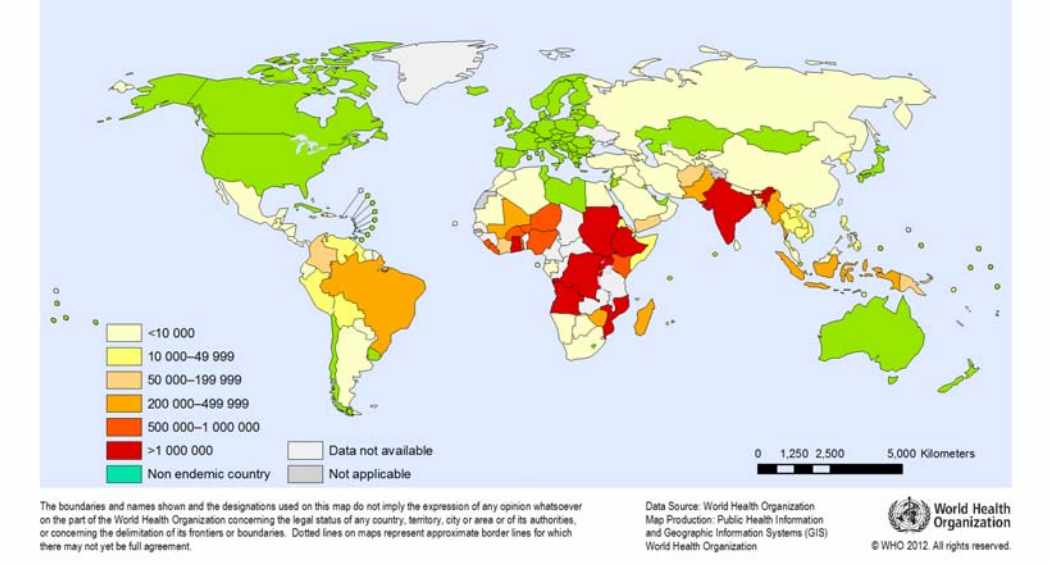

Figure 1. Nombre de cas confirmé de paludisme dans le monde en 2010.
Source: WHO http://gamapserver.who.int/mapLibrary/app/searchResults.aspx.

Encadré. Quelques maladies infectieuses émergentes et réémergentes à moustiques.

Dengue: *Flavivirus* transmis par le genre *Aedes* (principal vecteur : *Ae. aegypti*). Quatre sérotypes du virus peuvent être responsable de la dengue. Une immunité envers un sérotype particulier se développe après la première infection. Mais lors d'une infection ultérieure par un autre sérotype une réaction immune complexe peut accroître les risques de développer une forme sévère de dengue, dont la forme hémorragique qui s'avère fatale dans 2.5% des cas (Holmes & Twiddy 2003; World Health Organization 2012a). La Dengue est l'arbovirose la plus répandue dans le monde; 40% de la population mondiale, répartie dans une centaine de pays, est désormais à risque (Guzmán & Kouri 2002; World Health Organization 2012a). Son incidence s'est considérablement accrue durant les dernières décennies avec l'apparition de nombreuses épidémies localisées. Il n'existe aucun vaccin ni traitement spécifique; seuls un diagnostic précoce et des soins médicaux appropriés permettent de réduire la morbidité de 20 à 1% (World Health Organization 2012a).

Filariose lymphatique (éléphantiasis): nématode de la famille des *Filariodidae* (*Wuchereria bancrofti, Brugia malayi, Brugia timori*), transmis par les piqures d'hématophages dont plusieurs genres de *Culicidae* (*Aedes, Anopheles, Culex*). 1.3 milliard de personnes sont estimées à risque, dans 72 pays des zones tropicales, principalement en Asie du Sud-Est, dans les îles du Pacifique, et en Afrique (World Health Organization 2012b). Elle est responsable d'anomalies lymphatiques entrainant des déformations douloureuses et souvent incapacitantes. Un traitement médical permet d'éliminer les microfilaires du système sanguin et de tuer les formes adultes.

Fièvre jaune: zoonose hémorragique causée par un *Flavivirus*, transmis au singe ou à l'Homme par le genre *Aedes* et *Haemogogus*. Un vaccin existe contre ce virus depuis 1932, cependant la fièvre jaune est considérée comme réémergente car un risque croissant d'épidémies existe dans les zones densément peuplées et pauvres d'Afrique et d'Amérique du Sud (Gardner & Ryman 2010). En 2010, 200 000 cas de fièvre jaune et 30 000 décès étaient recensés (World Health Organization 2011a).

Virose West Nile (WNV): *Flavivirus* transmis principalement par le genre *Culex* et responsable de l'encéphalite japonaise (létale dans 2 à 10% des cas). Parmi les personnes infectées, 80% ne développent pas de symptômes (World Health Organization 2011b). Zoonose essentiellement aviaire, l'Homme et le cheval sont occasionnellement des hôtes accidentels, ne contribuant pas à la transmission du virus. Un vaccin existe pour les chevaux mais pas encore pour les humains. Le WNV était prévalent sur les voies migratoires, en Afrique, Europe, Moyen-Orient, Asie de l'Ouest et Australie. Il s'est maintenant installé en Amérique du Nord et Centrale (Gould & Higgs 2009).

1.1.3 Le Chikungunya

Rarement mortel, l'Alphavirus responsable du chikungunya est transmis principalement par le genre *Aedes*; *Ae. albopictus* en étant le vecteur principal. Il n'existe aucun vaccin ni médicament curatif ; le traitement consiste en l'atténuation des symptômes (fièvre, douleurs articulaires fortes). Ce virus endémique d'Afrique de l'Ouest, s'est récemment étendu en Asie, dans le sous-continent Indien, en Europe et en Australie (Figure 2) (Chevillon *et al.* 2008; Simon, Savini, & Parola 2008; Staples, Breiman, & Powers 2009;

Thiboutot *et al.* 2010). La distribution du vecteur a elle aussi rapidement progressée, *Ae. albopictus* étant actuellement présent dans presque la totalité du bassin méditerranéen, ainsi qu'en Amérique (Figure 3) (Benedict *et al.* 2007). Le réchauffement climatique en Europe dont les hivers plus doux permettent une meilleure survie des œufs de moustiques, ainsi que les déplacements fréquents de populations humaines entre zones endémiques de maladies vers des zones où les vecteurs sont présents, seraient à l'origine de l'expansion rapide de la distribution géographique de ce vecteur. En 2005-2006 une importante épidémie a frappé La Réunion avec 1/3 de la population infectée (Pialoux, Gaüzère, & Strobel 2006; Reiter, Fontenille, & Paupy 2006; Renault *et al.* 2007). Le premier cas européen a été rapporté en 2007 dans le nord-est de l'Italie et a rapidement donné lieu à une épidémie localisée (Angelini *et al.* 2007; Bonilauri *et al.* 2008).

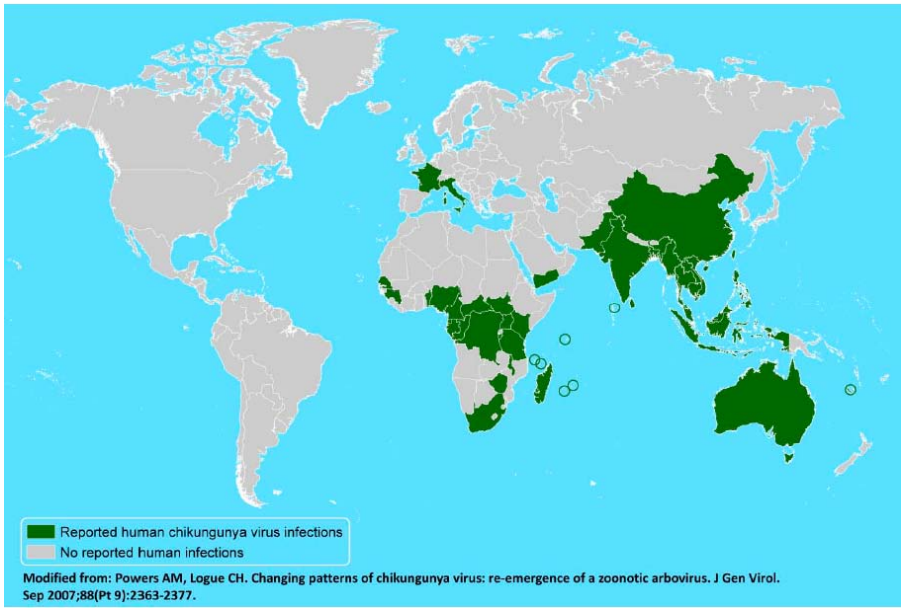

Figure 2. Pays où des cas d'infections au virus chikungunya ont été rapportés (mise à jour mai 2012).
Source: Centers for Disease Control and Prevention (CDC)
http://www.cdc.gov/chikungunya/pdfs/ChikungunyaMap.pdf.

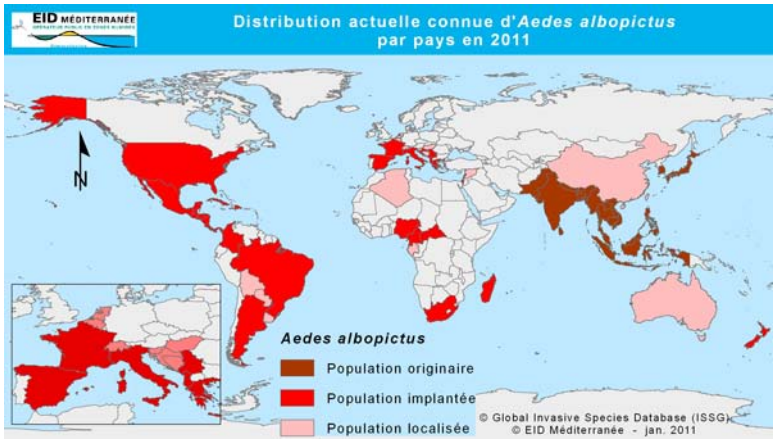

Figure 3. Distribution géographique d'*Aedes albopictus* en 2011.
Source: http://www.albopictus.eid-med.org/index.php/carte-actualisees-de-limplantation.

1.1.4 La lutte anti-vectorielle

Des stratégies de lutte chimique et biologique contre les moustiques ont connu plusieurs succès importants, mais se sont souvent révélées insuffisantes sur le long terme, à

cause de résistances aux insecticides, de ré-invasions par les populations voisines non traitées, ou d'une inadéquate gestion de l'environnement (Wilke *et al.* 2009).

Actuellement des stratégies de lutte anti-vectorielle (LAV) sont mises en place pour abaisser les niveaux de transmission de maladies vectorielles et minimiser les risques d'endémisation ou d'épidémisation. Un contrôle efficace des populations de vecteurs nécessite une gestion intégrée (Hendrichs *et al.* 2007), combinant pulvérisations intradomiciliaires à effet rémanent (il peut s'agir de pulvérisation de DDT uniquement dans le cadre de la lutte contre le paludisme (World Health Organization)), distribution de moustiquaires imprégnées d'insecticides (World Health Organization 2007), utilisation de larvicides, gestion de l'environnement pour réduire les gîtes larvaires, et pulvérisation d'insecticides dans les gîtes de repos. A cela, sont récemment venues s'ajouter des méthodes plus "vertes", notamment la technique de l'insecte stérile (TIS) et des méthodes de remplacement de population de vecteurs par des souches réfractaires au développement des agents pathogènes. Ces dernières techniques sont actuellement en phase d'étude, et seuls quelques essais à grande échelle ont permis d'établir leur efficacité.

1.1.5 Eléments de biologie des moustiques (Culicidae)

Il existe plus de 3 400 espèces de culicidés, réparties en trois sous-familles (Toxorhynchtinae, Anophelinae et Culicinae) et 42 genres, dont environ 50 transmettent des maladies à l'Homme, 20 étant d'une réelle importance épidémiologique.

Seules les femelles constituent une nuisance de part leur piqures et la transmission de maladies. Pour la majorité des espèces, les femelles requièrent un repas sanguin afin d'obtenir les nutriments nécessaires à la maturation des œufs. Quelques espèces, appelées autogènes, sont cependant capables de développer les œufs sans repas sanguin. En revanche, les mâles se nourrissent uniquement de nectar et de sécrétions sucrées.

La plupart des moustiques s'accouplent peu après émergence, le sperme transféré par le mâle étant stocké dans le ou les organes de stockage de la femelle (spermathèque). Les femelles sont généralement considérées comme monogames car un mâle transfère généralement suffisamment de sperme pour fertiliser tous les œufs qu'une femelle va pondre au cours de sa vie. Cependant des cas d'insémination multiples peuvent avoir lieu, notamment en condition d'élevage. Après le repas sanguin, 2 à 3 jours (en zones tropicales) sont nécessaires pour la maturation des œufs. Un lot de 30 à 300 œufs est ensuite pondu sur la surface de l'eau (pour *Anopheles* et *Culex*), sous une plante flottante (pour *Mansonia*), ou sur un substrat humide au dessus de la surface de l'eau (pour *Aedes*, *Psorophora* et *Haemagogus*). Une femelle est capable de faire plusieurs cycles gonotrophiques (repas sanguin, maturation des œufs et ponte) durant sa vie. Les œufs éclosent au bout de 2 à 3 jours. Les larves requièrent de l'eau pour se développer et, à l'exception de quelques espèces, doivent respirer à la surface de l'eau, à l'aide un siphon. Elles peuvent se nourrir en surface (Anophelinae) ou en profondeur, de bactéries, de levures, de protozoaires, et autres micro-organismes, ou de déchets de plantes et d'animaux. Le développement passe par quatre stades larvaires et sa durée varie entre 5 à 14 jours en fonction de l'espèce. Les larves se transforment en nymphes (ou pupes) : stade aquatique de 2 à 3 jours pendant lequel l'individu ne se nourrit pas. La fin du stade nymphal se traduit par l'émergence de l'adulte.

Les œufs des genres *Aedes* et *Psorophora* peuvent résister à la dessiccation pendant plusieurs mois et sont capables d'entrer en diapause pendant l'hiver, ce qui contribue à la forte capacité adaptative et à la large distribution de certaines de ces espèces. Les habitats larvaires peuvent être très diversifiés: larges retenues d'eau permanentes (marécages, rizières, etc.) ou temporaires (flaques, fossés, canalisations, etc.), habitats naturels (creux dans les arbres ou les rochers, bambous ou noix de coco coupés, feuilles de bananiers, etc.) ou artificiels et anthropiques (vases, réservoirs, pneus, etc.). Beaucoup d'espèces ne peuvent survivre dans un milieu trop riche en matière organique, alors que certaines espèces se développent dans un milieu très pollué et d'autres dans l'eau salée.

Selon les espèces, les femelles se nourrissent de sang humain (anthropophiles), d'oiseaux (ornithophiles), ou d'autres animaux (zoophiles). Elles peuvent se reposer à

l'intérieur des habitations pendant la maturation des œufs (endophiles) ou à l'extérieur (exophiles). Ces comportements ont une grande importance dans la transmission des maladies et pour la mise en place d'un contrôle anti-vectoriel. La compétence vectorielle (aptitude à assurer le cycle de l'agent pathogène) et la capacité vectorielle (aptitude à transmettre l'infection) varie selon les espèces, la densité du vecteur, le degré d'anthropophilie, la durée du cycle gonotrophique, la durée de vie du moustique, et la durée de développement de l'agent pathogène.

1.2 La Technique de l'Insecte Stérile (TIS)

1.2.1 Principe

Contrairement aux méthodes de lutte traditionnelles qui se basent sur l'altération de la survie, le principe de la Technique de l'Insecte Stérile (TIS ; voir Article 1) repose sur l'altération de la fertilité d'un insecte pour contrôler sa population. Quatre stratégies de lutte peuvent être distinguées: éradication, suppression d'une population nuisible, prévention de sa propagation ou prévention de son introduction (Hendrichs *et al.* 2005).

Les mâles représentent les agents actifs de cette technique, la stérilité étant introduite dans une population sauvage au travers de lâchers massifs de mâles stériles. Les mâles stérilisés produisent un sperme viable (sperme motile, capable d'induire les réponses post copulatoires normales chez les femelles) et doivent être capables de s'accoupler pour transférer aux femelles ce sperme, qui sera utilisé pour la fertilisation des œufs. C'est l'incapacité du zygote à se développer du fait de mutations létales qui est responsable de la stérilité.

Il existe plusieurs moyens pour induire de la stérilité. L'action mutagène des rayonnements ionisants induisant des lésions aux cellules reproductrices (Muller 1927) est la méthode la plus communément utilisée. Cependant les cellules somatiques peuvent également subir des dommages, se traduisant dans certains cas par une altération de la longévité et de la compétitivité des insectes relâchés par rapport aux insectes sauvages (Bakri, Mehta, & Lance 2005; Robinson 2005). Des produits chimiques stérilisants ont également été couramment utilisés dans les années 1950 et 1960 (Campion 1972; Weidhaas 1972), leur avantage étant de ne pas affecter la compétitivité de l'insecte. Cependant leur utilisation est actuellement limitée à cause de craintes de contaminations environnementales par des résidus de certaines substances cancérigènes, et à cause des problèmes de gestion des déchets issus du traitement (Bartlett 2009). Le recours à l'hybridation de deux espèces différentes, ou à la stérilité partielle des souches de sexage génétique (voir paragraphe 1.2.2.2), sans apport supplémentaire de stérilité par irradiation, peut également permettre de diminuer les populations sauvages en transférant des chromosomes anormaux à la population native, et ainsi y introduire une semi-stérilité (Carpenter & Bloem 2005). Cependant cette méthode présente le risque du réaccroissement de la population cible dès l'interruption des lâchers, si celle-ci n'est pas isolée (Laven & Cousserans 1971). Enfin la stérilisation peut aussi être obtenue par génie génétique (voir section 1.3).

L'application de la TIS se fait préférentiellement dans une zone de traitement assez large et isolée pour exclure la possibilité d'immigration de femelles sauvages inséminées, provenant de zones non traitées. Les îles représentent des sites de lâchers idéaux; cependant des résultats positifs peuvent être obtenus sur des zones plus larges en progressant dans une ou plusieurs directions selon le "principe du tapis roulant" ou le "principe de la vague" (Hendrichs *et al.* 2005).

1.2.2 Différentes étapes de la TIS

1.2.2.1 Élevage de masse

Après colonisation, l'insecte nuisible cible doit être élevé en masse afin de permettre une production généralement supérieure au million de mâles par semaine ou par jour. Dans le cadre des programme de TIS contre la mouche méditerranéenne des fruits (*Ceratitis capitata* (Wiedemann) ; Diptera : Tephritidae), la quantité de mâles stériles relâchés par semaine peut dépasser le milliard, comme c'est le cas pour l'usine d'élevage d'El Pino au Guatemala (Hendrichs *et al.* 2002). Un certain degré d'automatisation est requis pour de tels élevages de masse : pour cela le développement d'équipements adaptés à l'élevage de larves et d'adultes ainsi qu'à la séparation des différents stades est requis. La nourriture larvaire représente un des facteurs les plus importants, qui doit permettre de maximiser les performances des insectes tout en minimisant le coût de l'élevage de masse (Parker 2005).

De nombreux facteurs doivent être pris en compte afin que l'élevage de masse n'affecte pas la fécondité et la survie des insectes, minimise la durée de développement larvaire, et optimise la qualité des mâles à relâcher.

1.2.2.2 Séparation mâle / femelle

Dans de nombreuses espèces d'insectes nuisibles, seules les femelles sont responsables des dégâts causés aux fruits ou animaux à travers la ponte des œufs, ou de la transmission d'agents pathogènes lors d'un repas sanguin. Il est donc généralement préférable de ne relâcher que les mâles dans le cadre de la TIS. Par ailleurs, la présence de femelles stériles lors des lâchers peut s'avérer préjudiciable si elles s'accouplent avec les mâles stériles (Hendrichs, Franz, & Rendon 1995). Leur accouplement avec des mâles sauvages n'aurait qu'un effet mineur puisque ces derniers ont la capacité d'inséminer plusieurs femelles.

La séparation des sexes est donc une étape cruciale de la TIS, qui doit se faire préférentiellement le plus tôt possible, afin de diminuer les coûts d'élevage. Pour certaines espèces, un dimorphisme sexuel des nymphes permet une séparation à l'aide de tamis de différentes tailles de mailles. En revanche, lorsque la différence de taille entre mâles et femelles est insuffisante, la création d'une souche à sexage génétique (SSG) est requise afin d'éviter une séparation manuelle, inadaptée à l'élevage de masse.

Les SSG se basent sur des allèles existants dans une population sauvage, qui confèrent une résistance à une substance chimique toxique ou à un traitement physique (par exemple, haute température). Cet allèle marqueur de sélection doit être transloqué sur un seul des chromosomes sexuels, généralement le chromosome du sexe mâle. Pour cela les mâles de la souche résistante sont irradiés à faible dose afin de créer des mutations aléatoires, et sont croisés avec des femelles de la souche sensible jusqu'à ce qu'un croisement résulte en une descendance où seulement les mâles possèdent le caractère de résistance. Le traitement (chimique ou physique selon le marqueur de sélection) des œufs, larves ou adultes permettra d'éliminer les femelles avant stérilisation ou lâchers.

1.2.2.3 Stérilisation des mâles par rayonnement

Les irradiateurs les plus communément utilisés sont ceux à rayonnement X ou gamma. Les irradiateurs gamma (Figure 4) utilisent les rayons produits lors de la désintégration radioactive de radio-isotopes. Dans le cadre de la TIS, les plus couramment utilisés sont le Cobalt 60 et le Cesium 137. La courte demi-vie du ^{60}Co (5,2 ans) necessite un remplacement plus régulier de la source que s'il s'agit de ^{137}Cs (demi-vie de 30 ans), cependant l'énergie photonique d'une source du ^{60}Co est environ quatre fois supérieure à celle du ^{137}Cs (Bakri, Mehta, & Lance 2005). Les rayons X sont des rayonnements électromagnétiques produits par accélération d'électrons et ne nécessitent pas de changement de source. L'effet stérilisant des rayonnements gamma et X est

Figure 4. Irradiateur à rayons gamma (^{60}Co). (IPCL, Seibersdorf)

similaire, cependant les irradiateurs à rayons X apparaissent de plus en plus comme l'alternative aux irradiateurs gamma, notamment pour des raisons de sûreté lors du renouvellement des sources radio-isotopiques (Mastrangelo *et al.* 2010; Mehta & Parker 2011).

L'irradiation peut permettre de rendre un individu stérile en endommageant ses gonades. Elle provoque la fragmentation des chromosomes des cellules germinales à travers des mutations létales dominantes, translocations, ou autres aberrations chromosomiques. La fécondation d'une femelle par un mâle stérilisé produira des gamètes déséquilibrés, inhibant ainsi la mitose et le développement de l'embryon.

Les cellules somatiques sont moins sensibles à l'irradiation que les cellules germinales reproductrices car étant différentiées, elles ont perdu leur capacité à se diviser. L'effet du rayonnement ionisant sur les cellules somatiques pourra, en fonction de la dose absorbée, se traduire par une diminution de la longévité, ou de la capacité de vol et/ou d'accouplement. De façon à limiter les lésions somatiques, il est recommandé de procéder à l'irradiation le plus tard possible dans le développement de l'insecte, lorsque le nombre de divisions cellulaires est minime (Robinson 2005).

Une courbe dose-stérilité doit être établie afin de déterminer la dose de radiation nécessaire pour atteindre le niveau de stérilité voulu. L'effet d'un lâcher de mâles stériles sur une population sauvage dépend à la fois de la compétitivité des mâles (*i.e.* leur capacité à s'accoupler et à inséminer les femelles par rapport aux mâles sauvages) et de leur stérilité. Dans certains cas, la dose optimale d'irradiation sera un compromis entre stérilité génétique et qualité des mâles (Robinson 2005).

1.2.2.4 Lâchers

La TIS doit être intégrée dans un programme de gestion d'une espèce nuisible, en combinaison avec d'autres méthodes de lutte conventionnelle. Les lâchers de mâles stériles se font préférentiellement à une période de l'année où la densité d'insectes est la plus basse. Ils peuvent également être précédés par l'utilisation d'insecticides afin d'abaisser la population sauvage et d'augmenter le ratio de mâles stériles par rapport aux sauvages, ou être accompagnés d'un lâcher de parasitoïdes spécifiques de l'insecte cible afin d'accroitre l'efficacité de l'intervention (Klassen 2005).

Les lâchers sont soit statiques à partir de réceptacles basés au sol s'il s'agit de nymphes, ou, dans le cas d'adultes, mobiles à partir de véhicules ou de moyens aériens (Dowell, Worley, & Gomes 2005). Le développement d'outils adaptés pour estimer les densités de populations sauvages, avant et après lâchers, est primordial afin de déterminer les quantités et la fréquence des lâchers, et d'évaluer leur efficacité. Le ratio de mâles stériles par rapport aux mâles sauvages peut varier de 7:1 à 100:1 selon les programmes (Vreysen 2005). Le ratio critique étant déterminé selon de nombreux facteurs, dont l'occupation spatiale de l'habitat, la qualité des mâles stériles à se disperser, leur compétitivité sexuelles, mais aussi la densité de la population sauvage (Vreysen 2005). L'utilisation de modèles mathématiques permet d'estimer les paramètres de lâchers importants pour le succès de la TIS en fonction des différents facteurs biologiques (Barclay 2005; Dumont & Tchuenche 2011; Anguelov, Dumont, & Lubuma 2012).

1.2.3 Principaux programmes utilisant la TIS

Cette technique a été conçue en 1937 par EF Knipling, et a été utilisée pour la première fois pour le contrôle d'un insecte nuisible dans les années 1950 (Klassen & Curtis 2005). Ce programme visait la lucilie bouchère, *Cochliomyia hominivorax* (Coquerel) dont les larves se nourrissent de tissus vivants et sont responsables de myiases des plaies et de nombreuses pertes de bétail. Il s'agit d'un des programmes les plus réussis, qui a permis l'éradication de la lucilie de Curaçao en 1954, du Sud des Etats-Unis, du Mexique, d'Amérique centrale et de Panama, sur plus de 50 ans (Wyss 2000). La mouche tsé-tsé

Glossina austeni (Newstead), vectrice du parasite trypanosome, responsable de la "maladie du sommeil" chez les Hommes et de pertes de bétail considérables, a également pu être éradiquée de Zanzibar grâce à l'utilisation de cette technique (Msangi *et al.* 2000; Vreysen *et al.* 2000). L'utilisation de la TIS pour l'éradication de populations de mouches des fruits, nuisibles aux cultures agricoles, a eu de nombreux succès, notamment contre la mouche méditerranéenne des fruits au Chili et en Argentine, et contre la mouche du melon *Batrocera cucurbitae* (Coquillett) sur l'archipel d'Okinawa (Japon) (Enkerlin 2005). Actuellement de nombreux projets de TIS sont en cours pour diminuer ou maintenir en dessous d'un seuil économique les populations de mouches des fruits ou de lépidoptères nuisibles (Hendrichs *et al.* 2005; Bloem *et al.* 2007; Reyes *et al.* 2007).

Plusieurs tentatives d'application de la TIS contre les moustiques ont eu lieu pendant les années 70 et 80 pour contrôler *Ae. aegypti*, *Ae. albopictus, Culex pipiens* (Linnaeus), *Culex quinquefasciatus* (Say), *Anopheles albimanus* (Wiedemann) et *Anopheles gambiae* (Giles), dont certains essais à grande échelle qui ont pu démontrer qu'une réduction des populations était possible. Toutefois aucun de ces projets n'a pu être mené à grande échelle (Benedict & Robinson 2003; Dame *et al.* 2009). L'échec de certains de ces programmes a été attribué à une baisse significative de la compétitivité des mâles lâchés ou à l'immigration de moustiques provenant de zones non traitées. Cependant ce sont des raisons politiques qui ont mis fin aux deux projets les plus prometteurs qui avaient pour but la suppression d'*An. albimanus* au Salvador et d'*Ae. aegypti* et *Cx. faticans* en Inde (Lofgren *et al.* 1974; Curtis 2007). Par la suite, aucun projet de TIS contre les moustiques n'a plus été entrepris jusqu'au début des années 2000. Un regain d'intérêt pour cette technique est apparu car les moyens disponibles ne sont pas suffisants pour lutter contre les réémergences de maladies infectieuses et contre le paludisme (Collins & Paskewitz 1995; Beier *et al.* 2008; Klassen 2009; Feachem *et al.* 2010). La Division jointe de la FAO/IAEA porte actuellement un projet d'élimination d'*An. arabiensis*, vecteur du paludisme, au Soudan Nord via la TIS classique, utilisant la stérilisation par irradiation (Robinson *et al.* 2009). Un programme semblable est également en cours dans la région de Bologne, en Italie, et a permis de démontrer la possibilité de réduction des populations sauvages d'*Ae. albopictus*, vecteur du Chikungunya, dans des villages tests (Bellini *et al.* 2007).

En outre, diverses recherches sont menées actuellement pour améliorer la TIS dite classique, et pour utiliser les méthodes de génie génétique ou d'incompatibilité cytoplasmique afin de lutter contre les moustiques vecteurs de maladies.

1.3 Autres approches pour l'utilisation de la TIS contre les moustiques

1.3.1 Moustiques génétiquement modifiés (GMM)

L'utilisation de la génétique pour stériliser les populations de moustiques nuisibles est actuellement en vogue, en dépit des nombreuses inconnues sur l'impact écologique et de la réticence du public. Contrairement à l'usage des rayonnements par la TIS classique, la stérilité phénotypique induite par génie génétique peut être sujette aux variations biologiques, et l'évolution de résistance reste imprévisible.

Une première stratégie envisageable est une variante de la TIS classique, où la stérilité est induite par l'insertion dans l'ADN un gène létal dominant: "release of insects carrying a dominant lethal" (RIDL). Ce gène possède un caractère létal conditionnel qui permet d'entrainer la mort de la progéniture lorsqu'il n'est pas désactivé en élevant les larves en présence de l'antibiotique tétracycline (Thomas 2000). Afin de permettre de ne relâcher que des mâles, la létalité peut être appliquée uniquement aux femelles en plaçant ce gène sous la commande d'un promoteur spécifique du sexe femelle. La survie de la descendance mâle permettrait alors de propager l'effet stérilisant sur quelques générations (Curtis *et al.* 2006). Différents phénotypes létaux peuvent être induits (Nolan *et al.* 2010), tels que l'incapacité des femelles à voler (Fu *et al.* 2010) ou l'incapacité de produire du sperme par les mâles (Thailayil *et al.* 2011). Plusieurs études de faisabilité sont en cours et des essais préliminaires encourageants ont eu lieu aux îles Caïmans (Harris *et al.* 2011), en Malaisie (Lacroix *et al.* 2012), et au Brésil (Mumford 2012).

Une deuxième stratégie consiste en un remplacement de populations sauvages par une souche réfractaire à un agent pathogène, afin d'en stopper la transmission. Cette technique implique la fixation d'un gène réfractaire à l'agent pathogène dans la population vectrice sauvage, mais reste particulièrement vulnérable à la perte du caractère réfractaire après plusieurs générations suite à l'immigration de moustiques sauvages (Curtis *et al.* 2006).

1.3.2 Wolbachia *et incompatibilité cytoplasmique*

La technique de l'incompatibilité cytoplasmique (IC) se base sur l'infection de certains individus d'une espèce donnée par une bactérie endosymbiotique du genre *Wolbachia*. Un croisement entre une femelle non infectée et un mâle infecté (IC unidirectionnelle) ou entre un mâle et une femelle infectés par deux souches incompatibles de *Wolbachia* (IC bidirectionnelle), entrainera une IC et la mort des embryons (Bourtzis 2007; Brelsfoard & Dobson 2009). En revanche les croisements entre une femelle infectée et un mâle non infecté, ainsi qu'entre une femelle et un male infectés par la même souche, sont compatibles et viables. *Wolbachia* étant uniquement héritée de la mère, le lâcher de mâles infectés aura un effet stérilisant sur une population sauvage (Bourtzis 2007; Brelsfoard & Dobson 2009). Quelques essais ont permis de montrer l'efficacité de cette technique pour le contrôle de moustiques vecteurs, dont l'éradication d'une population isolée de *Cx quinquefasciatus* par le lâchers de mâles incompatibles avec la souche ciblée (Krishnamurthy & Laven 1976).

Une autre stratégie d'utilisation des *Wolbachia* est le remplacement de population, pour répandre un génotype particulier dans la population sauvage (Curtis & Sinkins 2011). Récemment, une souche de *Wolbachia* réduisant la durée de vie du moustique a été introduite dans une souche d'*Ae. aegypti*, afin que les femelles ne survivent pas suffisamment longtemps pour permettre la transmission du virus de la dengue à l'Homme (McMeniman *et al.* 2009; Popovici *et al.* 2010).

1.4 Le projet TIS Réunion

1.4.1 Contexte

1.4.1.1 Ile de La Réunion: géographie et faune culicidienne

La Réunion est située dans l'archipel des Mascareignes, dans l'hémisphère austral entre l'équateur et le tropique du Capricorne, dans la région sud-ouest de l'océan Indien (21°10" S, 55°30" E). La terre la plus proche est l'île Maurice à 170 km au Nord-Est; Madagascar est situé à 700 km à l'Ouest (Figure 5). D'une superficie de 2512 km^2, la Réunion présente un relief escarpé, constitué de nombreuses ravines à végétation dense et de massifs montagneux dont le plus haut sommet culmine à 3070 m.

Figure 5. Ile de la Réunion.

Le relief est responsable d'une multitude de méso-climats, constituant des zones géo-écologiques plus ou moins favorables au développement des culicidés et à la transmission de maladies. Une limite naturelle d'altitude stoppe la dispersion des

culicidés, et conditionne les zones de transmission des maladies. Le climat tropical favorise le développement des culicidés, avec des pics de densité pendant la saison des pluies de novembre à avril, où les fortes précipitations (jusqu'à 4 700 mm d'eau par an en moyenne à l'Est) favorisent l'apparition de nombreux gites larvaires temporaires. De plus, la haute température (21 à 30°C) et la forte humidité relative (70 à 98%) permettent une bonne survie des adultes.

1.4.1.2 Moustiques vecteurs de maladies à la Réunion

Douze espèces de Culicidae ont été recensées à La Réunion, appartenant à quatre genres: *Aedes*, *Anopheles*, *Culex* et *Orthopodomuia* (Hamon 1953). Quatre espèces d'*Aedes* sont présentes dont une espèce endémique, *Ae. dufouri* (Hamon), et deux espèces vectrices d'arboviroses, *Ae. aegypti* et *Ae. albopictus*. Le genre *Anopheles* est représenté par deux espèces, le vecteur potentiel de paludisme *An. arabiensis* et *An. coustani* (Laveran). *Ae. albopictus,* espèce dominante et responsable de la transmission de la dengue et du chikungunya, et *An. arabiensis* sont les principales espèces d'importance médicale à la Réunion, et font l'objet de mesures importantes de lutte (Figure 6, (Michault 1998). *Ae. aegypti*, vecteur potentiel de dengue*,* et *Cx. Quinquefasciatus,* vecteur potentiel de filariose et des virus de la Vallée du Rift et du West Nile, restent sous surveillance stricte.

Ae. albopictus, communément appelé "moustique tigre", est vecteur d'au moins 22 arboviroses dont le chikungunya et la dengue (Gratz 2004). Il est présent à la Réunion depuis le 17 ou 18ème siècle, et a rapidement colonisé les habitats occupés par *Ae. aegypti*, qui ne persiste aujourd'hui que sous forme de populations résiduelles (Delatte *et al.* 2008). *Ae. albopictus* est présent jusqu'à 1200 m d'altitude où il peut survivre à des températures moyenne de 13°C lors de l'hiver austral (Delatte *et al.* 2007). Il est capable de coloniser divers types d'habitats d'origine naturelle (trous d'arbres, tiges de bambous coupées, creux de rocher) ou anthropique (vase à fleurs, pneus, divers récipients domestiques et péridomestiques), aussi bien urbains et péri-urbains que ruraux (Delatte *et al.* 2007). Il est présent en forte densité dans les forêts des ravines comme dans les jardins domestiques et les cimetières. Ce moustique pique le jour, principalement au crépuscule, et préférentiellement à l'extérieur des habitations. *Ae. albopictus* a été à l'origine de l'épidémie de dengue de 1977 qui a concerné 30 à 35% de la population réunionnaise (Kles *et al.* 1994; Michault 1998); un nouvel épisode épidémique concernant 228 personnes a également eu lieu en 2004. Alors que seuls quelques cas sporadiques ont été signalés en 2007, 2008 et 2010 (D'Ortenzio *et al.* 2011), la dengue a ré-émergé depuis janvier 2012 avec plusieurs cas autochtones, faisant craindre une épidémie de plus grande ampleur du fait de la forte densité du vecteur et de la faible immunité de la population (Larrieu *et al.* 2012b). *Ae. albopictus* a également été responsable d'une des plus importantes épidémies de chikungunya en 2005-2007, concernant 38% de la population, qui a nécessité le renforcement des mesures de lutte anti-vectorielle (Pialoux, Gaüzère, & Strobel 2006; Bâville *et al.* 2012; Larrieu *et al.* 2012a); quelques cas de transmission autochtone du virus sont réapparus en 2010 (Dehecq *et al.* 2010; Vilain *et al.* 2012).

La population d'*Ae. aegypti* à la Réunion présente un comportement opposé à celui communément observé dans le reste du monde, elle se trouve limitée aux gîtes sauvages et ne pique l'homme qu'exceptionnellement (Salvan & Mouchet 1994). Les campagnes de traitement au DDT pour lutter contre *An. arabiensis* (1949-1953), et des interactions compétitives avec *Ae. albopictus* auraient contribué à la réduction de sa population et à son déplacement vers les zones rurales (Bagny *et al.* 2009). Bien que cette espèce ne représente pas actuellement une menace pour la santé humaine, il faut toutefois rester prudent quant à l'évolution de sa population et son implication dans des épidémies, si l'on parvient à réduire ou éliminer la population d'*Ae. albopictus*.

Le paludisme serait arrivé à la Réunion vers la fin du 19ème siècle, probablement importé de Madagascar et d'Afrique de l'Est. Après la 2ème Guerre Mondiale, une campagne de lutte a débuté à travers la pulvérisation d'insecticides (dont le DDT et le temephos), l'administration massive de médicaments antipaludéens, et des mesures d'assainissement. L'Organisation Mondiale de la Santé (OMS) a déclaré l'éradication du pathogène *P. falciparum* de la Réunion en 1979 (Denys & Isautier 1991; Michault 1998). Bien que l'unique

vecteur *An. arabiensis* soit toujours présent, sa population, très structurée génétiquement (Morlais *et al.* 2005), reste à en milieu rural et à un niveau de densité faible, et a développé un comportement plutôt exophile et zoophile (Girod *et al.* 1999). Ses gîtes sont distribués principalement dans trois larges zones en dessous de 1 200 m d'altitude, sur les étendues marécageuses et agricoles irriguées du littoral, et dans les flaques d'eau créées par les rochers des ravines et des bords de rivières (Gouagna *et al.* 2011). En dépit des mesures anti-larvaires fréquentes, la population se maintient sur l'île (Girod, Salvan, & Denys 1995). Des cas importés de paludisme sont reportés chaque année, chez des voyageurs en provenance des îles voisines Comores, Mayotte et Madagascar; bien que leur nombre soit en diminution durant les dernières années, le risque d'introduction du parasite reste fort et impose une surveillance épidémiologique et entomologique constante, ainsi qu'un système d'information des communautés à risque sur les recommandations prophylactiques (D'Ortenzio *et al.* 2010).

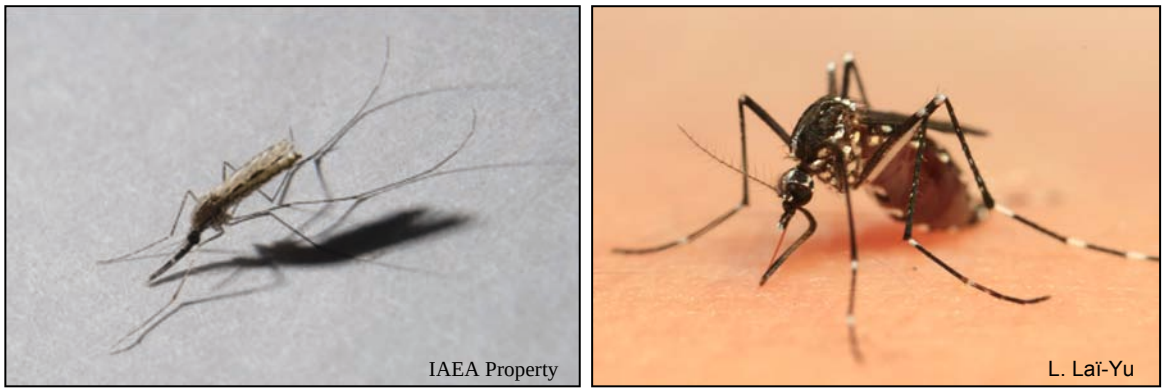

Figure 6. Femelles d'*An. arabiensis* (gauche) et d'*Ae. albopictus* (droite).

1.4.1.3 La LAV à la Réunion

Des moyens de lutte importants sont mis en œuvre par l'Agence Régionale pour la Santé (ARS) de la Réunion, afin de limiter les populations de moustiques vecteurs et d'éliminer localement les populations vectrices lors de la découverte d'un foyer de maladie (lutte péri-focale).

Suite à l'éradication du paludisme en 1979, l'objectif principal était la lutte contre la réintroduction de cette maladie (Denys & Isautier 1991). Cependant après les récentes épidémies de dengue (2004) et de chikungunya (2005-2007), des mesures d'éducation sanitaire, de surveillance et d'élimination *d'Ae. albopictus* en milieu urbain ont été prises. Depuis décembre 2004, un réseau de surveillance entomologique mesure de façon mensuelle (puis hebdomadaire à partir de 2007) des indices d'infestation larvaire dans les différentes zones de l'île (Delatte *et al.* 2008). Une importante campagne de sensibilisation de la population a été mise en place en 2006 afin d'impliquer la société réunionnaise dans les actions de démoustication au niveau familial, associatif et collectif (Delatte *et al.* 2008).

À une lutte anti-larvaire préventive à base de *Bacillus thuringiensis israelesiensis* (Bti), s'ajoutent, en fonction des indices entomologiques et des cas d'infections, des actions de destruction ciblées des gîtes larvaires et des traitements adulticides (deltaméthrine). Cependant ces mesures se heurtent aux difficultés d'accès à certains gîtes dans les ravines ou jardins privatifs (Delatte *et al.* 2008). Une lutte mécanique est également mise en place afin d'assainir l'environnement et de limiter les gîtes larvaires.

Les épidémies de dengue et de chikungunya ont mis en exergue la nécessité de développer des moyens efficaces de lutte contre les moustiques vecteurs, et de maintenir une veille sanitaire afin de pouvoir intervenir rapidement.

1.4.2 Le projet TIS Réunion

La lutte chimique et mécanique actuelle est insuffisante pour permettre de contrôler sur le long terme les populations d'*Ae. albopictus* et d'éliminer les derniers foyers d'*An. arabiensis.* Or les risques d'épidémies de chikungunya et de dengue, ainsi que de réintroduction du paludisme sont forts. Afin d'apporter un moyen de lutte complémentaire, la possibilité d'utiliser la TIS a été mise en avant. Le projet TIS Réunion pour le contrôle d'*Ae. albopictus* et d'*An. Arabiensis,* piloté par l'Institut pour la Recherche et le Développement (IRD) et le Centre de Recherche et de Veille sur les Maladies Émergentes dans l'Océan Indien (CRVOI), financé par le Ministère de la Santé et des Sports, les Fonds Européens de Développement Régional (FEDER) et la Région Réunion, et soutenu par la division jointe de l'Organisation des Nations Unies pour l'Alimentation et l'Agriculture et l'Agence Internationale de l'Énergie Atomique (FAO/IAEA), a débuté en 2009. La première phase de ce projet consiste en une étude de faisabilité de quatre ans afin de déterminer si cette méthode peut s'adapter scientifiquement, techniquement et socialement à la Réunion.

Ce projet s'inscrit également dans une demande de moyens de lutte plus respectueux de l'environnement et spécifiques de l'espèce nuisible, qui permettrait de limiter l'usage à long terme d'insecticides polluants pour l'environnement et toxiques pour les espèces non-cibles.

L'île de la Réunion représente un site idéal pour l'application de la TIS, du fait de son isolement géographique limitant la ré-invasion, de la présence d'une seule espèce vectrice de chikungunya et de dengue, ainsi que d'une seule espèce vectrice de paludisme (Morlais *et al.* 2005; Gouagna *et al.* 2011; Boyer 2012). En outre, elle bénéficie de l'organisation et de l'expérience du service de LAV.

Le projet s'articule sur quatre volets concomitants. Le volet 1 est consacré à l'étude de la biologie et de l'écologie des vecteurs, notamment le comportement, la distribution géographique, et la phylogénie. Le volet 2 vise à développer les techniques d'élevage de masse, de sexage, de stérilisation et de lâchers, et de s'assurer de la qualité et de la compétitivité des mâles stériles. Le volet 3 permettra de modéliser mathématiquement les lâchers en fonction des données rassemblées dans les volets 1 et 2, afin d'estimer l'envergure et la fréquence des lâchers ainsi que leur impact à court et à long terme. Enfin, le volet 4 a pour but d'évaluer l'impact socio-économique de la TIS à la Réunion et son acceptation par la population réunionnaise.

1.5 Travaux de thèse de doctorat

1.5.1 Problématique

Comme nous venons de le voir, le développement de la TIS nécessite des connaissances sur le comportement et la biologie reproductrice du vecteur ciblé, afin d'optimiser les paramètres d'élevage, de stérilisation et de lâcher. Cette thèse de doctorat s'inscrit dans l'étude de faisabilité de la TIS à La Réunion, plus particulièrement dans le volet 2, en focalisant sur l'étude de la souche de sexage génétique d'*An. arabiensis* et sur le comportement reproductif des mâles *Ae. albopictus* irradiés. Les études présentées ici ont été réalisées en partie au laboratoire IPCL de la Division jointe FAO/IAEA à Seibersdorf (Autriche), et au laboratoire de l'IRD/CRVOI à Sainte Clotilde (La Réunion).

D'autre part, ce travail apporte des connaissances importantes sur la biologie des vecteurs étudiés, qui peuvent avoir également des implications dans d'autres stratégies de lutte anti-vectorielle.

1.5.2 Objectif général

Ce travail de thèse vise à **améliorer les connaissances sur le contrôle des moustiques vecteurs de maladies par la technique de l'insecte stérile en étudiant la souche de sexage génétique d'*Anopheles arabiensis* et le comportement sexuel des mâles stériles d'*Aedes albopictus.***

1.5.3 Stratégies, objectif spécifiques et structure de la thèse

Cette thèse s'articule autour de deux parties principales. Une introduction et une discussion en français résume les travaux menés dans chaque partie et discute les résultats rapportés. Chaque chapitre correspond à un sujet de recherche développé dans une publication de langue anglaise. Une conclusion générale en anglais permet de mettre en perspective les résultats des travaux de chaque partie par rapport à l'application de la TIS pour éliminer les populations d'*An. arabiensis* et d'*Ae. albopictus,* et de discuter les réponses apportées sur la biologie de ces vecteurs. Une analyse des obstacles à surmonter et des perspectives de recherche future est proposée à la lumière de ce travail.

*1.5.4 Étude des souches d'élevage et de la souche de sexage génétique d'*Anopheles arabiensis

Bien que la lutte contre le paludisme ait été et reste au centre des préoccupations depuis de nombreuses décennies, les stratégies actuelles ne parviennent pas à limiter l'incidence de cette maladie. L'utilisation de la TIS pour lutter contre les insectes nuisibles a montré de nombreux succès depuis les années 1950, et son intégration dans les stratégies de lutte contre le paludisme fait l'objet d'un fort intérêt depuis la dernière décennie. Une revue est donc proposée dans un premier temps afin d'identifier ce qui a conduit à envisager l'utilisation de la TIS pour lutter contre le paludisme, les potentialités de cette nouvelle stratégie et les démarches permettant d'assurer l'acceptation de tels projets. Celle-ci rassemble la littérature sur le paludisme, son incidence sur la santé publique, les interventions de lutte passées et actuelles, ainsi que sur la TIS, son principe et son application générale et contre les moustiques en particulier, et enfin sur l'impact du public sur de tels programmes de santé publique (Article 1).

Les colonies d'insectes maintenues en laboratoire pouvant, au fil des générations, se différencier des souches sauvages (Huettel 1976), il est important de déterminer comment ces variations peuvent avoir un impact sur l'efficacité des lâchers et comment prendre en compte ces paramètres durant l'élevage de masse. Le deuxième chapitre de cette partie a mis en évidence l'impact d'une longue colonisation en conditions de laboratoire sur la maturation sexuelle d'*An. arabiensis,* et en définit les implications pour l'élevage de masse et les lâchers (Article 2).

Un des avantages d'utilisation de la TIS contre les moustiques est la possibilité de ne relâcher que les mâles, qui ne piquent pas car se nourrissant uniquement de sucre. Pour cela, il est nécessaire de disposer d'un système de sexage fiable. La différence morphologique entre nymphes mâles et femelles chez les espèces d'*Anopheles* n'étant pas suffisamment marquée, il est indispensable de créer une souche à sexage génétique (SSG) (voir section 1.2.2.2). La SSG "ANO IPCL1" a été créée avec succès pour *An. arabiensis* à partir d'une souche sauvage naturellement résistante à l'insecticide Dieldrine et d'une souche sensible. La souche ANO IPCL1 étant celle destinée aux lâchers, cette étude s'est ainsi attachée à comparer les traits d'histoire de vie d'ANO IPCL1 à ceux des souches sauvages parentales (Article 3) et à en déduire les implications pour l'élevage de masse et les lâchers.

La création de SSG implique une translocation d'un gène de résistance, or les mâles portant cette translocation ont une stérilité induite partielle, directement proportionnelle à la complexité de la translocation (Franz 2000; Robinson 2002a). Grâce à cette stérilité partielle, la dose de radiation nécessaire pour stériliser les mâles pourrait être réduite. Les mâles de souches sauvages d'*An. arabiensis* sont complètement stériles après irradiation à une dose supérieure à 100 Gray (Gy) (Helinski, Parker, & Knols 2006) ; il s'agit de déterminer les modalités de stérilisation par irradiation d'ANO IPCL1 (Article 4).

Un de nos objectifs initiaux était l'évaluation de la compétitivité de mâles irradiés ANO IPCL1 face à des mâles d'*An. arabiensis* provenant d'une souche de la Réunion, en

conditions semi-contrôlées à la Réunion; malheureusement les difficultés de colonisation de la souche sauvage réunionnaise d'*An. arabiensis* n'ont pas permis d'avoir un élevage suffisamment conséquent pour pouvoir effectuer de tels tests.

*1.5.5 Étude de la capacité de reproduction des mâles stériles d'*Aedes albopictus

Les stratégies de reproduction varient beaucoup entre les insectes, les mâles tendant à optimiser leur stratégie selon les quantités de sperme et de sécrétions séminales disponibles, et la disponibilité et le statut reproductif des femelles (Wedell, Gage, & Parker 2002). L'objectif premier est ici d'étudier la stratégie de reproduction des mâles d'*Ae. albopictus*, la gestion du sperme par des mâles non traités ou irradiés et par des femelles vierges, en condition de laboratoire (Article 5).

L'évaluation de la qualité des mâles stériles est une étape fondamentale au succès de la TIS, qui permet la détermination et l'adaptation des paramètres d'élevage, de stérilisation et de lâchers. Pour cela, la longévité des mâles stériles a été comparée à celle de mâles sauvages en conditions semi-contrôlées, et en fonction de la disponibilité d'une source sucrée à l'émergence, et de la présence de femelles (Article 5). Enfin, l'effet de l'irradiation sur la compétitivité sexuelle des mâles face à des mâles sauvages a été déterminé en conditions semi-contrôlées à la Réunion, selon deux ratios de lâchers, avec ou sans une période préalable de repos en laboratoire (Article 7).

PARTIE I. ÉTUDE DES SOUCHES D'ÉLEVAGE ET DE LA SOUCHE À SEXAGE GÉNÉTIQUE D'*ANOPHELES ARABIENSIS*

Introduction

An. arabiensis, membre du complexe d'espèces *An. gambiae* Giles, est l'une des trois espèces les plus importantes dans la transmission du paludisme en Afrique (Figure 7). L'efficacité vectorielle d'*An. arabiensis*, d'*An. funestus* et d'*An. gambiae s.s*. tient de leur préférence marquée pour les hôtes humains et leur capacité d'adaptation rapide aux changements d'environnements et aux interventions de contrôle (Collins & Besansky 1994). Plusieurs types de substances insecticides ont été utilisés pour éliminer les populations de vecteurs de paludisme ; cependant les substitutions d'insecticides ont continuellement été suivies d'évolution de résistance de la part des moustiques (revue par Hemingway & Ranson 2000). L'utilisation de champignon entomophathogènes semble à présent être la plus prometteuse alternative aux insecticides chimiques (Farenhorst *et al.* 2009). Cependant la mise en place de techniques non ou peu sujettes au développement de résistance devient impérative.

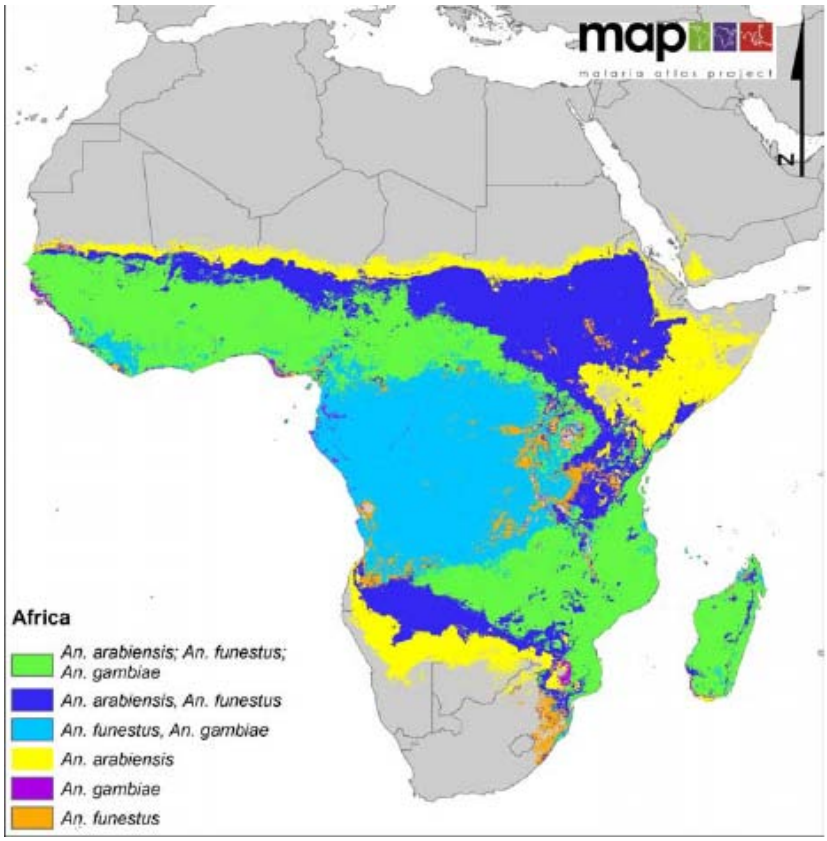

Figure 7. Distribution des espèces vectrices de paludisme dominantes en Afrique.
 (Sinka *et al.* 2012).

À la demande de plusieurs États Membres, la Division jointe de l'Organisation des Nations Unies pour l'Alimentation et l'Agriculture et l'Agence Internationale pour l'Énergie Atomique (Joint FAO/IAEA Division) a initié le développement de moyens d'élevage de masse, de stérilisation et de lâchers afin d'apporter un support technique à des projets de TIS. Elle soutient actuellement plusieurs projets d'étude de faisabilité de l'utilisation de la TIS contre des espèces de moustiques vectrices d'agents infectieux, dont la mise en place de la TIS pour lutter contre le vecteur de paludisme, *An. arabiensis,* au Soudan, et l'étude de faisabilité de l'utilisation de la TIS pour lutter contre *An. arabiensis* et *Ae. albopictus* à l'île de la Réunion.

Au Nord du Soudan, le paludisme sévit toute l'année, et représentait 52% des patients hospitalisés et 9% des décès en 2006 (FMOH 2006). La vallée du Nil, située entre Khartoum et la frontière Soudano Égyptienne, n'est pas considérée comme l'une des zones les plus affectée par le paludisme. Cependant cette région a une importance nationale croissante grâce aux projets hydroélectriques et agricoles, et l'endémicité du paludisme a motivé le développement d'un projet d'utilisation de la TIS, soutenu par la Division jointe FAO/IAEA, pour éliminer les populations d'*An arabiensis* (Ageep *et al.* 2009). La région Nord du Soudan a un climat extrêmement chaud et aride, cependant les populations d'*An. arabiensis* sont présentes le long du Nil; leur densité est maximale de novembre à juin, lorsque le niveau de la rivière décroit et laisse de nombreuses retenues d'eau favorables au développement larvaire (Dukeen & Omer 1986). *An. arabiensis* est l'unique vecteur présent et sa distribution est restreinte aux bords du Nil du fait de l'encerclement par le désert, ce qui en fait un site idéal pour l'application de la TIS (Ageep *et al.* 2009).

A l'île de la Réunion, *An. arabiensis* est l'unique vecteur potentiel de paludisme, et la possibilité de réintroduction du parasite sur l'île par les voyageurs a motivé la considération d'utiliser la TIS pour contrôler efficacement les dernières populations présentes. Les populations d'*An. arabiensis* se retrouvent dans un large spectre d'habitats anthropiques et naturels, désormais restreints sur trois zones géographiques de basse altitude, ce qui offre une situation favorable pour des interventions de contrôle (Gouagna *et al.* 2011).

Revue du contexte

L'intérêt et l'importance de ces projets s'inscrivent dans le besoin actuel de nouvelles méthodes de contrôle du paludisme, revu dans l'Article 1**.** Le paludisme reste l'un des plus grands défis du 21ème siècle. Depuis la découverte des agents vecteurs (moustiques du genre *Anopheles*) et des agents infectieux (parasites du genre *Plasmodium*) à la fin du 19ème siècle (Cox 2010), la lutte contre le paludisme a pu être plus ciblée et plus efficace. D'importantes mesures de drainage, assainissements et traitements médicamenteux, ont permis à de nombreux pays d'éradiquer cette maladie (Konradsen *et al.* 2004). Avec la découverte des propriétés du DDT pendant la seconde guerre mondiale, l'approche chimique a été largement développée et a permis des interventions efficaces contre les vecteurs du paludisme (de Zulueta 2000). Dans les années 1970, le paludisme avait disparu en Europe, en Amérique du Nord, et en Australie (Kager 2002). Depuis 2000, la transmission de cette maladie a décliné en Amérique du Sud et en Asie, mais elle reste fortement prévalente en Asie du Sud-Est, dans les îles du Pacifique Ouest, et surtout en Afrique (Article 1, Figure 1).

En 2010, l'Organisation Mondiale de la Santé (OMS) estimait à 174 millions les cas de paludisme en Afrique et à près de 600 000 les décès de personnes dus à cette maladie, dont 86 % d'enfants (World Health Organization 2012c). Cette maladie a aussi un poids considérable sur la croissance économique d'une nation et contribue à maintenir la pauvreté à cause des pertes de productivité et de revenus associés, et des frais inhérents aux infrastructures médicales, à la gestion des populations de vecteurs, à la distribution de protections et de médicaments, etc. (Gallup & Sachs 2001; Sachs 2001; Sachs & Malaney 2002; Malaney, Spielman, & Sachs 2004; Thuilliez).

De nombreuses interventions visant à éradiquer le paludisme se sont succédées en Afrique, mais la variété des situations épidémiologiques, les vastes étendues, les faibles moyens de communications, un système de santé insuffisant, et le développement de résistances aux insecticides, ont contribué au découragement et à l'abandon des campagnes d'éradication en 1969. Ce n'est qu'après 1997 qu'un regain d'intérêt de la part des gouvernements africains et de la communauté internationale a permis de soulever des fonds pour soutenir la recherche contre le paludisme (Breman, Alilio, & Mills 2004). Le plan d'action mondial contre le paludisme (Roll Back Malaria) lancé par l'OMS en 1998 a pour objectif d'éliminer la menace du paludisme dans le monde d'ici 2015 (World Health Organization 1999). Bien que ce but puisse apparaître très ambitieux trois ans avant échéance, d'encourageantes réductions de la mortalité liée au paludisme ont été rapportées dans certaines régions d'Afrique (World Health Organization 2012c).

Les mesures actuelles de contrôle des populations de vecteurs consistent principalement en des pulvérisations d'insecticides à l'intérieur des habitations, en l'utilisation de moustiquaires imprégnées d'insecticide, et l'administration de médicaments curatifs (Beier *et al.* 2008; World Health Organization 2012c). Cependant l'éradication du paludisme s'avère difficile dans de nombreux pays tropicaux en développement, du fait de perpétuelles adaptations des moustiques aux insecticides et des parasites aux médicaments. En outre, l'usage de certaines molécules insecticides efficaces a été interdit à cause d'incontestables considérations écologiques et sanitaires. Afin d'améliorer la gestion du paludisme et de ses vecteurs, il est à présent urgent d'apporter des tactiques complémentaires qui soient non seulement efficaces mais aussi économiques et respectueuses de l'environnement.

Ainsi l'utilisation de la TIS intégrée à des programmes plus larges de gestion des populations de moustiques vecteurs fait l'objet d'un regain d'intérêt depuis quelques années (Klassen 2009; Malcolm *et al.* 2009; Robinson *et al.* 2009). La possibilité de ne relâcher que des mâles, qui ne piquent pas, rend cette technique attractive puisque les risques de nuisances et transmissions de maladies sont inexistants. De plus, de grandes quantités de moustiques peuvent être obtenus sur une courte période de part leur fécondité élevée et leur court cycle de vie. Les programmes antérieurs étudiant l'utilisation de la TIS contre les moustiques (de 1960 à 1991, voir 1.2.3) ont permis des avancées techniques et scientifiques considérables. Le projet du Salvador dans les années 1970 a été l'un des plus prometteurs : un premier essai terrain de 5 mois a permis une diminution de 99% de la population indigène d'*An. albimanus*. Il a été suivi d'un essai à plus grande échelle sur une zone de 150 km^2 qui a résulté en la diminution de 97% de la population locale après 4 mois de lâchers quotidiens de plus d'un million de males stériles (Lofgren *et al.* 1974; Dame *et al.* 2009). Bien qu'aucun de ces programmes n'ait pu arriver à un stade d'application à grande échelle, pour des raisons techniques ou politiques, les connaissances qu'ils ont permis de rassembler ouvrent la voie, dans un futur proche, à l'utilisation de la TIS contre les moustiques à un niveau opérationnel.

Le succès de tels projets ne dépend cependant pas seulement des études biologiques et techniques mais la perception du public joue un rôle majeur. L'exemple malencontreux du projet d'utilisation de la TIS en Inde (1969-1975) a montré la fragilité d'un programme d'intervention contre des vecteurs de maladies face au poids de controverses animées par une méconnaissance du programme et par des raisons politiques (Anonymous 1975; Curtis 2007). Une insuffisante communication entre les scientifiques et les porteurs de projet d'un coté et les médias et le gouvernement de l'autre a empêché d'atténuer les doutes sur le bien fondé du programme et les tensions avec la population locale. Plus récemment, les essais de lâchers de moustiques génétiquement modifiés aux îles Caïmans et en Malaisie ont aussi été sujets à controverses car la mise en place des lâchers a été perçue comme peu transparente par la communauté scientifique internationale ou par le public local (Subbaraman 2011; Anonymous 2011).

Les approches alternatives de lutte contre les insectes vecteurs sont souvent encouragées par des pays développés pour être mises en place principalement dans des pays du Sud, or il est intéressant de voir que les chercheurs de pays tropicaux en développement seraient plus enclins à favoriser une implication du public que les chercheurs du Nord (Boëte 2011). Afin de favoriser le succès d'un programme de gestion des populations d'insectes vecteurs intégrant la TIS, l'approche de communication doit prendre en compte l'influence culturelle locale sur la perception des maladies, des interventions de gestion des vecteurs, des nouvelles technologies, ainsi que les variables économiques et politiques, la situation sanitaire et les contraintes techniques.

*Effet de l'élevage sur des souches d'*An. arabiensis

L'étude de la biologie de l'espèce cible est une étape fondamentale à la mise en place d'une TIS, qu'il s'agisse de la souche sauvage ou de la souche d'élevage destinée aux lâchers. Dans le cadre des projets de TIS au Soudan et à l'île de la Réunion, une souche

d'*An. arabiensis* originaire de Dongola, Soudan Nord, a été élevée au laboratoire IPCL, Seibersdorf, depuis 2004. L'élevage peut induire une sélection de certains caractères biologiques dûe au confinement en petite cage, telle que mise en évidence au Article 2.

Lors de tests impliquant l'utilisation de femelles d'*An. arabiensis* vierges, une évolution de la maturation sexuelle des adultes de la souche de laboratoire Dongola a été suspectée. La séparation des sexes peut se faire au stade de nymphe sous loupe binoculaire ou au stade adulte à l'œil nu. Le temps nécessaire à la maturation sexuelle des mâles *Anopheles*, qui était considéré comme supérieur à 24 h, permettait de procéder au sexage au stade adulte dans les 16-20h suivant l'émergence. Cette technique avait été utilisée avec succès à de nombreuses reprises au sein du laboratoire IPCL afin de collecter des femelles vierges destinées à des tests de stérilité (Helinski & Knols 2008; 2009). Cependant 4 ans et plus de 100 générations plus tard, les mêmes protocoles ne permettaient plus d'assurer la virginité des femelles. Cette méthode a été comparée au sexage au stade de nymphe, plus laborieux et chronophage. Des mâles stérilisés à diverses doses de radiation ont été accouplés à des femelles séparées des mâles selon le protocole habituel au stade adulte ou à des femelles isolées au stade de nymphe. La stérilité totale et donc la virginité des femelles ont pu être obtenues uniquement grâce à la dernière méthode de séparation (voir Article 2, Figure 2). Ce résultat indique que les mâles âgés de moins de 16 h sont déjà sexuellement matures et les femelles sexuellement réceptives.

Les mâles moustiques ne sont pas sexuellement matures dès l'émergence, car leur *terminalia* (trois derniers segments abdominaux des *genitalia*) est tourné vers le dos et les *fibrillae* des antennes ne sont pas dressées. Le temps nécessaire à la rotation à 180°C du *terminalia* (voir Article 2, Figure 1) varie selon les espèces de quelques heures à quelques jours. La durée de rotation n'avait jamais été rapportée pour des espèces d'*Anopheles*, toutefois des études indiquaient que les mâles d'*An. arabiensis*, d'*An. gambiae s.s.* et d'*An. stephensi* n'étaient pas sexuellement matures avant 24 h post émergence. L'observation du *terminalia* des mâles *An. arabiensis* Dongola montre que 42% des mâles ont complété cette rotation déjà 11 h après émergence, et plus de 90% d'entre eux après 14 h (Article 2, Figure 3). Des nymphes mâles sauvages ont été collectées sur le terrain à Dongola, Soudan Nord; les premiers mâles à compléter la rotation sont observés seulement 23.5 h après émergence. Les proportions de mâles, âgés de 12.5 h ou 17 h, ayant atteint les trois derniers stades de rotation sontt significativement différentes entre la souche Dongola de laboratoire et la souche sauvage (Article 2, Tableau 1). Immédiatement après émergence, des mâles d'*An. arabiensis* Dongola de la souche de laboratoire ont été mis en contact avec des femelles vierges pendant diverses durées allant de 11 à 18.5 h. Dans chaque groupe au moins une femelle est inséminée.

En outre, la large proportion de descendance semi stérile (53%) confirme la forte probabilité d'inséminations multiples des femelles d'*An. arabiensis* maintenues en petite cage (Article 2, Figure 2). Une seconde insémination aurait pu être favorisée par un transfert partiel de sperme par les mâles récemment émergés.

Comparaison de la SSG aux souches parentales sauvages

Dans le cadre des projets TIS soutenus par l'IAEA pour le contrôle des populations d'*An. arabiensis*, une souche à sexage génétique (SSG) “ANO IPCL1,” destinée aux lâchers, a été créée. Elle permet la séparation des sexes à divers stades de développement grâce à une translocation liant un allèle de résistance à l'insecticide dieldrine (organochloré) au chromosome Y. L'évaluation et la comparaison des traits d'histoire de vie d'ANO IPCL1 et des souches sauvages parentales permet de déterminer l'existence de différences biologiques entre ANO IPCL1 et les souches sauvages ayant des conséquences sur les performances des mâles sur le terrain et la production de masse ; elles sont rapportées au Article 3.

Une résistance à l'insecticide dieldrine existe naturellement dans une souche d'*An. arabiensis* de Sennar, Soudan Nord (Gaddal *et al.* 1985; Du *et al.* 2005). Une faible dose d'irradiation gamma a été utilisée pour induire des mutations aléatoires dans le but de transloquer l'allèle de résistance sur le chromosome Y. Des rétrocroisements entre les mâles

candidats et des femelles vierges de la souche Dongola, sensibles à la dieldrine, ont permis d'identifier une famille dont seule la descendance mâle était résistante à l'insecticide. La SSG ainsi créée en 2008, doit être régulièrement "purifiée" en croisant les mâles résistants à des femelles Dongola, afin d'éliminer les hétérozygotes (voir description de la création en Article 4). Nous avons considéré les souches parentales Sennar et Dongola, élevées au laboratoire IPCL, comme des références raisonnables pour comparer les traits d'histoire de vie de la SSG, et déterminer comment l'élevage peut être adapté en fonction de leur différences afin de parvenir à des niveaux suffisants de production d'adultes.

Des différences mineures au niveau des paramètres de développement (taux de survie et temps de développement entre les différents stades, taille et longévité des adultes, fécondité des femelles) sont observées entre les souches parentales et ANO IPCL1, mais aucune n'affecte particulièrement ANO IPCL1. Cependant le processus de translocation a induit une très faible fertilité naturelle chez ANO IPCL1: 27% contre 82 et 95% respectivement pour Sennar et Dongola (Article 3, Tableau 1). Cela se traduit par un plus faible taux intrinsèque d'accroissement (r) de la population d'ANO IPCL1, en dépit de temps de génération similaires (Article 3, Tableau 3). Une population d'ANO IPCL1 est capable de doubler sa taille en 7,8 ± 0,4 jours, alors que Dongola et Sennar peuvent le faire en respectivement 4,9 ± 0,3 et 5,6 ± 0,4 jours.

*Stérilisation de la SSG d'*An. arabiensis

La souche ANO IPCL1 est destinée à être utilisée pour les lâchers de mâles stériles. La semi-stérilité naturelle de cette souche laisse supposer que les doses de radiations nécessaires pour stériliser les mâles pourraient différer des souches normales d'*An. arabiensis*. L'étude de la stérilisation par irradiation aux rayons gamma des nymphes et des adultes ANO IPCL1 est présentée dans l'article en Article 4.

L'irradiation doit être effectuée le plus tard possible afin de réduire les lésions somatiques, c'est pourquoi la stérilisation des adultes permet de préserver la compétitivité des mâles (Helinski & Knols 2008). Cependant, pour des raisons de facilité de manipulation, l'irradiation au stade nymphe est généralement favorisée. Les protocoles d'irradiation des adultes décrits dans la littérature, nécessitaientt leur anesthésie par le froid et pouvait résulter en une mortalité importante dûe aux nombreuses manipulations nécessaires. Nous avons mis au point un conteneur permettant la stérilisation des adultes sans anesthésie et permettant une irradiation homogène et sans mortalité (Figure 8). Les divisions en tissu noir permettent aux adultes de rester immobiles durant l'irradiation.

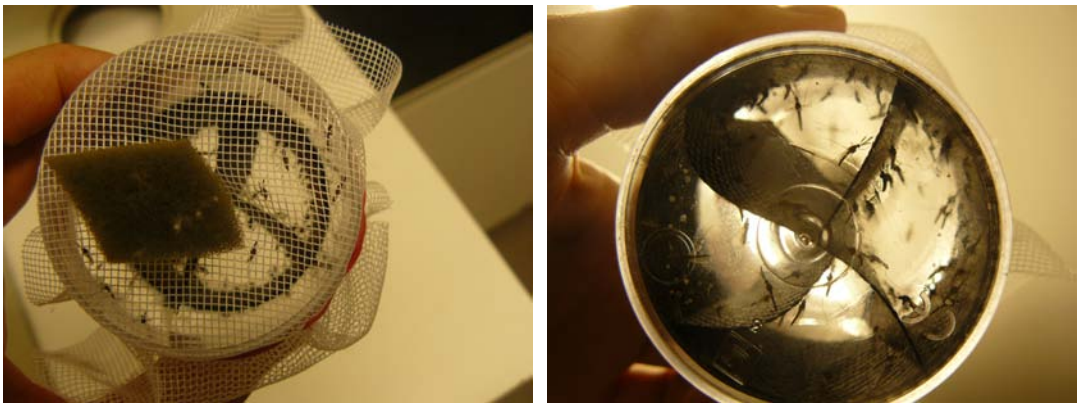

Figure 8. Conteneur pour irradiation des adultes.

L'irradiation du parent mâle au stade nymphe (âge > 20 h) à plus de 75 Gy ou au stade adulte (âge < 15 h) à plus de 90 Gy, permet l'éclosion de moins de 5 % des œufs (Article 4, Tableau 1). Le suivi de la survie de la descendance jusqu'à l'émergence de l'adulte a permis de mettre en évidence une forte mortalité au stade larvaire L1. En effet 20%

des œufs éclos meurent peu après éclosion chez les mâles non traités. Cette mortalité augmente avec la dose de radiation reçue par le père, allant jusqu'à 64% lorsque le mâle était irradié à 105 Gy au stade nymphe. Une mortalité plus tardive a été observée aux différents stades de développement, pour résulter en un très faible nombre final de descendants adultes viables. En effet, moins de 1.5% des œufs, issus d'un père irradié à plus de 75 Gy, a pu se développer jusqu'au stade adulte. Si l'on considère la fertilité comme étant la proportion d'œufs produisant des adultes (sexe ratio environ égal à 1), alors la stérilité moyenne d'un mâle ANO IPCL1 irradié à plus de 75 Gy est supérieure à 98%.

Article 1. The malaria struggle and the sterile insect technique: a review

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The stakes surrounding the control of malaria using the sterile insect technique: public health, technical and social perspectives.

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Abstract

The female mosquito transmitting malaria is still considered to be one of the greatest killers across half of the world. Malaria was efficiently eradicated in the 1970s from a large number of countries through impressive measures of sanitation, draining, insecticide use, and drug treatments. However, current interventions are still facing difficulties in Africa and South-East Asia, despite numerous successes with insecticide impregnated bed-nets and residual indoor spraying. The struggle is constantly slowed down by recurring resistance of the parasites and vectors to commonly used and new drugs and insecticides. Efficient control methods against the malaria vectors are desperately needed. Whilst a future malaria vaccine is being regarded as the 'magic bullet' against malaria, it seems that the development of an effective vaccine is moving at a slow pace. Control strategies for malaria that rely on the transfer of sterile or genetically-engineered sperm by released males to wild virgin females are currently being developed and are approaching field implementation, while their use against a dengue vector has already been tested in the open field. However, the success of this strategy in pest control or health management programmes strongly depends on gaining public understanding and acceptance. Here we attempt to consider what are the stakes involved in the use of the sterile insect technique against malaria from technical, public health, and social perspectives.

Keywords: Malaria Control; Mosquito Management, Sterile Insect Technique; Social; Public Support

Introduction

Malaria has been threatening human health for thousands of years; it was first mentioned in the Antiquity by Hippocrates (de Zulueta 1973). But it was not until the late 19th century that the causative agent responsible for the disease was discovered to be a protozoan parasite of the genus *Plasmodium* present in the blood or tissues, and that transmission to humans occurred through bites of female *Anopheles* mosquitoes (Cox 2010). In developing countries, especially in Africa, malaria remains one of the greatest global public health challenges of the 21st century (Grépin and Reich 2008, Lammie, et al. 2006, Mathers, et al. 2007, Stratton, et al. 2008, Walther and Walther 2007). The implementation of impressive sanitary improvements and the massive use of insecticides have enabled the malaria map around the tropics to be shrunk (Hay, et al. 2004), (Feachem, et al. 2010), but half of the global population remains at risk of contracting this disease (WHO 2011).

Traditional vector control approaches, including insecticide spraying, drug administration to cure the disease, and more recently the use of bed-nets have not yet succeeded in solving the malaria problem in numerous tropical developing countries (Beier, et al. 2008). Development of resistance of the mosquito vectors to insecticides and of the parasites to drugs has impeded eradication of this disease, despite the commitment of international and national authorities to reduce the malaria toll. In addition, some of the most efficient insecticides have been banned for malaria vector control, mainly because of indisputable ecological and health concerns. It is obvious that there is an urgent need for additional control tactics that are not only effective and economical but also environmentally friendly. The possibility and potential of using the sterile insect technique (SIT) as part of area-wide integrated pest management programmes (AW-IPM) against mosquitoes has gained considerably in importance in recent years (Robinson, et al. 2009, Wilke, et al. 2009). The SIT is a form of "birth control" for pest insect populations: mass-reared sterile males are released into a wild population and through mating with virgin females they induce sterility in the wild population that will decrease the number of progeny, therefore progressively reducing the population size.

In view of the successes of previous AW-IPM programmes that included an SIT component against other pest insects (Bloem, et al. 2005, Enkerlin 2005, Feldmann, et al. 2005, Vargas-Teràn, et al. 2005), the technique seems to be reliable and robust, and we anticipate that its use against disease-vector mosquitoes should be well accepted by the public. However, experience has shown that promising public health programmes can unexpectedly fall apart (Torny 2006, Trostle 2005) as the perception of diseases (Herzlich and Augé 1984), risks (Douglas and Wildavsky 1982), or even of nature (Descola 2005) vary between cultures, and do not always tie in with the views of researchers, health policy makers, or politicians, creating mutual misunderstandings. As underlined by Faith et al (2005), and encapsulated in the quote "*Public trust is essential in promoting public health*", more research is needed to investigate which might be the concerns and fears of people that could threaten the implementation of future mosquito SIT programmes.

This paper undertakes to review the technical, public health and social challenges surrounding the use of the SIT for controlling malaria. It focuses on the burden of malaria, the history of malaria control, and the development and principles of the SIT for insect pest management and more specifically for mosquitoes. Finally, we explore how the relationship between science and society can impact on the successful completion of these programmes.

The malaria burden: a worldwide public health concern

Malaria is considered by many experts to be the most important insect-transmitted disease (Gilles and Warrell 1993). Since 2000, its transmission has declined in the Americas, Asia, and in some countries of the European region (as defined by the World Health Organization (WHO): Russian Federation, Azerbaijan, Kyrgyzstan, Tajikistan, Turkey, Georgia, and Uzbekistan). However, it is still highly prevalent in most African countries, in the WHO Eastern Mediterranean region (Afghanistan, Djibouti, Pakistan, Somalia, Sudan, South Sudan, and Yemen), in the WHO South-East Asian region (Bangladesh, India, Indonesia, and Myanmar), and in the WHO Western Pacific region (WHO 2011). In 2010, 3,300 million people were estimated to be at risk of contracting malaria (WHO 2011) with the burden of disease being especially high in developing countries and on children. The WHO estimates that malaria killed 655,000 people in 2010, 91% of whom were in Africa, and 86% children under 5 years old (WHO 2011).

Malaria undoubtedly helps to maintain a state of poverty in affected regions (Kitua 2003, Thuilliez 2009, WHO 1999), hugely affecting a nation's economy indirectly through the losses of productivity and income that are associated with illness and death (Gallup and Sachs 2001, Sachs and Malaney 2002). In countries with a high prevalence of malaria it is estimated that the disease could be responsible for a 1.3% (or more) reduction in economic growth per year (Sachs 2001). Families have to invest in insecticide-treated nets (ITN) for beds and curtains, antimalarial drugs, and transport to health facilities, while governments need to invest in health care infrastructure, vector control, education, and research, via public and private sectors (Malaney, et al. 2004). Besides the morbidity it causes, *Plasmodium falciparum* malaria can hamper children's schooling and social development through both absenteeism and permanent neurological and other damage associated with the disease (Breman, et al. 2004, Holding and Snow 2001, Sachs and Malaney 2002). The infection of pregnant women with malaria is also particularly serious, since it can lead to child mortality (Sachs and Malaney 2002). In addition, co-infection with human immunodeficiency virus (HIV) and other parasitic, bacterial or viral diseases has now clearly been linked to increased malaria parasitemia (Breman 2009, Breman, et al. 2004, Brooker, et al. 2007, Hay, et al. 2004, Wongsrichanalai, et al. 2003). This highlights the overwhelming health threat malaria poses to mankind in many parts of the tropical world. The intolerable burden of malaria, when faced with high levels of drug resistance, increasing insecticide resistance and meagre

resources at national levels, remains a great public health challenge to governments and the research/control community.

Whilst a malaria vaccine has often been regarded as the 'magic bullet' against malaria, it is becoming increasingly unlikely that an effective vaccine can be deployed before 2020 at the earliest. The complexity of the malaria parasites is still preventing the development of an effective vaccination strategy (Desowitz 2000), and there is concern that a "leaky" vaccine would promote the evolution of more virulent pathogens (Sedwick 2012). However, the complete genomes of one of the main vectors *An. gambiae* (Holt, et al. 2002) and of the parasite *P. falciparum* (Gardner, et al. 2002) have been sequenced. With these recent genomic advances further new technologies may be added to the already impressive arsenal of malaria control tactics (Ashburner 2002, Morel, et al. 2002, Sachs 2002, Touré, et al. 2004, Wellems 2002). One example might be the genetically modified mosquito strain refractory to the malaria parasite that has recently been developed (Isaacs, et al. 2012).

The old story of malaria control: evolution of the current global political situation

For hundreds of years malarial disease was known to be linked with swamps, and control efforts mainly consisted of drainage interventions for sanitation purposes (Gilles and Warrell 1993). After the key discoveries of the causative agents of malaria and its vectors at the end of the 19th century (Cox 2010), more specific control tactics have been developed through management of the environment (Konradsen, et al. 2004). In addition, the discovery of the efficient and affordable curative drug chloroquine considerably reduced malarial morbidity and mortality for decades (Krafts, et al. 2012, Wellems 2002). The eradications of malaria from Brazil (Soper 1965) and Italy (Romi, et al. 1997) are some of the great historical examples of successful sanitation projects in the pre-DDT era (see de Zulueta 2000, Killeen, et al. 2002), where a quasi military approach was used to rigorously remove *An. gambiae* from their breeding and resting sites, leading to an interruption of malaria transmission. It was only during the Second World War that the chemical insecticide approach was widely developed, when the residual insecticide properties of DDT were discovered, allowing the implementation of efficient large scale interventions against malaria vectors (de Zulueta 2000, Gahan, et al. 1945, Najera 1989, Wright , et al. 1972). However, by 1947 the first mosquitoes resistant to insecticides had already been discovered (Brown 1986), while resistance of the parasite to chloroquine first occurred in the late 1950s, and by 1970s had spread to all malarious regions (Wellems 2002).

In 1955, the Global Malaria Eradication Program (GMEP) mandated the WHO to provide technical advice and coordinate resources to attempt the complete eradication of malaria (Najera, et al. 2011). By 1969 eradication of the disease was achieved in Europe, northern America, and northern Australia by rigorous larval control measures integrated with all other available tools; a significant reduction in disease prevalence was observed in Asia, but no effective results could be maintained in Africa (Kager 2002). Indeed, the concomitant evolution of resistance to drugs and to insecticides, together with economic issues, conflicts, and environmental changes, led to a great resurgence of malaria in Africa (Greenwood and Mutabingwa 2002, Nchinda 1998), and the variety of epidemiological situations found across the vast areas of tropical Africa had not been foreseen by the GMEP (Najera, et al. 2011). In 1969, eradication of malaria in Africa was considered not to be feasible in the short term: the GMEC had failed in Africa and was abandoned (Molineaux and Gramiccia 1980, Najera 1989, Najera, et al. 2011, WHO 1969). Though a suppression and containment policy was conducted in the ensuing decades, the impetus to manage malaria was lost, as eradication appeared impossible. Geopolitical factors probably also contributed to this lack of interest as all the more wealthy countries had already been freed from malaria, and support to the African continent to achieve the same was not high among their priorities (Sachs 2002). Consequently, financial and research support declined and the malaria situation quickly deteriorated (Greenwood and Mutabingwa 2002).

In 1992, a Ministerial Conference on Malaria Control developed new policies to encourage early diagnosis and treatment, to apply selective and sustainable preventive measures, to contain epidemics, and to promote local research. These ambitions were endorsed and encouraged by the World Health Assembly in 1993 (Trigg and Kondrachine 1998). After 1997, control interventions finally regained importance and an increasing number of organizations raised funds to support malaria research (Breman, et al. 2004). The WHO launched the Roll Back Malaria (RBM) initiative in 1998, together with the World Bank, United Nations Development Program, and United Nations Children's Fund. Following the RBM, the Multilateral initiative on malaria (MIM) was created in 1997 with the aim of fighting malaria through concerted international cooperation efforts, communication, and utilization of research findings to inform malaria prevention, treatment, and control (Rugemalila, et al. 2007). The ambitious RBM initiative’s target was the elimination of the malarial threat by 2015 (Nabarro and Taylor 1998, Nabarro and Mendis 2000), which at the time of writing is only three years away: how far from this target are we? The WHO

reported a 17% global reduction of an estimated malaria incidence between 2000 and 2010, which is lower than the intended target of 50% (WHO 2011). This reveals that many hurdles still remain to be overcome. However, an encouraging reduction of 33% in the malaria specific mortality rate was observed in the African region (WHO 2011). The RBM initiative foresees the need for continuation of malaria control efforts after 2015 to ensure that the malaria burden continues to decrease, until global malaria eradication can be achieved in the long-term.

Controlling *Anopheles* populations

The long history of malaria control has demonstrated that the efficacy and sustainability of interventions is often highly undermined by the resistance developed by parasites to drugs and vectors to insecticides. Despite this, all the currently recommended intervention strategies rely entirely on those drugs and insecticides. To counteract this strategical weakness and to work towards the RBM goal of freeing the world of malaria, the concomitant use of other control strategies should be considered (Beier, et al. 2008). As stressed by Killeen et al. (2002), the significant success of domestic adulticide tactics over the last few decades has diverted interest from other approaches, including environmental management, which was historically successful in eradicating malaria in Brazil (Soper and Wilson 1943) and Egypt (Soper 1966) in the pre-DDT era, and in Zambia between 1929 and 1949 (Utzinger, et al. 2001).

Insecticides have dominated the pest control sector for decades, and are still widely used nowadays, despite the known harmful consequences to non-target insect species of the exclusive use of broad-spectrum insecticides, and the direct or indirect negative impact on animal and human health (Carson 1962, Repetto and Baliga 1996). Due to increased concerns about the long-term toxicity of DDT residues and its bio-magnification in the food chain (Tren and Bate 2001) it has been banned for agricultural purposes in most developed countries since the 1970s (Müller 1992). However, DDT remains available for the control of some disease vectors and is used in 13 countries, accounting for 20% of all insecticides used for malaria vector control; although mosquito resistance to DDT is prevalent worldwide, its efficiency as a control tool is clear (Berry-Cabàn 2011, WHO 2011).

Current WHO policies favor rapid diagnostic tests (RDTs) and treatment (artemisinin-based combination therapy) of malaria patients, mass campaigns to distribute long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), and larval control measures (Beier, et al. 2008, Curtis and Townson 1998, Floore 2006,

Noor, et al. 2009, Pluess, et al. 2010, WHO 2011). The WHO goal of universal coverage of all malaria affected regions with ITNs (WHO 2007) is far from being reached, although impregnated bed-nets are commonly seen in the homes of many affected areas (WHO 2011). IRS has been effective in rapidly interrupting malaria transmission and is increasingly used in many malaria-endemic countries since 2006 (Pluess, et al. 2010, WHO 2011). Between 2000 and 2010, ITN and IRS coverage has greatly increased in countries such as Rwanda, Ethiopia and Zambia, resulting in a decline of malaria cases by more than 50% in some instances (WHO 2011). The convincing impact of these tools on childhood mortality and morbidity has been extremely useful not only because of the lives directly protected but also because it has restored confidence in vector control as a valid prevention tool against malaria.

The involvement of three living beings (parasite, mosquito, and human) and the complexity of their respective environments complicate the reduction of malaria spread. The evolutionary history of malaria vectors is intimately linked to human activity. Selection for highly anthropophilic and domestic species, for insecticide resistant strains, and their widespread expansion and adaptation to changing environments have been linked to major milestones throughout human history, such as the establishment of agriculture through deforestation (Ayala and Coluzzi 2005, Wolfe, et al. 2007), human urbanization and the accompanying industrialization and intensified agricultural activities (Coluzzi 1994, Robert, et al. 2003). Efforts to control malaria have to consider the changing habits of the human host and mosquito vector, and the high adaptability of the vector to insecticides and of the parasite to drugs to get successful results (Enayati and Hemingway 2010). To complement the available tools for the suppression of mosquito vectors, there is renewed interest in the potential of the sterile insect technique (SIT) (Curtis and Townson 1998, Jayaraman 1997, Klassen 2009, Malcolm, et al. 2009, Robinson, et al. 2009).

Emergence of new biological tools: principles of the SIT

The SIT was conceived in 1937 by E.F. Knipling, but it took until the 1950s for the technology to be first implemented. The mutagenic activity of X-rays was described by Muller in 1927 (Muller 1950), and the potential to damage the reproductive cells so as to induce sterility in a target insect pest was demonstrated 30 years later by entomologists (Knipling 1959, Knipling 1955). Independently, A.S. Serebrowskii, and F.L. Vanderplank (in: Klassen and Curtis 2005) indicated that the sterility arising from hybridization between different species or strains could be exploited for population control. Knipling understood that the integrity of the hereditary machinery of insect populations is critical for its survival, and that pest

populations can be reduced by compromising their fertility (Knipling 1959). This was in contrast to traditional methods of pest control that usually relied on compromising the survival of the insect pest (Vreysen and Robinson 2011).

The SIT relies on the mass-production of the target pest species males, their sterilization by means of ionizing radiation or chemicals, and finally their release in a sustained way in the target area (Knipling 1959). Alternative methods of sterilization rely on *Wolbachia*-mediated cytoplasmic incompatibility or genetically modified mosquitoes (GMM) (See Alphey, et al. 2010 for review). The success of SIT implementation obviously requires that the released insects are able to mate efficiently with their wild counterparts. Although the technique is often promoted for geographically isolated areas where no immigration of the wild pest can occur (Curtis 2002), ecological isolation is not a requirement, provided the approach is area-wide and a phased strategy (using the "rolling carpet" or "wave" principles) is implemented (Hendrichs, et al. 2005). To be effective, the release of sterile insects needs to be integrated with other control tactics as part of AW-IPM approaches (Hendrichs, et al. 2007, Knipling 1955, Knipling 1979, Mangan 2005).

Including an SIT component in an AW-IPM approach is complex, from a technical and managerial point of view (Vreysen, et al. 2007) as the research phase must include the development of efficient mass rearing, sterilization, and release techniques that ensure a good quality and competitiveness of the released insects (Bakri et al. 2005; Calkins and Parker 2005; Parker 2005). A high initial population density of the target pest, landscape complexity, and the possibility of the migration of pests from other populations into the release site can all affect a programme (Hendrichs, et al. 2005, Itô and Yamamura 2005, Lance and McInnis 2005). The sterile insects are usually released in over-flooding ratios to rapidly and efficiently transfer sterility into the wild population (Dame, et al. 2009, Mahon 1996), and the release period is usually adapted to seasonal density variations, or preceded by conventional management tactics to achieve an initial reduction in the pest density (Lofgren, et al. 1974).

The first successful application of classical SIT resulted in the eradication of the New World screwworm from Curaçao in 1954 (Baumhover, et al. 1955). The population of this insect could not be managed with existing control methods, contributing to its devastating impact on the livestock industry in the southern US every year. The programme ultimately culminated in arguably the most successful eradication effort of an insect pest ever, achieving the eradication of the New World screwworm from Florida, the southern part of the United States, Mexico, Central America and Panama (Klassen and Curtis 2005). Although the total screwworm

eradication campaign over the course of 50 years came with a price tag of USD 1,000 million, its annual benefits for the region now equal its total costs (Vreysen and Robinson 2011, Wyss 2000). This success prompted scientists to investigate the feasibility of using the technique for dealing with other insect pests (Knipling 1998). The SIT allowed successful eradication of several fruit fly species (Enkerlin 2005, Gonzalez and Troncoso 2007) and tsetse fly (Vreysen, et al. 2000), and is now used more and more for suppressing a target population below an economic threshold, especially against fruit flies (Reyes, et al. 2007), but also for certain Lepidoptera species (Bloem, et al. 2007). Used as part of containment programmes to prevent the spread or the establishment of a pest, the SIT also offers a cost effective alternative to the previous strategy of dealing with outbreaks using bait sprays (Hendrichs, et al. 2005).

In comparison to many other approaches to pest suppression (for example the use of chemical and biological products), the SIT is generally considered to have the least risk of unexpected environmental consequences, as it is self-limiting and non-toxic (Nagel and Peveling 2005). In the case of disease vector insects such as mosquitoes, reduction of a vector population density, even without eradication, could reduce the probability of contact between the host and the vector sufficiently to result in the overall decrease or total suppression of disease transmission (Anguelov, et al. 2012, Dumont and Tchuenche 2011). Used as a preventive approach (Dowell, et al. 2000), SIT may be one of the most appropriate and useful tools to prevent the spread of disease in previously mosquito free areas facing re-invasion, or in areas recently infected by disease-vector mosquitoes.

The use of SIT against mosquitoes

The integrated use of the SIT in AW-IPM programmes against mosquitoes is attractive, as only males would be released; therefore there is no associated risk of increased biting nuisance or disease transmission.

Between 1960 and 1991, several small scale SIT programmes, using various sterilizing approaches (chemosterilization, ionizing radiation, cytoplasmic incompatibility (CI), or chromosome translocation), were partially successful in local elimination of several mosquito species (Klassen and Curtis 2005). Although none of the trials ever reached the operational level, several had as their objective the reduction of small-scale wild populations: *Ae. aegypti* in Florida and Kenya, *Cu. pipiens* in France, *Cu. quinquefasciatus* in Myanmar, Florida and India, *Cu. tarsalis* in California, *An. albimanus* in El Salvador, *An. gambiae* in Burkina Faso, and *An. quadrimaculatus* in Florida (See Benedict and Robinson 2003, Klassen and

Curtis 2005 for review). In addition, various other trials have been carried out where the final goal was not population suppression but to conduct specific research experiments (Benedict and Robinson 2003, Klassen and Curtis 2005). These pilot trials allowed several technical issues to be overcome, and produced valuable information that could lead to a mosquito AW-IPM programme with an SIT component reaching an operational level in the near future (Dame, et al. 2009).

Amongst the most successful programmes was the operational release of chemo-sterilized male *An. albimanus* in El Salvador in the 1970s. In an initial field trial, sterile males were released for five months over a 14-15 km^2 area and the isolated indigenous population was reduced by 99% (Lofgren, et al. 1974). During a larger trial over a 150km^2 area, daily releases of more than 1 million (almost fully competitive) sterile males, together with larval control measures, resulted in a 97% reduction of the wild *An. albimanus* population after 4 months (Dame, et al. 2009, Weidhaas, et al. 1974). Although the programme experienced some set-backs, such as immigration from untreated populations into the release area, the trial demonstrated that this mosquito species could be suppressed using the release of sterile males. Unfortunately the project was aborted prematurely due to the civil war (Benedict and Robinson 2003, Dame, et al. 2009, Klassen and Curtis 2005). The programme against *Cx. quinquefasciatus*, a vector of filariasis and other human diseases, on a Florida island, USA is another example, where daily releases of 8,400 to 18,000 chemo-sterilized males over a 10-week-period successfully suppressed a native mosquito population in an isolated area (Patterson, et al. 1970).

Unfortunately, despite some success, the failure of many of the projects, attributed either to a lack of competitiveness of the males, immigration of fertilized females from untreated area, or political decisions, has slowed down subsequent research efforts (Asman, et al. 1981, Benedict and Robinson 2003). It is only recently that the SIT has regained attention as a control tactic against mosquitoes (Jayaraman 1997, Wilke, et al. 2009), since available control tools are not sufficient for the eradication of malaria and mostly only contain disease transmission (Beier, et al. 2008, Collins and Paskewitz 1995, Feachem, et al. 2010, Klassen 2009). Upon the request of several member states, the International Atomic Energy Agency and the Food and Agriculture Organization (Joint FAO/IAEA Division) initiated the development of methods and equipment for mass-rearing and sterilization, and are assisting several national and regional projects to assess the feasibility of the use of the SIT against some of the major disease-vector mosquito species, such as the malaria vector *An. arabiensis* in Northern Sudan and dengue vector *Ae. albopictus* in Reunion Island (Malcolm, et al. 2009, Robinson, et al. 2009). Recent technological

advances have already overcome some of the key issues such as mass rearing and the need for sorting devices (Balestrino, et al. 2012, Balestrino, et al. 2012), genetic sexing and sterilization methodologies (Helinski, et al. 2006, Helinski and Knols 2009, Yamada, et al. 2012), and optimization of the immature and adult rearing parameters (Gilles, et al. 2011, Oliva, et al. 2012). In addition, small-scale field trials performed in Northern Italy against the chikungunya and dengue vector *Aedes albopictus* have shown encouraging results: the fertility of the wild population was reduced despite a relatively low number of released sterile males and high immigration of females from untreated areas (Bellini, et al. 2007).

Public perceptions and their impact on vector management

In the history of mosquito releases for vector-borne disease control, similar projects have had very different impacts on the local or international communities, even though a communication strategy with the public was usually deployed.

An ambitious SIT programme in India in the 1970s, launched in 1969 by the WHO, the Indian Council of Medical Research (ICMR) and the USA, to eradicate malaria, yellow fever, and filariasis, experienced unfortunate setbacks (Curtis and Reuben 2007). Some tensions had already developed between scientists and part of the Indian population who opposed an initial field release of chemosterilized mosquitoes in 1962 (Anonymous 1976, Krishnamurthy, et al. 1962). An educational programme was then developed to determine the most suitable approach to inform the local communities about the goals of this programme, and good acceptance and cooperation from the village populations had been reported (Singh, et al. 1973). However, from 1972 to 1975, the project faced resistance again, as the Indian press, and later some Indian politicians, raised doubts about the scientific foundations of this programme, and diffused the idea of a foreign conspiracy intending to spread yellow fever disease in India (Curtis and Reuben 2007). The involvement of the USA and the WHO, which had dominant positions in the world, reinforced the fear that mosquitoes were being tested as biological weapons in India. Unfortunately, this rumour was not countered in time by the WHO or the Indian government, and the international scientific press also questioned the validity and transparency of the research (Anonymous 1976). The controversy escalated very fast and further (unfounded) criticisms were levelled, fanned by the interactions between internal and external politics, scientists, and the media. The project was abandoned in 1975 despite considerable technical and biological achievements. The WHO later acknowledged that a weakness of the project had been the lack of communication with the public via the media (Anonymous 1976).

To avoid similar failures different strategies can be developed upstream of the final open release of mosquitoes. A strong collaboration between institutional partners and local communities in the early stages of a programme is thought to be essential (El Sayed, et al. 2009). However this may prove to be insufficient in some context. Since 2009 several releases of genetically modified mosquitoes (GMM) have been conducted and have unleashed much debate. Prior to and during the open-field trial releases of GMM *Ae. aegypti* in the Cayman Islands, approval was granted by the relevant governmental institutions, and information about the project had been provided to the local population through media and personal contact (Harris, et al. 2011). Likewise, various actions were undertaken as part of the GMM *Ae. aegypti* open-field release carried out in Malaysia to inform the community (information displays, a public meeting), and to get the approval from the local government authorities and the National Biosafety Board (Lacroix, et al. 2012). Despite these measures to gain community engagement and approval, those trials have been subjected to controversy in the international scientific press (Anonymous 2011, Enserink 2010, Subbaraman 2011). However, a similar programme of even larger scale releases in Brazil suffered less criticism and appeared to be welcomed by the local population (Oxitec 2012, Specter 2012). SIT pilot trials to suppress populations of the chikungunya vector *Ae. albopictus* are ongoing in Northern Italy and limited to small villages (Bellini, et al. 2007); the local authorities and citizens, were adequately informed and seemed to have accepted the releases without apprehension (Bellini R., pers. comm.).

The discrepancies around the social impact between these programmes can obviously not be easily explained, since each project site is characterized by a unique ensemble of political factors and differences in social history and norms, regulatory environment, health situation and perception of it, environmentalist involvement, public expectation and acceptance of applied science, etc.

From a social perspective

As SIT programmes are strategies to be employed in the open-field, they entail various essential ethical considerations (McNaughton 2012) and could be subjected to a variety of challenges and setbacks before they can be implemented.

Social aspects of science were regarded until recently as “exteriors” (Beck 1992), however they are now considered as being a part of the whole process (Ferretti 2007). Callon et al. (2001) describe science as a "translation" process which occurs in three phases. The first one addresses the reduction of a global issue into a scientific question. A scientific framework is then built around donors, political actors,

and priorities. It is only in a second phase that scientists explore and resolve the questions, relying on current knowledge and techniques, on a small laboratory scale. Finally, the most perilous phase is the return to the "macro-world" with the operational programme, which will test the strength of the alliances made in the first step. That is the decisive moment when the solution developed in the laboratories will face politics (identification of the priorities and rationale), beliefs, social norms, and even the environment in an attempt to transform the “macro-world”. In each step, social and anthropological factors can propel the project into oblivion or collapse, or be a key to its success.

Scientific projects such as health programmes focussing on the reduction of malaria incidence are of worldwide concern (Hay, et al. 2004), and gather a community of networked researchers (Breman, et al. 2011), and international donors (Alphey, et al. 2010). They are largely initiated by developed countries to be used mainly in endemic countries of the South; interestingly, researchers from the tropical malarial areas of the South would favor a deeper involvement of the public than would researchers from the North (Boëte 2011). Disease reduction programmes intend to promote public health and the preservation of the environment according to international norms. However, the views of outside stakeholders are often different from the local perceptions of health and public health policy (Trostle 2005). What is considered by some actors as a "good-in-itself" is not necessarily universal (Chateauraynaud 2011). Perceptions of the disease, its associated risks, and related health behaviour are strongly dependant on the local culture and beliefs, and the political context (Jones and Williams 2004).

Gaining actual public support requires understanding and the consideration of a wide range of sensitive ethical issues (McNaughton 2012). The development of a good working relationship between scientists and institutional partners will not be sufficient, and a chance must be given for the general public to consider and understand the issue in order to avoid eroding its trust (Boëte 2011, Corraliza and Berenguer 2000, Jegede 2007, Robinson and Hendrichs 2005, Sandman 1987). The two year social research programme undertaken in northern Australia, as part of a potential trial release of *Wolbachia*-infected *Ae. aegypti*, reported that public engagement is an essential upstream step for developing trust and authorization of a release (McNaughton 2012). The examples of the previous section demonstrated that communicating with the local communities would not ensure their long-term acceptance and an easy implementation of a vector control programme, however it

appears important that the local community be part of the final decision (Boëte 2011, McNaughton 2012).

Education is often considered to be a privileged means of information exchange between scientists and the wider public, and some educational interventions have been very positive, such as the example of a community-based education programme in Sri Lanka which led people to adopt environmentally sound measures to limit mosquito density (Yasuoka, et al. 2006). However this approach should not be considered as a panacea. Interactive means of mediation have the potential to integrate these issues into a democratic discussion, through public debates, consensus conferences or face-to-face presentations. This approach is being considered in Reunion Island and, although it will not guarantee the public community consent to implementation of SIT, a three year research programme is currently underway to attempt to unravel the specific cultural issues and complexities surrounding public health issues and vector-borne diseases. Undertaking thorough long-term social research prior to any trial release appears to be the most appropriate approach to understanding the context, knowledge of the disease, and expectations at various scales. This understanding will allow the design of situation-specific engagement strategies and communication materials (McNaughton 2012).

Conclusion

There are now compelling arguments in favour of using the SIT against malaria vectors, from an ecological, economic and biological point of view. Given the considerable burden of this vector-borne disease, the current international effort employed to address this key health issue, and the possibility of success of an SIT strategy with tangible benefits, it is important that the potential areas for integration of an SIT component into mosquito control programmes are selected carefully. Increased participation of the public and private sector would probably help to ensure an effective implementation. In contrast to the conventional vector control methods, which tend to opportunistically respond to problems and thus require minimal planning, and are implemented independently of populations' involvement, implementation of an SIT programme requires proper planning over several years and a well-considered and appropriate organization.

Potential sites for implementation of mosquito SIT programmes are varied and culturally different; for that reason the provision of information and public interaction strategies have to be well adapted. Releases of millions of sterile insects may be impressive and even frightening, particularly if they happen close to inhabited

areas; good communication between the programme staff and the public is necessary to maintain their participation and support and to keep the work oriented towards the public health objectives.

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Figures

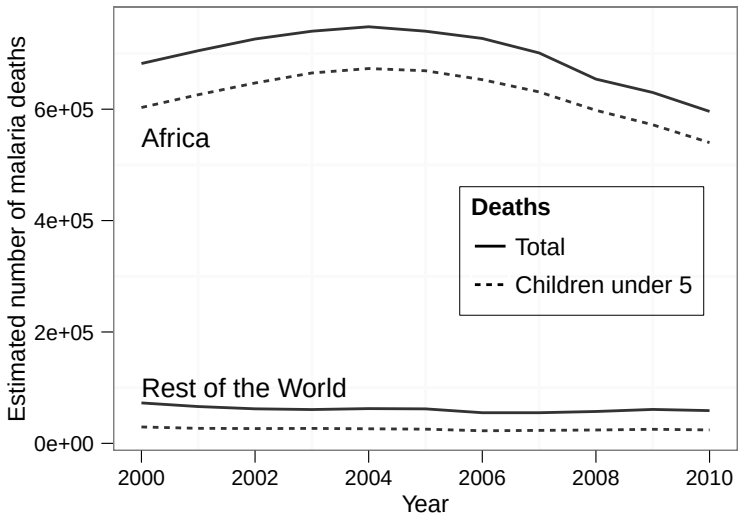

Figure 1. Estimated number of malaria deaths in Africa and the rest of the world from 2000 to 2010. From data from the Global Health Observatory (GHO) data repository (http://www.who.int/gho/database/en/).

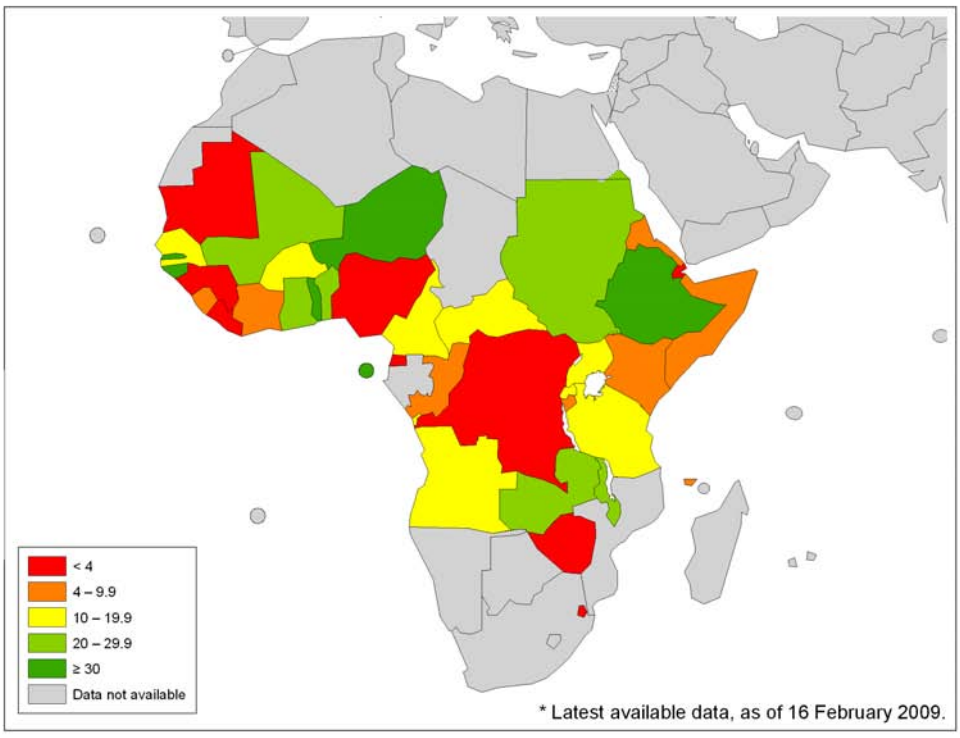

Figure 2. Proportion of children under 5 sleeping under insecticide-treated bed-nets (%) in Africa, 2000-2006. Courtesy from the World Health Organization (WHO 2009).

Article 2. Laboratory selection for an accelerated mosquito sexual development rate

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Laboratory selection for an accelerated mosquito sexual development rate

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Abstract

Background: Separating males and females at the early adult stage did not ensure the virginity of females of *Anopheles arabiensis* (Dongola laboratory strain), whereas two years earlier this method had been successful. In most mosquito species, newly emerged males and females are not able to mate successfully. For anopheline species, a period of 24 h post-emergence is generally required for the completion of sexual maturation, which in males includes a 180° rotation of the genitalia. In this study, the possibility of an unusually shortened sexual maturity period in the laboratory-reared colony was investigated.

Methods: The effect of two different sex-separation methods on the virginity of females was tested: females separated as pupae or less than 16 h post-emergence were mated with males subjected to various doses of radiation. T-tests were performed to compare the two sex-separation methods. The rate of genitalia rotation was compared for laboratory-reared and wild males collected as pupae in Dongola, Sudan, and analysed by Z-tests. Spermatheca dissections were performed on females mated with laboratory-reared males to determine their insemination status.

Results: When the sex-separation was performed when adults were less than 16 h post-emergence, expected sterility was never reached for females mated with radio-sterilized males. Expected sterility was accomplished only when sexes were separated at the pupal stage. Observation of genitalia rotation showed that some males from the laboratory strain Dongola were able to successfully mate only 11 h after emergence and 42% of the males had already completed rotation. A small proportion of the same age females were inseminated. Wild males showed a much slower genitalia rotation rate. At 17 h post-emergence, 96% of the laboratory-reared males had completed genitalia rotation whereas none of the wild males had.

Conclusion: This colony has been cultured in the laboratory for over one hundred generations, and now has accelerated sexual maturation when compared with the wild strain. This outcome demonstrates the kinds of selection that can be expected during insect colonization and maintenance, particularly when generations are non-overlapping and similar-age males must compete for mates.

Background

Malaria is the most important insect-transmitted disease with half of the world's population at risk of disease and mortality. In 2008, malaria led to nearly 900,000 deaths [1]. The high impacts on human health and on countries' economies have motivated campaigns for the eradication of the disease through methods controlling either the parasite *Plasmodium* spp. or its vectors. *Anopheles arabiensis* is one of the major African vectors of malaria. A feasibility study of using the sterile insect technique (SIT) for *An. arabiensis*, as part of an area-wide integrated pest management project [2] for population suppression, is currently being conducted in Northern Sudan and in Réunion [3]. SIT is based on the release of large numbers of sexually sterile males, which would mate with wild females and transfer their sterile spermatozoids for the fertilization of the eggs. If the sterile males successfully compete for mates, the wild population size will progressively diminish. A lower probability of contact between the vector and humans is, therefore, expected, and as a result pathogen

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transmission and disease incidence will decrease. Sexual sterilization can be accomplished by male mosquito exposure to ionizing radiation, resulting in random dominant lethal mutations in the germinal cells that cause the death of the developing embryos after fertilization.

Part of the research work for the implementation of SIT requires mating of radio-sterilized males with virgin females so as to identify the effect of irradiation on those males. Until 2 years earlier, the sex-separation method routinely used in this laboratory for *An. arabiensis* Dongola, consisted of separating females from males as adults, less than 18 h after emergence [4,5]. This procedure consistently ensured virginity of the females. However, recent experiments suggested the occurrence of early matings between males and females separated from one another between 12 to 16 h after their emergence.

Like many Diptera species, male mosquitoes are not immediately sexually mature after emergence: maturation includes a permanent 180° rotation of their genitalia [6]. The male mosquito genitalia consist of the 8^{th} to 10^{th} abdominal segments. Claspers tipped with claws enable the male to grasp the female for copulation and are located on segment 10^{th} [7]. When males emerge these claws are rotated dorsally, which prevents them from copulating until the rotation occurs. Rotation is driven by two sets of opposed and crossed muscles [8] and can happen equally frequently either clockwise or counter-clockwise [8,9]. The time to complete this event is species-specific. Aedine species show a great variation: *Aedes iriomotensis*, *Aedes albopictus*, *Aedes atriisimilis* were shown to rotate 180° respectively around 12, 22 and 40 hours after emergence [10]; 18 to 24 h were required for *Aedes aegypti* [8]; 30 h for *Aedes taeniorhynchus* [11] and nearly 4 days for *Aedes provocans* [12]. *Culex tritaeniorhynchus* and *Culex quinquefasciatus* completed the rotation in 19 h [13,14]. Finally, the species *Culiseta inornata* needed only 6 to 12 h [7]. The rate of genitalia rotation for anopheline mosquitoes is not yet reported. Only observations of the insemination status demonstrated that at least 24 h post-emergence are required for mating in *An. arabiensis* and *Anopheles gambiae s.s.* [15] as well as for *Anopheles stephensi* [16]. Besides the requirement for male maturation, females of most mosquito species are unreceptive during the first 30-60 h after emergence; although they may allow copulation, they will not become inseminated [17]. Mahmood and Reisen [16] showed that females of *An. stephensi* reached sexual maturity by the 2^{nd} night of life, though a very low proportion of females were inseminated by older males less than 12 h after their emergence.

In order to investigate the issue of sexual maturity in this laboratory colony, experiments were performed to determine the rate of sexual maturation over time in the current laboratory colony of *An. arabiensis* Dongola. This was compared with wild specimens collected directly in the field. The forces for laboratory selection due to the stock-keeping method and the possible consequences of this outcome on insects rearing and research are discussed.

Methods

Mosquito stock and rearing

Laboratory colony

All experiments used the Dongola strain of *An. arabiensis* [18] colonized in 2004 from specimens collected near the village of Dongola in Northern State, Sudan. The Dongola colony has been reared in the Insect Pest Control Laboratories, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, since that time. Approximately 18 generations of *An. arabiensis* can be reared per year as the development of one generation takes around 20 days from egg hatching to reproductive adult stage. Therefore, this strain has been maintained for approximately 125 generations in discrete generations. The Dongola strain was reared in a climate-controlled room maintained at a temperature of 27 ± 1°C and 60 ± 10% relative humidity. The light regime was LD 12:12 h photoperiod, including dusk (1 h) and dawn (1 h). The same environmental conditions were used for all laboratory experiments. Larvae were reared in plastic trays (40 × 29 × 8 cm) at a density of approximately 500 first instar larvae (L1) per tray that contained ± 1.5 litre of deionised water and fed a diet of finely ground (224 μ-sieved) Koi Floating Blend® (Aquaricare®, New York, USA). Pupae were collected and placed in small plastic cups inside a fresh adult cage for emergence. Adults were kept in standard 30 × 30 × 30 cm insect cages (Megaview Science Education Services Co, Ltd, Taiwan) and continuously supplied with 10% [w/v] sucrose solution with 0.2% methylparaben [19] Females were blood-fed weekly on defibrinated bovine blood. Gravid females were allowed to oviposit in plastic cups with black lining containing a wet sponge over which a filter paper was placed.

Wild Dongola strain

Pupae were collected along the Nile River bank in 2010 (Dongola, Northern State, Sudan) and allowed to emerge in laboratory cages. Room temperature was ca 40°C, because no air-cooling system was available.

Experimental set up

Effect of male-female separation methods on virginity

In the first group, females of *An. arabiensis* Dongola (generation F_{118}) were separated from males at the adult stage, 12 to 16 h post-emergence, similarly to the method of Helinski & Knols [4]. A second group consisted of females that were isolated from males at the

pupal stage to ensure their virginity. Sex-separation at the pupal stage was based on observation of the genitalia under a binocular microscope (MR4, 2009). Those two groups of females were mated with males (1:1) subjected to various sterilizing doses of radiation. Male pupae were collected from trays within a 4 h interval. Males were exposed to gamma rays emitted by a Cobalt-60 source (Gammacell220, MDS Nordion, Ottawa, Canada) with a dose rate of ca 9 Gy/min. Doses of 0, 40, 70 or 120 Gray (Gy) were applied 18-22 h after pupation. Pupae were placed on a wet filter paper at the centre of the chamber. A dosimetry system was used to measure the dose received by the lot based on Gafchromic® dosimeter film HD-810 (International Specialty Products, NJ, USA); three dosimeters were included with each lot of insects and read after irradiation with a Radiachromic® reader (Far West Technology, Inc., California, USA). Each treatment consisted of two replicates of 100 males and 100 females for the first group, and 50 males and 50 females for the second group. Females were blood-fed on a membrane with human blood collected in an anticoagulant tube. They were then placed in tubes for individual oviposition. Hatch rates of each family (*i.e.* progeny of one female) were recorded.

Observation of male genitalia rotation The degree of genitalia rotation was recorded for males of different ages using the following divisional markers to categorize five stages of the rotation of the male genitalia [11,14], stage 0: no rotation (pleura of segments 7 and 8 continuous); stage 1: ≤45° rotation; stage 2: >45° - ≤90° rotation (basistyles perpendicular to the pleuron of segment 7); stage 3: >90° - ≤135° rotation and stage 4: >135° to complete rotation of 180° (pleura of segments 7 and 8 once more continuous, Figure 1). In all individuals that had completed rotation, the pleural line of the 7th and 8th segments of the genitalia were perfectly aligned only on one side but slightly shifted on the opposite side depending on whether rotation occurred clockwise or counter-clockwise. Thus, the genitalia had to be observed from both sides to allow determination of the final rotation stage: when claspers were ventrally rotated and the pleural lines were aligned on one side, the male was recorded as fully rotated. Such jagged lines have previously been reported and are due to the elasticity of the inter-segmental membranes [11]. According to the side that presented a perfect alignment, the rotational direction (clockwise or counter-clockwise) could be determined.

Emergence of all males spanned ca 2 h, all the males from one cage were removed at different time and frozen for later examination of the genitalia rotation stage. For the laboratory strain, these males were 0, 2.5, 3.5, 4.5, 5.5, 11, 12.5, 14, 15.5, 17 and 19.5 h, and 1 week old. Males aged from 2.5-5.5 h were obtained from a batch of pupae whose emergence was delayed to the morning by cooling the pupae at 15°C overnight, as a cooling treatment reduces the metabolic rate of pupae [5,20]. Concerning the wild males collected as pupae in the field, the degree of genitalia rotation was recorded only for 5 different ages due to a low number of individuals available: 0, 5.5, 13, 17 and 23 h.

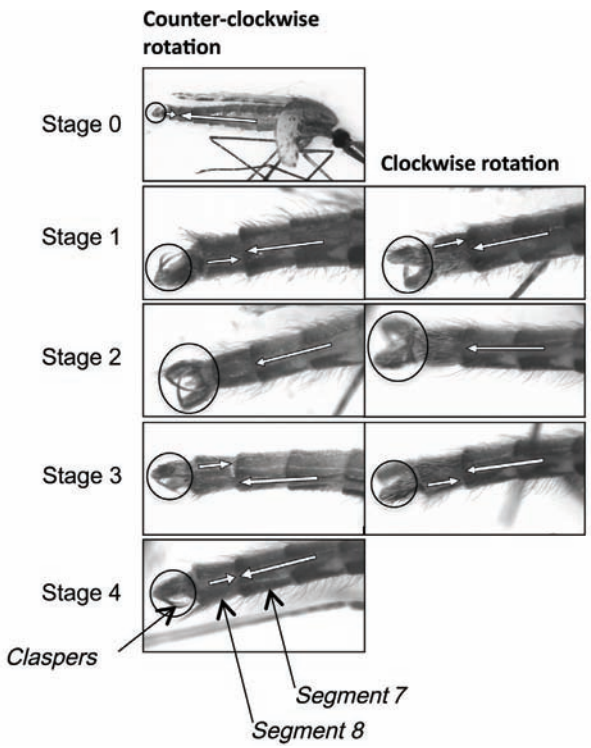

Figure 1 Stages of genitalia rotation for *An. arabiensis*. Clockwise or counter-clockwise rotation, when the specimen is observed on its right side. Arrows indicate the position of pleuron 7 and 8.

Female insemination by laboratory reared males Newly formed pupae of the laboratory reared Dongola strain (F_{126}) were collected within a 4 h interval and sexed manually under a microscope. 65 male pupae and 55 female pupae were placed in each of 6 cages. Females were removed from the cages after a cohabitation period with the males of 11, 12.5, 14, 15.5, 17 and 19.5 h. In each cage a sample of five females was dissected and the spermatheca was examined for insemination, the remaining females were blood-fed and allowed to oviposit *en masse* in an egg cup. A batch of 40 female pupae was kept in a cage without any males as a control. They were blood-fed after seven days and an egg cup was introduced for eventual oviposition.

Statistical analysis Normality and homoscedasticity of egg hatch data were examined using Shapiro and Bartlett tests. ANOVA was performed to test differences within one group between the mean egg hatch rates

for each radiation treatment. T-tests (with Welch correction) were used to compare the mean egg hatch rates between the two groups of females and to compare the mean rotation stages between wild and laboratory reared males for a given time. Chi-squared tests (with Yates correction) were used to compare the proportions of wild and laboratory reared males for a given rotation stage and time. For all tests, the alpha level was $P < 0.05$. Statistical analyses were performed using Microsoft Excel (Microsoft, USA) and the open source package R [21].

Results

Effects of male-female separation methods on virginity

In the group where females were separated from males <16 h post-emergence at the adult stage, the mean egg hatch rate in the control was 91.0 ± 1.2% (mean ± sem); no reduction of fertility was visible among the irradiated treatments and full sterility was never reached (Figure 2). The mean fertility was significantly different between 70 and 120 Gy ($F_4 = 63.318$, $P < 0.001$) but was still as high as 41.7 ± 3.3% and 21.2 ± 3.8% (mean ± sem) respectively. The distribution of individual egg hatch rates for the various radiation doses was similar and ranged over ca 80% indicating a high number of intermediate hatch rates (*i.e.* all values ranging between the expected sterility value and the control value). However this distribution was different in the group where females were separated from males at the pupal stage, and the intermediate hatch rates described earlier were no longer observed. In this group, results were in accord with the sterility curve obtained by Helinski *et al* [22]. Control mean fertility was 84.1 ± 3.2% (mean ± sem), and a proportional reduction was observed up to a dose of 120 Gy, where fertility was close to zero. Statistical comparisons between the two groups of females showed high significant differences between treatments where males were irradiated at 40 ($t_{14.5} = 4.16$, $P < 0.001$), 70 ($t_{48.7} = 6.21$, $P < 0.001$) or 120 Gy ($t_{37.8} = 4.59$, $P = 4.768e\text{-}05$).

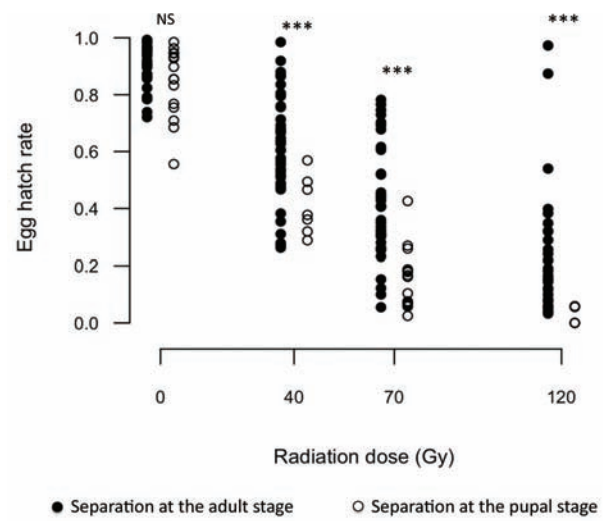

Figure 2 Distribution of the egg hatch rates after mating with irradiated males. Individual egg hatch rates for females separated as adults (black dots) or as pupae (circles) and mated with males irradiated at different doses. NS indicates no significant difference and *** indicates a significant difference ($P < 0.001$) between the two groups of females.

Observation of male genitalia rotation and female insemination

All the 0-3.5 h old laboratory-reared males had non-rotated genitalia; rotation was evident at 4.5 h after emergence at which time only very few individuals had reached stage 1 (Figure 3). 11 h old males were distributed within the stages 2-4 in the following proportions: 42%, 17% and 42%. After 14 h post-emergence, all males had their genitalia rotated at least 135° (stage 3), and more than 90% of them were already fully rotated. The examination of one-week-old males revealed that all males fully completed the rotation.

Genitalia rotation of the wild-caught specimens started a few hours after emergence as 40% of them were already in the second stage of rotation when 5.5 h old (Table 1). After 12.5 h more than 60% of the males had reached stage 3. The length of stage 3 lasted for ca 10 h as complete rotation was recorded only 23.5 h post-emergence, and this for only 40% of them. Comparison of the rotation degree between laboratory and wild males was made for the shared sampling times between the two male populations, *i.e.* 5.5 h, 12.5 h and 17 h post-emergence. A statistically significant difference was found when comparing the mean rotation stage at 12.5 h ($t_{5.3} = 3.48$, $P < 0.01$) and 17 h ($t_{7.3} = 7.34$, $P < 0.001$) (Table 1).

In this experiment, the frequencies of clockwise and counter-clockwise rotation were not significantly

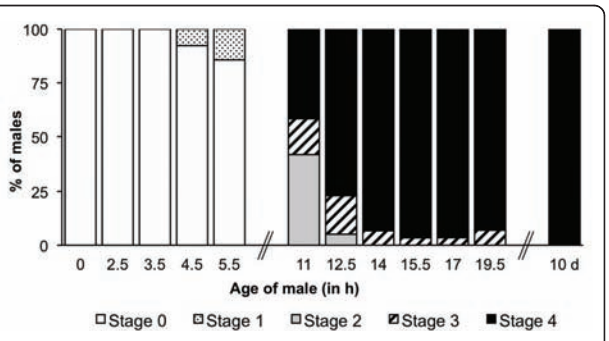

Figure 3 Genitalia rotation over time. Percentage of laboratory-reared males with the terminalia rotated in the different stages as a function of age.

Table 1 Stages of terminalia rotation for laboratory and wild males at different time after emergence.

Age (hrs)	Stage 0			Stage 1			Stage 2			Stage 3			Stage 4		
	Lab	Wild		Lab	Wild		Lab	Wild		Lab	Wild		Lab	Wild	
5.5	86	60	NS	14	40	NS									
12.5				0	12.5	NS	0	25	*	6.7	62.5	***	93.3	0	***
17							0	25	***	4	75	***	96	0	***

Percentage of laboratory colony (Lab) and males collected on the field (Wild) with the terminalia rotated in the different stages. NS indicates a non significant difference between laboratory and wild males; * and *** indicate a significant difference ($P < 0.05$ and $P < 0.001$ respectively). Sample sizes were 14, 39 and 57 for the laboratory males and 5, 8 and 8 for the wild males at 5.5, 12.5 and 17 h post-emergence respectively.

different for each observation point, with mean clockwise and counter-clockwise rotation frequencies of 44.6% and 55.4% respectively. As expected, the control group of virgin females held in a cage without males did not lay any eggs. Female insemination was checked for the groups in which males aged 11 to 18.5 h old. At least one female was found inseminated in all groups; the proportion of spermathecae containing sperm varied from 20% to 40%. Oviposition occurred in all the cages with a low number of eggs laid.

Discussion

The establishment of disparities between reared and wild insects during colonization can result from selection, genetic drift and inbreeding [23]. Possible methods to avoid and mitigate these in mosquitoes have been discussed but few have been empirically demonstrated to be effective [24]. Several studies reported modifications in the sexual behaviour of long-term laboratory reared insects (house flies [25], screw-worm flies [26,27], bud worms [27] and a shortened sexual maturity period (Mediterranean fruit flies [28]).

Between 2004 and 2009, more than one hundred generations of the Dongola strain have been reared under laboratory conditions, allowing the possibility that selective pressures would lead to purifying selection of particular traits. The present work was prompted by unexpected data obtained during irradiation experiments where the expected sterility levels were not reached. The mating of radio-sterilized males with females separated from males less than 16 h post-emergence never allowed the achievement of full sterility with the five-year-old laboratory colony. A high proportion of females were inseminated at this age as mosquitoes from the laboratory colony were shown to be already sexually competent a few hours after emergence. As early as 11 h post-emergence, males were able to copulate and females were receptive. In spite of a much higher temperature, wild males collected in the field as pupae required twice as much time as laboratory males to complete the rotation of their genitalia. This observation and comparison with previous results suggest a rearing induced selection of the males to sexually mature more rapidly.

Sexual maturity in male mosquitoes is reached after a 180° rotation of the genitalia and the maturation of sexual organs and antennal fibrillae [29]. Approximately 20 h would be required for wild males collected in the field to become sexually mature which is in agreement with the data reported by Mahmood & Reisen [16]. A deceleration in genitalia rotation beyond 90° has been reported for an aedine species [11]; a similar pattern seems to exist in the Dongola wild males, but was not evident in the laboratory males. Provost *et al* [11] reported an increase of the rotation rate with temperature. As the wild males observations had to be conducted at ca 40°C, one could suppose that the completion of this process would be even slower in typical laboratory conditions e.g. 28°C.

Very few females inseminated by 11 to 19.5 h old males laid eggs though spermatheca dissection showed that 20 to 40% of them were inseminated. The low number of ovipositions would suggest that the quantity of sperm actually transferred by males may not always be sufficient to permit oviposition. Indeed, it has been shown with *An. gambiae s.s.* that the oviposition behaviour is triggered by a spermatheca filled with sperm [30], although more recent work demonstrates that the mating plug may alone be sufficient [31]. However, during the mating experiments with sterile males irradiated at 120 Gy, in which females were separated at the adult stage, 6% of them laid fertile progeny (i.e. > 60% fertile eggs) resulting from the early insemination by same age un-irradiated males. This result indicates that those females received enough sperm before the sex-separation process to fertilize and lay viable eggs. Besides, 53% of those females laid egg-batches that were semi-sterile (i.e. >10 and <60% fertile eggs) indicating that fertile then sterile males inseminated them successively and that they used both sperm to fertilize the eggs. The high rate of semi-sterility corroborated the fact that a relatively high proportion of the young emerged males were able to transfer their sperm in the first hours after emergence. It seems likely that most of them inseminated the females only partially thus allowing a subsequent double mating by a sterile male. Mahmood and Reisen [16] suggested that the high rate of multiple matings observed in caged *An. stephensi* and *Anopheles culicifacies* would be induced by an

incomplete transfer of sperm, which would mostly occur when the male's reproductive system is partially depleted. They reported the depletion of the accessory glands in newly emerged males or following a mating. However once sexual maturity was reached, the accessory glands were fully filled with secretory cells and male accessory gland fluid. The observation of semi-sterility however suggests that the sperm transferred by young emerged males was already mature, as it has been used to fertilize at least some of the eggs. A possible hypothesis is that the quantity of sperm in the sperm reservoir or the quantity transferred during the mating might be low for the < 18.5 h old males. An alternative explanation could be that newly emerged males were not able to transfer a mating plug after the sperm transfer, and females would eject part of the sperm. Rogers *et al* [31] demonstrated that in anophelines the seminal secretions (mating plug) produced by the male accessory gland and transferred during insemination, promote sperm storage. They suggested that when males failed to transfer the mating plug, females would actively eject the sperm or part of it. It would be of interest to investigate whether the failure of most of the females to oviposit is due to an insufficient quantity of sperm transferred or to the non-transfer of a mating plug.

There are at least five reasons to support the hypothesis of rearing selection pressures for accelerated sexual maturation in males: (*i*) variation in rotation time exists within males of the same age for a given temperature [9]; (*ii*) the observation of males attempting to mate before their genitalia had sufficiently rotated to ensure a successful copulation has been reported for *Ae. aegypti* [9]; (*iii*) the small cages used in laboratory rearing might not allow males to execute a mating swarm and hence favour individual mating attempts [16]; (*iv*) the management of the stock in the laboratory consists of a situation in which there is no overlapping of generations and all males are of a similar age range of approximately three days; (*v*) genetic selection on insects due to rearing pressures have already been reported [32,33]. Such behaviours could favour males that would complete early sexual maturation and this phenomenon would be purified over generations.

The precocious sexual maturity we observed in males may have occurred as well in females. Indeed, they both showed a reduced sexual maturity period as compared with typical values from the literature [15,16]. However the data presented here do not allow us to state whether males and females evolved simultaneously or if one sex was already pre-adapted for early mating. Lima *et al* [34] mentioned the evolution of male *Anopheles albitarsis* mating ability after ca. 10 years of rearing under laboratory conditions, with an improvement of the mating capacity and insemination rates. They suggested that this evolution did not involve females, as the difficulties to mate in a confined space concerned males only. However, Gwadz and Craig [35] reported that females *Ae. aegypti* from four two-year-old laboratory strains showed a significantly shorter refractory period than two young colonized strains, but they did not mention male receptivity. In females *Aedes atropalpus*, Gwadz [36] showed that early receptivity was inherited as it is linked to a juvenile hormone. It is not known however if sexual maturity in males is also moderated by hormones.

Difficulties in establishing colonies of anopheline mosquitoes are often reported and attributed to the incapacity of male swarm formation in a confined space [24]. The adaptation to rearing conditions would necessitate individual mating capacities of the males. Because *Anopheles* mosquitoes' sex ratio is 1:1, it is likely that not all males have an opportunity to mate. Variation among males that may be under selection and that could affect reproductive success include size [37] and the quality of the male accessory gland fluids and sperm [38-40] and genetic factors whose phenotypic effects are unknown [41]. This study showed that, in laboratory settings in which similarly aged males must compete for mates, maturation rate is apparently under selection. As mentioned by Howell & Knols [29], laboratory rearing can lead to a bottleneck because of selective pressures. Negative side effects of such unintentional behavioural selections might present themselves when the reared insects are released in the field and strong differences to their wild counterparts might compromise their survival or mating capacities [42]. Nevertheless the early capacity of mating found in males *An. arabiensis* Dongola might not be a negative attribute in an SIT project, as the released males would be reproductively capable only a few hours after emergence, assuming that they are also able to join or initiate a mating swarm. As part of an SIT program, it is of primary importance that the released sterile males are able to copulate and successfully inseminate wild females as early as possible, so that predation pressures and survival capacities would not have a negative impact on their performance. Indeed, when releasing sterile insects, it is often recommended to wait until adults are sexually mature before their release so that they would be immediately effective in the field. But if a selection of males able to mate shortly after emergence can be induced by the rearing procedure, the possibility of release at the pupal stage becomes advantageous, as the handling can be easier than for adult releases.

Conclusion

This is the first study describing the temporal process of genitalia rotation for an anopheline species and

highlighting the apparent shortening of the sexual maturation period for males of a mosquito species due to rearing conditions. Females were possibly subjected to the same kind of selection but the present study design could not detect it. This result has consequences on laboratory experiments involving virgin females where special care must be taken for the sex-separation process. In addition, rotation of the genitalia is used to determine the age of males collected in the field, using as a reference a correlation between rotation degree, temperature and known age of laboratory reared males [11]. The outcome reported in this study implies that these biological indicators must be used carefully, preferably with a newly colonized strain in order to avoid any discrepancies. Finally, the potential of early mating by freshly emerged males and females of a mass reared strain should be evaluated regularly as it may reflect the evolution and adaptation of the strain in response to laboratory conditions.

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Authors' contributions
CFO, JG and MQB developed the design of the study. CFO carried out the experimental work, performed the statistical analysis and drafted the manuscript. JG, MQB and GL supervised manuscript preparation and contributed significantly to the final draft of the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Article 3. Comparisons of life history characteristics of a genetic sexing strain with laboratory strains of *Anopheles arabiensis* (Culicidae: Diptera) from Northern Sudan

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Comparisons of Life-History Characteristics of a Genetic Sexing Strain With Laboratory Strains of *Anopheles arabiensis* (Diptera: Culicidae) From Northern Sudan

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ABSTRACT A genetic sex separation strain (GSS) has been created for *Anopheles arabiensis* (Patton) (Diptera: Culicidae), one of the major African malaria vectors, for use in controlling wild populations of this species via the sterile insect technique (SIT). This GSS strain, "ANO IPCL1," allows sex separation by a translocation linking a dieldrin resistance allele and the Y chromosome. Differences between ANO IPCL1 relative to wild strains might reflect its field performance and therefore are of concern. Of more immediate interest is how differences might affect production during mass rearing. Life-history parameters were measured for the ANO IPCL1 strain and the two wild strains from which it originated. Although developmental rate differences were found among them, none were large. However, a major observed variation was the very low intrinsic fertility of ANO IPCL1 because of the translocation itself. This resulted in a much lower rate of increase: ANO IPCL1 was able to double its population size, in 7.8 ± 0.4 d, whereas Dongola and Sennar strains could do so in 4.9 ± 0.5 and 5.6 ± 0.4 d. The presence of the Y-autosome translocation mainly affected the natural fertility of the males, and this will require amplification steps during mass rearing.

RÉSUMÉ Dans le cadre d'un projet de contrôle des populations de l'un des principaux vecteurs du paludisme en Afrique, une souche d'*Anopheles arabiensis* (Patton) (Diptera: Culicidae), "ANO IPCL1," permettant une séparation des sexes de façon génétique (GSS) a été créée pour le développement de la technique de l'insecte stérile. Cette séparation est possible grâce à une translocation liant au chromosome Y un allèle de résistance à la dieldrine. L'existence de différences entre ANO IPCL1 et les souches sauvages pourrait refléter les performances des mâles sur le terrain et il est donc important de les évaluer. Il est fondamental de comprendre comment ces différences peuvent affecter une production de masse. Les traits d'histoire de vie ont été mesurés pour ANO IPCL1 et les souches sauvages parentales. Hormis des différences mineures concernant les paramètres de développement, le processus de translocation a induit une très faible fertilité naturelle chez ANO IPCL1 entrainant un plus faible taux intrinsèque d'accroissement de la population. Une population d'ANO IPCL1 était capable de sa taille en 7,8 ± 0,4 jours, alors que Dongola et Sennar pouvaient le faire en 4,9 ± 0,5 et 5,6 ± 0,4 jours. La translocation ayant principalement affecté la fertilité des mâles, elle aura un impact important sur l'élevage de masse.

KEY WORDS genetic sexing strain, sterile insect technique, life history, fitness

The high impacts of malaria on human health and on countries' economies continue to motivate control campaigns targeting either the parasite or the vector. The female mosquito *Anopheles arabiensis* (Patton) (Diptera: Culicidae) is one of the major African vectors of malaria (Coetzee et al. 2000). A feasibility study of the use of the sterile insect technique (SIT) for *An. arabiensis* as part of an areawide integrated pest management project (Klassen and Curtis 2005) is currently being conducted in northern Sudan and La Reunion (Robinson et al. 2009, Boyer et al. 2011). SIT effectiveness in reducing diseases is based on the prediction of a lower probability of contact between the vectors and humans resulting from the progressive reduction of the vector population. The means to accomplish this purpose is to release large numbers of mass-reared and sterilized males in situ where they would mate with wild virgin females. For insects, such as mosquitoes, in which only females bite and thus are

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able to transmit diseases, it is mandatory that only males be released. Beside the public health concerns, the release of sterile females together with males has been shown to reduce the dispersal and the mating efficiency of sterile male fruit flies (Hendrichs et al. 1995, Rendon et al. 2000). Indeed, when both females and males are released, males can mate with the released females and therefore do not necessarily need to fly farther to mate with wild females. It is therefore useful to be able to efficiently remove females before release.

In contrast to aedines and culicines, anopheline pupal size differences between sexes are not large enough to allow accurate mechanical separation (Dame et al. 1974). So far, besides separating adults (e.g., providing toxicant in the bloodmeal; Lowe et al. 1981), the only alternative is the use of a genetic sexing strain (GSS) that confers a certain resistance only to males. For that purpose, the GSS named "ANO IPCL1," based on dieldrin resistance, has been selected for *An. arabiensis* at the Food and Agriculture Organization/International Atomic Energy Agency Insect Pest Control Laboratory (IPCL, Seibersdorf, Austria). Natural resistance to dieldrin exists in the strain of *An. arabiensis* from Sennar, Sudan (El Gaddal et al. 1985, Du et al. 2005). A translocation linking the resistance allele with the Y chromosome was induced via gamma irradiation and identified by backcrossing candidate males with virgin females from the Dongola strain, which has no resistance to any insecticide.

The success of SIT projects is based on the survival and mating capacity of the released males, and it is important to understand how these are altered by the translocation in the GSS compared with the wild parents. Ultimately, this can only be determined by large cage trials or open field releases. In the context of mass rearing, it is fundamental to understand how differences can be accommodated to achieve sufficient levels of production. The assumption is that the wild strains from which the GSS is produced is a reasonable comparator for a GSS. Here, we compare the three closely related Sudanese strains of *An. arabiensis:* Dongola, originating from Sudan; Sennar originated in the Gezira irrigation area, Sudan; and ANO IPCL1, derived from both. The comparison of life-history traits between these strains is discussed in the context of mass production.

Materials and Methods

Mosquito Stocks and Rearing Methods. The experiments were conducted using three strains of *An. arabiensis.* The Dongola strain (available from the Malaria Research and Reference Reagent Resource Center (MR4) as MRA-856) was colonized in 2004 from specimens collected near the village of Dongola, Sudan, and has been maintained for 125 generations at the IPCL. It is pure breeding for a dieldrin susceptibility trait. Sennar (MRA-334) was first colonized in 1969 by the Malaria Training Center in Sudan. Sennar, which carries the resistance to dieldrin, was used to establish ANO IPCL1. The ANO IPCL1 is maintained by backcrossing resistant males to Dongola females. All three strains were reared in a climate-controlled room maintained at 27 ± 1°C, 60 ± 10% RH, and a photoperiod of 12:12 (L:D) h, including dusk (1 h) and dawn (1 h). Larvae were reared in plastic trays (40 by 29 by 8 cm) at a density of ≈500 first-instar larvae (L1) per tray that contained ≈1.5 liter of deionized water and were fed larval diet consisting of finely ground (224-μm sieved) Koi Floating Blend (Aquaricare, Union Hill, NY). Pupae were collected and placed in small plastic cups inside a fresh adult cage for emergence. Adults were kept in standard 30 by 30 by 30-cm insect cages (Megaview Science Education Services Co., Ltd., Taichung, Taiwan) and continuously supplied with sugar water (10% wt:vol sucrose solution with 0.2% methylparaben, Benedict et al. 2009). Females were blood fed weekly on defibrinated bovine blood and provided a standard oviposition cup consisting of plastic cups with black lining containing a wet sponge over which a filter paper was placed.

Immature Development of Three Strains. To determine the egg hatch rate, 400–500 eggs (24 h old) of each strain were hatched in deionized water at 27 ± 1°C. This was replicated three times for each strain, with eggs originating from three different cages. The hatch rate was determined by microscopic examination of eggs after 48 h.

Larval development and survival were determined by transferring 100 larvae (<4 h old) of each strain to 20 by 20 by 8-cm plastic trays filled with 500 ml of deionized water. This was replicated three times for each strain, with larvae originating from egg batches collected from three different cages. Larval diet was provided on a daily rate per tray (days 1 and 2, 25 mg; days 3 and 4, 50 mg; day 5, 100 mg; and days 6 and 7, 150 mg).

Trays were rearranged daily within the space being used for the experiment to randomize the effects of local conditions within the room. Pupae were removed on the day they formed and transferred into individual tubes for emergence; eclosion time, sex, and survival were recorded. Digital photos of the left wings (or right where left wings were damaged) were taken and measured using analysis B software (Olympus Soft Imaging Solutions, Münster, Germany). Wings were measured from the distal edge of the alula to the end of the radius vein (excluding fringe scales).

Adult Fecundity and Longevity. One hundred newly emerged males and females (ratio 1:1) were placed in 30 by 30 by 30-cm plastic cages (Megaview Science Education Services Co., Ltd.) with constant access to sugar water. Starting at day 8, a mechanically defibrinated bovine bloodmeal was provided weekly through a Parafilm membrane (American Can, Neenah WI). A standard oviposition cup was added in the cage 48 h after blood feeding for en masse oviposition. The egg paper was removed the following day, and eggs were counted under a microscope. Dead adults were removed daily and their sex determined. Three replicates were performed for each strain.

Statistical Analyses. The analyses were conducted using MINITAB statistical software (Minitab, State

Table 1. Egg hatch rate, L1 survival to pupa and adult, and sex ratio (all means with CI in parentheses) for the three *An. arabiensis* strains studied

Strain	Egg hatch rate	Survival rate from L1 to pupa	Survival rate from L1 to adult	Sex ratio[a]
Dongola	0.95a (0.91–0.98)	0.81a (0.76–0.85)	0.78a (0.72–0.82)	0.50a (0.41–0.58)
Sennar	0.82b (0.78–0.85)	0.92b (0.88–0.94)	0.85c (0.80–0.88)	0.53a (0.51–0.55)
ANO IPCL1	0.27c (0.26–0.27)	0.92b (0.88–0.95)	0.91b (0.88–0.94)	0.50a (0.37–0.63)

Within columns, values followed by different lowercase letters are statistically different; ANOVA was performed for egg hatch rate and sex ratio analysis, and logistic regression was performed for survivorship analysis ($P < 0.05$).

[a] Sex ratio was calculated as the proportion of males out of the total number of adults.

College, PA), Excel (Microsoft, Redmond, WA), and R (R Development Core Team 2011). Egg hatch rates and sex ratio data were arcsine transformed and compared between strains by using two-tailed one-way analysis of variance (ANOVA) and Tukey's post hoc tests ($P < 0.05$). Survivorship of *An. arabiensis* larvae (from first-instar larva [L1] to pupa) was compared between strains using a logistic regression ($P < 0.05$). Mean time to pupation was defined as the average duration (in days) from the L1 until pupation. Fecundity was calculated as the number of eggs laid per female per day. One-way ANOVA and Tukey's post hoc tests were used to compare the developmental duration between strains for each sex, the wing measurements between strains and sexes, and female fecundity between strains. Kaplan–Meier survival analyses ($P < 0.05$) were conducted to determine adult survivorship differences between the strains.

The net reproductive rate, R_0, was calculated for each strain based on the daily survivorship and fecundity. *R0* was defined as the average number of offspring a female produced in her lifetime and was calculated as $R_0 = \Sigma(l_x m_x)$, where l_x is the age-specific survivorship, and m_x is the age-specific fecundity. Per-capita intrinsic growth rate, r, defined as the number of progeny born to each female mosquito per unit of time, was calculated using the Euler–Lotka equation $1 = \Sigma_{x=0}\, e^{-rx}\, l_x m_x$ (Hedrick 1984, David et al. 1995), where x was the mosquito age. The generation time in days, T_c, is defined as the average length of time between the hatching of an individual and the hatching of its offspring and was calculated as $T_c = \Sigma\, x(l_x m_x)/\Sigma(l_x m_x)$. The doubling time for the population size was calculated as $T_d = \ln 2/r$. One-way ANOVA and Tukey's post hoc tests ($P < 0.05$) were used to compare differences in the net reproductive rate, generation time, intrinsic growth rate, and time for population size doubling between the strains. Results are expressed as mean ± SEM.

Results

Development of *An. arabiensis* Immatures. Under similar conditions, the mean fertility of the three genotypes differed significantly ($F = 234.33$, df = 2, $P < 0.001$; Table 1). The mean percentage of egg hatch of ANO IPCL1 was low (26.8 ± 0.2%) compared with those of Dongola (94.5 ± 1.8%) and Sennar (81.8 ± 1.7%).

The mean survivorship from L1 to pupa was 92% for Sennar and ANO IPCL1 (Table 1). In contrast, Dongola survival rate to the pupal stage was significantly lower than that of Sennar ($Z = -3.95$, df = 299, $P < 0.001$) and ANO IPCL1 ($Z = -4.10$, df = 299, $P < 0.001$). The survival rate to adult eclosion followed the same pattern. For the three strains the sex ratio was similar ($F = 0.120$, df = 2, $P = 0.89$) and averaged 51.0 ± 2.4%.

Mean time to pupation was ≈6 d for the three strains. However ANO IPCL1 showed a significantly faster development compared with Dongola and Sennar for both females and males ($F = 29.27$, df = 2, $P < 0.001$ and $F = 63.19$, df = 2, $P < 0.001$, respectively; Table 2). The same pattern was found for the duration time until emergence, because eclosion from pupae collected in the morning occurred in the evening following the day of pupation.

Wing lengths were significantly shorter for both males ($F = 15.95$, df = 2, $P < 0.001$) and females ($F = 4.57$, df = 2, $P < 0.05$) of the Sennar strain compared with both Dongola and ANO IPCL1 (Fig. 1); however, there was no difference between Dongola and ANO IPCL1. The difference between sexes was significant for Dongola ($t = 4.82$, df = 19.9, $P < 0.001$), ANO IPCL1 ($t = 8.17$, df = 50.9, $P < 0.001$), and Sennar ($t = 5.32$, df = 26.7, $P < 0.001$).

Adult Fecundity and Longevity. Four blood-feedings and ovipositions occurred, each separated by 1 wk; they are referred to as the four gonotrophic cycles (GCs) of the females. Because mortality was checked daily, the mean fecundity per female could be calculated as the number of eggs laid en masse divided by the number of females alive in the cage. For all strains, the mean fecundity per female was low on the first oviposition opportunity and was the highest on the second (Fig. 2). A strong decrease of fertility was

Table 2. Developmental duration (mean ± SEM) from L1 to pupa formation and to adult emergence

Strain	Mean time to pupation (d)		Mean time to eclosion (d)	
	Female	Male	Female	Male
Dongola	6.4 ± 0.05a	6.2 ± 0.04a	7.8 ± 0.04a	7.5 ± 0.05a
Sennar	6.6 ± 0.05c	6.3 ± 0.04a	7.8 ± 0.05a	7.4 ± 0.05a
ANO IPCL1	6.1 ± 0.05b	5.8 ± 0.04b	7.4 ± 0.05b	6.9 ± 0.04b

Within columns, values followed by different lowercase letters are statistically different ($P < 0.05$; ANOVA).

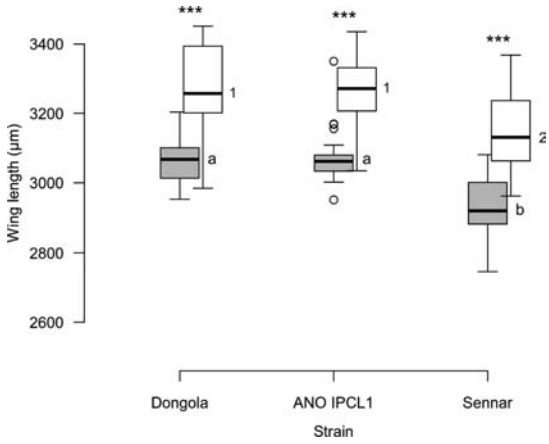

Fig. 1. Box plots of wing length measurements for males (gray box) and females (white box). The line represents the median of the sample, the box itself shows the upper and lower quartiles, the whiskers show the range (i.e., the largest and smallest values), and circles indicate outliers. Boxes with the same letter or number were not statistically different from each other, $P < 0.05$ (ANOVA between strains). Asterisks (***) indicate a significant difference between sexes within the same strain ($P < 0.001$).

observed on the third and fourth GCs. There was no significant difference between strains except during the fourth GC where Sennar showed a higher fecundity than the two other strains ($F = 11.9$, df = 2, $P < 0.01$).

Small but significant differences were observed when comparing the Kaplan–Meier estimates of the adult survival curves between the three strains (Fig. 3). Survival of Dongola males was slightly higher compared with Sennar males ($\chi^2 = 4.2$, df = 1, $P < 0.05$) and ANO IPCL1 males ($\chi^2 = 7.8$, df = 1, $P < 0.01$). When the Peto & Peto modification of the Gehan–Wilcoxon test was used to give more weight to the early deaths, it seemed that in both cases the difference was significant only for the early days of mortality. Concerning the longevity of females, no difference was observed between the three strains ($\chi^2 = 2.6$, df = 2, $P = 0.272$).

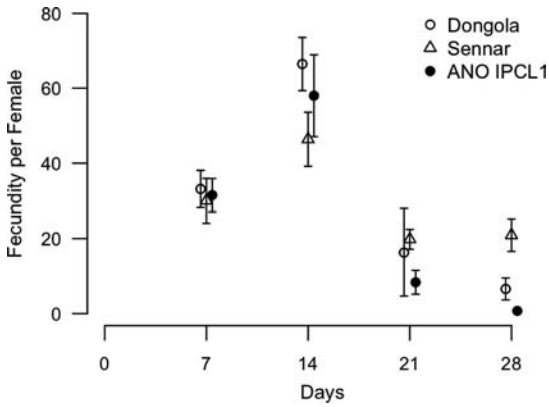

Fig. 2. Fecundity (mean ± SEM) per female for the four gonotrophic cycles, for the three strains of *An. arabiensis*.

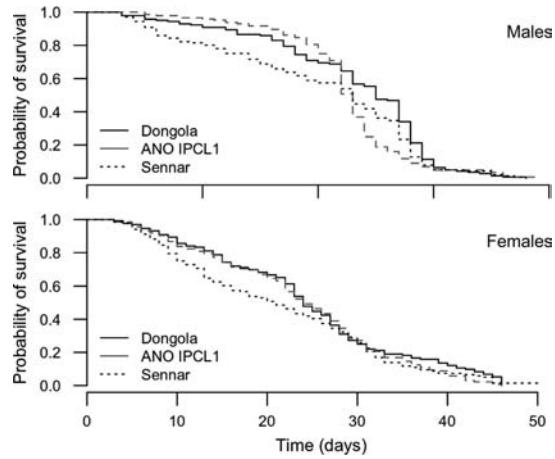

Fig. 3. Adults survival curves for the three strains of *An. arabiensis*. Kaplan–Meier curves (estimate of the survivor function) for males and females.

Life–History Parameters. The variations of the main life-history parameters of different strains are given in Table 3. The generation time (T_c) values were close for the three strains, although a significant difference was found ($F = 10.45$, df = 2, $P < 0.05$) between the strains ANO IPCL1 and Sennar at a level of significance of 0.01 (Tukey's post hoc honestly significant difference [HSD] test). The net reproductive rate (R_0) varied greatly between the strains ($F = 8.95$, df = 2, $P < 0.05$), ranging from 8.70 for the ANO IPCL1–31.62 for the Dongola strain. Tukey's post hoc HSD tests indicated a significant difference between these two groups at the 0.05 level of significance. The intrinsic rate of natural increase (r) was a more comprehensive measurement of fitness (Birch 1948) and was significantly lower for ANO IPCL1 compared with Dongola and Sennar ($F = 14.87$, df = 2, $P < 0.01$). Therefore, the ANO IPCL1 strain was theoretically able to double its population size in 7.8 ± 0.4 d, whereas Dongola and Sennar strains could do so in 4.9 ± 0.5 and 5.6 ± 0.4 d, respectively. The difference between ANO IPCL1 and the two other strains was significant ($F = 18.35$, df = 2, $P < 0.01$).

Discussion

The ANO IPCL1 strain was created from a cross between males from the Sennar strain and females

Table 3. Calculated generation time (in days; mean ± SEM), net reproductive rate, intrinsic rate of increase, and doubling time of the population for the three strains

Strain	Avg T_c	Avg R_0	Avg r	Avg T_d
Dongola	25.5 ± 0.4ab	31.6 ± 5.7a	0.14 ± 0.01a	4.92 ± 0.28a
Sennar	26.6 ± 0.2a	21.9 ± 3.4ab	0.12 ± 0.01a	5.60 ± 0.36a
ANO IPCL1	24.9 ± 0.2b	8.7 ± 1.0b	0.09 ± 0b	7.82 ± 0.41b

Within columns, values followed by different lowercase letters are statistically different ($P < 0.05$; ANOVA).

from the Dongola strain of *An. arabiensis*, with the purpose of mass rearing, sterilization, and release for SIT programs against this malaria vector. The chromosome rearrangement has obvious effects on the egg hatching rate, but it is possible that other undetected effects, including behavioral, developmental, and neurological effects, might exist.

With the exception of fertility rate, the observed life history of ANO IPCL1 is similar to that of Dongola and Sennar. ANO IPCL1 however showed a slightly higher larval-to-adult survivorship and shorter developmental time, which will impact positively on a mass production system. The sex ratio for the three strains was 50%, demonstrating similar survivorship for male and female larvae. Similar probabilities of survival over time were observed for adults in laboratory cages and at a low density. Sennar adult size, reflected by wing length, was significantly smaller than for the two other strains. A high larval survivorship can lead to a lower food availability and thus a lower food intake, which results in smaller size (Agnew et al. 2002, Gilles et al. 2011). In the case of Sennar, the larval survival rate was similar to the one of ANO IPCL1; however, its development took slightly longer. It has recently been documented that longer larval development was strongly correlated with shorter wing length in this species (Gilles et al. 2011) and in *Aedes aegypti* (L.) (Agnew et al. 2002), which is concordant with the developmental data for the Sennar strain. When comparing strains heterozygous for dieldrin resistance with homozygous resistant or susceptible strains of *Anopheles gambiae* Giles and *Anopheles stephensi* Liston, Rowland (1991) reported a faster development for heterozygous larvae, but no difference concerning adult survival and size were observed. In both his study and ours, no strong developmental effects seem to be attributable to the dieldrin resistance itself.

In ANO IPCL1, the females do not carry the translocation; hence, the genetic background of these females is similar to that of Dongola females to which ANOP IPCL1 males are often backcrossed. It is then not surprising to observe no difference in terms of egg number or adult female survivorship between the two strains. However, all GSS based on a Y-autosome translocation show a reduction of male fertility, attributable to the genetic behavior of the translocation during meiosis. This inherited sterility of translocation-carrying males is proportional to the complexity of the translocation, i.e., the more autosomes involved the higher the sterility level (Franz 2000, Robinson 2002). The egg-hatching rate of ANO IPCL1 was low; however, 90% of the first instar larvae survived until adulthood.

In a mass-rearing facility, a high number of broodstock adults will be required for the egg production to counteract the low fecundity; thus, it will be essential to optimize the survivorship of the immature stages. This emphasizes the importance of adjusting the number of females present in a mass-rearing cage according to the operational sex ratio (i.e., the ratio of sexually active males to receptive females at any time) so that an adequate number of females produce progeny. Although the generation time remains similar, the translocation of the ANO IPCL1 induced an important fitness cost regarding the demographic parameters with a lower intrinsic rate of increase and a longer period needed to double the population size. The demographic parameters values for the Dongola and Sennar strains were similar to those reported for different strains of this species in Kenya, which varied from 0.08 to 0.169 according to the landcover types (Afrane et al. 2007). A similar study on *An. gambiae* indicated a higher intrinsic rate of increase, ranging from 0.205 and 0.230 according to the various landcover types (Afrane et al. 2006). However, the comparison of demographic parameters between different studies remains complex because of variations between protocols, highlighting a need for standardization. We estimate that starting from a small GSS colony in which undesired recombinants are eliminated by selection and manual sex separation for crossing, two generations of amplification will be required to produce a sufficient number of mosquitoes for production of 10 million males per week (unpublished data). If one of the wild-type strains could be used for this purpose, or a GSS with higher fertility were available, one amplification stage could probably be eliminated, thus reducing the cost of production.

The use of male-linked translocation systems for population control has often been investigated. The semisterile males from GSS strains released in a wild population can affect directly the rate of increase of the latter by the decreased average fertility levels, as shown in several models (Serebrovsky 1940, Curtis and Hill 1968, Laven 1969). McDonald and Rai (1971) developed a model showing the possibility of eradicating a wild population of *Ae. aegypti* after six generations following successive releases of 50% sterile male-linked translocated males. High sterility levels of translocated males might be required for a successful control program as the initial impact on the reduction of the wild population fertility would then be high and would avoid the compensation because of a decreased larval competition in the natural larval sites (Curtis 1975, Krishnamurthy et al. 1975, Service 1985, Yakob et al. 2008). Laven et al. (1971) reported a progressive and rapid reduction of wild population of *Culex pipiens* L. from southern France after 1 yr of releases of a translocated strain in which males were 50% sterile. However, during the years following the end of the releases, the number of larvae carrying the translocation rapidly decreased, suggesting immigration, dilution, or both by remaining wild individuals by failure to completely replace normal male karyotypes (Cousserans and Guille 1974), underlining the difficulty of controlling population densities when the population is not isolated and if releases are not continuous. The fitness costs associated with the semisterility of ANO IPCL1 might put this strain at a disadvantage with the wild strains; therefore, its use for population replacement does not seem viable. However, under continuous releases, this natural semisterility might be sufficient to progressively reduce a wild population. In addition, the larvae resulting from matings between

wild females and semisterile males would maintain the larval competition for food in the larval sites and thus avoid creating conditions of overcompensatory dynamics that might improve the survival of wild larvae (Yakob et al. 2008). If the released males were fully sterilized by irradiation, these fitness costs would only affect the mass-rearing production, because no progeny would survive in the field.

In conclusion, this study of these life-history parameters detected few differences between the ANO IPCL1 and its parental strains Dongola and Sennar in the laboratory. Although important characteristics of ANO IPCL1 such as competitiveness remain to be determined and there is natural sterility of 73%, the strain shows potential for reduction of wild population size over generations. The life-history characteristics of ANO IPCL1 as determined in the laboratory show qualities consistent with the requirements for a successful SIT program such as fast development, good immature and adult survivorships, and high fecundity.

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Article 4. Sterilization parameters of the *Anopheles arabiensis* genetic sexing strain ANO IPCL1

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Genetic sex separation of the malaria vector, *Anopheles arabiensis*, by exposing eggs to dieldrin

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Abstract

Background: The sterile insect technique (SIT) has been used with success for suppressing or eliminating important insect pests of agricultural or veterinary importance. In order to develop SIT for mosquitoes, female elimination prior to release is essential as they are the disease-transmitting sex. A genetic sexing strain (GSS) of *Anopheles arabiensis* was created based on resistance to dieldrin, and methods of sex separation at the egg stage were developed. The use of this strain for SIT will require sexually sterile males: useful radiation doses for this purpose were determined for pupae and adults.

Methods: For the creation of the sexing strain, dieldrin-resistant males were irradiated with 40 Gy using a ^{60}Co source and were subsequently crossed to homozygous susceptible virgin females. Individual families were screened for semi-sterility and for male resistance to dieldrin. For sex separation, eggs of a resulting GSS, ANO IPCL1, were exposed to varying concentrations of dieldrin for different durations. Percent hatch, larval survival, and male and female emergence were recorded. Radiation induced sterility was determined following adult and pupa exposure to gamma rays at 0–105 Gy. Mortality induced by dieldrin treatment, and levels of sterility post radiation were investigated.

Results: ANO IPCL1 contains a complex chromosome aberration that pseudo-links the male-determining Y chromosome and dieldrin resistance, conferring high natural semi-sterility. Exposure of eggs to 2, 3, and 4 ppm dieldrin solutions resulted in complete female elimination without a significant decrease of male emergence compared to the controls. A dose of 75 Gy reduced the fertility to 3.8 and 6.9% when males were irradiated as pupae or adults respectively, but the proportions of progeny of these males reaching adulthood were 0.6 and 1.5% respectively

Conclusion: The GSS ANO IPCL1 was shown to be a suitable strain for further testing for SIT though high semi-sterility is a disadvantage for mass rearing.

Keywords: Genetic sexing, *Anopheles arabiensis*, Sterile insect technique, Dieldrin resistance, Sterility

Background

The sterile insect technique (SIT) [1,2] as part of area-wide integrated pest management (AW-IPM) programmes has celebrated many successes in suppressing, and eliminating several agriculturally and economically important insect pests in many regions of the world [3]. There is renewed interest in using sterile insects for managing endemic, as well as emerging or re-emerging vector-borne diseases, thus providing new momentum for developing SIT in the field of infectious disease control [4]. In spite of a successful SIT programme against *Anopheles albimanus* in El Salvador in the 1970s [5] most mosquito SIT programmes were either too small to demonstrate effectiveness or simply failed [6]. The development of the SIT for use in mosquito AW-IPM programmes is, therefore, in its infancy, and many fundamental components of the technique still need to be developed, validated and optimized. These include aspects of the mass-rearing of the vectors in question, the quality of the sterile males produced, and methods

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of handling, transporting and releasing the sterile insects within the targeted geographic region [7].

One of the many essential steps in mass production of mosquitoes for the SIT is the elimination of females, since even sexually sterile females can transmit disease pathogens. As the manual separation of the sexes based on their morphology is time and labour-intensive and with some risk of error, genetic sexing strains (GSS) based on an artificially induced sex linkage of a selectable marker are required [8].

In recent years, novel strategies for genetic sexing have been developed involving genetic modification (GM) through germ-line transformation, including systems involving testes-specific expression of enhanced green fluorescent protein [9] and a tetracycline repressible dominant lethal [10]. Various arguments are routinely made to promote the merits of individual systems [4], but leaving aside the debate on field release of GM mosquitoes, it is apparent that even the most sophisticated of novel approaches suffers some disadvantages and there remains considerable scope for conventional non-GM systems [11].

The classical approach for creating a GSS is to link a conditionally lethal allele to the Y chromosome through irradiation-induced chromosome rearrangements [8]. This is technically a genetic modification, but does not require the introduction of foreign DNA via modern biotechnology: the resulting organisms are not considered GM. Most systems previously developed in mosquitoes have been based on genes conferring insecticide resistance, where the male is heterozygous for resistance by virtue of a Y-translocation, whereas females are homozygous susceptible. Resistance to dieldrin (*Rdl*), an insecticide that blocks γ-aminobutyric acid receptors inhibiting transport of chloride ions, is the locus of first choice for *Anopheles arabiensis* for the following reasons: no other conditional lethal beside insecticide resistance has been identified in this species. The resistance is due to a single amino acid substitution in the target site [12] and can be easily detected by the PCR [13]. Both dominant and semi-dominant alleles have been identified [14], allowing homozygous susceptible and heterozygous resistant insects to be easily distinguished by a discriminating dose of insecticide in larvae and adults. The use of dieldrin for insect control has been banned since the 1970s, so the accidental introduction of resistance into mosquito populations is only important if cross-resistance becomes an issue. As dieldrin resistance is already widespread in mosquito populations and in some cases remains high [15], it is unlikely that other GABA-gated chloride channel antagonists, such as fipronil, will be used for mosquito control. However, there is evidence that fipronil can still be effective against insects carrying *Rdl* [16].

GSSs based on dieldrin resistance have been produced in the past for the experimental organism in this study, *An. arabiensis* [17] and its sibling species, *Anopheles gambiae* [8], but the strains no longer exist, so it was necessary to attempt the creation of a new strain for programmes supported by the International Atomic Energy Agency (IAEA). Previously, the necessary chromosome translocations were created with relative ease in *An. arabiensis*, with only 60 semi-sterile families screened [18]. In contrast, 216 *Anopheles stephensi* semi-sterile families were screened to recover the GSS [19].

Advantages of an *Rdl*-based GSS are that females can be eliminated at an early life stage with minimal handling, ensuring that mass production costs are low and that males of optimal quality are produced. At the same time, a stable inbreeding GSS strain can be reared under standard conditions also ensuring reduced costs. Lines and Curtis [17] demonstrated that elimination of females from the previously created *An. arabiensis* GSS could be effectively achieved by exposure of first instar larvae to dieldrin, and despite approximately 1% recombination, the strain was maintained with minimal additional selection. The creation of a new *An. arabiensis* GSS strain is reported here, but with a lower level of recombination. The strain has been used to demonstrate the separation of the sexes in which eggs, rather than larvae, are exposed to dieldrin. This not only eliminates the need to mass rear female larvae, but also greatly simplifies the separation step reducing handling of the insects and requirements for materials and equipment. Because, an SIT project requires that the released males be sterile and ANO IPCL1 is intrinsically semi-sterile, the cumulative effect of semi-sterility and induced sterility of the GSS males through irradiation is reported.

Methods

Mosquito stocks and rearing

Two pure-breeding stocks of *An. arabiensis* were used for creation of the GSS and other experiments. Both strains and details of their characteristics are available from the Malaria Research and Reference Reagent Resource Center under the numbers indicated. The SENNAR strain (MRA-334) contains a semi-dominant allele for resistance to dieldrin [12] and DONGOLA (MRA-856) contains only the dieldrin-susceptible allele. Although the formal symbol for dieldrin resistance is Rdl^R, it will be referred to as the homozygous resistant, susceptible and heterozygous individuals as RR, SS and RS respectively. The GSS described in this manuscript has been maintained since 2008 in the Insect Pest Control Laboratory (IPCL) of the FAO/IAEA Agriculture & Biotechnology Laboratories, Seibersdorf, Austria. All strains were reared in a climate-controlled room maintained at a temperature of 27 ± 1 °C and 60 ± 10% relative humidity. The light regime was LD 12:12 h photoperiod, including dusk (1 h) and dawn (1 h). Larvae were reared in plastic trays (40 x 29 x 8 cm) at a density of approximately 500 first instar larvae (L1) per tray that contained ± 1.5 L of

deionized water. Larvae were fed a diet of finely ground (224 μm-sieved) Koi Floating Blend® (Aquaricare ®, Victor, New York, USA, no longer available). Pupae were collected and placed in small plastic cups inside a fresh adult cage for emergence. Adults were kept in standard 30 cm cubic insect cages (Megaview Science Education Services Co, Ltd, Taiwan) and continuously supplied with 10% [w/v] sucrose solution with 0.2% methylparaben [20]. Females were blood-fed weekly on de-fibrinated bovine blood using the Hemotek feeding apparatus (Discovery Workshops, Accrington, Lancashire, UK). Gravid females were allowed to oviposit in plastic cups with black lining containing a wet sponge over which a filter paper was placed. Eggs were collected from individual females by placing them in a plastic medicine vial lined with filter paper and plugged with a cotton ball. For egg hatching rates, the filter paper was removed and examined under a dissecting microscope.

Dose response of larvae

A dieldrin (291218, Sigma-Aldrich, St. Louis, MO, USA) 1,000 ppm stock solution was prepared in acetone, and all further dilutions were prepared from this. The original selection and confirmation of the resistance status of DONGOLA and SENNAR was performed by exposure of batches of 50 L3 and L4 larvae to 100 ml of dieldrin solutions (in plastic cups) of various concentrations ranging from 0.001 ppm to 10 ppm for 1 h at room temperature (approximately 25 °C). Any larvae that pupated within 1 h after the end of the dieldrin exposure were discarded, as pre-pupae are more resistant than earlier stages (data not shown). In addition to the pure-breeding susceptible DONGOLA and resistant SENNAR strain, heterozygous F1 larvae were created by crossing these strains. They will be referred to as F1 or heterozygotes.

Creation of the GSS ANO IPCL1

Late pupae of the resistant (SENNAR) and susceptible (DONGOLA) strains were separated into males and females based on genital morphology and placed in holding cages for emergence. About 100 SENNAR males (<24 hours post emergence) were irradiated with 40 Gy using a cobalt-60 (^{60}Co) source (Gammacell220, MDS Nordion, Ottawa, Canada) [21] and crossed to about 200 homozygous susceptible virgin females. The F1 males were then backcrossed to susceptible virgin females *en masse*. Females were blood-fed and placed in a holding cage for two days. For each screening round, 60 to 100 single females were placed in 2.5 x 7.5 cm glass flat bottom vials, the bottom two thirds of which was lined with filter paper and sealed with a cotton wool plug. Distilled water was added to about one third of total volume. The backcross and egg collection procedure was repeated two or three times for each of three irradiation experiments conducted.

The eggs were allowed to hatch within the vial. One day after the first larvae were observed, the empty egg cases and unhatched eggs were counted under a dissecting microscope. In the latter, where possible, the presence of an eyespot was looked for to confirm embryonic death. Only semi-sterile (<50% hatch) lines were maintained. Three approaches were taken to screening with dieldrin depending on the numbers of larvae in each line. Most lines were screened by exposing batches of 25 or fewer fourth instar larvae to 0.2 ppm dieldrin in 150 ml of distilled water in standard 210 ml plastic cups. Occasionally, a line was inbred and the test postponed to the next generation on the grounds that a promising line would show very little recombination. The third approach used, again rarely, was to expose adult males after mating to standard WHO 0.4% dieldrin papers. Only lines showing a markedly higher than expected survival of males were maintained for further analysis, which involved out-crossing resistant males to DONGOLA females. The karyotype of the finally selected strain (ANO IPCL1) was determined by examination of salivary gland chromosomes by a method described by Cornel [22].

Routine GSS strain purification

To avoid the accumulation of undesirable recombinants (dieldrin-resistant females and males that carry the dieldrin-resistance allele in repulsion to the aberration), a pure stock was maintained by regularly out-crossing dieldrin-resistant ANO IPCL1 males to virgin DONGOLA females in a three-step process: 1) larvae of the most recently back-crossed ANO IPCL1 were exposed to 0.1 ppm dieldrin solution for 1 h and surviving (resistant) males were kept. Ten crosses were set up in small cages, each containing three resistant males and 10 virgin DONGOLA females. Egg batches were collected *en masse*, hatched and the larvae were exposed to dieldrin as described above. Entire batches of progeny containing any females were discarded and the remaining batches pooled; 2) with these males, another 10 crosses were then set up as stated above. Again, eggs were collected and larvae treated. Those batches containing no females were kept and pooled; and, 3) 100 of these males were then crossed with approximately 300 virgin DONGOLA females. Cages were kept at densities no higher than approximately 400 adult mosquitoes. The routine mode of maintaining purity of the stock repeated the last two steps, in which males surviving dieldrin treatment are backcrossed to virgin DONGOLA females. This should be done every generation to maintain a pure colony.

Effects of dieldrin exposure on GSS eggs

To determine the effects of dieldrin exposure on GSS eggs, females of ANO IPCL1 were blood fed, and oviposition cups were placed in the cage overnight and removed the following morning (aged ≤ 12 h). The eggs were concentrated by rinsing them off of the filter paper

into plastic cups lined with filter paper, to which they adhere. The eggs were counted and separated into batches of 400–600 eggs per exposure tube (made of plastic, 2 cm in diameter, the bottom of which was sealed with fine netting). These tubes allow simple and rapid exposure and rinsing of batches of eggs. The tubes containing the eggs were then placed into 50 ml of 0.5, 1, 2, 3, 4 and 5 ppm dieldrin at a constant temperature of 25 °C for 1, 2, 6, or 24 hours. After exposure, the eggs were collected and rinsed before placing them into white cups lined with filter paper containing de-ionized water and 640 μl of 1% FAO/IAEA larval diet, consisting of 0.1 mg of bovine liver powder, 0.1 mg of tuna meal and 92 μg of Vanderzant Vitamin Mix mixture per larva per day [23]. The hatch rates were observed under a dissecting microscope, and the number of L1 larvae noted. Pupae were collected by pipetting once daily and transferred to emergence tubes (BioQuip Products Inc. 2321 Gladwick Street, Rancho Dominguez, CA 90220, USA). The number of emerged male and female adults was recorded. Adults that eclosed incompletely or were unable to fly were counted as dead.

Effects of temperature on dieldrin treatment efficacy

ANO IPCL1 eggs collected as stated above were exposed to 1, 2 or 3 ppm dieldrin for 2 h at 25 °C (ambient temperature in the treatment laboratory) or 30 °C in a water bath (TECHNE, TE-10A, Bibby Scientific Ltd., Stone, Staffordshire, ST15 0SA, UK). There were three replicates for each treatment. Effects on hatch rates, number of surviving males and females were observed.

Effects of egg age on treatment efficacy

It was hypothesized that because fresh eggs are white and soft and progressively melanize and sclerotize that their permeability to dieldrin would change with age. Therefore, eggs were collected at intervals of less than two hours to ensure a defined narrow age range. All eggs were still white or whitish yellow in colour when collected. These eggs were then exposed to 1 ppm and 3 ppm dieldrin solutions when "young" (<12 hours old) and "old" (≥24 hours old). Treatment was stopped and the batch discarded if eggs began to hatch.

Radiation-induced sterility

ANO IPCL1 pupae (n = 50) were collected within a six-hour interval after pupation and irradiated 20 h later. They were placed on a wet net, in a 4 cm diameter cup at the centre of the irradiation chamber. Approximately 15 hour-old adult males (n = 50) were placed in a 4 cm diameter container using a buccal aspirator without anaesthesia. The container was then put in contact with ice for several minutes to chill the males before irradiation. The container was maintained on ice to keep the males immobilized during the irradiation process. All treatments were left on ice for the maximum irradiation time so that the chilling effect on all groups would be similar. Pupae and adults were exposed to gamma rays emitted by a ^{60}Co source at 0, 60, 75, 90, and 105 Gy (dose rate ca. 9 Gy/min). The Gafchromic HD-810 film (International Specialty Products, NJ, USA) dosimetry system was used to measure the dose received by the lot; three dosimeters were included with each lot of insects and read after irradiation with a Radiachromic reader (Far West Technology, Inc., California, USA). Males were offered virgin females from the wild strain DONGOLA in a 1:1 ratio. Virginity of females was ensured by separating them from males at the pupal stage. After five days, females were blood-fed with human blood from a volunteer and allowed to oviposit in individual tubes. Egg hatch rates were then recorded, as well as the number of L1 alive. For the treatments 60, 75, 90 and 105 Gy, all L1 were transferred to Petri dishes for rearing. Density was less than two larvae/ml and feeding was standardized (0.2 mg of diet/larva/day) for all treatments. The number of pupae and emerging adults was recorded for each family.

Statistics

The analyses were conducted using R [24]. Results of resistance assays were analysed by logistic regression using the DR routines of the R statistics package. The dieldrin-induced mortality on eggs, larvae and adults was corrected from the control mortality levels. The female or male production rates were calculated as the number of emerged adults out of the total number of eggs. Egg eclosion rates, mortality rates, and adult production rates were square-root-transformed to achieve normal distribution; ANOVA ($P < 0.05$) and Tukey Post-hoc tests were used to compare treatments.

Egg hatch rate data were square-root-transformed and compared between treatment using ANOVA and Tukey Post-hoc tests. Within one treatment, Kruskall-Wallis rank sum tests were used to compare the proportions of hatching eggs, L1 or emerging adults resulting from the progeny of irradiated males ($P < 0.05$).

Results

Dose response of larvae

The three dieldrin genotypes were easily distinguished by dieldrin exposure in the larval stage. Briefly, all SS larvae are susceptible to 0.1 ppm dieldrin and RS individuals survived doses up to 1.0 ppm (Figure 1) for 1 h. RR individuals survived doses exceeding 1.0 ppm. On the basis of these susceptibilities, a discriminating dose of 0.1 ppm was chosen to select RS and RR individuals and to kill SS larvae.

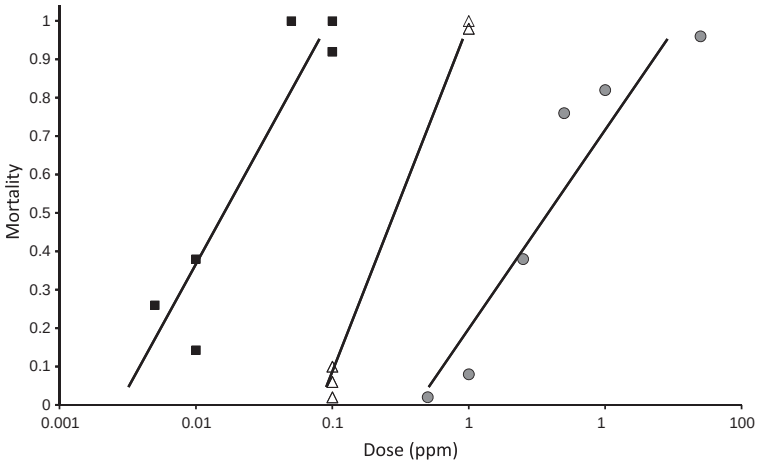

Figure 1 Larval dose–response curves. Left to right are DONGOLA (SS), F1 hybrids and SENNAR (RR). The dose 0.1 ppm dieldrin for 1 h was selected to eliminate susceptible larvae based on these analysis and was used for selection of the GSS.

Creation of the GSS ANO IPCL1

Approximately two-thirds of 750 females from the three irradiation experiments oviposited. As the focus was the isolation of a useful GSS rather than an evaluation of the procedure, investigations of individual lines were minimal. The cut-off for the initial screening for semi-sterility was applied at 50% hatch or less; however, lines in the higher end of this range rarely showed semi-sterility in the next generation, a characteristic that would be expected for an appropriate chromosome rearrangement. Three lines in this category were found amongst 19 initially classified as semi-sterile from three rounds of screening in the second irradiation experiment. A further two lines did not survive rearing in sufficient numbers to maintain the lines. Ten were discarded after first, or second, generation dieldrin assays. Amongst a few lines that had not yet been fully evaluated was 5–33. The initial bioassay was performed on 10 adult males after mating, only one died. It was particularly difficult to amplify the line to obtain sufficient numbers for bioassays and ensure its survival, since egg yield and egg hatching were low. Line 5–33 (ANO IPCL1) was kept for about nine months without selection, but with an occasional supplement of virgin DONGOLA females. A bioassay was then conducted on 500 early fourth instar larvae using a concentration of 0.1 ppm under standard conditions. Mortality after the 24-h holding period was 48%. Only two females were obtained amongst the survivors indicating a recombination frequency of 0.4% or less. A subsequent experiment in which approximately 3,000 first instar larvae were exposed *en masse* in one large tray to 0.1 ppm dieldrin resulted in no female survivors. The strain shows high semi-sterility, with an average percent hatch of eggs at 26.7% regardless of the data collection method (family data, 95% CI = 0.015, n = 220; *en masse* egg-collection data 95% CI = 0.023, n = 34).

The karyotype of the GSS is complex, a finding consistent with the low hatching rate: neither the X nor 3 L chromosome is involved (Figure 2). Determining the exact positions of the break points was not possible because of the complexity of the translocation and the resulting difficulties to obtain properly spread chromosomes. Furthermore, the only existing photographic chromosome map [25] has incorrect arm and band assignments (V Petrarca, pers. comm.). The best interpretation is that there is a peri-centric inversion including much of chromosome 2R with a break at 9A, and on 2L in division 22. The breakpoint may be common with a Y-chromosome translocation having a breakpoint basal on 3R. It is quite possible that the aberration is even more complex. For example, an alternative explanation is that it contains a chromosome 2–3 translocation. The dieldrin resistance allele in on chromosome 2L in division 22A [12], i.e. approximately one-third of the arm length from the centromere, a location that would be well within the putative peri-centric inversion.

Effects of dieldrin exposure on ANO IPCL1 eggs

Two manifestations of dieldrin toxicity were expected as a result of egg exposure: failure of larvae to hatch and delayed mortality during the later stages of development. Several preliminary observations were made to determine the treatment parameters that would be most effective. To ensure that the acetone concentration of the solutions was not affecting hatch rates or larval survival, <12-h old eggs were exposed to acetone solutions for 1 h at concentrations up to 1%, the highest concentration used in these experiments. No increases in larval mortality or change in hatch rates were observed.

Non-treated ANO IPCL1 eggs hatched an average rate of 25.7 ± 0.9%. The mean hatch remained between 22 and 29% up to a concentration of 3 ppm, then dropped

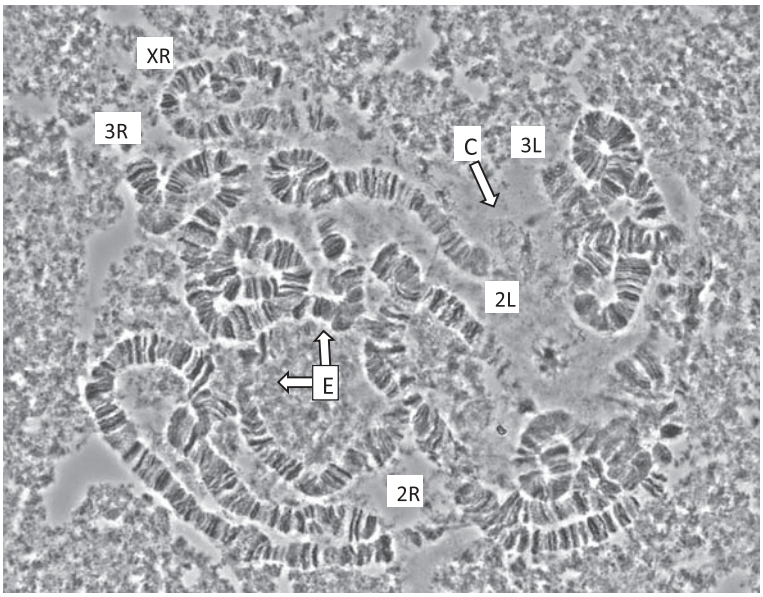

Figure 2 Karyotype of the GSS. C refers to the putative chromocenter, and E the vicinity of the exchange. Labels for chromosome arms have been placed in the vicinity of their telomeres. Because only one copy of the X chromosome is present in males, chromosome XR is expectedly narrower than the autosomes.

to 14.4 ± 0.9% (mean ± SEM) at exposures to 5 ppm dieldrin (Figure 3). No statistically significant differences in hatching rate were observed between control, 0.5, 1, 2 and 3 ppm dieldrin solution treatments for any of the treatment durations. However concentrations of 5 and 10 ppm significantly reduced the hatching rate ($F_{7,\ 76} = 19.41$, $P < 0.001$). No interaction was observed between the time and concentration of dieldrin treatment ($F_{13,\ 76} = 0.64$, $P = 0.81$). For each dose, the duration of exposures of 1, 2, 4, 6, or 24 h had no effect on hatch rate ($F_{4\ 76} = 2.31$, $P = 0.07$), thus all data from a same concentration were merged for the next analyses. It is suspected that some susceptible females died shortly after hatching as the numbers of L1 larvae counted were far lower than what would be expected based on the hatch rate.

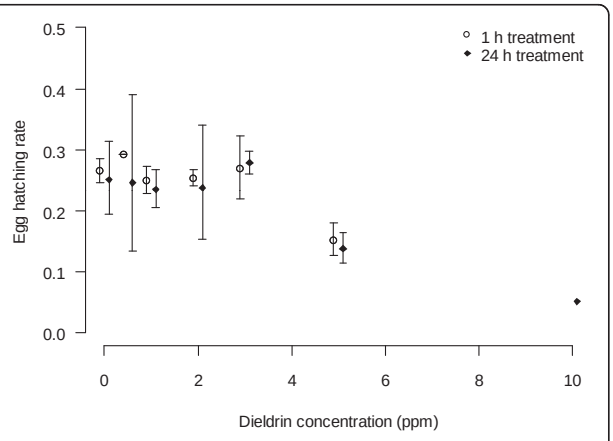

Figure 3 Effects of dieldrin treatments on egg eclosion of ANO IPCL1. Mean hatch rate (± CI) following exposure for 1 h or 24 h treatments.

In control treatments the survivorship from hatched eggs to L1 larva was 92.1 ± 2.3% but only 66.8 ± 5.3% of the hatched eggs survived to adulthood. The mortality rates of eggs (Figure 4, panel A), from hatched eggs to larvae (Figure 4, panel B) and from hatched eggs to adulthood (Figure 4, panel C) were corrected from the control values for the dieldrin treated groups. The dieldrin induced mortality increased with the dieldrin concentration for the various developmental stages. Although no females appeared after treatments at 5 ppm dieldrin, the number of males obtained was too small for this concentration to be useful. Concentrations greater than 2 ppm dieldrin induced an increase of 35 ± 5% of larval mortality and 33 ± 6% of adult mortality as compared to the normal mortality of the untreated batches.

Assuming an equal sex ratio with a natural fertility of 27%, this strain can produce a maximum of 13% of males from the initial number of eggs. This production fluctuated between 9 and 13% in treatments up to 3 ppm; and decreased to 6.4% when treated at 4 and 5 ppm (Figure 5). However there was no significant difference between all these treatments ($F_{6,\ 93} = 2.19$, $P = 0.051$).

Five of six egg batches treated at 0.5 ppm yielded some females, a result similar to batches treated at 1 ppm where 16 of 27 batches produced a mean 2.9 ± 0.6% of

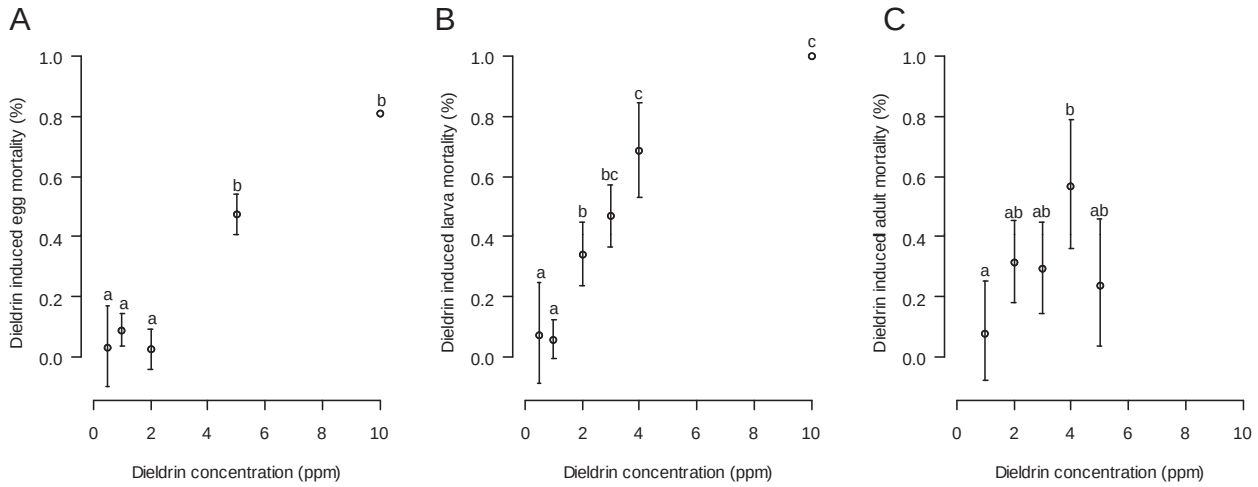

Figure 4 Dieldrin-induced mortality on eggs (A), larvae (B) and adults (C). Mean mortality rate (± CI) for different concentrations of dieldrin. Different letters indicate a significant difference ($P < 0.05$).

females from the initial number of eggs. Significant differences were observed between the treatments 0, 0.5, 1 ppm and the higher concentrations ($F_{6,\ 93} = 28.7$, $P < 0.001$). At 2 ppm, the production of females was significantly reduced to 0.4%; of 16 batches only three yielded ≥ 1% females. (These females, when selected on 1 ppm dieldrin paper survived, suggesting that they were most likely dieldrin resistant). At 4 ppm, none of the egg batches yielded any females, however the number of males obtained fell below 10% of the original number of eggs indicating that delayed mortality was occurring even among the RS individuals.

Effects of solution temperature and egg age at treatment on male and female emergence and egg hatch rate

The egg-hatching rate was significantly lower when treatment occurred at 30 °C as compared to 25 °C ($F_{1,\ 16} = 11.45$, $P < 0.01$); control batches hatched at 24.6% at 30 °C against 26.4% at 25 °C. No interaction between concentration of dieldrin and treatment temperature was detected. Temperature did not affect the number of adult females emerging after treatment ($F_{1,16} = 0.33$, $P = 0.58$).

The age of eggs when treated had a significant effect on the production of females ($F_{1,\ 16} = 42.0$, $P < 0.001$), however significant interactions were found between age and concentration ($F_{2,\ 16} = 4.19$, $P < 0.05$) and between age and time of treatment ($F_{1,\ 16} = 6.99$, $P < 0.05$). Of the 9 batches of young eggs treated at 3 ppm (for 1, 6 and 24 h treatments), all yielded males only (Figure 6). When more mature eggs (12 hrs or older) were treated, all three batches treated yielded females. It is therefore important to treat the eggs while the eggs are less than 12 hrs old, when the treatment is more effective in killing females.

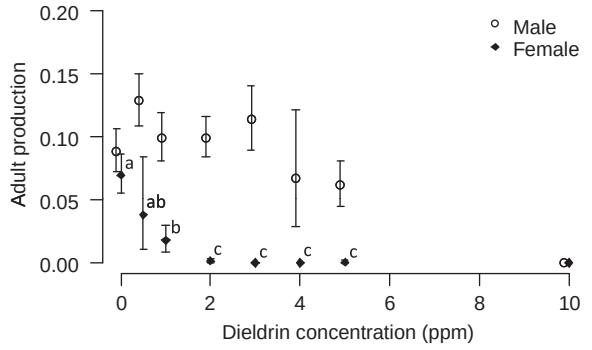

Figure 5 Efficiency of dieldrin treatments on male emergence and female elimination. Adult emergence (calculated as the rate of adults emerged out of the total number of eggs ± CI) of male and female adults out of the initial number of eggs as a function of dieldrin concentration.

Radiation induced sterility of ANO IPCL1

ANO IPCL1 males were irradiated at various doses either as late pupae or <15 h old adults. The mean natural fertility of the two control groups in these particular experiments was 29.7 ± 3.0% (Table 1). At a dose of 75 Gy ca. 95% sterility was observed when considering the egg hatch rate: the fertility did not differ significantly over 75 Gy for pupal irradiation and 90 Gy for adult irradiation. The reduction of fertility that could be attributed to gamma irradiation was similar for the pupal and adult stages, and they were similar to those observed on the wild *An. arabiensis* DONGOLA strain [26] and to those reported by Helinski *et al* [21] with the *An. arabiensis* KGB strain, originating from Zimbabwe. The survival of the progeny was followed until adult emergence. The mortality between hatched eggs and L1 for un-irradiated ANO IPCL1 males'

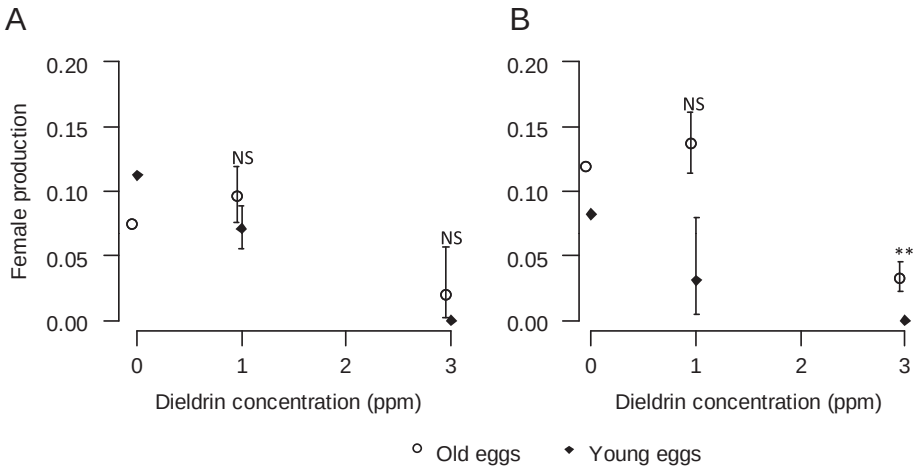

Figure 6 Effect of egg age on dieldrin toxicity. Female emergence (calculated as the rate of females emerged out of the total number of eggs ± CI) after treatment of 1 h (A) or 6 h (B) with old (> 24 h) or young (aged ≤ 12 h) eggs.

progeny was 20 ± 3%. Progeny larval mortality increased with the radiation dose received by the male. When male pupae were irradiated at 75 and 105 Gy, the progeny larval mortality rate was respectively 52 and 64%, and for adult irradiated at 75 and 105 Gy, it was 38 and 57% respectively. When adults were irradiated at 105 Gy, 96% of them had no viable adult progeny; only one emerged adult was found in two out of 29 broods. When males were irradiated as adults at 90 Gy, 78% of ANO IPCL1 did not produce any offspring that reached adulthood; the remaining 22% of the males produced only one adult offspring. When males were irradiated as pupae at 75, 90 or 105 Gy, more than 80% of them had no or only one surviving offspring. When considering the fertility as the proportion of eggs resulting in adults, the mean fertility was 3.1 ± 0.6% and 2.1 ± 0.4% respectively for the pupal and adult irradiation at 60 Gy. More than 98% sterility was reached from doses over 75 Gy for both pupal and adult irradiation.

Discussion

For ethical and public health reasons, female mosquitoes must be eliminated from releases. There are many advantages of a GSS that allows separation in the egg stage: cost reductions in the production process can be considerable if only half of the number of larvae is cultured [3], and almost exclusively male pupae and adults are immediately available for irradiation, transport and release. Treatment at the egg stage may not only

Table 1 Radio-sterilization of ANO IPCL1. Percentage of egg hatch, resulting in first instar larvae or in emerged adults in progeny from males subjected to different radiation doses at the pupal or adult stage.

Dose (Gy)	Stage of irradiation	Percentage of hatched eggs			Percentage of 1st instar larvae			Percentage of emerged adults		
		Mean	SEM		Mean	SEM		Mean	SEM	
0	Pupa	33.9	1.6	a	28.2	1.9	b			
	Adult	27.6	1.9	a	22.4	1.7	b			
35	Adult	12.9	1.2	a	9.6	1.3	b	2.4	0.8	c
60	Pupa	8.3	1.0	a	5.8	0.9	a	3.1	0.6	b
	Adult	5.8	0.8	a	3.6	0.6	a	2.1	0.4	b
75	Pupa	3.8	0.8	a	1.3	0.5	a	0.6	0.3	a
	Adult	6.9	1.1	a	3.8	1.0	b	1.5	0.5	b
90	Pupa	4.6	0.8	a	1.6	0.4	b	1.0	0.3	b
	Adult	2.6	0.6	a	0.8	0.3	a	0.2	0.1	b
105	Pupa	4.2	0.7	a	0.9	0.3	b	0.5	0.2	b
	Adult	1.2	0.3	a	0.2	0.1	b	0	0	b

Within one treatment (same dose and stage of irradiation), values followed by different letters are statistically significantly different ($P < 0.05$).
Emergence of adults could not be followed for control treatments.

improve the quality of the ANO IPCL1 males by minimizing damage to them due to handling during the larval stages [27] and eliminating unnecessary larval culture, but sex separation becomes more practical and accurate.

To these ends, a GSS for *An. arabiensis* was created and tested and methods for exposing eggs to eliminate females were developed. GSS utilizing a selectable marker with recombination frequencies <1% have been created in mosquitoes previously: *An. gambiae*, 0.25% [8]; *An. arabiensis* (no specific value given but well below 0.1%) [28]; *An. albimanus,* 0.3% [29]; *Anopheles quadrimaculatus,* 0.02% [30]; *An. stephensi,* 0.3% [19]; *Anopheles culicifacies* < 0.02% [31]. All of these used either malathion, dieldrin or propoxur as the selectable marker for obvious reasons: resistance is relevant to public health and is often quickly selected in wild populations and easily identified in stocks.

GSS creation depends on fortuitous isolation of aberrations that suppress recombination between the selectable marker and the Y chromosome. In the case of this GSS, the number of families screened was unusually large. In remarkable contrast, only 18 families were screened to identify a previously created GSS for *An. arabiensis* [28] which was also based on dieldrin resistance.

The conditions for egg exposure to dieldrin do not appear to be stringent. The ideal dieldrin concentration to eliminate all females during the egg stage lies between 2 and 3 ppm for a duration from 1–6 hrs in the temperature range of 25-30 °C. While these experiments demonstrated that exposing eggs when young is important, the degree of latitude that is possible is not known yet. Further trials are needed to assess the efficacy of the treatments when treating larger quantities of eggs being prepared for mass releases. Additional refinements to the technique such as the quantification of eggs volumetrically would greatly enhance efficiency and accuracy when treating larger quantities on a daily basis. Such methods applied to a GSS of *An. albimanus* [27], including egg treatment, greatly facilitated production of this species, and similar benefits are expected for *An. arabiensis*.

The ANO IPCL1 shows high intrinsic sterility of 73%, which results in the production of a maximum of 13% males from the total number of eggs. This puts great pressure on the brood stock production level for mass production, but this should not be an insurmountable obstacle. The MACHO GSS strain of *An. albimanus* showed sterility of 50%, and yet they were able to produce one million sterile males per day [27]. Balancing this limitation is the potential advantage of fairly high sterility inherited from GSS males by any male progeny in the field. This allows for the possibility that the irradiation dose can be reduced to attain the same level of population suppression that would require greater irradiation when using a GSS with higher fertility. A reduced dose generally improves competitiveness. Indeed, as part of an integrated pest management approach, release of a semi-sterile strain subjected to radio-sterilization has been considered: this is known as the "Combi-Fly concept" [32,33]. Full radio-sterilization of wild strains usually leads to a lower competitiveness of males as compared to non-irradiated ones [34]. Irradiation produces dominant lethals, which lead to a dose-dependent lethality among the offspring. This death would occur predominantly at the very early stage of embryonic development: Laven and Jost [35] reported that embryos could not be detected in most of the non-hatching eggs fathered by irradiated male *Culex pipiens*. However, irradiation affects similarly normal sperm and sperm carrying a translocation, hence the fully sterilizing dose should not differ greatly between a wild strain and a GSS [36]. As a matter of fact, the radiation-induced sterility in ANO IPCL1 showed the same rate of increase as the wild strain DONGOLA [26]. However, a greater difference between the wild and the ANO IPCL1 strain appears when looking at the survival of the progeny. An average of 19.7% first instar larvae died soon after hatching in the progeny from ANO IPCL1 un-irradiated males and this mortality rate increased greatly with the radiation dose. What really matters in the release of sterile males is the final number of adults that would result from the mating of wild females and sterile males. Thus, the sterilizing dose should be chosen accordingly and sterility rates of genetic sexing strains should not be evaluated only as the egg hatch rates but rather as the proportion of eggs leading to adults. Considering this, ANO IPCL1 shows > 96% sterility at a radiation dose of 60 Gy and a dose of 75 Gy appears sufficient to lead to > 98 % sterility. It was reported as well for other GSSs that, in addition to a reduced egg hatch, males usually sire progeny with a reduced survival rate during the later developmental stages [37]. This lethality could be explained by the presence of triplication carrying individuals that resulted from adjacent segregation during meiosis in the male parent [34]. The chromosomal study showed that the translocation was complex in this GSS; this is consistent with the high lethality observed in the various stages of the progeny fathered by ANO IPCL1 irradiated males. This later mortality brings the advantage of maintaining larval competition in the breeding sites and thus maintain a low wild larval survivorship through density-dependence effects [38].

Though genetic recombination in the ANO IPCL1 occurs at a low rate, it requires management. Leaving recombinants unchecked runs a risk of deterioration of the strain. Therefore, there is a need to periodically purify the strain by keeping a homozygous susceptible stock to outcross ANO IPCL1 males to on a regular basis. At this time, there is no data demonstrating the accumulation rate of breakdown progeny of the strain.

While insecticide-resistance alleles are widely available, systems based on chemical toxicity can be disadvantageous

for several reasons: contamination of the susceptible rearing colony is always an immediate danger; use of insecticide requires that residues, contamination, and waste management are all issues that must be dealt with appropriately. Furthermore, dieldrin solutions become less potent once used [39]. It is presumed that the dieldrin molecules are absorbed and/or adsorbed by eggs as well as onto the surfaces of containers in which the treatments are performed. Therefore the solutions should not be reused for consecutive treatments.

In order to avoid the disadvantages of having a toxicant in the insectary, it is desirable to develop a sex separation system that relied on a physical selection treatment such as one based on a temperature sensitive lethal mutation. This has been accomplished in *Culex tritaeniorhynchus* [40] similar to the system used for medflies in mass production facilities [37]. The *Cx.* temperature-sensitive lethal isolation depended on an array of genetic markers that were available during the heyday of classical mosquito genetics, but these are no longer extant for any mosquito species, so the difficulty of isolating additional lethals should not be underestimated.

There may be an intrinsic loss of vigour related to the dieldrin resistance gene. The biological quality of RR males and females of *An. gambiae* Giles and *An. stephensi* Liston have been compared to RS and susceptible SS males and females [36,41]. It was found that the females of resistant strains were less responsive to oviposition stimuli, produce fewer eggs per unit of blood, fly less when seeking hosts or oviposition sites and respond slower to simulated predators. The males were generally less successful in competing for females. It is thought that perhaps the mating success of RR males was poorer because of their reaction to female swarms (as to predator movements) was generally slower. These results should be considered carefully as there was no attempt to distinguish strain from resistance gene effects. However, in the light of other findings, the general fitness and quality of ANO IPCL1 must be scrutinized with a series of experiments to ensure that there are not prohibitive reductions in competitiveness.

Conclusion

The GSS reported here provides a suitable strain to proceed toward releases and has been used in a small scale field release in northern Sudan primarily concerned with evaluating logistics. Its performance characteristics will have to be tested in detail, but mating competition studies in large cages and semi-field conditions using sterilized males from the susceptible parental strain were very encouraging [42]. It is certain that without a GSS, releases on an operational scale cannot occur. While transgenic methods for sex-separation [43] and sterilization [44] are being developed, it is not yet clear that their potential advantages will be realized.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
HY carried out the experiments and collected the data for the egg-dieldrin exposures, and drafted the manuscript. JG as project supervisor, oversaw the design of the egg- exposure study, as well as the progress of the manuscript, and managed the collaboration between authors. CAM together with SMS created the GSS ANO IPCL1 and provided the manuscript for this section. MQB performed the dose–response assays, provided essential expertise for the egg exposure experiments and contributed greatly to the development and editing of the manuscript. CFO designed and performed the radiation induced sterility study and completed the statistical analysis for this study, as well as for the egg exposure experiments in this manuscript. All authors read and approved the final manuscript.

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Discussion générale

Ce travail a permis d'apporter des connaissances fondamentales sur le développement et la stérilisation de la souche à sexage génétique d'*An. arabiensis,* et représente une étape préliminaire importante au développement d'une technique de l'insecte stérile visant gérer les populations de ce vecteur de paludisme.

L'élevage peut-il avoir un effet négatif sur les souches ?

Nous avons pu mettre en évidence les pressions de sélection existantes sur la maturation sexuelle des mâles d'*An. arabiensis* en conditions d'élevage.

La maturation sexuelle des mâles de la souche de laboratoire Dongola s'est avérée très précoce par rapport à la même souche quatre années auparavant et par rapport à la souche sauvage. Il existe de nombreux exemples de sélections génétiques sur les insectes dues aux pressions d'élevage. Les conditions d'élevage de la souche d'*An. arabiensis* ont pu permettre de favoriser les tentatives d'accouplements individuels au dépend de la formation d'essaims pour s'accoupler, à cause de la petite taille des cages. De plus la gestion des stocks dans le laboratoire ne permettant pas la superposition des générations, les femelles récemment émergées ne peuvent donc être inséminées que par des mâles d'âge similaire. Étant donné que la durée de rotation des génitalia varie entre des individus de même âge pour une même température, il apparaît vraisemblable qu'une sélection de mâles sexuellement précoces ait pu se faire au fil des générations. Cette évolution a pu concerner également les femelles, cependant les données présentées ne permettent pas de le mettre en évidence.

Très peu de femelles inséminées par ces jeunes mâles ont effectué une ponte, laissant supposer que la quantité de sperme transféré ait pu être insuffisante pour induire la stimulation de l'oviposition ou que les mâles n'aient pas pu transférer les sécrétions séminales nécessaire à la formation d'un bouchon copulatoire ("mating plug").

La survenue de goulots d'étranglements dûs à l'élevage pourrait avoir des conséquences négatives dans le cadre d'une TIS si les différences entre mâles relâchés et mâles sauvages diminuent les capacités d'accouplement et de survie des premiers. Cette découverte fait ressortir la nécessité d'évaluer fréquemment les souches d'élevage afin de déceler d'éventuelles adaptations comportementales qui pourraient se révéler néfastes aux mâles lâchés sur le terrain. Par ailleurs cela souligne également l'attention particulière à apporter au sexage lors d'expériences impliquant des femelles vierges.

La souche ANO IPCL1 est-elle apte à être utilisée dans le cadre d'une TIS ?

L'élimination des femelles avant lâchers et la stérilisation des mâles sont des étapes critiques et obligatoires dans la TIS. Du fait de l'impossibilité de séparer les sexes de façon mécanique chez les *Anopheles*, la faisabilité de la TIS dépend pleinement de l'existence d'une souche de sexage génétique.

L'étude de la SSG ANO IPCL1 a démontré qu'elle possède de nombreux atouts tels qu'une bonne survie des stades immatures et adultes, un développement rapide et une forte fécondité. Les paramètres démographiques des souches Dongola et Sennar correspondaient à ceux de plusieurs souches Kenyanes, toutefois la comparaison entre différentes études reste délicate du fait des variations entre les protocoles, ce qui souligne également un besoin de standardisation.

La création d'une SSG entraine inévitablement une réduction de la fertilité qui a des conséquences indéniables sur l'élevage de masse. Le réarrangement chromosomique a principalement affecté la fertilité des mâles, cependant il n'est pas impossible que des effets

non détectés existent aussi au niveau comportemental et neurologique. Une réduction de la fertilité des mâles est observable chez toutes les SSG impliquant une translocation sur le chromosome Y. Cette semi-stérilité est attribuée au comportement génétique de la translocation au cours de la méiose et est proportionnelle à la complexité de la translocation (un grand nombre d'autosomes impliquera un plus haut niveau de stérilité). Cette faible fertilité aura un impact conséquent sur l'élevage de masse, toutefois le fort taux de survie larvaire, nymphal et adulte, ainsi qu'un temps de développement relativement court et une forte fécondité, permettront de faciliter la production de masse. La production moyenne de descendants mâles par femelle croisée avec un mâle ANO IPCL1 est de 13%. Afin de contrebalancer cette faible production et afin qu'un nombre adéquat de femelles contribue aux pontes, il sera nécessaire d'optimiser la survie et d'ajuster le sex-ratio opérationnel (ratio de mâles sexuellement actifs / femelles réceptives). A partir d'une petite colonie d'ANO IPCL1, il est estimé que deux générations d'amplification seront nécessaires pour atteindre un nombre suffisant d'adultes permettant la production de 10 millions de mâles stériles par semaine (M. Benedict, comm. pers).

L'utilisation de mâles semi stériles (issus de systèmes de translocation sur le chromosome mâle) a été proposée pour diminuer les densités de population d'insectes nuisibles, en remplaçant le caryotype sauvage normal par celui transloqué, et sans avoir recours à une stérilisation totale par irradiation (Serebrovskii 1940; Curtis & Hill 1968; Laven 1969; McDonald & Rai 1971). Les larves résultant d'accouplements entre mâles semi stériles et femelles sauvages permettraient de maintenir une compétition larvaire pour la nourriture dans les gîtes et ainsi d'éviter les effets de densité dépendance argués de permettre une meilleure survie aux larves sauvages (Yakob, Alphey, & Bonsall 2008). Toutefois, concernant ANO IPCL1, les coûts de fitness associés à la semi-stérilité semblent rendre son utilisation pour un remplacement de population non viable si les lâchers ne sont pas continus. En revanche, si les mâles sont totalement stérilisés par irradiation, ces coûts de fitness n'affecteraient pas l'effet des lâchers mais uniquement la production de masse, puisqu'aucune descendance ne survivrait.

ANO IPCL1 implique l'utilisation de la dieldrine, matière active toxique dont l'accumulation par des moustiques relâchés par millions dans la nature peut s'avérer problématique. De même, la gestion des eaux polluées par la dieldrine au moment du traitement des œufs pose de nombreuses questions. Le développement de procédures minimisant les résidus chimiques apparait donc capital. L'utilisation de méthodes transgéniques pour la séparation des sexes (permettant un tri par fluorescence, une élimination des femelles, ou une modification du sexe ratio) est vue comme une alternative aux SSG, cependant leur stabilité et les conséquences écologiques de leur utilisation ne sont pas encore élucidées.

Quelles sont les modalités de stérilisation de la souche ANO IPCL1 ?

L'irradiation induit des effets létaux dominants responsables d'une létalité dose dépendante chez la descendance. Cette mortalité se présente principalement durant les premiers stades du développement embryonnaire. Les taux de stérilité induite par l'irradiation chez les nymphes ou adultes d'ANO IPCL1 sont similaires à ceux induits sur les souches Dongola (Article 2, Figure 2) et KGB (Helinski, Parker, & Knols 2006). Cependant une différence considérable apparaît entre souches sauvages et cette SSG quant à la survie des descendants; ce phénomène a également été rapporté pour d'autres SSG (Robinson 2002b) et s'explique par la présence de triplications résultant de ségrégations adjacentes durant la méiose du parent mâle (Franz 2000). L'étude chromosomique rapportée dans l'Article 4, démontre la complexité de la translocation subie par ANO IPCL1, ce qui est cohérent avec la forte mortalité observée aux différents stades larvaires. Par ailleurs, cette mortalité larvaire tardive peut se révéler avantageuse suite aux lâchers de mâles stériles, en maintenant la compétition larvaire qui a lieu dans les gîtes larvaires sauvages.

Un des paramètres importants lors de lâchers TIS est la survie de descendants adultes suite à l'accouplement d'un mâle stérile et d'une femelle sauvage. La dose de radiation adéquate doit être choisie en conséquence. Ainsi la stérilité des souches de sexage

génétique devrait être évaluée en fonction de la proportion d'œufs se développant jusqu'au stade adulte plutôt que de la proportion d'œufs qui éclosent.

La forte stérilité héréditaire d'ANO IPCL1 possède un avantage potentiel puisqu'elle serait transférée aux descendants sauvages mâles suite à des lâchers. Par ailleurs, la radio stérilisation de souches semi stériles semble favorable dans le cadre d'une TIS ("Combi-Fly Concept" (Steffens 1982; Franz 2000)). En effet, la dose d'irradiation nécessaire à stériliser totalement les mâles d'ANO IPCL1 est plus faible que pour les mâles sauvages, du fait de la plus forte mortalité larvaire qui s'en suit. Une réduction de l'intensité des lésions somatiques peut ainsi être attendue, et la compétitivité des mâles stériles s'en trouvera améliorée.

La TIS contre An. arabiensis *à La Réunion ?*

Ces études préliminaires sur la souche ANO IPCL1 ont permis d'établir qu'elle possède un potentiel favorable pour permettre une réduction des populations sauvages. Toutefois les conditions d'élevage de masse, et notamment le ratio opérationnel mâles/femelles, devront être optimisées afin de contrebalancer une faible production de mâles.

La suite logique de ce travail sur *An. arabiensis* était l'étude du comportement des mâles d'ANO IPCL1 stérilisés face à des individus sauvages en conditions semi-contrôlées. Les difficultés de colonisation de la souche sauvage d'*An. arabiensis* à la Réunion n'ont malheureusement pas permis de réaliser ces tests de compétitivité. Ces complications constituent l'obstacle majeur au développement du programme TIS contre cette espèce à la Réunion, puisque les études de la souche ANO IPCL1 ainsi que les études comportementales et génétiques des populations locales d'*An. arabiensis* ont apportées des indications allant dans le sens d'une bonne probabilité de succès.

De façon à faciliter la colonisation d'une souche locale réunionnaise, il pourrait être envisagé de transférer une colonie d'*An. arabiensis* déjà adaptée aux conditions de laboratoire (telle que la colonie Dongola) à la Réunion afin de permettre des croisements avec les souches locales. Cela impliquerait d'évaluer les risques potentiels liés à l'introduction d'une souche exotique et nécessiterait le maintien en quarantaine.

PARTIE II. ÉTUDE DE LA CAPACITÉ DE REPRODUCTION DES MÂLES STÉRILISÉS *AEDES ALBOPICTUS*

Introduction

Ae. albopictus est présent en très forte densité sur l'île de la Réunion et les mesures actuelles de lutte anti-vectorielle ne parviennent pas à réduire ces populations. Ce vecteur de chikungunya et de dengue menace les populations humaines réunionnaises, et chaque année plusieurs cas de maladies sont enregistrés. Les services de lutte anti-vectorielle de l'ARS interviennent efficacement autour de chaque cas infectieux afin d'éviter la survenue d'épidémies. L'utilisation de la TIS pour lutter contre les populations d'*Ae. albopictus* à la Réunion semble constituer une voie prometteuse.

Suite à l'épidémie de chikungunya à la Réunion en 2005-2006, plusieurs études ont été menées de 2006 à 2009 sur la biologie, l'écologie, l'épidémiologie et le contrôle de ce vecteur à travers le programme Entomochik (Delatte *et al.* 2008). Le projet TIS, conduit par l'IRD/CRVOI, permet la réalisation d'études supplémentaires afin d'établir si l'utilisation de la TIS serait envisageable à la Réunion pour réduire efficacement les populations d'*Ae. albopictus*. Le laboratoire IPCL division jointe FAO/IAEA soutient le projet réunionnais ainsi que le projet TIS mené par le Centro Agricoltura Ambiente (CAA) à Bologne, Italie, sur *Ae. albopictus*, à travers le développement de méthodes appropriées pour l'élevage de masse, la stérilisation et les lâchers.

La TIS interférant avec la reproduction de l'insecte cible, il est essentiel afin de la mettre en œuvre de comprendre certains aspects de la biologie, de l'écologie du comportement reproductif du vecteur. Cette deuxième partie s'attache donc à étudier la biologie de la reproduction et l'effet de la stérilisation sur les performances des mâles d'*Ae. albopictus,* afin d'évaluer la compétitivité des mâles stériles et d'optimiser les procédés de stérilisation et les conditions de lâchers.

La littérature existante relative à la reproduction des culicidés du genre *Aedes* concerne principalement *Ae. aegypti*. Le comportement et le système reproductif des aedines diffèrent en plusieurs points de celui des anophelines. Les mâles ne requièrent pas la formation d'essaims afin de s'accoupler mais recherchent les femelles près des hôtes (lors du repas sanguin), des gîtes larvaires ou de repos (Gubler & Bhattacharya 1972). Lors de l'accouplement le mâle transfère un mélange de sperme et de secrétions des glandes accessoires dans la *bursa inseminalis* de la femelle (Jones 1968). Après quelques minutes, le sperme est transféré dans les spermathèques, au nombre de trois chez la plupart des espèces d'*Aedes*. Peu après leur dépôt dans la *bursa*, les secrétions deviennent plus visqueuses et de larges sphérules apparaissent et solidifient l'ensemble de la *bursa*; le contenu de la *bursa* se dissout dans les 48 h suivant l'insémination (Spielman 1964; Jones & Wheeler 1965). Ce contenu solide empêcherait la femelle de s'accoupler à nouveau (Spielman, Leahy, & Skaff 1967), de la même façon que le bouchon copulatoire chez les anophelines (Giglioli & Mason 1966; Rogers *et al.* 2009), ou le spermatophore chez d'autres espèces d'insectes (Landa 1960; Gerber 1970). Les femelles d'*Aedes* ont généralement été considérées comme monogames (Roth 1948; Weidhaas & Schmidt 1963; George 1967), mais de récentes études ont rapporté des taux de polygamies de 14% chez des femelles d'*Ae. aegypti* en conditions semi-contrôlées (Helinski *et al.* 2012b), et 26% chez des femelles d'*Ae. albopictus* capturées sur le terrain à la Réunion (Boyer *et al.* 2012).

*Stratégie de reproduction des mâles d'*Ae. albopictus *et effet de l'irradiation*

La stratégie de reproduction des insectes mâles est généralement optimisée en fonction de la quantité de sperme ou autre produit éjaculatoire disponible et de la disponibilité et du statut des femelles. L'étude de la stratégie des mâles d'*Ae. albopictus* est présentée dans l'Article 5. Ceux-ci s'accouplent généralement près des hôtes où il existe une forte probabilité de rencontrer des femelles vierges à leur premier cycle gonotrophique comme des femelles non vierges avant chaque cycle gonotrophique. Cette haute probabilité de rencontre des femelles pourrait résulter en une gestion parcimonieuse du sperme de la part des mâles. Nous avons examiné la stratégie de reproduction et l'utilisation du sperme

par les mâles et femelles vierges d'*Ae. albopictus* élevés en laboratoire, ainsi que l'effet de l'irradiation sur l'utilisation du sperme et sur les possibilités d'accouplements multiples.

Un mâle d'*Ae. albopictus* (souche Rimini, Italie) est capable de s'accoupler en succession rapide avec jusqu'à 10 femelles en quelques heures, et insémine environ la moitié d'entre elles (Article 5, Figure 3). Au cours de chaque accouplement, le mâle transfère un mélange de spermatozoïdes et de sécrétion séminale dans la *bursa inseminalis* de la femelle (Article 5, Figure 1). Le sperme est ensuite transféré aux spermathèques durant les 2 à 6 minutes suivantes. Une large quantité de substance séminale (sécrétion et spermatozoides) reste enfermée dans la *bursa* où elle se solidifie rapidement servant probablement de bouchon copulatoire pendant une période de 24 à 48 h. Les mâles continuent de s'accoupler même lorsque leurs réserves de matériel séminal s'épuise, et n'inséminent alors pas ou seulement partiellement les femelles (Article 5, Figure 3).

Lorsque deux mâles copulent avec la même femelle dans un intervalle inférieur à 40 min, tous deux peuvent dans environ 15% des cas participer à la fécondation des œufs (Article 5, Figures 4 & 5). En revanche si l'intervalle entre les deux accouplements est supérieur à 40 min, seul le sperme du premier mâle est utilisé par la femelle.

Un mâle stérile présente une capacité d'accouplement et d'insémination similaire à un mâle non traité, toutefois il n'était capable d'inséminer qu'un maximum de 7 femelles au cours de sa vie. Alors que les mâles d'*Ae. albopictus* sont capables de régénérer les réserves séminales épuisées après plusieurs inséminations, les mâles stériles ont perdu cette capacité. En dehors de cet effet, l'irradiation n'altère pas la capacité des mâles à s'accoupler et à transférer suffisamment de substance séminale pour empêcher une insémination ultérieure de la femelle.

*Survie des mâles stériles d'*Ae. albopictus *en conditions semi-contrôlées*

Dans le cadre d'une TIS, il est primordial que les mâles stériles puissent survivre suffisamment longtemps pour accomplir efficacement leur tâche de transmission de stérilité. Ce trait de vie a été examiné dans l'Article 6. Dans la nature, de nombreux facteurs peuvent altérer la survie des mâles, tels que la prédation, la disponibilité des ressources sucrées (Foster 1995; Gary, Cannon, & Foster 2009), ou les conditions de température et d'humidité (Alto & Juliano 2001). Le procédé de stérilisation et l'activité d'accouplement peuvent également influer sur leur capacité de survie.

La plupart des études de longévité existantes ont été menées en conditions de laboratoire, et ne reflètent donc pas la situation de terrain (Hawley 1988). L'estimation de la longévité sur le terrain ne peut se faire qu'à travers des études de marquage-recapture, toutefois une approche en conditions semi-contrôlées semble être la méthode la plus adaptée et la plus fiable pour étudier l'impact de divers paramètres sur la longévité d'un insecte dans un environnement naturel non contrôlé (Knols *et al.* 2003; Okech *et al.* 2003; Winkler *et al.* 2006).

Afin de prendre en compte l'effet des variations climatiques naturelles sur la survie des adultes d'*Ae. albopictus*, nous avons étudié la longévité des mâles sauvages (issus d'œufs collectés sur le terrain) et stériles en utilisant de grandes cages installées sur une clairière entourée d'arbres et de buissons. L'effet de l'apport de sucre dès l'émergence ou avec un retard de 48 h, ainsi que l'effet de la présence de femelles et donc de l'activité d'accouplement, ont été testés (Article 6, Figure 1).

Dans ces conditions semi-contrôlées, les mâles sauvages ont une longévité moyenne de 15,5 jours en l'absence de femelles et avec un apport de sucre immédiat. Dans cette situation, la longévité des mâles stériles est similaire (Article 6, Figure 3 & Tableau 1), cependant l'irradiation augmente significativement le risque de décès d'un individu (Article 6, Figure 5 & Tableau 2). La reproduction a un coût évident pour les mâles stériles comme sauvages car elle induit une augmentation du risque de décès. Toutefois, l'apport immédiat de sucre permet de compenser l'effet négatif de l'irradiation et de la reproduction. La durée de vie des mâles stériles ayant la possibilité de s'accoupler augmente de 5,5 à 11,6 jours lorsqu'ils sont approvisionnés en sucre dès l'émergence.

*Compétitivité des mâles stériles d'*Ae. albopictus *en conditions semi-contrôlées*

La réussite de l'utilisation de la TIS dépendant fortement de la compétitivité des mâles relâchés, celle des mâles stériles d'*Ae. albopictus* a été étudiée en conditions semi-contrôlées (Article 7). Les lésions somatiques engendrées par l'irradiation entrainent généralement une perte de la compétitivité des mâles, qui doit alors être compensée par l'augmentation du ratio de mâle stériles lâchés ou des mesures optimisant la qualité des mâles (additif à la nourriture larvaire, phéromones, période de repos et d'alimentation pré lâchers, irradiation au stade adulte au lieu de nymphe, etc.) (Calkins & Parker 2005).

La stérilisation des mâles de la souche réunionnaise d'*Ae. albopictus* a été effectuée par irradiation gamma (Cesium 137) grâce à l'irradiateur de l'Etablissement Français du Sang (EFS) de Saint Denis, La Réunion. Une dose de 40 Gy permet de réduire la fertilité des mâles à 4%, ce qui correspond aux résultats obtenus sur la même espèce après irradiation au Cobalt 60 (Balestrino *et al.* 2010) ou aux rayons-X (Yamada *et al*., manuscrit soumis). Pour des raisons pratiques (manipulation de l'irradiateur par les personnels de l'EFS), la dose de radiation choisie pour certaines expériences a été de 35 Gy; cette dose confère 93% de stérilité, résultat non significativement différent de 40 Gy (Article 7, Figure 1). Les mâles ainsi stérilisés ne recouvrent par leur fertilité après plusieurs accouplements ou une période de repos.

L'effet de l'irradiation sur la maturation sexuelle des mâles a été examiné et est apparu non significatif. Toutefois un ralentissement de la vitesse de rotation du génitalia des mâles et une plus faible proportion de femelles inséminées est observée pour les mâles stériles âgés de 15 à 20 h, par rapport aux mâles non traités (Article 7, Figure 2). Les premiers individus physiquement sexuellement matures sont observés 11 et 13 h après émergence respectivement pour les mâles non traités et les mâles irradiés. Quelques mâles sont capables d'inséminer des femelles dès 15 h après émergence, et la moitié des femelles présentes dans les cages est inséminées 25 h après émergence par les mâles non traités ou stériles.

Le taux d'insémination des femelles après 48 h ne semble pas être affecté par l'irradiation ou l'âge des mâles, ni le sexe ratio; en moyenne 93% d'entre elles sont inséminées (Article 7, Tableau 1). Lorsque de nouvelles femelles sont quotidiennement présentées à un mâle, un schéma cyclique de périodes de 5 jours de forte fréquence d'insémination est observé. Un mâle stérile insémine légèrement moins de femelle qu'un mâle non traité, cependant ces différences sont significatives seulement après le 9ème jour (Article 7, Tableau 2). L'âge des mâles stériles (1 ou 5 jours) n'affecte pas leur capacité d'insémination.

Afin d'approcher la réalité du terrain, des tests de compétitivité ont été réalisés en conditions semi-contrôlées. Les mâles stérilisés à 35 Gy ont été mis en compétition avec des mâles sauvages pour l'insémination de femelles sauvages (mâles et femelles sont issus d'œufs collectés sur le terrain). Les adultes âgés de 1 ou 5 jours étaient relâchés dans de larges enceintes en toile de moustiquaire, installées sur un terrain bordé de grands arbres (Figure 9). Des ratios de lâchers de 1:1:1 (mâle stérile : mâle sauvage : femelle sauvage) ou 5:1:1 ont été testés. Après 7 jours, les œufs pondus par les femelles étaient collectés et leur taux de fertilité a permis de calculer l'indice de compétitivité C (dont la valeur 1 indique une compétitivité égale à celle des mâles témoins) des mâles stériles (Article 7, Table 3).

Les mâles stériles relâchés avec un ratio 1:1:1 le jour de leur émergence, ont un indice de compétitivité faible (C=0,14). En revanche, une nette amélioration de cet indice (C=0,53) est observée lorsque les adultes sont maintenus en insectarium (avec un accès plus facile à une source de sucre) pendant 5 jours avant les lâchers. La fertilité de la population sauvage est alors réduite de 93 à 62%. Enfin, l'augmentation du ratio à 5:1:1 permet de réduire la fertilité de la population sauvage de moitié.

Figure 9. Enceintes en toile moustiquaire pour tests de compétitivité.
(CIRAD La Bretagne, Ile de La Réunion)

Article 5. Are male *Aedes albopictus* sexually prudent?

Submitted to PLoS Biology.

Are *Aedes albopictus* Mosquitoes Sexually Prudent?

Running head: Are *Aedes albopictus* Sexually Prudent?

PLOS Biology

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Abstract

Male insects have an interest in optimizing their reproductive strategy according to the availability of sperm or other ejaculatory materials, and to the availability and reproductive status of females. Here, we investigated the reproductive strategy and sperm management of male and virgin female *Aedes albopictus*, a mosquito vector of chikungunya and dengue fever. The dynamics of semen transfer to the bursa inseminalis and spermathecae were observed. The mating behavior of males was examined during a rapid sequence of copulations. Double-mating experiments were conducted to study the effect of time lapsed between two matings on the likelihood of a double insemination and the use of sperm for egg fertilization; untreated fertile males and radio-sterilised males were used for this purpose. Irrespective of the available sperm supply or accessory gland secretion material, male *Ae. albopictus* would mate with several females in rapid succession, sometimes resulting in partially inseminated, or uninseminated females. When two males consecutively mated the same female within a 40 min interval, the second male was sometimes capable of transferring semen, and only in ca. 15% of the cases did both males sire progeny. When the intervals between the matings were longer, sperm transfer to or sperm storage in the spermathecae was inhibited by the first insemination and all progeny were offspring of the first male. Our results provided no evidence of a prudent or parsimonious sperm use of *Ae. albopictus* males, who invest in multiple matings and copulate with pre-mated females with a low probability of transferring their genes to the next generation. *Ae. albopictus* females showed a steep decline in the possibility of being reinseminated although their willingness to mate appeared continuous. After the first insemination, the sperm transfer and storage within the spermathecae appeared inhibited by a short-term physical barrier followed by a long-term biochemical barrier. The outcomes of this study provide as well some essential insights with respect to the sterile insect technique (SIT) as a vector control method.

Blurb: Male *Ae. albopictus* invest in frequent copulations and copulate with pre-mated females, both with a low probability of success.

Introduction

The male insect's reproductive strategy shapes its fitness through the production and management of sperm, the capacity to acquire mates, ability to compete with other males, female choice, and investment in offspring. Male insects commonly invest little in individual sperm but instead increase reproductive fitness by maximizing both the number of sperm produced and the number of matings [1,2]. Nevertheless, sperm production can be costly and some species appear to have limited amounts of sperm at emergence [3]. In addition, sperm depletion after several matings has been demonstrated in numerous insect species [4,5,6,7,8]. In male invertebrates, mature sperm is stored in seminal vesicles or in testis before mating and is usually delivered to females either freely (as ejaculates) or in a package (as spermatophores). The resources needed to package sperm could be a limiting factor for male fitness [9,10,11,12]. Males thus have an interest to optimize semen usage according to the availability of sperm or other ejaculatory materials, to the availability and reproductive status of females (e.g. virgin or mated), and to patterns of female sperm utilization [9,13].

In mosquito species such as *Aedes aegypti* and *Anopheles arabiensis*, males can produce several thousand sperm cells, and total sperm production increases with age [14,15]. It has been shown that mating depletes the stock of spermatozoa in *Anopheles* mosquito males, but also prompts the testis to produce and mature new spermatocysts [16,17]. In some insect species, males appear not to be limited by sperm production during their lifetime [18], but in others spermatogenesis during the adult stage is negligible [19].

Contrary to *Ae. aegypti*, little is known about *Aedes albopictus* mating strategies, despite the fact that it is rapidly becoming a growing threat in Europe and other parts of the world making the development of control strategies for this pest insect increasingly pertinent. Fergusson et al [20] underlined the need for greater knowledge on male dipteran mating biology; even if male mosquitoes are not directly involved in disease transmission – understanding their mating behavior has critical consequences for vector control programmes that include the sterile insect technique (SIT) (classical SIT, or SIT using *Wolbachia*-modified or genetically modified mosquitoes) [21]. *Ae. albopictus* males can acquire mates in pair matings or through the formation of small swarms [22]. Both strategies generally occur near blood-meal sources [22], giving the male a high probability to meet either gravid females seeking a mate or non-virgin females seeking a blood-meal for a second or subsequent gonotrophic cycle. In such situations the likelihood of mating with non-virgin females is high, irrespective whether the females had their first mating a few days or an instant ago. In *Aedes* species, males inject sperm and a secretion produced by the male

accessory gland (MAG) into the female bursa inseminalis (BI) [23]. In *Ae. aegypti* a proportion of this sperm is then transferred to the spermathecae after a few minutes, with the rest solidifying in the bursa during the hour following copulation [24,25,26]. The solidification of the BI contents in *Aedes* mosquitoes [24] is thought to prevent further insemination, similarly to the mating plug in *Anopheline* species [27,28] or to the spermatophore in other insect species [29,30]. It is unknown whether males can make a distinction between virgin or inseminated females during the copulation act; however, the BI content may also provide evidence of recent mating for the successive males or sperm storage cues for the female. The influence of the relative availability of virgin or non-virgin females in addition to the risk of remating an already inseminated female will likely shape sperm allocation in male *Ae. albopictus.*

Aedes females are generally considered to be genetically monogamous, even though they may copulate several times [31,32,33]. In the laboratory, Gwadz and Craig [34] reported that 7.5% of female *Ae. aegypti* exposed to several males produced offspring fathered by multiple males. However, the significance of multiple inseminations occurring in the field has been questioned as the data seem to indicate that the second of subsequent matings are a result of either incomplete insemination in a previous mating [34] or the two mating events occurring within a few hours of each other [24]. Recent studies have reported that polyandry might not be so uncommon in nature as 14% of *Ae. aegypti* females were found to be carrying sperm from two males after 48 hours in semi-field enclosures in one study [35], and 26% of wild-caught *Ae. albopictus* females produced multi-sired progeny in another [36]. The occurrence of multiple inseminations could have consequences for the efficiency of sterile males during programmes with an SIT component if the sperm from both males is not equally competitive, and might require the use of a higher release ratio. It is therefore important to assess whether females inseminated by sterile males are more likely to remate than those inseminated by wild males, and to determine the use of both sperms for eggs fertilization. For other insect species, an increased incidence of remating by females mated with sterile males relative to fertile males has been ascribed to a smaller quantity of sperm transferred by the sterile males [37], or to a reduced efficacy of sterile males' accessory glands product to inhibit female receptivity [38].

The purpose of this work was to study sperm transfer in the female reproductive tract of *Ae. albopictus* and the management of sperm by conspecific males when mated in rapid succession or according to the female reproductive status. Sperm transfer and management in twice mated females were investigated as well. In order to differentiate which male had inseminated the females, untreated (fertile) and sterilised (X-ray ionized) males were used.

Materials and methods

Rearing procedures

The colony of *Ae. albopictus* used for the experiment originated from field collections in Rimini, Northern Italy and has been maintained under laboratory conditions at the Centro Agricultura Ambiente, Bologna, Italy.

The strain was transferred to the FAO/IAEA Insect Pest Control Laboratory, Austria in 2010, where adults were kept in a climate-controlled room maintained at 27 ± 1°C and 60 ± 10 % relative humidity with a light regime of LD 12:12 h photoperiod, including dusk (1 h) and dawn (1 h). Adults were kept in standard 30 x 30 x 30 cm cages (Megaview Science Education Services Co, Ltd, Taiwan) and continuously supplied with 10% wt:vol sucrose solution with 0.2% methylparaben [39]. Females were blood-fed weekly on defibrinated bovine blood using the Hemotek feeding apparatus (Discovery Workshops, Accrington, Lancashire, United Kingdom) and were allowed to oviposit in plastic beakers containing deionized water and lined with crêpe paper (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Five days after the blood meal, the crêpe paper with eggs attached was removed from the cage and left to dry at ambient conditions for 24 hours. The eggs were allowed to embryonate for at least one week. To hatch, eggs were counted and put in a closed, 1-litre jar with 0.7 litre of deionized water, 0.25 g of Bacto Nutrient Broth® and 0.05 g of yeast. Hatched larvae (less than 4 hours old) were transferred to plastic trays (40 x 29 x 8 cm) containing 2 litres of deionized water and fed a diet of finely ground (224 µm-sieved) Koi Floating Blend® (Aquaricare®, Victor, New York, USA). Pupae were collected and placed in small plastic cups inside a fresh adult cage for emergence.

Male sterilisation

Male pupae were irradiated in an X-ray irradiator (RS 2400, Rad Source Technologies Inc.) containing horizontal cylindrical canisters, which rotate around an X-ray tube. Pupae were maintained with minimal water using plates in a cylindrical canister designed specifically for irradiation of mosquito pupae. Male pupae, aged 24-40 hours, were irradiated with 40 Gy; at this dose male fertility averaged 5.7 ± 1.6 % (SEM). After irradiation, males were allowed to emerge in a laboratory cage and were provided with sugar solution.

Hereafter, fertile and irradiated males are referred to as untreated and sterile males, respectively.

Pair mating procedure and parameters measured

For all of the following experiments pair matings were carried out in an emergence tube (diameter 11.4 cm, height 9.7 cm, BioQuip, Rancho Dominguez, CA). One male and one female were introduced into the tube and the tube was gently shaken from time to time to stimulate copulation. Two observers were operating simultaneously on all treatments in order to minimise any observer effect. The latency period before copulation and copulation duration were recorded with a stopwatch, with the insertion of the *aedeagus* being considered the start of copulation. Immediately after copulation either the male or female was removed, depending on the type of experiment, and isolated in a small tube (diameter 2 cm, height 10 cm). In some cases, the male made several terminalial touches, i.e. brief genital contacts without seizing the cerci, before achieving coupling. The occurrence of this phenomenon was also recorded in all experiments.

Males and females used in the experiments were 2 days old, except when otherwise specified; all mosquitoes used in these experiments originated from the main colony with larvae reared under standard conditions.

Experiment 1. Copulation of virgin males and insemination success

The relationship between copulation and effective insemination was determined using virgin females in 105 couples involving untreated males and 88 couples involving sterile males. Each individual was only used for one mating. After mating, males were immediately frozen for later wing size measurements. Females were killed by freezing at least 1 hour after mating to allow sufficient time for sperm to reach the spermathecae. The spermathecae and BI were then dissected in a drop of saline solution. The number of inseminated spermathecal capsules was recorded as well as the presence of seminal fluid in the BI.

Experiment 2. Sperm transfer dynamics in the female reproductive tract

To determine the structural changes in BI contents following insemination and the dynamics of sperm transfer from the BI to the spermathecae, pairs consisting of a virgin female and either an untreated or sterile male were allowed to mate. After copulation, the female was killed by exposure to ether vapors [26] and the spermathecae and BI were dissected. Mosquitoes were dissected 1-6 min (n = 84), 15-30 min (n = 15), 40 min-1 h (n = 21), 6 h (n = 18), 24 h (n = 20) or 48 h (n = 34) after the start of copulation (half of the males dissected at each time period were untreated and half were sterile).

Experiment 3. Transfer of semen in once- or twice-mated females

To determine if a female that copulated twice possessed more semen in the BI and and in the spermathecae compared to once-mated females, the BI surface was measured and the spermathecae were dissected. Females were mated with either: 1) one untreated male (n=61); 2) one sterile male (n=15); or 3) two untreated males (n=66).

First copulations were considered when uninterrupted and lasting more than 30 seconds (decided based on the results from Experiment 1), and the male was then removed. Some females were then allowed to mate with a second male who was introduced immediately after removal of the first. In all cases the two mating opportunities were provided within a 1-hour interval. Females were dissected one hour after the first copulation; the number of inseminated spermathecal capsules was recorded as well as the presence or absence of seminal fluid in the BI, and a picture of the bursa was taken. The surface area of the bursa was used to estimate the quantity of seminal fluid in the BI using the formula $\pi \times 1/2a \times 1/2b$, where *a* and *b* were the two axes of the oval capsule (Figure 4A). Digital pictures and measurements were taken using 'analySIS B' software (Olympus Soft Imaging Solutions, Germany). Mating duration was also recorded for both first and second copulations.

Experiment 4. Fertility of females when mated twice

The effect of the time between two copulations on the use of sperm for egg fertilization was studied; a second mating opportunity was offered to a mated female, either "immediately after mating" (before solidification of the BI contents), "after 3 h"(when BI contents was solid), "after 48 h" (when BI contents had dissolved) or "after oviposition" (to test the effect of egg laying on female mating behavior). For each treatment two combinations were considered, where either an untreated followed by a sterile male ("untreated-sterile sequence") or vice versa ("sterile-untreated sequence") were offered to the female. Only females that copulated uninterrupted for more than 30 seconds in a first mating were offered a second mating opportunity. Each female was kept in a separate tube (diameter 2.5 cm) to assess individual fertility over several gonotrophic cycles (GC). Tubes were covered with thin netting, which allowed females to blood feed daily on the arm of a human volunteer. The bottom of the tube was lined with crepe paper and a small amount of deionized water to moisten the paper for oviposition. After each oviposition, the female was transferred into a new tube and the eggs were matured before hatching. The egg hatch rate was recorded for each gonotrophic cycle. High fertility indicated the use of sperm from untreated males and low fertility was associated with use of sperm from sterile males. Intermediate fertility values indicated the use of sperm from both untreated and sterile males for egg fertilization. After death, the female was dissected and the number of full

spermathecal capsules was recorded. Each interval treatment was replicated between 10 and 18 times with untreated and sterile males in each combination, using different cohorts. When the number of eggs oviposited by a female remained below 30, oviposition was considered incomplete and the fertility value was not taken into account for data analysis.

Experiment 5. Male's insemination capacity over multiple matings in rapid succession

Twenty-three untreated males and 25 irradiated males were used to determine the number of successive females that a male can fertilize in rapid succession. Four mating period were performed separated by resting periods of three days. Each 48-hour-old male was provided with 10 virgin females one at a time in succession, which were then removed after copulation before the next female was immediately added. Males were then allowed to rest in the emergence tube for periods of 3 days between subsequent mating periods; a cotton ball dipped into a 10% sugar solution was available. When males were 5, 8 and 11 days old, they were provided with 5 virgin females in rapid succession, in the same way as the initial 10 matings took place. If no mating occurred for one hour after addition of a female the test was stopped and the male was once again isolated and allowed to rest. Each mated female was isolated in a tube and frozen 1 hour after mating; the BI and spermathecae were dissected, and the surface of the BI was measured if well enough preserved.

Ethics Statement

No specific permit was necessary for the experiments carried out. Human volunteers for the blood-feeding were part of the team and co-authors.

Data analysis

All statistical analyses were carried out using R version 2.15.1. For all the tests, the alpha level was $P<0.05$. Shapiro and Bartlett tests were performed to test the normality and the homoscedasticity of the data, respectively.

The distribution and the average duration of copulation events were compared between untreated and sterile males using a two-sample Kolmogorov-Smirnov test and a two-tailed paired Student's t-test, respectively. The effect of sterilisation, copulation duration, or terminalial touches on the insemination success was tested using binary logistic regressions. The proportion of successful inseminations for each class of copulation duration and the proportion of females with 1, 2 or 3 filled spermathecae were compared between untreated and sterile males using a proportion test with Yates correction and a Pearson's chi-squared test, respectively. Logistic regressions with a three level categorical variables were used to test the effect of male irradiation, copulation duration, and the number of

previous matings on the number of full spermathecae. Differences between the duration of first and second copulations were tested using a two-tailed paired Student's t-test. Proportions of copulations lasting less than 30 seconds and proportions of males making terminalial touches were compared between copulation of once-mated females and second copulation of double-mated females using proportion tests with continuity correction. BI surface measurements were square root transformed to reach normality and homoscedasticity, prior to performing a one-way ANOVA to test the effect of the female mating status. For the double-mating experiment, the effects of the gonotrophic cycle, male treatment and the interaction of both, on female fertility and fecundity were tested using repeated-measures two-way ANOVAs. For the male rapid succession mating experiment, the effects of number of previous matings by the male (i.e. mating history), male treatment and the interaction of both on copulation duration were tested using repeated-measures two-way ANOVAs; their effects on copulation success (n observation=775), insemination success (n=557), and spermathecal fill (n=556) were tested using a generalized linear mixed model. The success of insemination for the first three females of each of the three remating periods was compared between untreated and sterile males using a logistic regression. Proportions of females with 1, 2 or 3 spermathecae filled were compared between untreated and sterile males using a Pearson's chi-squared test.

Values in the text are expressed as mean ± SEM.

Results

Experiment 1. Copulation of virgin males and insemination success

Copulation duration of untreated males varied from 11 to 338 s with one third lasting from 30 to 50 s (Fig. 1). Sterile males showed a similar frequency distribution of copulation duration (two-sample Kolmogorov-Smirnov test, D = 0.075, *P* = 0.95). The mean mating time was not significantly different between untreated and sterile males (two-tailed paired Student's t-test, t = 0.603, df = 191, *P* = 0.547); median values indicated a similar value of ca. 45 s (Table 1). Duration of copulation was separated into classes of 10 s, except when the number of observation was lower than 10.

Overall, ca. 80% of the copulations were successful (i.e. resulted in insemination) for both groups of males, sterile and untreated, and there was no effect of the irradiation treatment (binary logistic regression, z = -0.18, df = 182, *P* = 0.86). For a given duration class, the proportion of matings which resulted in insemination did not differ significantly between untreated and sterile males (proportion test with Yates correction, X^2 = 6.67, df = 9, *P* = 0.67). However, the probability of a successful insemination was significantly affected by

the duration of copulation (binary logistic regression: z = 2.17, df = 182, $P<0.05$); both short and very long copulations were less successful. For untreated and sterile males, respectively, 9.5 and 11.4% of all the copulations observed lasted ≤ 20 s and only 10% of these matings resulted in insemination (Fig. 1). Copulations lasting between 30 and 100 s were successful in 91.3 ± 3.4% and 93.3 ± 3.3% of the cases for untreated and sterile males, respectively. Twelve and 10% of the copulations from untreated or sterile males, respectively, lasted longer than 100 s; of these, 87% resulted in insemination for untreated males compared with 60% for irradiated males, however this difference was not significant (Pearson's chi-squared test, $X^2 = 1.11$, df = 1, $P = 0.29$).

When males made several terminalial touches before copulating with the female, the insemination success was significantly reduced to 52.4 and 38.9% for untreated and sterile males respectively (binary logistic regression, untreated males: z = 5.71, df = 98, $P<0.001$; sterile males: z = 5.38, df = 83, $P<0.001$).

Of all the successful matings, 91% resulted in the filling of two spermathecal capsules (Table 1); the proportions of females with 1, 2, or 3 filled spermathecae did not differ between untreated and irradiated males (Pearson's chi-squared test, $X^2 = 0.0998$, df = 3, $P = 0.99$). There was no effect of irradiation (logistic regression with three-level variable, z = 5.74, df = 146, $P = 0.88$) or copulation duration (z = 4.32, df = 146, $P = 1$) on the number of spermathecal capsules filled. Less than 5% of females had all 3 spermathecal capsules filled, and in half of these females only a small portion of the third capsule was filled with sperm.

Experiment 2. Sperm transfer dynamics in the female reproductive tract

After copulation, the transferred sperm was first stored in the empty BI (Fig. 2A & B). The transfer of sperm cells from the BI to the spermathecae was initiated 2 to 3 minutes after the start of copulation, and was terminated for all females 6 minutes after the start of copulation. A large quantity of sperm cells were not transferred to any spermathecae but remained trapped in the bursa (Fig. 2C).

The semen content inside the bursa started to solidify during the first fifteen minutes after copulation. The solidification could be visually observed as the granular mass appeared denser and the movement of the sperm cells became sparser, starting at the base of the BI, close to the junction with the common oviduct. All the BI observed 40 min to 6 h after copulation appeared completely solid. The beginning of the dissolution of the BI contents was not observed, but 80% of the females had an empty BI after 24 h, and depletion was complete in all the females dissected 48 h after copulation. After dissolution of the BI contents, a very small quantity of remnant material could be observed in the BI (Fig. 2D).

Experiment 3. Transfer of semen in once- or twice-mated females

No difference was found between the mean duration of the second copulation of twice-mated females as compared to that of once-mated females (two-tailed paired Student's t-test, t = 0.307, df = 81.1, $P = 0.76$). However, the proportion of copulations lasting less than 30 s differed significantly (48.5% for second copulation of twice-mated females, and 4.5% for once-mated females; proportion test with continuity correction: $X^2 = 31$, df = 1, $P<0.001$).

The number of matings significantly affected the BI surface area in females (one-way ANOVA, $F_{(4, 120)} = 9.93$, $P<0.001$; Fig. 3). The BI surface area of once-mated females with either an untreated or a sterile male was significantly lower as compared to twice-mated females, when the interval between the two matings was less than 40 min (Tukey Post-Hoc tests, $P<0.001$ for both untreated and sterile mates). Around 11% of the females mated twice in an interval of less than 40 minutes showed a higher BI surface area than the maximum surface observed in once-mated females. In a few twice-mated females two distinct bodies of semen were visible in the BI (Fig. 4). When the second mating occurred more than 40 minutes after the first one, the BI surface area was not significantly different than that of females mated once or females that were mated twice in an interval of less than 40 minutes. The number of matings did not affect the number of filled spermathecal capsules in the females (logistic regression, z = 1.48, df = 126, $P = 0.52$).

Experiment 4. Fertility of females when mated twice

Figure 5 represents the mean fertility of each mating treatment over several GCs, according to the mating sequence. In the single mating treatment, the average fertility of females mated by untreated or sterile males was 92.9 ± 1.7 and 1.3 ± 0.7%, respectively, for the first GC, and remained similar for all the following GCs (repeated-measures ANOVA, untreated controls: $F_{(1,27)} = 1.14$, $P = 0.3$; sterile controls: $F_{(1,7)} = 0.49$, $P = 0.51$).

Twice-mated females showed a similar fertility to their respective controls except in the situation where the second mating occurred immediately after the first one (one-way ANOVA, untreated-sterile sequence, $F_{(4,164)} = 4.07$, $P<0.01$; sterile-untreated sequence, $F_{(4,105)} = 9.21$, $P<0.001$). In the treatment where females mated twice within an hour, fertility of the females was intermediate i.e. 13.3% and 16.7% in the untreated-sterile and sterile-untreated mating sequences respectively (i.e. fertility at the first GC between 10 and 75%). This was indicative for sperm being used both from sterile and untreated males to fertilize the eggs. The fertility of females from the untreated-sterile mating sequence decreased during the second and subsequent GCs (repeated-measures ANOVA, $F_{(1,4)} = 36.4$, $P<0.01$), whereas the fertility of females from the sterile-untreated mating sequence remained stable

during the second and subsequent GCs ($F_{(1,2)}$ = 1.57, *P* = 0.34). All other twice-mated females were inseminated only by one male, as judged by either high fertility or high sterility which remained stable during all the GCs (repeated-measures ANOVA, $F_{(1,289)}$ = 3.65, *P* = 0.057).

The intervals separating the two copulations of twice-inseminated females ranged from 4 to 35 minutes. The mean fecundity was 64 ± 5 eggs per female for the first GC, decreasing slightly to a mean of 51 ± 7 eggs at the 5th GC. The fecundity varied significantly over the GCs (repeated-measures ANOVA, $F_{(1,301)}$ = 873, *P*<0.01), except during the first two ($F_{(1,219)}$ = 3.62, *P* = 0.058). The fecundity was independent from the father(s) treatment status ($F_{(4,298)}$ = 2.04, *P* = 0.088).

The number of spermathecae filled, observed after the death of the females, appeared not to be related to the number of GCs completed. 74% of females had 2 full spermathecae whether they had completed 0 or 7 GCs; all the females dissected after 6 GCs had still two spermathecae filled.

Experiment 5. Male's insemination ability over multiple matings in rapid succession

The first sequence of 10 copulations in rapid succession lasted from 1 to 6 h for a single male, with a mean of 3.5 h for both untreated and sterile males. The time separating two successive copulations ranged from 0 to 2 h with an average of 15 min for both untreated and sterile males. Copulation duration was significantly affected by the male treatment (repeated-measures ANOVA, $F_{(1,544)}$ = 5.00, P<0.05) but not by the number of previous matings across all mating periods ($F_{(1,544)}$ = 1.72, *P* = 0.191).

Refusal or failure of males to copulate increased with the number of previous matings (Fig. 6A). Male copulation success (regardless of the insemination success) was significantly affected by irradiation (generalized linear mixed model, log likelihood = -230, z = 2.0, *P*<0.05) and number of previous matings across all mating periods (z = -12.44, *P*<0.001). During the first mating period, more than 90% of the males whether untreated or sterile, copulated with all of the first 6 females. When subsequent females were offered there were fewer attempts or success to copulate for sterile males as compared to untreated ones. More than half of the males copulated during the second mating period, but the percentage of copulations strongly decreased over time and with each mating period.

In all copulation attempts, untreated males were able to inseminate females during each mating period, although a cyclic pattern of decreasing and increasing insemination success was observed (Fig. 6B). During the first mating period, the insemination success of a 2-day-old untreated and sterile male averaged 80 ± 9.2% and 95.8 ± 4.2%, respectively,

for the first female offered. This percentage decreased progressively to 16.7 ± 9% and 17.6 ± 9.5% for the 10th copulation with untreated and sterile male respectively. During the next three mating periods, semen was transferred at least to the BI of the females in on average 70.6 ± 1.1% of the first copulations with untreated males against 42.9 ± 1.1% during matings with sterile males. The insemination success of males was significantly affected by male treatment (generalized linear mixed model, log likelihood = -351, z = 2.99, P<0.01), number of previous matings across all mating periods (z = -6.4, P<0.001), and the interaction of both (z = -4.42, P<0.001).

The spermathecae and BI filling varied greatly over the series of copulations (Fig. 6B), and was significantly affected by male treatment (generalized linear mixed model, log likelihood = -303, z = -4.86, P<0.001), number of previous matings across all mating periods (z = -2.37, P<0.05), and the interaction of both (z = -6.46, P<0.001). During all mating periods untreated and sterile males were able to transfer sperm to at least one spermatheca of a maximum of 11 and 7 females, respectively, and to transfer semen to the BI (but not spermathecae) of an additional maximum of 9 and 8 females, respectively. More than 80% of the first five females inseminated by an untreated male during the first mating period had 2 spermathecae filled with sperm. This percentage decreased to less than 50% for the sixth and subsequent females in the sequence. After the 6th female an increasing proportion of females (from 18% for the sixth, to 66.7% for the tenth) had some semen in the BI but no sperm was transferred to any spermathecae. After a resting period of three days, untreated males recovered the capacity to fill two spermathecae in 100% of the first two females presented in the second and third mating periods. During the fourth mating period, fewer females were inseminated and of those only half of the matings resulted in 2 spermathecae being filled. The proportions of females with 0, 1 or 2 spermathecae filled with semen were not significantly different between females mated with sterile and untreated males over the first mating period (Pearson's Chi-squared test, X^2 = 1.6, df = 3, P = 0.66). However, during the following mating periods the number of spermathecae filled in each mating continued to decrease, and the proportions differed significantly between females that mated with sterile and untreated males (X^2 = 38.8, df = 3, P<0.001). Fifty, 83 and 100% of the females inseminated by a sterile male had semen only in the BI during the second, third and fourth mating periods, respectively. In some instances where only the BI was filled with semen, observations under the microscope indicated the presence of sperm cells but little granular mass in the BI.

Discussion

To maximize the overall lifetime reproductive success, males from numerous species have evolved prudence mechanisms in ejaculate allocation. For example, partitioning sperm between each of a rapid series of mating events might prevent all mature sperm from being released at once [9], thus enabling the successful insemination of more than one female. However, in species with a short lifespan that are subjected to a high predation risk, such as insects, it might not be as crucial to parcel sperm ejaculate but rather more effective to transfer the maximum amount during the first matings to ensure paternity.

This study has shown that *Ae. albopictus* males transferred a large amount of accessory gland (AG) substance together with motile sperm during mating; this package is stored in the BI before migration of the sperm cells to the spermathecal capsules. The transfer of sperm did not occur immediately after coupling, as was evidenced by the absence of sperm transfer during most of the copulations that lasted less than 30 s. This suggests that the first moments of the copulation act are probably devoted to concentrating sperm and accessory material in the male reproductive organs before being transferred to the female. A similar mechanism of voluminous material transfer has been observed already for *Ae. aegypti* [24,40] but such a period of sperm concentration does not appear to be necessary for this species since Spielman et al [24] reported that the transfer of semen from the male to the female BI occurred after only 4 s of contact, and successful insemination occurred after 6 s. The average duration of copulation observed in this present study was 45 s for our *Ae. albopictus* strain in emergence tubes under free mating conditions. On the other hand, the duration of successful matings of *Ae. aegypti* was only 13 s as measured in a lantern chimney [40] and 16 s in larger cages [31]. The time for sperm to migrate from the BI to the storage organs was variable in our experiments, but for all females it was completed within the first 6 minutes after the start of copulation, similarly to *Ae. aegypti* [40]. The solidification of the BI contents started a few minutes after insemination and was finished around 40 minutes to 1 hour after insemination, with a decreasing motility of the sperm cells trapped in the seminal fluid being observed over time.

A large amount of the material transferred during copulation remained trapped within the BI by solidification of the granular content. The numerous sperm cells trapped appeared mostly inactive a few hours after copulation. Jones and Wheeler [40] estimated that 62% of the sperm transferred by *Ae. aegypti* reached the spermathecae, the remaining cells being trapped in the MAG secretion, and even when the presence of this sperm mass was observed in the BI, the third spermatheca was filled with sperm in only ca. 4% of the female *Ae. albopictus* inseminated by virgin males in this study. It is still unclear why the transfer of spermatozoids usually stops after the filling of two spermathecae despite large amounts of

sperm cells remaining in the BI [26]. In evolutionary terms, the persistence of this behaviour to not use all the transferred sperm can only be explained by males gaining benefits greater than the cost associated with this behavior or the unused sperm must lead to no costs being incurred, therefore causing no counter selection to occur.

One of the benefits of this unused sperm is that the solidification of the BI content may act as a physical barrier preventing a second insemination [41]. In our study, a male *Ae. albopictus* was unable to induce an already mated female to store his sperm if his mating event took place more than 40 minutes after the female's first matin, which corresponds to the duration of complete solidification of BI content. In this situation, we observed no visual signs of semen transfer. However, when the period separating two matings was shorter than 40 minutes the larger surface area of the BI in ca. 11% of twice-mated females compared with females mated only once suggested that the second male transferred semen as well as the first. The storage in the spermathecae and use for the egg fertilization of this second mass of sperm was observed in 15% of females mated twice in an interval shorter than 1 h. Craig [33] likewise reported that a second successful insemination might be possible for *Ae. aegypti* if mating occurred before the solidification of the BI content. The fertility of those twice-inseminated female *Ae. albopictus* ranged from 30 to 65% in the first GC. Over successive GCs, fertility of individual females decreased or remained stable, but no common pattern could be observed. We can hypothesize that variation in fertility level depends on the respective amounts of sperm transferred by each male, which might vary with the interval between the two mating events. After insemination, the female probably transfers a portion of the sperm from the BI to store it in the spermathecae. It is not known whether the sperm cells from each mating are distributed randomly between the spermathecae, nor if the sperm from each spermatheca is used equally for egg fertilization. Yet, when dissecting females that had completed up to 7 GCs, often two spermathecae were found filled with semen, suggesting that the sperm used for fertilizing the eggs did not originate from one spermatheca only.

However, the physical barrier created by the solidified mass in the BI is not the only mechanism that would prevent a second male from siring offspring. We reported the eventual dissolving of the BI content twenty-four hours or more after copulation, which was similar to what has been reported for *Ae. aegypti* [41]. Even after dissolving the content of the BI in female *Ae. albopictus*, the uniformity of the fertility rate of a female's progeny over several GCs indicated that only one type of sperm was present in the storage organs. Our results suggest that a second insemination was still not effective even after a blood-meal and oviposition. A similar situation was reported in *An. gambiae* females over five GCs [42]. Williams & Berger [43] reported that female *Ae. aegypti* became receptive once more after the 4th and particularly 5th GC (*i.e*. 6 and 22% of the females, respectively), although they

observed a transfer of semen to the BI, they could not demonstrate that the sperm had migrated to the spermathecae and was used for egg fertilization.

In other mosquito species, the MAG substances transferred to the BI are known to induce various genetic, physiological, and behavioral changes in the female [44,45] such as the inhibition of female receptivity to further matings through the action of a hormone called matrone [34,44,46,47,48,49]. Spielman et al [24] reported that during copulation with non-virgin females or females implanted with MAG substances, males were not capable of transferring semen to the BI. They hypothesized that the physical condition of the bursa was responsible for the absence of subsequent inseminations, as the seminal mass could not be retained in the female BI or would be withdrawn by the male at the termination of coitus. However Gwadz et al [50] reported no evidence, using radioactive labeled males, of semen wastage after coupling and no transfer of any seminal material to refractory females. Rather than a female preventing further insemination by seminal expulsion, they theorized that males would ejaculate only when they detected female sexual receptivity. Male *Ae. aegypti* have been observed to prematurely terminate copulation when mating with non-virgin females [32]. We reported that 10 times more second copulations of *Ae. albopictus* females lasted less than 30 s compared to first copulations. In addition, the average BI surface area of females mated twice in an interval of time longer than 40 minutes in this study was not significantly larger than the BI surface area of females mated only once, suggesting that semen may not be transferred or stored. In bumblebee and mite species a copulation with pre-mated females results in a transfer of a smaller quantity of sperm [51,52]. In addition, it has been shown that in female *An. gambiae* insemination induces molecular and structural changes a few hours after the mating, which suggests that the atrium tissue would become hermetic to further insemination [53]. A recent study with *Ae. albopictus* and *Ae. aegypti* reported that females injected with MAG proteins remained virgin for a long-term, but this effect was delayed of 2-3 days post-injection [48]. It therefore appeared that, similarly to tephiritids [54,55], the possibility of re-insemination of *Ae. albopictus* females is inhibited in the short term by sperm presence and the solidification of the BI contents, but a delayed longer-term biochemical inhibition is also induced by the accessory gland products.

Although pre-mated female *Ae. albopictus* showed a steep decline with time in the possibility to be reinseminated, no decline in the willingess to mate was observed as they readily accepted copulation in our experiments. Most of the published literature concerning *Aedes* species states an inhibition of female sexual receptivity following the first insemination, however we argue that this term is inappropriate, it is rather the sperm transfer to the spermathecae that appears to be inhibited by the first insemination. Considering the outcomes of our study and the recent report of multiple-inseminated female *Ae. albopictus* encountered in the field [36], we can assume that virgin females are subjected to several

mating attempts by different males in a short period of time and probably soon after emergence or during their first blood-meal. Female *Ae. albopictus* receive a large quantity of sperm cells, which is sufficient for the fertilization of the eggs during her lifetime and should favor a monoandrous behaviour; on the other hand, they also receive a large amount of secretion from the MAG, which in some insect species has a nutritional value as well and thus would favor polyandry [56]. In other insect species, female sexual receptivity is dependant on the spermathecae filling; as for most of the females *Ae. albopictus* the third spermathecae remains empty, it is possible that not enough "anti-receptivity" factor was transferred.

In the rapid sequence mating experiment, male *Ae. albopictus* usually copulated with all 10 females they were offered, but fully inseminated only the first five. The amount of semen transferred to most of the last five females decreased progressively, resulting in only one spermatheca filled or none at all in the end, even though the BI was always filled. A similar phenomenon was reported for *Ae. aegypti* in rapid sequence matings [34,57]. No experiment was done to determine if the depletion of MAG substance or sperm supply was responsible for this lack of insemination. However, Jones [58] reported the complete depletion of sperm from the seminal vesicles, *vas deferens* and *vas efferens*, and depletion of secretory material from the accessory glands in *Ae. aegypti* after 5 successive inseminations. The MAG secretion is believed to serve as a transport medium for the spermatozoa [41], therefore a lack of secretion might make migration of spermatozoa impossible. The impact of such mating without insemination is unknown, though even when the spermathecae are not filled, the presence of MAG substances in the BI of *Ae. aegypti* and *Ae. albopictus* females has been shown to be sufficient to provide an oviposition stimulus [59]. This probably diminishes the female propensity to remate, therefore increasing the relative fitness of the first male she mated. Such a phenomenon has been suggested in the case of copulation of sperm-depleted hymenoptera males [60]. On the other hand, an incomplete transfer of sperm or MAG substance, following insemination by a partially-depleted male or an interrupted copulation is responsible for multiple inseminations in female *Ae. aegypti* [34] and *An. freeborni* [61]. Gamete management has evolved in male insects in response to factors such as the number, quality and spatial and temporal dispersion of the reproductive opportunities that adults encounter [3]. In *Ae. albopictus*, females (mating opportunities) appear to be aggregated spatially and temporally around blood meal sources. The ability of males to successively inseminate at least 5 females during a day would enable them to take advantage of such high probabilities to encounter females.

After a resting period without sexual activity, male *Ae. albopictus* were once again able to fully inseminate females in our study. It has been shown in some mosquito species, including *Ae. aegypti,* that the maturation of new sperm cells as well as a replenishment of MAG secretory material occurs after depletion [16,58,62,63]. This ability to recover after multiple matings may be reduced by a smaller number of gonial cells in the later maturing spermatocysts which would not be identical for all males [16]. We observed an increasing number of refusals or failures of males to copulate over the course of successive remating periods. Jones [58] reported a similar celibate behavior or failure to ejaculate in depleted-but-replenished male *Ae. aegypti*, but the reason for this behavior is still unknown. As for many insect species, *Ae. albopictus* females in these experiments accepted copulations with males that had exhausted their sperm supply. The cost of this male strategy to females and/or the probability of this type of mating are probably low because otherwise behaviors that would protect females against mating with sperm-depleted males would likely be observed.

Our results provided no evidence of a prudent or parsimonious sperm use in *Ae. albopictus* males. Indeed, males invested in frequent copulations even when their semen supplies were depleted, and they copulated with already mated females even though these matings had a low probability of fertilizing embryos. No signals appeared to have evolved in males to detect the status of their own semen supply or the mating status of females. Because of the limitation of the BI capacity, the amount of semen transferred by the second male to attempt mating highly depends on the quantity transferred by the first male, which itself depends on the male's mating history. If they cannot detect the non-virginity of the female, it appears that the logical strategy of both the first and second male would be to transfer as much semen as possible in order to maximize the chances of paternity. However, in view of the short life span and the high competition amongst males, this behavioral tactic is likely an adaptive solution to successful mating.

The outcomes of this study provide as well some insights with respect to the SIT as a vector control method. Copulations with sterile males did not differ from those by untreated males in duration or quantity of sperm transferred, as estimated from the BI surface. However, sterile males were not able to replenish their reproductive stock once depleted. Over its lifetime, one sterile male might fully inseminate (fill at least one spermatheca of) a maximum of 7 females, and might transfer a partial amount of semen (filling only the BI) to a further average of 8 females. Irradiation damage is higher in the earlier stages of spermatogenesis (spermatocytes and spermatogonia) than in mature sperm cells, impeding the immature spermatocysts from developing further [64]. For other insect species, a reduction of sperm quantity or quality [65,66,67,68] and a faster emptying of males' testis

have been reported [37,69]. Although we observed that the total number of females inseminated by a sterile male was lower than for a wild male, the ability to copulate and inseminate *per se* appeared to be unaltered by the irradiation. Similar outcomes have been reported for the La Reunion strain of *Ae. albopictus* during the first 9 days of mating; the subsequent reduction in mating ability observed was likely due to a depletion of semen in sterile males [70]. However, it might be of interest to further investigate whether the induction of sterility is effective when a sterile male transfers only low quantity semen to females after previously copulating several times.

In the context of SIT it is critical to understand how sperm dynamics of irradiated males can influence female reproductive and remating behavior, and determine whether multiple mating, caused by unequal insemination ability (quality and quantity of sperm and MAG products transferred) in sterile and wild males, could decrease the efficiency of SIT [71]. In the current study, sterile *Ae. albopictus* males proved able to inseminate females similarly to untreated males, at least in the first few copulations, and apparently transferred enough semen to the females to also prevent a further insemination. Mating by a sterile male did not lead to an increased chance of a second insemination of the female than if the first mating was with an untreated male. Therefore in an SIT programme released sterile males could be expected to compete equally with wild ones, providing that they survive well and are able to locate the wild females under the natural conditions. However, this highlights the necessity of identifying the mating sites for this species in order to optimize the sterile males release strategy. Furthermore, if the final goal of the use of SIT is eradication, the occurrence of multiple mating may result in the necessity to increase the release ratio.

These laboratory-based investigations involved colonized mosquitoes, and the behavior of males and females in response to a first mating has to be verified in wild mosquitoes. However, this study allowed greater understanding of the mechanisms of sperm transfer in various situations and gives an indication of the sexual strategy of male *Ae. albopictus* and on the likelihood of multiple insemination of females of the species.

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Tables

Table 1. Duration of copulation, insemination success and spermatheca fill for untreated and irradiated male *Ae. albopictus*. 105 and 88 copulations were observed for untreated and sterile males, respectively. There was no significant difference between untreated and sterile male values for any of these parameters ($P<0.05$)

Male	Copulation duration (s)		Successful insemination (%)	Spermathecae fill per copulation as proportion of total (%):		
	Mean ± sem	Median		1 capsule filled	2 capsules filled	3 capsules filled
Untreated	61 ± 4.7	44	80.8	5	91.3	3.8
Sterile	56.3 ± 3.9	47.5	79.8	4.5	91	4.5

Figures

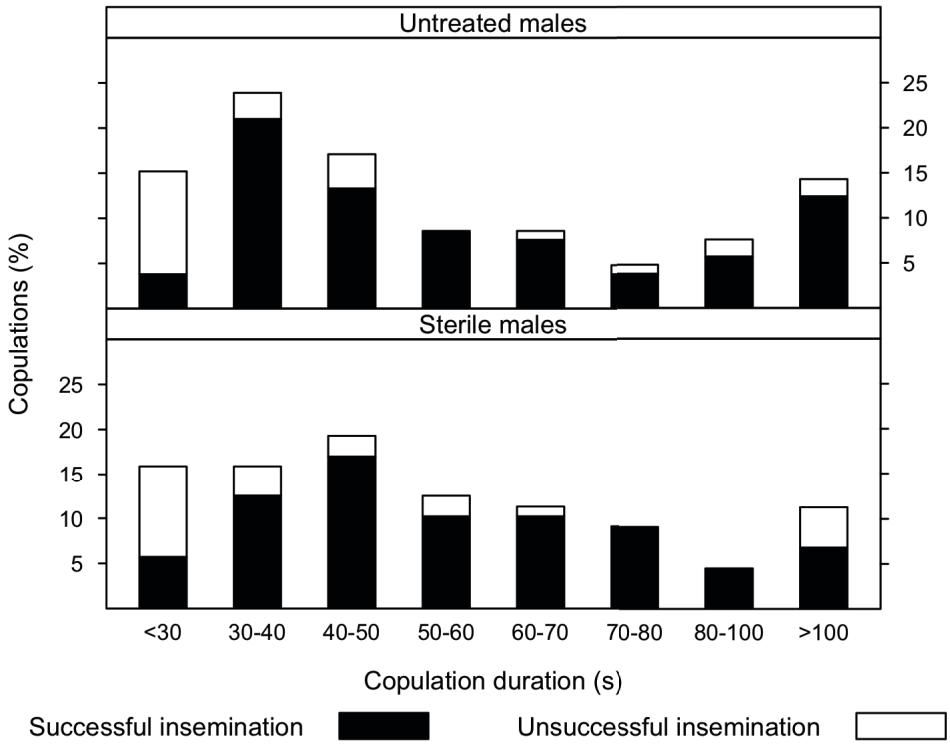


Figure 1. Copulation of virgin male *Ae. albopictus* and insemination success. Percentage of copulations with untreated or sterile males leading to successful (black bars) or unsuccessful (white bars) insemination, in relation to copulation duration.

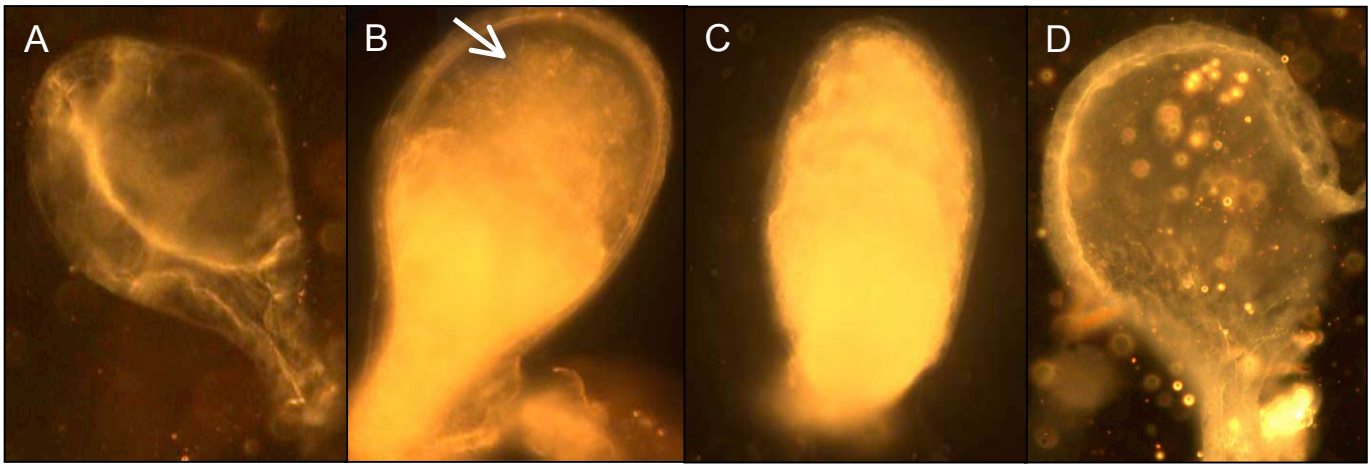


Figure 2. Photographs of the bursa inseminalis (BI) of a virgin female *Ae. albopictus* (A), and inseminated females dissected after 5 min (B), 1 hour (C), and 48 h following insemination (D). The arrow indicates sperm cells still motile in the BI.

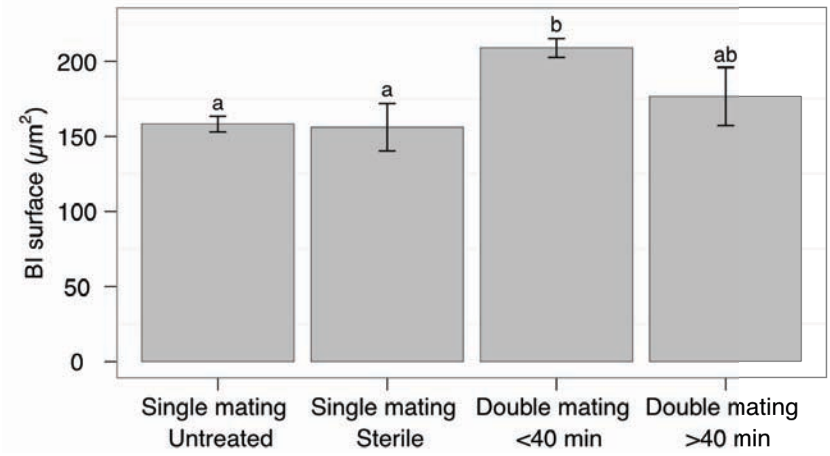


Figure 3. Sperm transfer to the *Ae. albopictus* female bursa inseminalis (BI): Mean (+ SEM) BI surface area (μm^2) according to the mating status of the female. N was 61, 15, 57 and 9, respectively, for females inseminated once by an untreated male or a sterile male, and females inseminated twice in an interval shorter or longer than 40 min. Bars with different letters are significantly different: ANOVA, P<0.05.

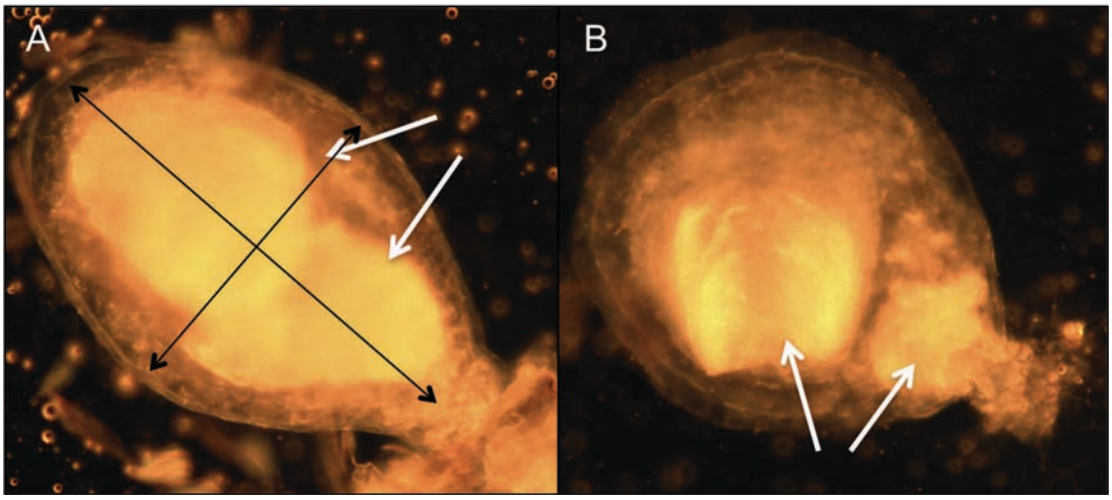
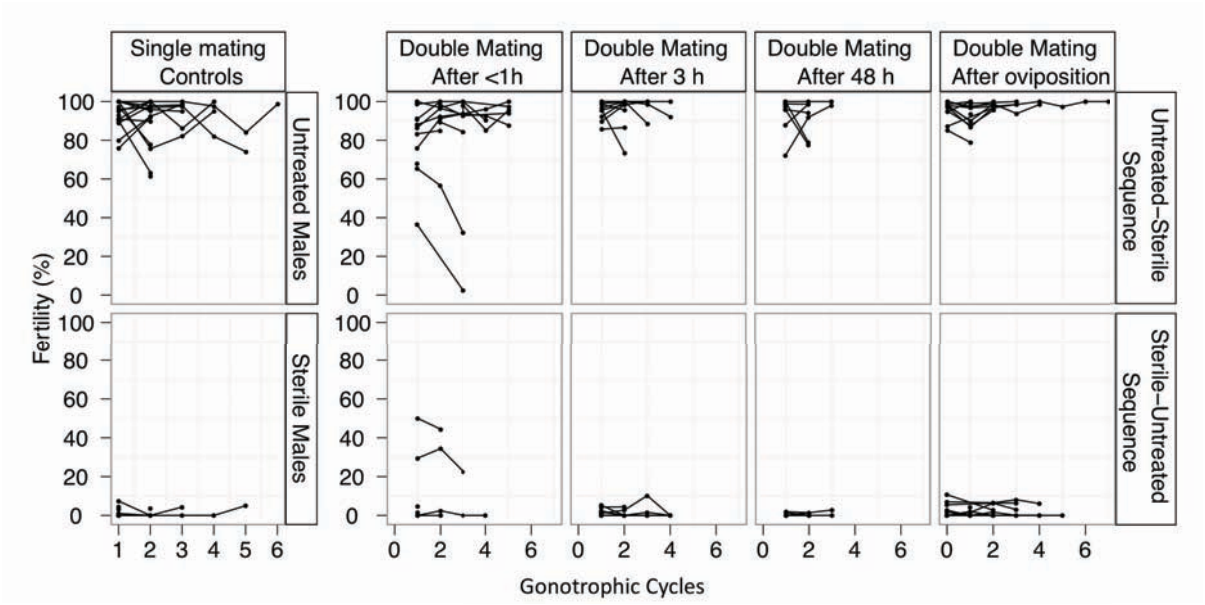


Figure 4. Photographs of the bursa inseminalis (BI) of female *Ae. albopictus* mated twice. The white arrows indicate the two distinct masses of semen transferred successively by two males. The black arrows in A indicate the measurements taken for the bursa surface analysis.



846

847 **Figure 5. Fertility of female *Ae. albopictus* mated once with an untreated or sterile**
848 **male, or twice with males in untreated-sterile or sterile-untreated mating sequences.**
849 Individual fertility of females over multiple gonotrophic cycles, after a single mating or two
850 matings separated by different intervals of time.

851
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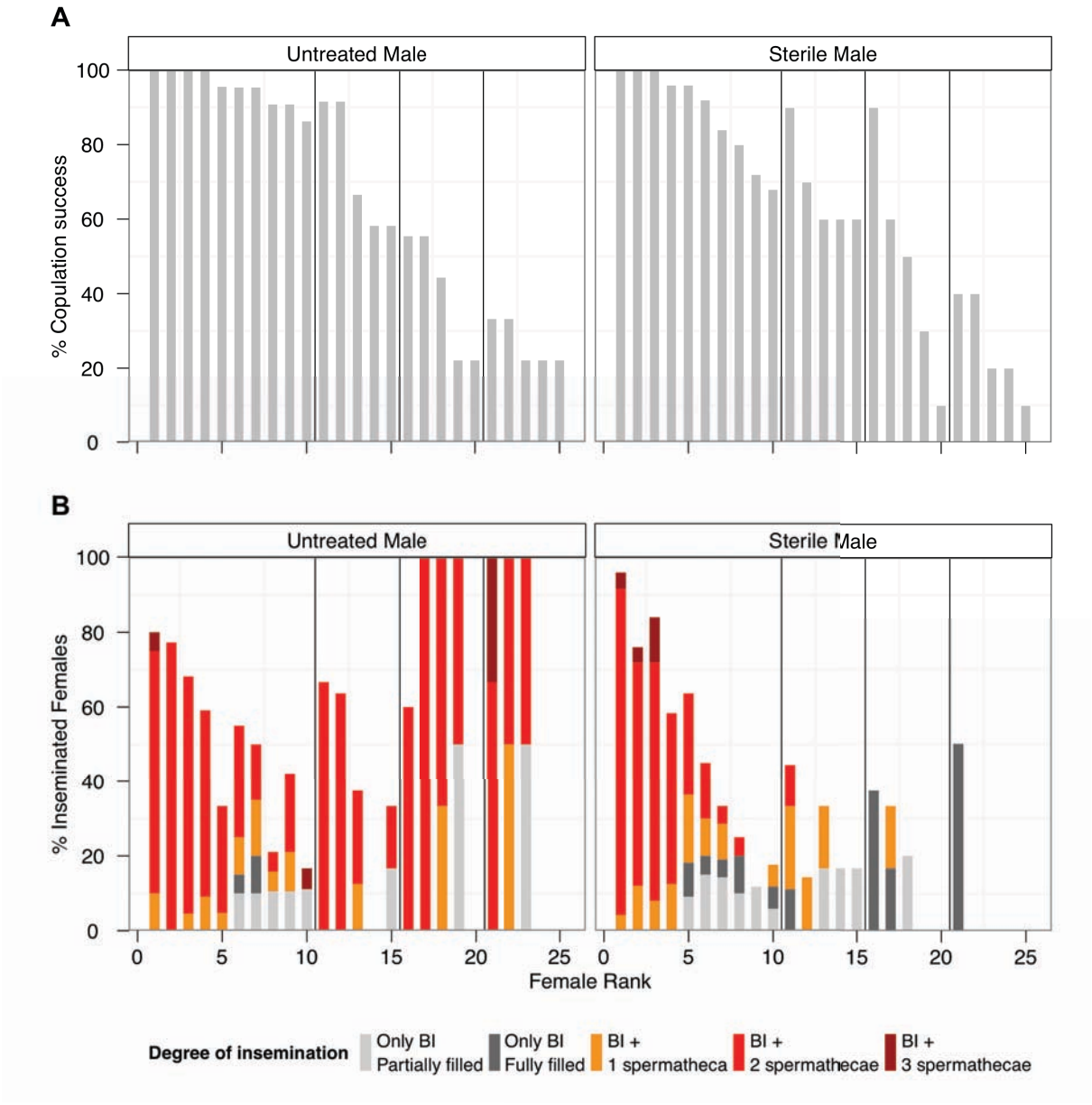

853

854 **Figure 6. Insemination capacity of a male *Ae. albopictus* mated with several females in**
855 **rapid succession.** Percentage of copulation success on all trials (A) and percentage of
856 insemination success of all copulations (B) according to order of mating opportunities, for
857 untreated and sterile males. Various degrees of female insemination are represented by
858 different coloration within the bars. The vertical lines within the graphs divide the four mating
859 periods.

Article 6. Control of *Aedes albopictus* in Reunion Island by the sterile insect technique: effects of irradiation, presence of females, and immediate sugar supply, on males longevity under semi-field conditions

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Effects of irradiation, presence of females, and sugar supply on the longevity of sterile males *Aedes albopictus* (Skuse) under semi-field conditions in Reunion Island.

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Abstract

BACKGROUND: The development of the sterile insect technique (SIT) for reducing populations of *Aedes albopictus* (Skuse), (the vector of Chickungunya and Dengue fever), was studied in Reunion Island. For some mosquito species the sterilization process and mating activity may alter male survival. Most previous studies were carried out in the laboratory and may inadequately reflect the field situation. We conducted a semi-field experiment to evaluate the impact of sugar supply and mating activity under natural climatic conditions on wild and sterile male *Ae. albopictus* longevity, using large cages set up in an open clearing between trees and shrubs in Reunion Island.

RESULTS: Wild males had a mean longevity of 15.5 days in the absence of females and with an immediate sugar supply; longevity in sterile males was similar. The presence of females greatly reduced both wild and especially sterile male lifespan; however, an immediate sugar supply could counteract this effect and allow sterile males to live an average of 11.6 days.

CONCLUSION: The outcomes indicate that sugar feeding could compensate for sterilization-induced damage, and that mating activity is not deleterious for well-fed males. This study stresses the critical importance of providing suitable sugar sources prior to release during SIT programmes.

Keywords. Sterile insect technique – longevity – semi-field – irradiation – sugar – mating

1. Introduction

Recent outbreaks of dengue fever in Hawaii (Hayes et al. 2006) and Chickungunya (CHICK) in Reunion Island and Mauritius (Reiter et al. 2006) have demonstrated that the 'Asian tiger mosquito' *Aedes albopictus* (Skuse) is a primary vector of arthropod borne viruses. Historically, many have considered *Ae. albopictus* to be a "secondary vector" due to its ecology and behaviour, that is, being less anthropophilic and not well adapted to urban environments (Sullivan et al. 1971, Rodhain et al. 1997, Schuffenecker et al. 2006). However, this species was responsible for major outbreaks of dengue fever in Reunion Island (Coulanges et al. 1979), in southern China (Qui et al. 1981) and in the Seychelles (Metselaar et al. 1980). In 2006, a CHICK outbreak occurred in Reunion, which accounted for over 266,000 cases, with an attack rate of 35% (Gerardin et al. 2008). These outbreaks suggest a modification in vector competence status of *Ae.*

albopictus, that is, from a secondary to a primary vector of dengue and CHICK and can be attributed to numerous factors including: presence of the vector in large numbers and viral adaptation to *Ae. albopictus* midgut epithelial cells leading to more efficient dissemination of the virus and transmission by the mosquito (Tsetsarkin and Weaver 2011).

The recent expansion of the geographical distribution of *Ae. albopictus* from the forest of South-East Asia (Smith 1956) has been systematically reviewed (Shroyer 1986, Gratz 2004) as well as the role shipping played in the global dissemination of this vector in used tires (Hawley 1988). In addition, the literature suggests that the establishment and spread of *Ae. albopictus* breeding populations was facilitated by its invasive behaviour (Benedict et al., 2007; Juliano and Lounibos, 2005), by anthropogenic means (Shroyer 1986) and its ability to adapt to a range of environmental conditions including natural, agricultural, peri-rural as well as urban areas (Knudsen, 1995). These adaptation and behavioural mechanisms are major risk factors for the introduction, establishment and dispersal of both *Ae. albopictus* and the CHICK virus.

Following the 2006 CHICK outbreak in Reunion Island, numerous studies were conducted on the biology, ecology, epidemiology and control of vector *Ae. albopictus* as part of the French national Agency for Research project Entomochick from 2006 to 2009 (Delatte et al. 2008, Boyer et al. 2012). However, since many vector control approaches including source reduction, larviciding and adulticiding strategies all failed to control the disease spread or its vector, it became apparent that alternative strategies should be developed. A 4-year feasibility study was launched in 2009 to investigate alternative strategies including the use of sterile insect technique (SIT) as part of an area-wide integrated pest management (AW-IPM) programme to control *Ae. albopictus* (Robinson et al., 2009). SIT relies on mass production and release of sterile males, which would confer sequential sterility into the wild target population when inseminating females. The goal of this "birth-control" strategy is to reduce the population density until a threshold is reached so that the disease transmission cycle from female mosquitoes to humans is broken (Anguelov et al., 2012).

To successfully develop and implement a vector control program with an SIT component it is essential that information on all aspects of the biology and ecology of the vector is known (e.g. survival of males in the field). In nature, different factors may affect the survival of adults such as predation, availability of food resources, mating activity, and meteorological conditions (Gratz

2004). Relative humidity and temperature play a vital role in adult mosquito survival (Alto and Juliano, 2001a) by enhancing mosquito metabolism and kinetics which regulate body fluids and can possibly explain the ability of *Ae. albopictus* to outlive other species at high temperatures and low humidity, which contributes to its establishment and spread (adaptation) in tropical, Mediterranean and temperate zones (Hylton, 1969). Experiments using diurnal variations in temperatures similar to that observed in the field revealed that at minimum ambient temperatures high mortality rates were observed (Löwenberg Neto and Navarro-Silva, 2004). In contrast, studies carried out in the laboratory, where optimum humidity and temperature conditions are maintained, revealed good adult survival rates which may possibly overestimated adult life spans in nature (Hawley, 1988). Many workers use the number of gonotrophic cycles completed by field collected mosquitoes to estimate the longevity of females (Clements and Paterson, 1981; Detinova, 1968; Dickson and Nielsen, 1983), whereas male longevity can only be estimated via mark-release-recapture studies (Clements and Paterson, 1981; Estrada-Franco and Craig, 1995; Hawley, 1988; Mori, 1979; Walker et al., 1987). Therefore, a semi-field approach appears to be the most suitable and reliable method to study the impact of various parameters on insect longevity under natural and uncontrolled environmental conditions (Knols et al., 2003; Okech et al., 2003; Winkler et al., 2006).

Male mosquitoes only feed on sugar sources, and it has been shown for *Anopheles gambiae* Giles that male mating performance and longevity are highly dependent on sugar-feeding (Foster, 1995; Gary et al., 2009). After emergence a male mosquito possesses enough energy reserves for 2-4 days of flight and survival (Foster, 1995). In addition, a high loss of energy can be associated with insect mating behaviour (Bailey et al., 1993; Clutton-Brock and Langley, 1997; Kotiaho and Simmons, 2003; Watson et al., 1998), though the distinction between the costs due to copulation itself, courtship, semen transfer, or male-male competition are difficult to estimate. The effect of sexual activity on male mosquito lifespan has not yet been documented, but it appears to alter the longevity of male *Drosophila melanogaster* Meigen (Cordts and Partridge, 1996; Partridge and Farquhar, 1981) and *Saltella sphondylli* Schrank (Martin and Hosken, 2004).

The use of ionizing irradiation to sterilize males has been found to impact the longevity of mosquitoes, depending on the radiation dose and the stage at which the insects were irradiated

(Proverbs, 1969, Sharma 1978, Curtis 1976, Helinski 2006). Indeed, radiation-induced damage to the reproductive cells is responsible for the induced sterility, however, according to the dose received, the somatic cells may also be affected, altering some physiological traits such as longevity, flight performance or competitiveness (Helinski et al., 2009). Male Ae*. albopictus* sterilized with low radiation doses of 35-30 Gy did not adversely affect their longevity. (Balestrino et al., 2010). However, estimating the survival rate and lifespan of males is essential in the context of the SIT in order to determine release procedures and frequencies. The present study was conducted to determine the survival rates of irradiated males compared to wild ones, under semi-field conditions in Reunion Island, and to investigate the additional effects of food supplies on emergence, mating and longevity of *Ae. albopictus* mosquitoes..

2. Material and methods

2.1. Mosquito rearing

The strain of *Ae. albopictus* used in the experiments (excluding the wild adults used in the competitiveness tests) originated from Saint-Benoît, Reunion Island. Mosquitoes were reared in a climate-controlled room maintained at 27 ± 2 °C and 75 ± 2% relative humidity; the light regime was LD 12:12 h photoperiod. Generations F3 and F4 were used for this study. Egg hatching was triggered using dehydrated rabbit food in deionised water (Haypellet, Compagnie des Grains du Capricorne, Le Port, Reunion Island). Larvae were reared at a density of approximately 500 first instar larvae (L1) per tray (30 x 40 cm) containing 1 litre of water and supplied rabbit and fish food (Sera Koi Food, Sera, Heinsberg, Germany) every two days. Pupae were collected using pipettes, released into small plastic cups and placed into mosquito cages (30 x 30 x 30 cm) for emergence. Adults were fed *ad libitum* with a sucrose solution (1 g litre $^{-1}$) placed into vials and dispensed by cotton wicks. Females were blood-fed on restrained mice, eggs were collected and kept at room temperature for a three-day period (for maturation) and placed in closed plastic containers for storage. Mice were reared according to the recommendations of the Guide for the Care and Use of laboratory animals (National Research Council and Committee for the Update of the Guide for the Care Use of Laboratory Animals, 2011); the rearing procedure was approved by the Institutional Animal Care and Use Committee, Veterinary Services Direction, Saint Pierre, Reunion Island.

Wild male and female pupae used in the experiments originated from eggs collected in the field close to the semi-field cages, La Bretagne, Reunion Island, and were therefore already adapted to the local environmental conditions. Larvae were reared under the same conditions as for the laboratory colony.

2.2. Irradiation procedure

Pupae were collected less than 9 h after the appearance of the first pupa and were irradiated 24 to 30 h after the last pupation occurred. They were maintained in cups 4 cm in diameter filled with water at the centre of the irradiation chamber. They were exposed to a sterilizing dose of 35 Gray (Gy) (Balestrino et al., 2010; Oliva et al. submitted manuscript), using a cesium-137 irradiator (IBL 437, Cis Bio International, Germany); the dose rate was ca 2.35 Gy min^{-1}. All males treated using this procedure is referred in the text as “sterile males”.

Semi-Field trials

The study was conducted in Saint-Denis, Reunion Island (20°52 S; 55°27 E) located in the western part of the Indian Ocean. Experimental cages (50 x 50 x 50 cm) were set up on a lawn bordered with trees which provide shade and raised 20 cm above ground level using breezeblocks (Fig. 1). The periphery of these cages was covered with fine netting to protect the cages from strong winds and flying objects. A cup containing cotton wicks soaked with a sugar solution (1 g litre $^{-1}$) was placed inside each cage and surrounded with water to prevent access by ants; the cotton wicks were changed every second day. Fifty male pupae were allowed to emerge in each cage; they were either sterilized colony males or untreated wild males. Three replicates were set up for each of the 8 combinations of conditions: (1) wild males alone supplied with sugar 48h after emergence; (2) wild males with females, supplied with sugar 48h after emergence; (3) wild males alone supplied with sugar at emergence; (4) wild males with females, supplied with sugar at emergence; (5) Sterile males alone supplied with sugar 48h after emergence; (6) Sterile males with females, supplied with sugar 48h after emergence; (7) Sterile males alone supplied with sugar at emergence; (8) Sterile males with females, supplied with sugar at emergence (Fig. 1). Depending on the treatment, the sugar source was available either on the day of emergence or 48 h after emergence; in this later treatment, water from the emergence cup was still available. In addition,

data was also analyzed according to treatments: radio-sterilized males (1) or wild males (0), males provided with sugar at emergence (1) or 48 h after emergence (0), and males left with females (1) or alone (0). For half of the experiments 50 females were added 48 h after the emergence of the males. Dead mosquitoes were counted and removed twice a day, at dawn and dusk.

2.3. Statistic analysis

All data were analysed using R 2.15.0 (R Development Core Team, 2010) software. One-way analyses of variance (ANOVA) were performed to compare the mean lifespan and mean survival rates between the various treatments. Survival curves were prepared and analysed using a stratified Cox proportional hazards model. The best models were selected based on the Akaike Information Criteria (AIC) (i.e., all possible main effects and interactions), of the models (backward-selection). This approach allowed us to examine the most important parameters influencing adult longevity. The reference group, i.e. wild males without females and delayed sugar supply was labelled as treatment 1. To account for the differences between the replicates due to the meteorological conditions, independent baseline hazards were computed but we assumed that these effects would be equal for all groups. Ties were handled by computing the exact partial likelihoods instead of using the Breslow or Efron methods. All computations were done in R using the package "survival", version 2.36-12.

3. Results

3.1. Meteorological data

The experiment was conducted during the rainy season (December 2010-March 2011). No substantial variation in temperature, humidity or precipitation occurred over this period, except for two occurrences of heavy rain (Fig. 2). The mean temperature was 26.5 °C (22.4 - 32.7 °C) and the average relative humidity was 71.6% (46 – 97%).

3.2. Male survival

Average survival rates of wild males were similar in all treatments except in treatment 2 where the presence of females and an absence of a sugar meal until 48 h post-emergence, significantly

decreased the mean lifespan ($F_{7,798}$ = 12.01, P < 0.001; Table 1). In the least restrictive conditions, where males were supplied with sugar at emergence and no mating activity was possible (treatments 3 and 7), the mean longevity of sterile males was similar to wild ones (15.5 ± 1.1 and 15.5 ± 1.1 days respectively) (Tukey Post-Hoc test, P > 0.01; Table1), and their survival curves did not differ significantly (X^2_1 = 0.1,, P =0.76) (Fig. 3). However, when analysing the risk of death and considering data from treatment 1 (wild males, delayed sugar supply and absence of females) as baseline (i.e. variation in the risk of death overtime equalled one), the sterilization process significantly increased the hazard of dying (P < 0.001) (Table 2 & Fig. 5). The presence of females greatly increased the mortality rate for males regardless of their irradiation status (P < 0.001); however, this did not significantly reduced mortality rates when males were supplied sugar meals at emergence (P = 0.815). When the interaction of the three factors was considered, the results showed that the availability of sugar reduced the mortality rate even more than the absence of females (P = 0.004). That is, when male mosquitoes were deprived of sugar until 48 h after emergence, mortality of both wild and sterile males increased over the first few days post emergence (Fig. 4). Among sterile males treatments, the mean longevity was significantly reduced when sugar supply was delayed regardless of the presence of females (Table 1).

The average survival rates did not differ significantly between treatments during the first 7 ($F_{7,15}$ = 1.35, P > 0.01) or 14 days ($F_{7,15}$ = 1.17, P > 0.01).

3.3. Female survival

In all the four treatments where females were present, the only varying parameter was the irradiation of the male partners; no significant difference was observed in female survival curves (X^2_3 = 0.7, P = 0.89), in mean lifespan (15.0 ± 0.5 days; $F_{3,340}$= 2.1, P = 0.1) or in the mean survival rates over the first 7 (0.81 ± 0.02; $F_{3,7}$ = 0.07, P = 0.98) or 14 days (0.68 ± 0.02; $F_{3,7}$ = 0.08, P = 0.97), indicating a similar effect of wild or sterile males on the female survival.

4. Discussion

The results of the present study conducted under semi-field conditions in Reunion Island clearly demonstrated that sterilization by irradiation and mating activity can both reduce *Ae.*

albopictus male survival, although within the same treatments the mean longevity of wild and sterile males was similar. However, these negative effects were largely reversed or nullified by sugar-feeding immediately after emergence. Wild male mean lifespan was 15.5 days, when supplied with sugar at emergence and in the absence of females. Similar results have been observed under laboratory conditions using another strain of *Ae. albopictus* from Reunion Island with mean longevity of 15 days when reared at 35 °C and 17 days at 30 °C (Delatte et al., 2009). An average of 22 days longevity was reported for a strain from Italy studied under laboratory conditions with mean temperature of 27 °C (Balestrino et al., 2010), and a mean longevity of 35.3 days for a strain from Brazil reared under a cyclic temperature regimen of 27/20 °C (Löwenberg Neto and Navarro-Silva, 2004), whereas studies conducted under uncontrolled conditions reported a mean longevity of 12.5 days for male *Ae. albopictus* mosquitoes from Malaysia (Aida et al., 2008). These variations among strains of the same species demonstrate how environmental conditions, and particularly the temperature, greatly influence the survival of mosquitoes.

In the present study, males irradiated at 35 Gy had a similar longevity as wild males within each treatment, however the risk of death was significantly increased by irradiation. Balestrino et al. (2010) reported no significant effects of a 40 Gy radiation dose on the longevity of male *Ae. albopictus* from an Italian strain under laboratory conditions. That is, when irradiated males were caged with females and with a constant sugar supply; however longevity was reduced when males were irradiated with higher doses (60 Gy). Previous studies show that irradiation can reduce the longevity of male *An. stephensi* (Sharma et al., 1978), but to have no significant effects at low doses on the longevity of *An. pharoensis* (Abdel-Malek et al., 1966) and *An. arabiensis* (Helinski et al., 2006). However, our study showed that the supply of sugar immediately after emergence allowed sterile males to survive longer. These results suggest that sugar-fed sterile males subjected to mating activity can be expected to live for an average of 11.6 days in the field. The daily survival rate observed for the first week averaged 0.82 and 0.85 for sterile and wild males respectively; these values are comparable to the ones reported after mark-release-recapture studies in Italy (Bellini et al., 2010) and in Japan (Hawley, 1988; Mori, 1979). However a mark-release-recapture study in Reunion Island estimated a daily survival probability of 0.95 (Lacroix et al., 2009), suggesting an ability of (non sterilized) released males to find suitable shelters and

sugar sources.

The results suggest that a 48 hr delay in the sugar supply mimicked a realistic situation which released males may encounter in the field, which is, foraging for a sugar source immediately after being release. This condition was found to be the most important parameter affecting *Ae. albopictus* male longevity suggesting that energy accumulated shortly after emergence would be crucial for survival of males especially if mating occurs. Newly emerged male and female mosquitoes commonly seek carbohydrate sources soon after eclosion as their energy reserves are limited; the rapid acquisition of sugars is critical for survival, swarming, mating, and dispersal (Briegel and Kaiser, 1973; Foster, 1995; Magnarelli, 1983). Sugar in addition to blood-feeding has frequently been shown to enhance female survival (Briegel and Kaiser, 1973; Briegel et al., 2001; Foster, 1995; Nayar and Sauerman Jr, 1971), whereas males solely depend on sugar sources for their energy reserve from which they draw lipids, glycogen, and sugar required for survival and flight (Foster, 1995).

Data from the present study revealed that the presence of females in the cages had a negative impact on male survival. A decreased longevity due to the presence of females was also reported for males of the species *Ae. aegypti* (Liles and De Long, 1960). Bargielowski et al. (2011) attributed a reduction of 43% in the lifespan of male *Ae. aegypti* to the courting and insemination of females. In the experimental conditions used in our study, mosquito density was twice as high in the cages with both sexes compared to the cages with males only. Therefore, in addition to the mating, it is quite possible that density of mosquitoes in cages could affect male longevity. Similar results were reported by Alto & Juliano (2001b) who observed that adult mortality was density independent. It is clear that the density resting surface (DRS) factor is considered to have important consequences for mating, feeding, and longevity of mosquitoes and Gerberg et al. (1994) reported that adult mosquitoes required a space of 1.8cm^2/ mosquito. During the present study mosquitoes were provided a DRS of 100 cm^2 / mosquito for the highest density, that is, at least 50 times higher DRS than required and this should have eliminated the detrimental effect of density on adult survival.

Interestingly, the survival rate of males provided a sugar meal at emergence was observed to be unaltered by the presence of females. These results suggest that males were able to

compensate for the energy spent during mating activity when resources were available. On the other hand, the energy spent when copulating during the first days after emergence could not be recovered by late feeding, that is, when sugar was provided 48 hours post emergence. Clements (1999) reported a reduction of 15% fecundity per gonotrophic cycle in mosquitoes while Partridge et al. (2005) suggested that the decrease in reproductive activity was related to the reallocation of the resources to repair damage and ensure a longer lifespan. Stone et al. (2011) reported that female *An. gambiae* mosquitoes favour survival over reproduction when one considers the relationship between the sugar resource availability and gonotrophic state. In addition, there seems to be a cost associated with reproduction in female *An. gambiae* but this involves copulation and not insemination, while males seem to suffer substantial mating cost as their longevity is reduced by 2 days compared to virgin males (Dao et al., 2010). Similar results have been reported for *Ae. aegypti*, with mated females having a longer lifespan than virgin ones whereas the contrary was true for males (Liles and De Long, 1960). Studies conducted by Leisnham et al. (2008) showed female *Ae. albopictus* mortality increases with reproductive output but decreases with cumulative blood feeding. Data from the present study showed that male *Ae. albopictus* appeared not to restrain their mating activity when deprived of a sugar diet, even at the apparent risk of dying more quickly. Considering the short average lifespan of wild adults and high predation risks, this may appear to the best adaptive behaviour or strategy.

In conclusion the results of this study showed that immediate availability of sugar after emergence was the most critical factor promoting longevity of males. In a natural environment where sugar supplies may be scarce, this effect may be even stronger in the field as released males will have to find a suitable sugar source. In addition, recent studies on the mating competitiveness of *Ae. albopictus* in Reunion Island also suggested that sugar feeding prior to release would greatly enhance the competitiveness of sterile males (Oliva et al submitted manuscript). Fruit fly SIT programmes generally allow a 1-2 day pre-release period for sucrose and water feeding to ensure a better survival and mating activity of the released flies (Anonymous, 1996). Providing sterile mosquito males with sugar before SIT releases would seem to likewise ensure better efficiency.

Such semi-field tests appear to be good indicators of the survival that can be expected from

released mosquitoes. Further investigations of the seasonal variation in adult longevity would be of interest to determine the best release period. Nonetheless, the outcomes of this study bring important information for the development of an SIT programme, especially regarding the release process and the frequency of releases according to the estimated lifespan.

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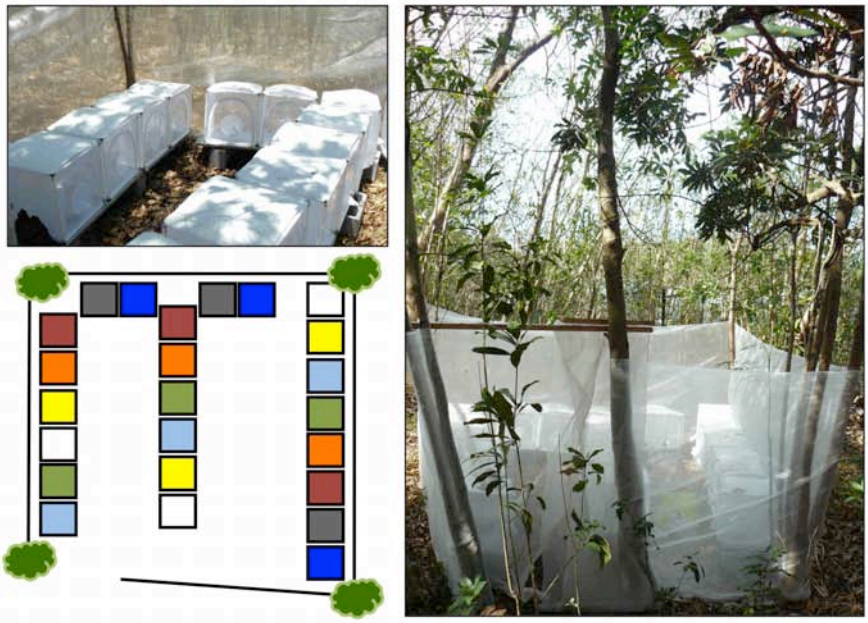


Treatment	1	2	3	4	5	6	7	8
Male irradiation	0	0	0	0	1	1	1	1
Sugar at emergence	0	0	1	1	0	0	1	1
Presence of females	0	1	0	1	0	1	0	1

Figure 1. Experimental set up. Pictures of the cage set up (top and right), schematic of the cage distribution (left), and combinations of the different treatments tested (table): radio-sterilized males (1) or wild males (0), provided with sugar at emergence (1) or 48 h after emergence (0), and left with females (1) or alone (0).

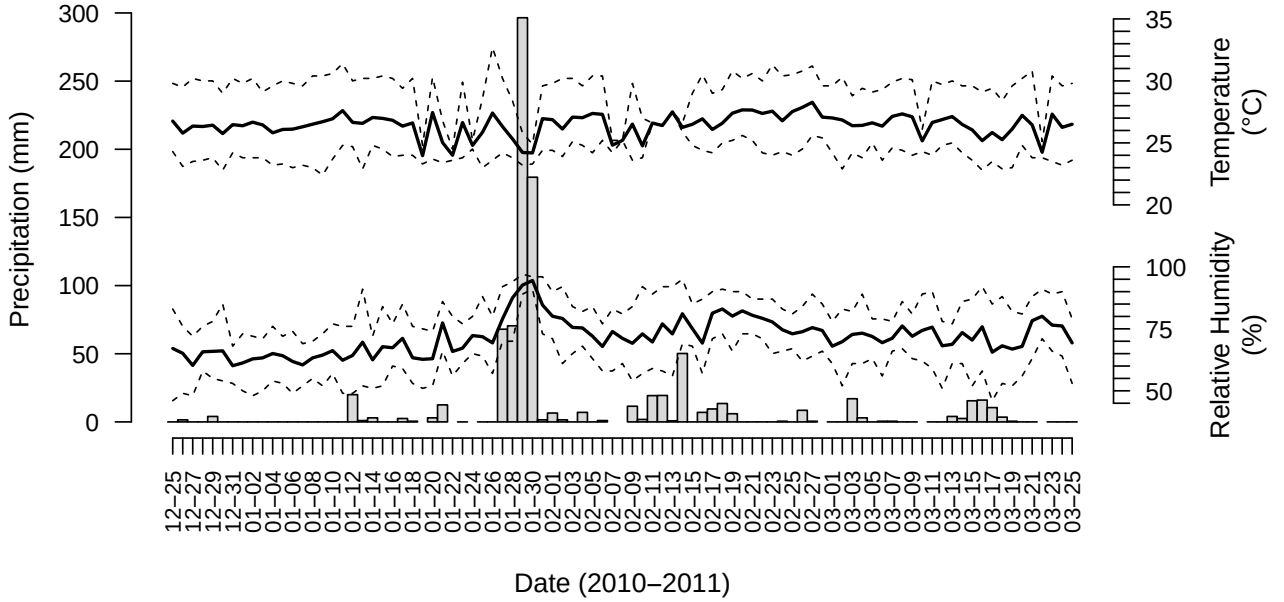


Figure 2. Climatic variations (precipitation, relative humidity and temperature) during the test period at the location of the semi-field cages.

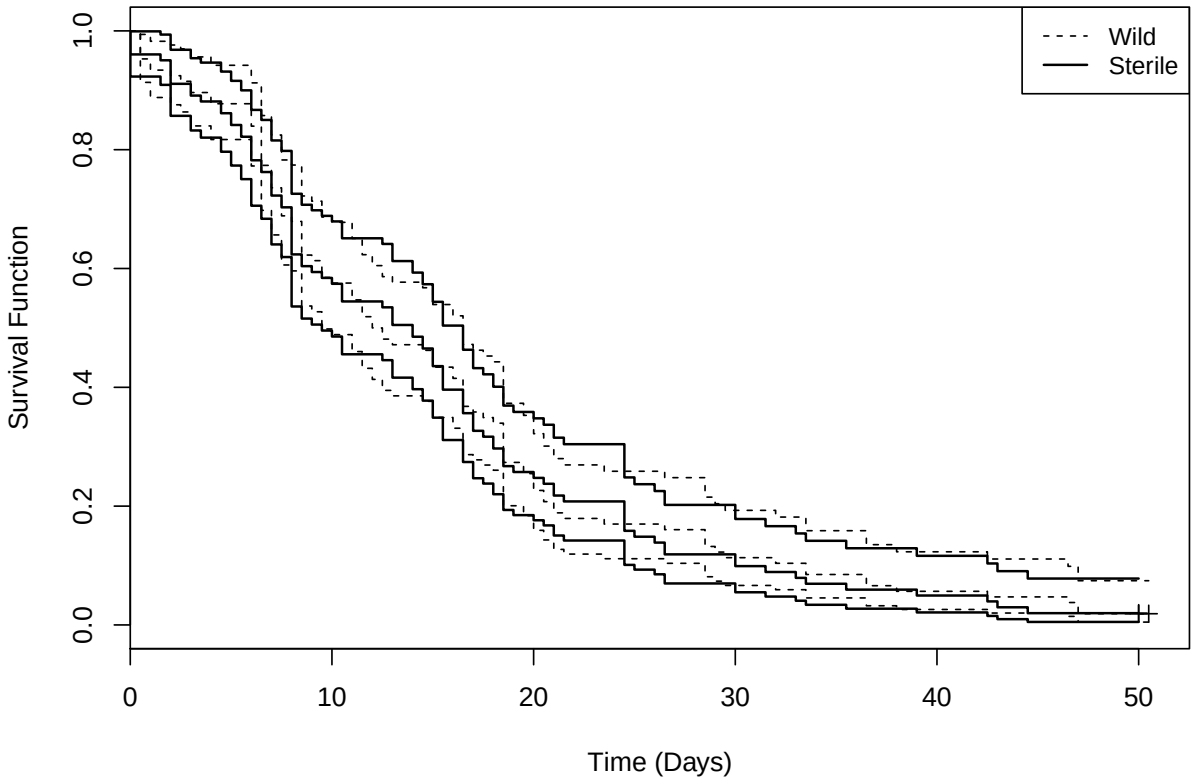
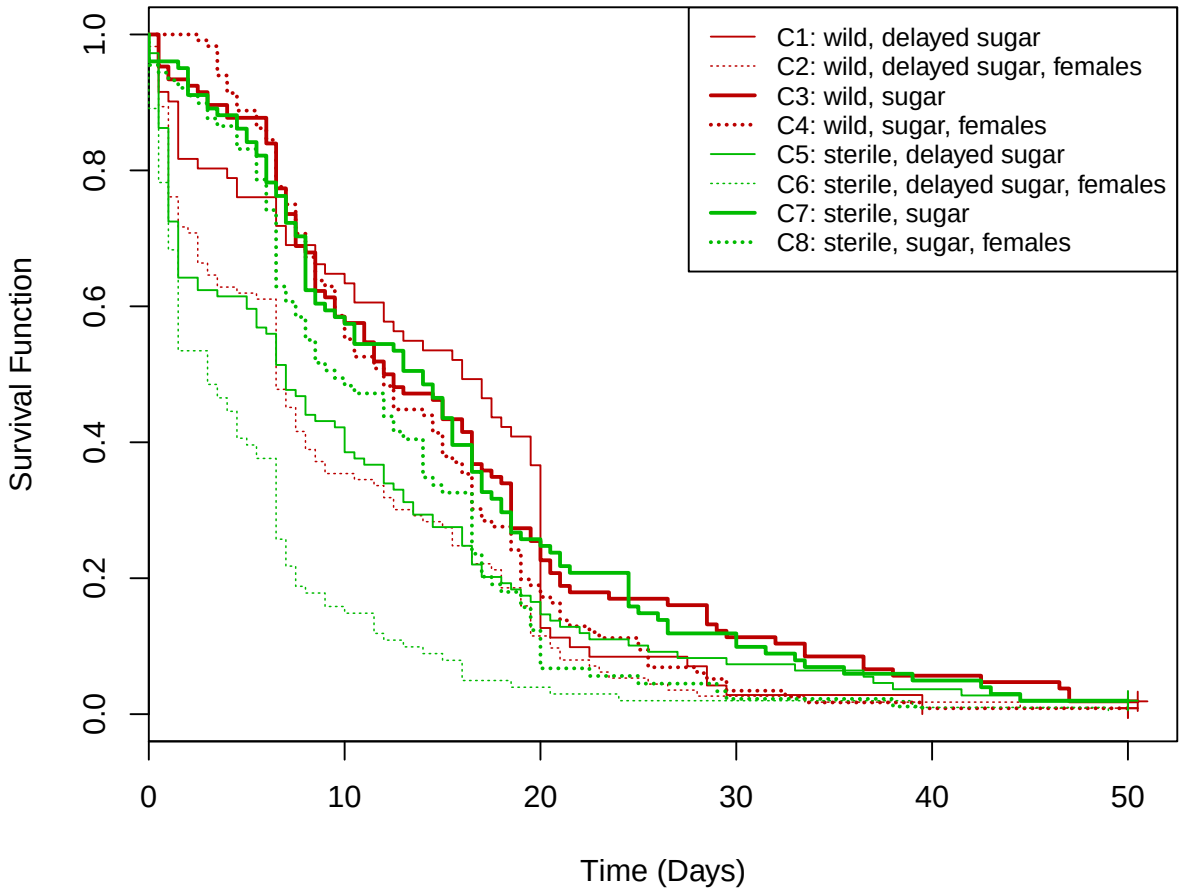


Figure 3. Kaplan Meier survival curves with 95% confidence bands, for wild and sterile males *Aedes albopictus* in the least restrictive conditions (sugar supplied at emergence and no mating activity possible).



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493 Figure 4. Kaplan-meier survival curves for *Aedes albopictus* males in all the treatments tested.

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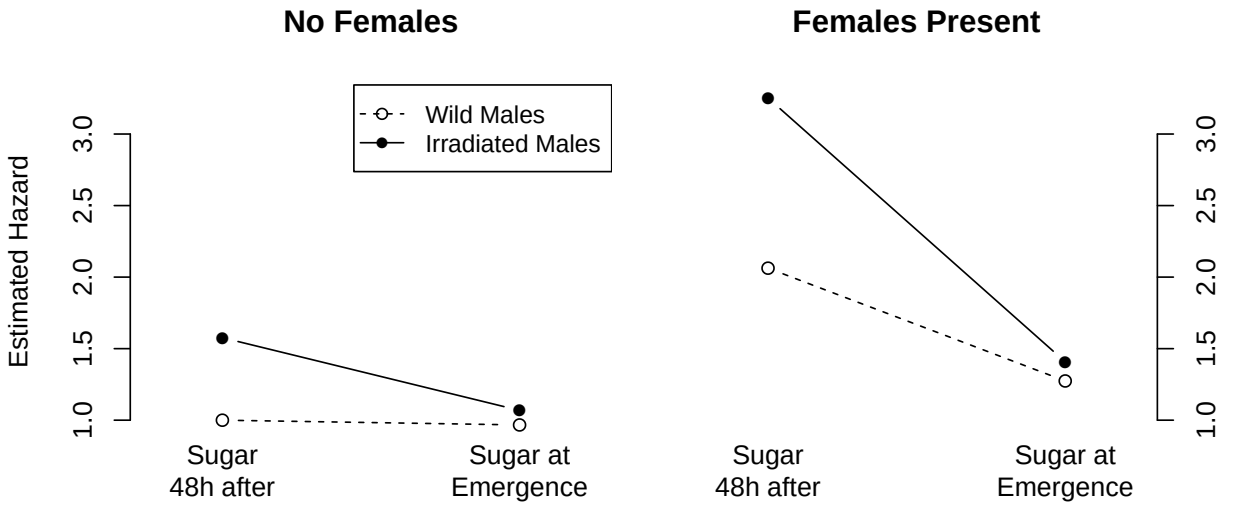

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495 Figure 5. Interaction plot of estimated death hazards from the Cox model for *Aedes albopictus*
496 males. The baseline hazard was treatment 1 (wild males, delayed sugar supply and absence of
497 females).

498

Table 1. Comparison of *Aedes albopictus* male longevity and survival rate for each treatment. Values followed by a similar letter are not statistically different from each other (Anova, $P < 0.05$).

Treatment	Parameters			Mean Longevity ± sem (days)	LT50 (days)	Mean survival rate ± sem	
	Males	Delay in sugar supply	Female presence			After 7 days	After 14 days
1	Wild	48 h	-	14.0 ± 1.1 ab	16	0.74 ± 0.16	0.71 ± 0.14
2	Wild	48 h	+	9.5 ± 0.9 cd	6.5	0.62 ± 0.27	0.48 ± 0.29
3	Wild	0 h	-	15.5 ± 1.1 ab	12.3	0.84 ± 0.11	0.70 ± 0.19
4	Wild	0 h	+	13.6 ± 0.8 ab	12	0.85 ± 0.10	0.67 ± 0.20
5	Sterile	48 h	-	10.5 ± 1.1 ac	7	0.63 ± 0.31	0.59 ± 0.25
6	Sterile	48 h	+	5.5 ± 0.7 d	3	0.58 ± 0.25	0.41 ± 0.31
7	Sterile	0 h	-	15.1 ± 1.1 b	14	0.83 ± 0.10	0.69 ± 0.17
8	Sterile	0 h	+	11.6 ± 0.8 abc	9.5	0.82 ± 0.14	0.65 ± 0.25

Table 2. Estimated hazards from the Cox model for the effect on Aedes albopictus male lifespan. The baseline hazard was treatment 1 (wild males, delayed sugar supply and absence of females), coefficients with z- and p-values indicating variations in the risk of death. Based on the AIC results, the interaction parameters "Irradiation * Sugar at emergence * Females" and "Irradiation * Females" were removed from the final model.

Effect	β	SE(β)	z-values	P	
Irradiation	0.455	0.113	4.042	< 0.001	***
Sugar at emergence	-0.034	0.144	-0.233	0.815	NS
Females	0.724	0.115	6.272	< 0.001	***
Irradiation * Sugar at emergence	-0.355	0.154	-2.301	0.021	*
Females * Sugar at emergence	-0.448	0.156	-2.877	0.004	**

Article 7. Mating vigour of sterilized male *Aedes albopictus* (Diptera: Culicidae) in Reunion Island

Clelia F Oliva, Maxime Jacquet, Jeremie Gilles, Guy Lempérière, Pierre-Olivier Maquart, Serge Quilici, François Schonemann, Marc Vreysen, and Sebastien Boyer. The Sterile Insect Technique for Controlling Populations of Aedes albopictus (Diptera: Culicidae) on Reunion Island: Mating Vigour of Sterilized Males. PLoS ONE, ***In press***.

The Sterile Insect Technique for Controlling Populations of *Aedes albopictus* (Diptera: Culicidae) on Reunion Island: Mating Vigour of Sterilized Males

PLoS ONE

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Abstract

Reunion Island suffers from high densities of the chikungunya and dengue vector *Aedes albopictus*. The sterile insect technique (SIT) offers a promising strategy for mosquito-borne diseases prevention and control. For such a strategy to be effective, sterile males need to be competitive enough to fulfil their intended function by reducing wild mosquito populations *in natura*. We studied the effect of irradiation on sexual maturation and mating success of males, and compared the sexual competitiveness of sterile versus wild males in the presence of wild females in semi-field conditions.

For all untreated or sterile males, sexual maturation was completed within 13 to 20h post-emergence and some males were able to inseminate females when 15h old. In the absence of competition, untreated and sterile males were able to inseminate the same number of virgin females during 48h, in small laboratory cages: an average of 93% of females was inseminated no matter the treatment, the age of males, and the sex ratio. Daily mating success of single sterile males followed the same pattern as for untreated ones, although they inseminated significantly fewer females after the ninth day. The competitiveness index of sterile males in semi-field conditions was only 0.14 when they were released at 1-day old, but improved to 0.53 when the release occurred after a 5-day period in laboratory conditions. In SIT simulation experiments, a 5:1 sterile to wild male ratio allowed a two-fold reduction of the wild population's fertility. This suggests that sterile males could be sufficiently competitive to mate with wild females within the framework of an SIT component as part of an AW-IPM programme for suppressing a wild population of *Ae. albopictus* in Reunion Island. It will be of interest to minimise the pre-release period in controlled conditions to ensure a good competitiveness without increasing mass rearing costs.

Introduction.

In Reunion Island (21°10" S; 55°30" E), *Aedes albopictus* Skuse can be found at very high densities and its habitat extends from urban areas to inhabited ravines [1]. Its wide distribution had a high impact on chikungunya disease transmission and thus leads to major health issues; a third of the Reunion human population was infected by the chikungunya virus during the 2005-2006 outbreak [2]. Research is being conducted to assess the feasibility of including the sterile insect technique (SIT) in an area-wide integrated pest management (AW-IPM) program targeting this species [3].

Ae. albopictus shows an efficient adaptative behaviour in Reunion Island as it can be found in various habitats [4]. As predation pressure cannot regulate its densities, the most limiting density-dependent effect on the population should be due to intra- or inter-specific competition [5]. Reproductive competition through the SIT could be an additional powerful tool to control populations of this pest: successive releases of sterile males would allow reducing the number of offspring in the following generations and may help controlling the density of this species in urban areas where it threatens the health of human populations [6]. Encouraging results were reported from a pilot trial during which sterile male *Ae. albopictus* were released in Northern Italy [7] .

The sexual competitiveness of the released sterile males could be a major limitation for the use of SIT to control mosquito populations. The competitiveness value of males may be linked to various parameters such as their survival, their aptitude to find females, mate and transfer semen and the number of females that a male is able to copulate with. Each of these parameters might be affected during the sterilization process. Irradiation causes random dominant lethal mutations in the germinal cells resulting in the death of the developing embryos after fertilization [8], but depending on the radiation dose and the stage of development of the insect, this process might affect somatic cells as well. Somatic damage is usually visible through a reduction of the longevity, sexual vigour and general activity of males [9]. This is a particularly important point of research for the success of the

SIT, as the released male's ability to find a female, mate and successfully transfer their sterile sperm must not be reduced in order to achieve highest efficiency. It is recommended to irradiate males as late as possible in the life stage as the sensitivity to radiation decreases because by then most of the cells would have finished their mitotic division [10,11]. The effect of radiation on insect pupae is higher than on adults, the latter generally leading to a sterile male competing almost equally with wild males [12,13]. Practical reasons however encourage the irradiation at the pupal stage for mosquito as handling of pupae is easier and adults are more fragile.

In addition to the irradiation process that can affect competitiveness [8], the rearing history of the colony and the lack of genetic diversity induced by the laboratory colonisation might also alter male's sexual vigour under field conditions [14]. Males adapted to mate in a confined space might not be able to behave as wild males under field conditions. Moreover, assortative mating with laboratory-reared females of a similar genetic background might result in a good competitiveness but would not necessarily reflect mating with wild females [15,16,17,18]. This emphasizes the importance of determining the competitiveness in semi-natural conditions with females and males coming from wild larvae or eggs.

The purpose of this research was to assess the effect of irradiation on various parameters of sterile male reproductive value, in the context of the development of the SIT for the control of *Ae. albopictus* in Reunion Island. We compared the mating success between sterile and untreated males in laboratory conditions and we performed competitiveness tests against wild specimens under semi-field conditions.

Material and Methods.

Ethics Statement

According to the French legislation (decree 2001-464 from May 29, 2001) experiments carried out on invertebrates are not considered as animal testing and hence are not subjected to regulation. Similarly, as no experimentations were carried out on mice and

rabbits, they are not subjected to this regulation; their use concerned only the blood-feeding of the mosquitoes for which purpose one mouse was placed inside the mosquito cage for 20 min. Their rearing strictly followed the Guide for the Care and Use of laboratory animals [19] and was approved by the Institutional Animal Care and Use Committee, Veterinary Services Direction, Saint Pierre, Reunion Island, as well as their use in the described experiments. A daily monitoring ensured their wellness, and none of them were sacrificed for the experiments. Mice were kept three per cage (28 x 18 x 15 cm) and fed with hay pellets (Compagnie des Grains du Capricorne, Le Port, Reunion Island); rabbits were reared in pens (200 x l55 x 100 cm) but kept one per cage (100 x l55 x 44 cm) during three consecutive nights for the semi-field experiments, they were fed with rabbit food and occasionally fresh vegetables. The director of Cirad La Bretagne permitted the use of the land for the location of the semi-field cages. These semi-field studies did not involve any endangered or protected species. Human volunteers were part of the team and of the co-authors. The research protocols are approved by the Comité Consultatif de Déontologie et d'Ethique (CCDE) of the research center IRD.

Mosquito Stocks and Rearing.

The strain of *Ae. albopictus* used in the experiments (excluding the wild adults used in the competitiveness tests) originated from Saint-Benoît, Reunion Island. Immature and adult mosquitoes were reared in a climate-controlled room maintained at a temperature of 27 ± 2°C and 75 ± 2% relative humidity; the light regime was LD 12:12 h photoperiod. Generations F3 and F4 were used for this study. Egg hatching was triggered using a highly dehydrated rabbit food left overnight in the rearing water (haypellet, Compagnie des Grains du Capricorne, Le Port, Reunion Island). Larvae were reared at a density of approximately 500 first instar larvae (L1) per tray (30 x 40 cm) containing 1 litre of water. They were fed with rabbit-food and fish-food (Sera Koi Food, Sera, Heinsberg, Germany). Pupae were collected and placed in small plastic cups inside an insect adult cage (30 x 30 x 30 cm) for emergence. Adults were continuously supplied with 10% sucrose solution [w/v]. Females

were blood-fed on mice and eggs were kept at room temperature after a three-day period of maturation.

Irradiation procedure.

Pupae from the Saint-Benoît laboratory strain were collected less than 9 h after the formation of the first pupa and were irradiated when 24 to 30 h old. They were maintained in cups of 4 cm diameter filled with water at the centre of the irradiation chamber. They were exposed to gamma rays emitted by a cesium-137 irradiator (IBL 437, Cis Bio International, Germany); the dose rate was ca 2.35 Gy/min. In the text non-irradiated males are referred to as untreated males.

Sterility curve.

Male pupae were irradiated at 0, 15, 25, 35 and 40 Gy. After emergence 70 males were caged with 35 virgin females. After five days, females were blood-fed on mice and allowed to lay eggs in individual tubes. Eclosion rate of each egg batch was then recorded to assess the mean fertility. According to the results, it was decided to use the sterilizing dose of 40 Gy for mating success experiments. However, the irradiator available was owned by the hospital and the equipment set to deliver a dose of 35 Gy for blood irradiation. As each modification of the radiation dose required a time-consuming and complex handling process, we decided to use a dose of 35 Gy for the competitiveness tests, which required frequent use of the source, as a convenient alternative for the operators.

Recovery of fertility.

Nine experiments were conducted in which one 40 Gy-irradiated male was allowed to mate with 2- to 3-day-old virgin females during two periods of five days separated by a five-days resting period without females. A different group of 2- to 3-day-old virgin females was used for the male's second mating period. Females were blood-fed on a human volunteer arm and allowed to lay eggs in individual tubes; egg hatch rates were recorded.

151 ***Effect of irradiation on male sexual maturation.***

152 The stage of genitalia rotation [20] was recorded every hour from 0 to 30 h post-emergence
153 for untreated and 35 Gy irradiated males. For each time point, a different group of males was
154 frozen and observed under a binocular microscope. The following divisional markers were
155 used to categorize five stages of the rotation of the male genitalia: stage 0, no rotation; stage
156 1, ≤45° rotation; stage 2, >45° - ≤90° rotation; stage 3, >90° - ≤135° rotation and stage 4,
157 >135° to complete rotation of 180° [21]. The rotational direction (clockwise or counter-
158 clockwise) was also recorded.

159 Ten males emerging within 30 min were immediately caged with 10 females (4- to 5-day-old)
160 for 15, 20 or 25 h. All adults were then frozen. Females were dissected and the three sperm
161 storage organs (spermathecae) were observed in order to determine how many were
162 inseminated. Male terminalia rotation stage was also recorded. For both untreated and 35-
163 Gy-irradiated males, five replicates were carried out for 15 h old males and ten replicates
164 were carried out for 20 and 25 h old males; two different cohorts were used. Females were
165 frozen 2 h after the end of the mating period to give sufficient time for the transferred sperm
166 to be stored in the spermathecae [22].

167 ***Insemination rates.***

168 Small laboratory cages (30 x 30 x 30 cm) were filled with 50 one- or five-day old untreated
169 males and 50 virgin females. The same cages were set up with males sterilized at 35 Gy. A
170 last cage contained 250 one-day old sterile males together with 50 females. After 48 h, the
171 females were dissected and the spermathecae were observed to determine the insemination
172 status.

173 ***Daily mating success.***

174 One male was caged with 10 virgin females (2- to 4-day-old) for 15 days. Every 24 h,
175 females were removed, frozen, and replaced by 10 virgin females (2- to 4-day-old). They
176 were then dissected and checked for spermathecae insemination status. Wing lengths were

also measured. The experiment was replicated five times for both untreated males and 40-Gy-irradiated males.

Effect of the age on mating success of sterile males.

Ten experiments were performed in which one sterile male (irradiated at 40 Gy) was left together with 10 virgin females (2- to 4-day-old) for 5 days. In a first group, males were one-day old at the beginning of the experiment, and five-days old in the second group. After the mating period, females were dissected and checked for spermathecae insemination status.

Competitiveness of sterile males against wild males.

Three semi-field cages (6 x 3 x 2 m) were set up on gravel soil bordered with tall trees providing shadow. A table was placed in the cage to offer shelter to the insects. Three cups containing cotton dipped into a 10% sugar solution were surrounded with water to prevent the access of ants and honey drops were put into water to attract the mosquitoes towards the sugar sources. In order to render the experimental conditions as restrictive as possible for the sterile males, wild males and wild females originated from eggs collected in the field close to the semi-field cages, and were therefore already adapted to the local environmental conditions. Laboratory males were irradiated as pupae at 35 Gy as described above. Larvae were reared under the same conditions as for the laboratory colony. Males and females were released at the same time in the cage and a rabbit was left for three days in a small cage under the table to provide the necessary blood source for females. On the fourth day, the rabbit was removed and four oviposition cups were placed in each semi-field cage for three nights. Egg cups were removed on the seventh day and the remaining mosquitoes were trapped using BG-traps [23]. This experimental set up did not allow females to undergo two gonotrophic cycles as they did not have the possibility to blood-feed after oviposition. Thus all the egg batches collected on the 7^{th} day were laid by a different female. Eggs were maturated for three days and hatched under laboratory conditions. Hatching rates and numbers of larvae were recorded.

Three different conditions were tested: (experiment 1) ratio 1:1:1 (1 sterile male: 1 wild male: 1 female) with one-day-old adults, (experiment 2) 1:1:1 with five-days-old adults and (experiment 3) ratio 5:1:1 with one-day-old adults. When one-day-old adults were released, pupae were placed in a small cage inside the semi-field cage for emergence and males and females were released on the following morning. When 5-days-old adults were released, emergence occurred in the insectarium; males and females were kept separately and fed under laboratory conditions for five days before the release. When the release ratio was 1:1:1, 200 sterile males, 200 wild males and 200 wild females were released whereas when the ratio was 5:1:1, 500 sterile males, 100 wild males and 100 wild females were released. Experiments 1 and 2 were replicated 6 times, and experiment 3 was replicated 5 times. Controls for the fertility levels of wild and irradiated males were carried out under laboratory conditions.

The competitiveness index (C) was calculated as ((Hn-Ho)/(Ho-Hs))*(N/S); where Hn and Hs were respectively the hatch rate from eggs of females mated with untreated or sterile males, Ho was the observed egg hatch rate in the experiment and N and S were the numbers of untreated and sterile males respectively [24]. The variance of C was calculated according to Hooper & Horton [25].

Statistical analysis.

Shapiro and Bartlett tests were performed to test respectively the normality and the homoscedasticity of the data.

For the sterility curve experiment, differences between egg hatch rates as a function of radiation dose were analysed using a one-way ANOVA after a square-root transformation of the data. In the recovery of fertility study, the fertility rates of females mated by irradiated males was compared during the first or second mating period using a two-tailed paired Student's t-test after square-root transformation of the data. For the sexual maturation study, proportion tests with continuity correction were used to compare the paired proportions of

untreated versus sterile males for a given rotation stage and a given time; one-way ANOVA were used to compare the mean number of females inseminated after a given period. For the insemination rate study, the proportions of females inseminated by males in the different situations were compared using proportion tests with continuity correction. For the mating success tests, the mean number of females and inseminated spermathecae per female was compared between untreated and sterile males, as well as between young and old sterile males, using one-way ANOVA. A two-tailed paired Student's t-test was used to compare the number of females inseminated for a given number of days between untreated and sterile males; and proportion tests with continuity correction were used to compare the proportion of females with 1, 2 or 3 spermathecae filled. Logistic regression was used to test the effect of female wing size on their inseminating status.

For all the tests, the alpha level was $P<0.05$. The dataset was analysed using R software [26]. Values in the text are expressed as mean ± SEM.

Results.

Sterility curve.

Male *Ae. albopictus* from the Reunion strain were exposed to gamma radiation at various doses and crossed with non treated females. The control fertility was 97%. It was reduced to 7% and 4% by irradiation with 35 and 40 Gy, respectively (Fig. 1). As no significant difference existed between the sterility levels induced by these two radiation doses, the 35 Gy dose was used for the competitiveness experiments.

Recovery of fertility.

The persistence of male sterility after irradiation was tested. In this experiment, females mated with 40 Gy irradiated males aged 1 to 5 days had a mean fertility of 3.0 ± 0.1%, which was not different from the result of the sterility curve. Each male inseminated 1 to 4 females during this first mating period. During the second period of mating, the same males then aged 10 to 15 days old were able to inseminate 1 to 6 females; the fertility of all of these

females (N=17) was zero, which was significantly lower than during the first period (two-tailed paired Student's t-test, t = 6.32, df = 21, $P<0.001$). During these two 5-days periods an individual male was able to inseminate a total of 2 to 8 females.

Effect of irradiation on male sexual maturation.

The temporal sexual maturation process of freshly emerged untreated and sterile males was assessed by examining the terminalia rotation and the insemination ability.

Terminalia rotation could be detected 4 h post-emergence for untreated males. After 10 h, all males had started the rotation and the first males with the terminalia fully rotated were observed after 11 h (25%). After 17 h, all males were in stage 3 or 4. In groups aged 18 to 25 h old, 80 to 100% of the males had completed rotation.

The first irradiated males recorded in stage 1 were observed 3 h after their emergence, which was significantly earlier than for untreated males (proportion test with Yates correction, $X^2 = 4.02$, df = 1, $P<0.05$). Similarly to the untreated males, after 10 h every sterile male had started the rotation. However, a delay in the rotation speed was observed in sterile males aged 15 to 19 h compared with untreated ones. The first fully rotated sterile males were observed after 13 h (11%) and the rotation process was completed 20 h post-emergence. Significant differences between untreated and sterile males were observed at the age of 12 h ($X^2 = 1.56$, df = 1, $P<0.01$), 15 h ($X^2 = 9.77$, df = 1, $P<0.01$), 16 h ($X^2 = 12.8$, df = 1, $P<0.001$), and 18 h old ($X^2 = 6.89$, df = 1, $P<0.01$).

Very few males were able to inseminate females during the first 15 h of their adult life as only 1 cage out of 5 contained a female inseminated for both untreated and sterile groups. After 20 h post-emergence, untreated males had inseminated twice as many females as sterile males (one-way ANOVA, $F_{(1, 23)} = 5.66$, $P<0.05$; Fig. 2). However after 25 h, all the cages contained inseminated females and both untreated and sterile males had a comparable insemination capacity with respectively 43 and 40% of females being inseminated: 87% of these females had 2 spermathecae out of three filled with sperm.

Insemination rates.

The insemination rate of a group of 50 females caged for 48 h with 1- or 5-day old untreated or sterile males, at a 1:1:1 or 5:1:1 ratio was assessed. No statistical difference in insemination rates was observed in relation to age, irradiation treatment or sex ratio (Table 1): on average 93% females were inseminated after 48 h, and 92% of them had 2 filled spermathecae.

Daily mating success.

In order to assess male mating performance, a new group of 10 females was offered daily to one sterile or untreated male for 15 days.

Over the 15-day period, sterile males inseminated significantly fewer females (one-way ANOVA, $F_{(1, 104)} = 4.89$, $P<0.05$) per day compared with untreated males (Table 2). On the first day, a fertile male inseminated on average twice as many females than a sterile one, but this difference was not statistically significant (two-tailed paired Student's t-test, t = 2.78, df = 4, $P = 0.07$). The number of females inseminated per day decreased until the 5th day and increased again during the next 5 days; this cyclic pattern was observed for both untreated and sterile males. The mean number of females inseminated by a sterile male was always slightly lower than for an untreated male, but this difference was significant only after the 9th day (t = 2.36, df = 7, $P<0.05$). Most of the inseminated females had only one spermatheca filled, only 18 and 6% of them had two spermathecae filled when mated by untreated and sterile males respectively; this difference was not significant (proportion test with Yates correction, $X^2 = 2.98$, df = 1, $P = 0.084$). Female wing size was not correlated to the insemination status (logistic regression, $z = 1.56$, df = 659, $P = 0.16$ for untreated males; z = -0.176, df = 509, $P = 0.86$ for sterile males).

Effect of the age on mating success of sterile males.

In order to compare the mating performance of 1- and 5-day old sterile males, the number of females inseminated by one male after 5 days was assessed.

There was no significant difference between young and older sterile males for the number of inseminated females (one-way ANOVA, $F_{(1,17)} = 0.95$, $P = 0.34$) or spermathecae filled per female ($F_{(1,17)} = 1.82$, $P = 0.2$). Sterile males aged 1 to 5 days old inseminated on average 3.3 ± 0.5 females of which 15% and 67% had respectively one and two spermathecae filled. When aged 5 to 10 days old they inseminated on average 2.7 ± 0.4 females of which 17% and 79% had respectively one and two spermathecae filled.

Competitiveness of sterile males against wild males.

Experiments were carried out under semi-field condition to assess the fertility of the caged population and the competitiveness index of sterile males when they where competing with wild males for inseminating wild females; adults were released at 1- or 5-day old and in a 1:1:1 or 5:1:1 ratio.

The mean fertility of the wild males used for these experiments was 93 ± 1% and 5 ± 0.9% for males irradiated with 35 Gy. Preliminary tests in non-competitive situations were conducted to ensure that both wild and irradiated males were able to survive and to inseminate wild females in this experimental set-up under semi-field conditions.

In competitive situations, when males were released in the cage on the day following their emergence, with an equal ratio of wild and sterile males, the mean fertility was reduced to 82.7% (Table 3), which was significantly higher than for the two other experiments (one-way ANOVA, $F_{(2,14)} = 17.7$, $P<0.001$); this resulted in competitiveness indexes (C) ranging between 0.06 and 0.35. When males and females were kept 5 days under laboratory conditions before the release in the semi-field cages, the mean fertility was reduced to 62.2%, C values ranged between 0.44 and 0.76, and were significantly different from the two other experiments ($F_{(2,14)} = 9.71$, $P<0.01$). When increasing the ratio of 1-day-old sterile to wild males, the mean fertility was twice as low as the one of a wild population as it averaged 46.4 ± 7.6%. High variations of fertility and thus C were observed between the five replicates in experiment 3, C varying between 0.10 and 0.62.

The mean fecundity differed significantly between each experiment ($F_{(2,14)}$ = 17.37, P<0.001). According to the previous experiment, a female oviposited an average of 50 eggs. If this assumption is correct, the mean number of females participating in the egg laying was 108, 154, and 47 respectively for the experiments 1 to 3. In experiment 1 and 2, 200 females were released whereas only 100 were used for the third test; therefore half of the females would have laid eggs in experiments 1 and 3, and 75% of them in experiment 2. The age of the females during the whole test differed between these experiments, as they were aged 1 to 7 days old in experiments 1 and 3, and 5 to 12 days old in experiment 2.

Discussion.

The ^{137}Cesium irradiation of pupae from Saint Benoit (Reunion Island) strain of *Ae. albopictus* males induced a similar decrease of fertility levels as reported by Balestrino et al. [27] on the Rimini (Italy) strain of the same species. The sterility induced by a 40 Gy irradiation was permanent as successive matings and a resting period did not show any recovery of fertility in males. The fertility was even reduced, as all the females inseminated during the second mating period were completely sterile. Radiation damage is higher on the earlier stages of spermatogenesis than on mature cells [28]; assuming that the sperm used during the second mating period originated from sperm cells that were immature during the irradiation process, and hence more radiosensitive, it is likely that this would result in the total sterility of the sperm cells. Patterson et al. [29] also showed permanent sterility in radio-sterilized males *Cx. quinquefasciatus* Say during 2 weeks. However, our results are different from those obtained in Italy with the Rimini strain where male *Ae. albopictus* showed a slight but non significant increase in fertility with male age [30].

Sterile male mating vigour as shown by the mating success tests was not significantly affected during the first week of adulthood, but the males became less efficient thereafter. Although the male mosquitoes inseminated fewer females each day, the differences were significant only from day 9 onwards. After that period, untreated males inseminated 50%

more females than sterile ones. As the irradiation may interfere with the maturation of new sperm cells, it seems likely that sterile males might have fewer successful matings (i.e. leading to insemination), therefore a lower daily mating success might be expected after several matings. However, using a 1:5 male-female ratio during 4 h, Balestrino *et al*. [27] observed no effect of a 40 Gy irradiation on the mating performances of male *Ae. albopictus*, but they reported fewer successful matings for 50-Gy-irradiated males. Grover *et al*. [31] reported that chemosterilization slightly reduced the insemination rates of male *Ae. aegypti* for periods of 24 h or 48 h over 10 days; on the other hand, radiosterilized males *An. pharoensis* Theoblad [32] or ***C****x. Quinquefasciatus* Say [29] did not show a different mating success compared with laboratory males.

We observed that when sterile and untreated males were offered new receptive females daily, creating intensive mating opportunities, most of the females had only one spermatheca filled. This pattern was also reported by Boyer *et al.* [33] for untreated male *Ae. albopictus* from another strain of Reunion Island. However, we showed that when a male was enclosed with the same 10 females for a longer period or when several males and females were caged together, females had mostly two spermathecae filled. We hypothesized that males from this strain might inseminate most of the time only one spermatheca per mating attempt and when kept with the same females for several days they might have more opportunities to transfer more sperm to the same female thus filling two spermathecae. The filling of only one spermatheca should not prevent female egg laying. Contrary to anopheline [34], the oviposition behaviour in *Ae. albopictus* and *Ae. aegypti* females would not be dependent on the spermathecae containing sperm but would rather be triggered by proteins from the male accessory gland (MAG) secretion that are transferred together with the sperm in the *bursa inseminalis* [35,36,37]. In addition, as the MAG substance would diminish the female propensity to be inseminated by another male [22], a female with only one spermatheca filled should therefore not have a higher probability of multiple-insemination. However, this would need further investigation as recent studies

386 indicated the occurrence of multiple matings in the field for *Ae. albopictus* [38] and in semi-
387 field condition for *Ae. aegypti* [39]. In the case of the SIT, multiple inseminations would not
388 be prejudicial if sterile and wild males are equally involved, and when the sterile males
389 outnumber the wild ones. The conditions and duration of mating success tests in laboratory
390 might affect the possibility to highlight differences or not between sterile and untreated
391 males, while carrying out these tests in a semi-field environment can help getting a realistic
392 value of the males' sexual performance and capacity as outlined by Huho *et al*. [40].

393 In order to perform unbiased competitiveness tests, the time of sexual maturation of
394 both untreated and sterile males has to be similar so that neither one would have the
395 opportunity to mate with females earlier. The genitalia rotation of males was overall not
396 greatly affected by irradiation, although rotation was slightly slower for sterile males between
397 15 to 19h post-emergence. Similarly, sterile males inseminated fewer females during the first
398 20h but this difference was no longer visible after 25h post-emergence. However, this
399 difference observed between untreated and sterile males is only of concern for the
400 establishment of competitiveness tests protocols.

401 Assuming that 40 Gy-irradiated males were equally competitive with untreated males,
402 the fertility of the caged population should have averaged 49% (as the fertility of wild and
403 sterile control males was 93 and 5% respectively). However, sterile one-day-old males had a
404 low competitiveness index when competing in an equal ratio with wild males, and the wild
405 female population fertility was only reduced with 10%. In Italy, competitiveness studies on
406 *Ae. albopictus* indicated a good performance of sterile males irradiated at 30 Gy with a
407 competitiveness index equal to 1.00 ± 0.66; a high variability among replicates and between
408 years was however reported [30]. A different strain, experimental setting and the diverse
409 environmental conditions might affect the behaviour and survival of the mosquitoes, and
410 could explain the differences observed between this study and ours. We observed that
411 maintaining males for five days after emergence in the laboratory before the release greatly
412 improved their competitiveness, and allowed the decrease of the semi-field cage wild

population's fertility to 62%, which indicated a nearly equal participation of both groups of males for the inseminations of females. The age of females differed between the experimental tests 1 and 2, as they were the same age as the males. However, females are already fully receptive to mating when two-days-old, and the receptivity to males and ability to retain semen should not differ with age as mentioned by Spielman et al. [41] for *Ae. aegypti* females. This almost four-fold improvement of the competitiveness value of the five-day-old males does not seem to be due to an increase of intrinsic male mating ability since, in laboratory conditions, a difference in male age did not affect the number of females inseminated. The time spent with an easily reachable sugar source in the insectarium during the pre-release period could have increased males nutritional status and thus improved some of their traits such as survival and flight capacity. A similar age effect on the mating competitiveness of sterile males *Culex pipiens fatigans* Wiedemann was reported by Krishnamurthy et al. [42] who observed that 36-60 h old males from a highly sterile male-linked translocated strain competed almost equally, in semi-field cages, against same age males from an untreated strain, whereas 12-36 h old males had a reduced competitiveness. Similarly, 36-60 h old chemosterilized *Ae. aegypti* males were competing better, in semi-field, against wild males than did 7-8 days-old males [31].We reported that a five-fold ratio of sterile to wild males allowed a reduction of almost 50% of the wild females natural fertility, suggesting that a 10-fold ratio could bring total sterility in a wild population and continuous releases might have a rapid efficient impact on the reduction of vectors density in the field. Releases of sterile males were usually performed with “over flooding ratios” so that the impact on the wild population would be faster [43,44]. Laboratory studies on radiosterilized male *An. quadrimaculatus* Say allowed a reduction of 80% of the fertility of wild females when released in a ratio higher than 6:1:1 (sterile males : wild males : wild females), but no reduction was observed at a ratio equal or less than 4:1:1 [45]. A reduction of 95% in the fertility was possible in laboratory experiments with irradiated males *An. pharaoensis* Theobald competing in a ratio 10:1:1 [46]. More recently, an average 5:1 overflooding ratio

of engineered sterile male *Ae. aegypti* occasioned an 80% reduction of a wild local population in the Cayman Islands over a 23-week period [47].

The results of this study suggest that a 5:1 or higher sterile to wild male ratio should be combined with a pre-release period in an insectary to ensure the efficiency of a sterile male *Ae. albopictus* release. The *Ae. albopictus* density on Reunion Island is high and covers a wide range of habitats; although the ravines may be less easily accessible, releases near habitation gardens and parks should be straightforward. Releases at the pupal stage are often considered as more convenient, but it may be conceivable to use the emergence cage where the sterile males would be maintained during the pre-release period to bring them to the various release areas. Provided suitable aerial release systems can be developed and the surface of the treated area is large enough, aerial releases would ensure a cost-effective area-wide coverage. Furthermore, if the release at the adult stage is selected, it might then be of interest to irradiate males as adult in order to reduce the radiation induced somatic damages and thus improve their competitiveness [8]. The pre-release period may turn out to be an important cost factor in a mass-rearing facility; further studies should determine the minimal period required before release. The balance between sterility level and competitiveness of the sterile males is a major question for such programmes [48]. As in this study we chose a lower radiation dose ensuring a better competitiveness but not a complete sterility, the question remains whether the use of 5% fertile males is conceivable for a field release?

A reduction of competitiveness of radio-sterilized males is the key argument put forward to support a transgenic approach over classical SIT [49]. However, we showed that the effect of irradiation could be counteracted by adapting the release process, and does not prevent accomplishing an efficient reduction of an *Ae. albopictus* population's fertility. In the native habitat, the competitiveness of the released sterile males will also depend on the effect of rearing and handling, the location of the release sites and the distribution of the wild mosquitoes [50]; a field trial is therefore now desirable to put sterile males to the test in

conditions where they would also have to find sugar sources, locate female mates, and face predation risks.

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Figures

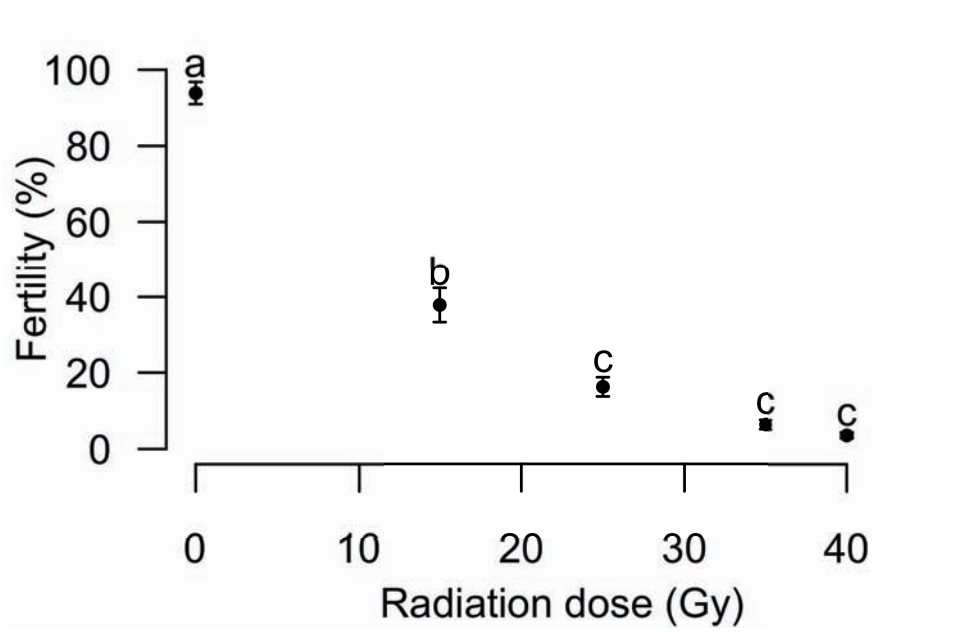

Figure 1. Sterility curve of male *Ae. albopictus* from Reunion Island. Mean fertility (%) as a function of radiation dose. Unlike letters indicate significant difference between the points (P<0.05).

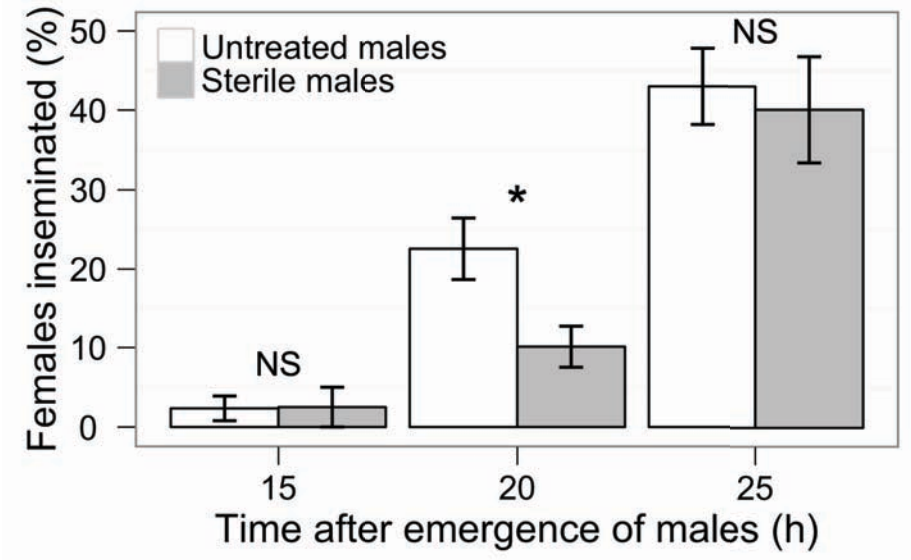

Figure 2. Insemination ability according to age and sterilization for male *Ae. albopictus*. Percentage (± SEM) of females inseminated in small cages (10 females and 10 males) for different durations after male emergence (3 replicates). NS indicates a non-significant difference and * stands for a significant difference (P<0.05) between sterile and untreated males.

626 **Tables**

627

628 **Table 1. Insemination rates of untreated or sterile male *Ae. albopictus* (50 females, 48**

629 **h).** Percentage of females with 0, 1, 2 or 3 spermathecae inseminated in laboratory cages in

630 the different situations of the competitiveness tests.

Male	Age (days) at release	Male:Female ratio	Number of spermathecae inseminated (% of females)			
			0	1	2	3
Untreated	1	1:1	2.0	0.0	91.8	6.1
Sterile	1	1:1	12.2	6.1	81.6	0.0
Untreated	5	1:1	6.5	4.3	84.8	4.3
Sterile	5	1:1	6.5	8.7	80.4	4.3
Sterile	1	5:1	6.4	0.0	89.4	4.3

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Table 2. Mating success of untreated or sterile male *Ae. albopictus*. Mean number of females inseminated per day, spermathecae filled per female per day, and cumulative number of females inseminated over a certain period, per male. Ten new females were given every day (5 replicates). NS indicates a non significant difference, * and ** stand for a significant difference between sterile and untreated males at $P<0.05$ and $P<0.01$ respectively.

	Untreated		Sterile males		Statistical difference
	Mean	**SD**	**Mean**	**SD**	
N inseminated females / male / day	1.5	0.5	1.0	0.3	*
N spermathecae inseminated per female / male / day	1.8	0.5	1.1	0.3	**
N inseminated females after: 1 day	2.4	0.5	1.2	0.8	NS
5 days	8.2	1.1	6.8	1.6	NS
9 days	14.6	3.8	9.5	1.3	*
14 days	19.2	4.1	12.0	-	-

Table 3. Competitiveness indices of sterile male *Ae. albopictus* under semi-field conditions. Mean fecundity and fertility values of wild females in the different conditions (ratio of sterile male: wild male: wild female and age at release varied) and competitiveness index (C) of sterile males. C was based on the fertility levels of control wild males (93.5 ± 0.4%) and sterile males (4.9 ± 0.3%). Unlike letters indicate significant difference between the row values (P<0.05).

	Conditions		
	Ratio 1:1:1 1 d old	**Ratio 1:1:1 5 d old**	**Ratio 5:1:1 1 d old**
N replicates	6	6	5
N adults released	200:200:200	200:200:200	500:100:100
Mean fecundity* (± SEM)	5395 ± 695 a	7685 ± 737 b	2339 ± 406 c
Mean % fertility (± SEM)	82.7 ± 2.8 a	62.2 ± 1.5 b	46.4 ± 7.6 b
Mean C (± SEM)	0.14 ± 0.14 a	0.55 ± 0.12 b	0.23 ± 0.19 ab

* Number of eggs collected in one semi-field cage.

Discussion générale

Ce travail a permis d'apporter des connaissances fondamentales sur certains aspects de la biologie de la reproduction d'*Ae. albopictus,* et contribue ainsi à mieux appréhender le comportement de ce vecteur de maladies dans le cadre d'interventions visant à la gestion de ses populations.

*Les mâles d'***Ae. albopictus** *sont ils prudents ?*

Les mâles et les femelles d'*Ae. albopictus* ont un comportement reproductif très actif et dynamique, tant en conditions d'élevage que sur le terrain, cependant peu d'accouplements résultent en une insémination réussie. Les mâles semblent favoriser de fréquents accouplements même si leurs réserves spermatiques sont épuisées. Les femelles ne refusent pas de s'accoupler même si elles ont déjà été inséminées et ont reçu une quantité de sperme suffisante pour accomplir la totalité des cycles gonotrophiques au cours de leur vie. Il semble qu'aucun mécanisme n'ait évolué pour empêcher un mâle de s'accoupler avec une femelle déjà inséminée, bien qu'il n'y ait alors qu'une très faible probabilité que son sperme soit utilisé pour la fertilisation des œufs.

L'inhibition du transfert et du stockage de sperme dans les spermathèques lors d'un second accouplement semble faire appel à des mécanismes complexes qui agissent efficacement et à long terme. La grande quantité de sécretion séminale transférée dans la *bursa inseminalis* de la femelle en même temps que le sperme, empêcherait une insémination ultérieure de la femelle dans un premier temps grâce au bouchon physique. L'action de protéines produites par les glandes accessoires mâles permettrait un effet à plus long terme (Helinski *et al.* 2012a). Une rapide succession d'accouplements avec une même femelle vierge, ayant lieu dans un intervalle de temps inférieur à 40 min, semble être l'unique possibilité pour qu'il y ait multiple transfert et stockage de sperme. Au vu des récentes études démontrant la possibilité d'inséminations multiples dans la nature (Helinski *et al.* 2012b; Boyer *et al.* 2012) et des résultats de cette étude, il semble que les femelles vierges sauvages soient exposées à de nombreuses tentatives d'accouplements de la part de différents mâles peu après émergence ou lors de leur premier repas sanguin. L'existence de mécanismes contrôlant le stockage du sperme apparait ainsi cohérent pour contrebalancer ce comportement reproductif pugnace des mâles d'*Ae. albopictus*.

Dans la littérature il est souvent fait état d'une inhibition de la réceptivité des femelles d'*Aedes*, suite à une première insémination. Or, nous soutenons que ce terme est inapproprié, puisque une femelle déjà inséminée ne refuse pas une nouvelle copulation et des signes d'un transfert de substance séminale dans la *bursa inseminalis* ont souvent été observés. C'est le transfert d'un second sperme jusqu'aux spermathèques qui semble être inhibé, physiquement ou chimiquement, par la première insémination.

La possibilité de double insémination d'une femelle ne semble toutefois pas être préjudiciable pour l'efficacité d'une TIS puisque cette potentialité existe aussi bien pour les mâles sauvages que stériles.

Les travaux menés sur les mâles d'*Ae. albopictus* des souches Rimini et Réunion ont démontré que l'irradiation n'affectait pas leur compétence à s'accoupler et à transférer leur sperme aux femelles. Cependant le nombre de femelles qu'un mâle stérile peut inséminer se limite à sept. Le nombre de femelles inséminées par un mâle sauvage dans la nature reste inconnu, toutefois des tests de laboratoire ont montré qu'un mâle d'*Ae. albopictus* Réunion s'accouplait en continu pendant une période maximale de 14 jours, inséminant jusqu'à 20 femelles (Boyer *et al.* 2011). La capacité totale d'accouplement des mâles stériles serait donc jusqu'à trois fois plus faible. Cela s'explique par la perte de la capacité de régénérer les réserves de spermatozoïdes et de sécrétion séminales chez les mâles stériles. En effet, les lésions engendrées par l'irradiation sont plus importantes sur les stades précoces de la

spermatogénèse (spermatogonies et spermatocytes) que sur les cellules matures, empêchant ainsi le développement des spermatocytes immatures (Proverbs 1969).

Nos résultats n'ont pas mis en évidence une gestion parcimonieuse et prudente des réserves de sperme de la part des mâles d'*Ae. albopictus*. En effet, ils investissent dans des copulations fréquentes même si les réserves de semence sont épuisées, et ils s'accouplent avec des femelles déjà inséminées en dépit de la faible probabilité de succès. Toutefois, du fait du haut risque de mortalité associé à une copulation proche d'un hôte de repas sanguin et de la forte compétition existante entre mâles, ce comportement est probablement la solution la plus adaptée pour optimiser leur fitness.

*Comment optimiser la survie des mâles stériles d'*Ae. albopictus *relâchés ?*

L'évaluation de la longévité des mâles d'*Ae. albopictus* en conditions semi-contrôlées a révélé une augmentation du risque de décès dû à l'irradiation (35 Gy). En revanche, en conditions de laboratoire, aucune différence significative n'a été observée quant à la longévité des mâles d'*Ae. albopictus* irradiés à 40 Gy par rapport à des mâles non traités (Balestrino *et al.* 2010). Le laboratoire permet de contrôler de nombreux facteurs influant sur la survie des adultes (taille de cage, nourriture, conditions climatiques), mais il est également nécessaire d'entreprendre des tests en conditions semi-contrôlées afin de refléter au mieux la survie des mâles relâchés.

Nous avons considéré qu'un délai de 48 h dans l'apport de sucre reflèterait une situation réaliste et stressante, où les mâles relâchés ne seraient pas capables de localiser immédiatement une source sucrée. Cette condition est apparue critique pour la longévité des mâles sexuellement actifs, ce qui suggère que l'énergie accumulée peu après émergence serait cruciale pour leur survie. Les données de cette étude indiquent que les mâles d'*Ae. albopictus* ne semblent pas restreindre leur activité reproductrice lorsqu'ils sont privés de ressources sucrées, même au risque de mourir plus rapidement. Il serait par ailleurs intéressant d'étudier l'activité de mâles privés de nourriture par rapport à des mâles nourris durant les premiers jours de leur vie.

Cette étude apporte des informations importantes et permet de préconiser l'apport de sucre lors des lâchers de mâles stériles d'*Ae. albopictus* à la Réunion. Des dispositifs de lâchers permettant l'accès à une source sucrée après émergence des nymphes ont été développés par le projet TIS du CAA, Bologne (Italie), cependant il pourrait s'avérer plus bénéfique de maintenir les mâles adultes en insectarium pendant au moins 24 h avant lâchers. Les programmes TIS contre les mouches des fruits prévoient généralement une période de pré lâchers de 1 à 2 jours permettant l'alimentation en eau et sucre, afin d'optimiser la survie et l'activité reproductrice des mâles stériles. Les résultats obtenus suggèrent que les mâles stériles d'*Ae. albopictus* pourraient être lâchés à une fréquence hebdomadaire, mais une modélisation serait nécessaire afin de le confirmer.

*Les mâles stériles d'*Ae. albopictus *seront-ils compétitifs sur le terrain ?*

L'étude des performances sexuelles journalière des mâles d'*Ae. albopictus* Réunion en condition de laboratoire n'a pas révélé de différence entre un mâle stérile et un mâle sauvage pendant les 9 premiers jours. La diminution ensuite observée de la capacité d'insémination des mâles stériles est cohérente avec les résultats obtenus en Article 5, indiquant une incapacité à régénérer les réserves spermatiques.

La majorité des femelles inséminées possédaient une seule spermathèque remplie, ce qui était cohérent avec les résultats rapportés sur une autre souche réunionnaise d'*Ae. albopictus* dans des conditions expérimentales similaires (Boyer *et al.* 2011). Cette situation expérimentale créait d'intenses opportunités d'accouplements pour un mâle puisque dix nouvelles femelles vierges et réceptives étaient substituées chaque jour. En revanche, lorsque les mêmes femelles restaient pendant plusieurs jours avec le même male ou lorsque mâles et femelles étaient présents dans une cage en forte densité, la majorité des femelles présentaient deux spermathèques remplies. Cela suggère que les mâles d'*Ae. albopictus* de

la Réunion auraient tendance a n'inséminer qu'une seule spermathèque par tentative d'accouplement, et la deuxième spermathèque étant inséminée lorsque la femelle s'accouple plusieurs fois.

En condition semi-contrôlées, les mâles irradiés à 35 Gy et relâchés dès émergence ont une faible compétitivité. Cependant les tests réalisés ont permis de démontrer qu'une période avant lâchers de cinq jours permettait d'augmenter considérablement la vigueur des mâles. Les tests d'insémination conduits en laboratoire avec des mâles stériles âgés de un ou cinq jours n'ayant pas reflété les différences observées lors des tests de compétitivité, seule la rigueur des conditions semi-contrôlées semble être à l'origine de la faible performance des mâles stériles. La période avant lâchers de cinq jours en insectarium a permis aux mâles d'avoir facilement accès à une source sucrée et peut ainsi avoir augmenté leur capacité de survie et de vol. Des effets similaires de l'âge sur la compétitivité ont été rapportés pour des mâles stériles d'*Ae. aegypti* (Grover *et al.* 1979) et de *Cx. Pipiens* (Krishnamurthy *et al.* 1975).

Bien que des études de compétitivité menées en Italie sur *Ae. albopictus* aient indiqué une bonne performance des mâles irradiés à 30 Gy, la variabilité de l'indice entre les réplications et les années était très grande (C = 0,95 ± 0,61). Des différences entre les souches d'une même espèce et les conditions environnementales influencent probablement la valeur compétitive des mâles. Cependant, le protocole expérimental permettant d'estimer la compétitivité se doit de minimiser les biais. Le protocole que nous avons suivi ici permet de relâcher les mâles une fois sexuellement matures et de s'assurer que chaque femelle ne ponde qu'une seule fois, ne biaisant ainsi pas le taux de fertilité observé. Une standardisation des protocoles d'étude de la compétitivité des moustiques permettrait une comparaison entre différentes études.

Dans le cadre des programmes TIS, les lâchers sont généralement fait avec des ratios inondatifs de façon à ce que l'impact sur la population sauvage soit rapide (Mahon 1996; Dame *et al.* 2009). Au vu de l'efficacité des mâles stériles relâchés en ratio 5:1:1, nous suggérons d'envisager, pour l'application de la TIS, l'utilisation de ce ratio en combinaison avec une période de repos au laboratoire avant le lâcher. Il sera toutefois nécessaire de minimiser la durée de cette période afin de ne pas accroître les coûts d'élevage de masse.

La TIS contre Ae. albopictus *à La Réunion ?*

Ces résultats des essais en condition semi-contrôlées sont encourageants et suggèrent une bonne potentialité de réduction des populations sauvages d'*Ae. albopictus* grâce à la TIS à la Réunion. Cependant compte tenu de la densité locale et de la large distribution du vecteur, des lâchers très massifs de mâles stériles seront nécessaires et l'utilisation de zones tampons indispensable pour permettre une avancé progressive des interventions. Cette étude apporte des informations fondamentales quant aux modalités de lâchers à favoriser pour l'utilisation de la TIS à la Réunion contre *Ae. albopictus*. Au vu de ces résultats, les lâchers au stade adulte sont à favoriser, il sera donc nécessaire d'étudier un dispositif adéquat pour la maintenance des adultes avant et lors des lâchers. De plus, à la lumière des tests de longévité et de compétition, il semble qu'une période de repos avant lâcher soit à recommander afin de permettre aux mâles stérilisés de faire des réserves en source sucrée. Sa combinaison avec un ratio de mâles stériles pour mâles sauvages de 5:1, devrait assurer une réduction efficace des populations sauvages.

Les études présentées ont pu démontrer que les mâles stériles ont une bonne survie (82%) en conditions semi-contrôlées pendant la première semaine (Article 6), et qu'ils sont capables d'inséminer un nombre maximum de 7 femelles (Article 5) en 5 à 9 jours (Article 7). Il semble donc que des lâchers hebdomadaires de mâles stériles soient à recommander dans le cadre d'une TIS contre *Ae. albopictus*.

Ce travail permet de conclure à un probable effet favorable de la TIS appliquée à *Ae. albopictus* à la Réunion, cependant des essais pilotes à grande échelle sont désormais

souhaitables afin de mettre les mâles stériles à l'épreuve dans un milieu naturel, où ils devront faire face aux risques de mortalité naturelle et être capables de localiser des sources sucrées et des femelles sauvages.

GENERAL CONCLUSION AND PROSPECTS

The objective of this PhD work was to participate to the feasibility study on the use of the sterile insect technique against the disease-vector species *An. arabiensis* and *Ae. albopictus*, and to bring biological and technical suggestions as to the use of this technique in Reunion Island.

What are the main results of this work?

The first part of this work delivered preliminary information concerning the colonized strains of *An. arabiensis*, but did not go so far as testing the competitiveness values of males from the strain ANO IPCL1.

The insect rearing procedure may often lead to a bottleneck because of strong selective pressures, and we demonstrated that a rapid evolution of the sexual maturation took place in the colonized strain Dongola at the Insect pest control laboratory (IPCL). Such a selection could influence the quality of the released males, and care should be taken to assess and identify differences between mass-reared and wild insects.

The feasibility of the SIT for *Anopheles* species entirely depends upon a good sex separation method, made possible thanks to a genetic sexing strain (GSS). ANO IPCL1 carries undeniable disadvantages because of the necessity of dieldrin treatments, frequent strain purification steps to eliminate recombinants, and low male progeny productivity. However, numerous assets confer favorable qualities for mass rearing such as good survivorship and fecundity, and rapid development. In addition, the possibility of using a lower dose of irradiation to sterilize the male ANO IPCL1, compared to wild males, suggests that their competitiveness should be preserved.

The progeny of irradiated ANO IPCL1 males showed higher mortality at the late larval stages. This indicates that the sterilizing dose of GSSs should be decided according to the proportion of eggs leading to viable adults. Considering this larval mortality, the possibility of choosing a partially sterilizing dose for SIT becomes advantageous, as the progeny of irradiated ANO IPCL1 males would maintain the density-dependent mortality in wild larval breeding sites. Indeed it is argued that the reduced larval density that follows sterile male releases, would enhance the wild larvae's survivorship (Yakob, Alphey, & Bonsall 2008).

The second part of my thesis work enabled us to gather important and useful information regarding the aptitude of sterile males *Ae. albopictus* to be used as population control agents.

Male mating behavior proved not to be prudent or parsimonious. They favor frequent copulation even when their semen supply is depleted, which does not lead to the insemination of the female. In addition, no mechanism seems to have evolved to prevent males from copulating with pre-mated females, even though they would have a very low probability of success. We showed that multiple inseminations of female *Ae. albopictus* were possible only if the subsequent matings took place within 40 min of the first one. Although cues of a second semen transfer to the female *bursa inseminalis* were observed in such a situation, the spermathecae were inseminated with the second sperm only in 15% of the cases. Female *Ae. albopictus* do not become unreceptive to copulation after the first insemination, however a long-term (physical or chemical) inhibition of the storage of sperm from the secondary male in the spermathecae occurs 40 min after the first mating. In the field, male *Ae. albopictus* try to copulate very actively with females while these are seeking a blood-meal. It thus appears plausible that some mechanisms exist to control the sperm storage in females.

The possibility of multiple inseminations of females should not be detrimental for SIT as it can involve wild and sterile males equally. A sterile male *Ae. albopictus* is able to successfully inseminate a maximum of 7 females in its lifetime, the semen supply is then exhausted and cannot be replenished, contrary to untreated males.

Tests carried out under semi-field conditions on Reunion Island clearly demonstrated that sterilization by irradiation and mating activity both increased the risk of death of *Ae. albopictus* males, although within the same treatments the mean longevity of wild and sterile males was similar. However, these negative effects were largely reversed by sugar feeding immediately after emergence. Similarly, the pre-release period in the insectary with constant access to sugar, allowed a remarkable improvement of the competitiveness value of the sterile males. A two-fold reduction of the wild population's fertility was possible when sterile males were released on the day of emergence in a 5:1:1 ratio. It can be supposed that the combination of a high release ratio and a pre-release period could allow an efficient reduction of the wild *Ae. albopictus* population.

What are the conclusions with respect to the SIT Reunion project?

Reunion Island represents an ideal site for the implementation of SIT as part of an AW-IPM programme due to its geographical isolation, a cool and dry season from May to September granting a natural decrease in adult mosquito populations, and its well-organized vector control and surveillance program. The feasibility phase of the SIT Reunion project will come to an end in December 2013, and the outcomes of the four work packages will allow for insight to the potential of implementing the SIT on Reunion Island to reduce populations of *An. arabiensis* and *Ae. albopictus.*

These two species show several ecological, behavioural, and physiological differences (see Klowden 2007) that will infer developing different SIT strategies for each species. Among those distinctives, *Ae. albopictus* is a container-breeding species and the larvae tolerate a wide range of conditions, whereas *An. arabiensis* is usually known as a "clean-water" species. As a direct consequence, the mass rearing technologies have to be adapted to each species. The adult reproductive behavior varies greatly among them, impacting the design of the mass-rearing cages and the release strategy. Male *An. arabiensis* aggregate in swarms and the conspecific females individually fly into them to mate, while male *Ae. albopictus* generally seek the mates close to blood-meal host. The latter are then more likely to encounter pre-mated females, which might impact the likelihood of multiple matings. The radiation doses necessary to sterilize males of both species differ significantly: around 40 Gy for *Ae. albopictus* compared to100 Gy for *An. arabiensis*. In addition, the use of a GSS is required for *An. arabiensis*, whereas male and female *Ae. albopictus* can be separated reliably enough at the pupal stage due to a marked size dimorphism. These are only few of the dissimilarities that can have profound implications for managing their control, and stress that each target species of a SIT has to be examined separately.

Concerning *An. arabiensis*, great technical and scientific progress has been achieved on the Sudanese strains at the IPCL, however some work remains to be accomplished on Reunion Island.

One of the biggest challenges of an SIT program is to develop cost effective technologies enabling the rearing of several thousands of sterile males per day. During the pilot program against *An. albimanus* in El Salvador, daily releases from 13,000 to 30,000 males were performed over a 5-month period, leading to a 99% reduction of the wild population accross a 14 km^2 area (Lofgren *et al.* 1974). The IPCL designed an easy-to-operate system of rack and trays adapted to the larval developmental characteristics of *An. arabiensis*, and which allows production of 140,000 to 175,000 larvae-pupae mixture per rack (Balestrino, Benedict, & Gilles 2012). For a same batch of larvae, the pupation can span over a two-day period with emergence occuring 24 to 36 h later. A reliable device for separating larvae and pupae before the sterilization step has been developed, based on the natural differences in buoyancy and behaviour between anopheline larvae and pupae; it allows processing a one million larvae and pupae mix per hour (Balestrino *et al.* 2011). A large rearing cage, optimising adult survival, mating, and fecundity is also under development. Studies are being conducted to optimize the operational sex ratio in order to ensure high fecundity of ANO IPCL1, with an approximate maximum capacity so far of 20,000 adults.

Though adult irradiation would induce a minor loss of competitiveness compared to pupal irradiation, the latter has often been favoured since the handling is easier. However the device used for the adult irradiation in this study caused no mortality and allowed homogenous irradiation. Attempts should be made to design a similar device for a greater quantity of males that could then be irradiated soon after emergence. The use of a low irradiation dose such as 75 Gy, which lead to ca. 99% of sterility (considering the progeny surviving to the adult stage), should ensure a fair competitiveness of the males ANO IPCL1.

Though the GSS available has a fair potential for mass rearing and appears to be able to produce good-quality sterile males, a semi-field test in Reunion Island is necessary to ascertain that sterile male ANO IPCL1 are able to survive, form or join swarms, and efficiently inseminate wild females. The colonization of wild strains of *An. arabiensis* in Reunion Island has been creating difficulties for several years. The on-going ecological studies will allow a better understanding of the adult behaviour, and will hopefully lead to success in their adaptation to laboratory conditions. In addition they should confirm whether the use of a unique strain would have an impact on the genetically structured populations of *An. arabiensis* (Morlais *et al.* 2005).

The distribution of *An. arabiensis* in Reunion Island is restricted to three large but discrete geographic regions (Gouagna *et al.* 2011), providing isolated areas that are appropriate for the use of the SIT. The austral winter leads to a decrease in population density and would thus be a suitable period for the releases. However, further studies are needed to determine the best release stage and process that would ensure good survival and efficacy.

In addition, detailed mating studies, similar to what has been carried out on *Ae. albopictus*, should be undertaken for *An. arabiensis*. It will be of importance to assess the effect of both a dieldrin treatment and a sterilisation process on the ANO IPCL1 male capacity to form or join a swarm and copulate, on the transfer of sperm and mating plug, and on its lifetime mating performance.

To conclude, the success of the SIT for eliminating the population of *An. arabiensis* in Reunion Island appears feasible, though semi-field tests are still required to confirm the suitability of the ANO IPCL1 strain and to assess the release procedure. In addition, improvements of the current methods for monitoring the anopheline adult densities are highly essential, in order to efficiently estimate the appropriate sterile to wild male ratio, and to assess the impact of the releases.

Ae. albopictus is an easier species to rear, and the mass rearing methodology developed by the IPCL in Seibersdorf has been efficiently adapted to this species in collaboration with the FAO/IAEA Collaborative Centre, CAA of Bologna (Medici *et al.* 2011). Small pilot tests suggested that populations of *Ae. albopictus* could be successfully controlled by incorporating the SIT (Bellini *et al.* 2007); during the last trials carried out in small towns of Northern Italy, the releases of ca. 1000 males per hectare per week induced up to 68% sterility in the released areas (Bellini et al, accepted manuscript).

The work reported here brings a better understanding of the reproductive behaviour of sterile males and demonstrates their great survival, reproductive, and competitive potential. The sterile males showed good survival capacity during the first week in semi-field conditions, and over this period they proved to be able to inseminate a maximum amount of 7 females. From these results it might be suggested to favour a weekly release of sterile males. Additionally, in order to improve male survival and competitiveness, we recommend that the releases be done at the adult stage, after a one- to two-day pre-release period in an insectary, allowing the sterile males to rest and feed with sugar. The release ratio of 5:1 leads to an efficient reduction of the wild population fertility; however, a higher release ratio might be necessary during the first period of the program, due to the high local densities of *Ae. albopictus* in some areas of Reunion Island. The sterile male sterility level should be balanced with their competitiveness value (Parker & Mehta 2007). At the beginning of the

SIT program, it might be advisable to favour a partially sterilizing dose that ensures a more efficient competitiveness (such as 30-35 Gy, ensuring 90-95% sterility), so as to have a high initial decrease of fertility. Subsequently, a fully-sterilizing dose would be necessary to reach a complete suppression of the vector population.

The outcomes of the reproduction study concerning *Ae. albopictus* has implication as well for the development of the mass rearing cages for this species. As pre-mated females cannot be re-inseminated after the first hour of her mating, it appears unnecessary to keep males alive in the cage for several days. Removing the sugar after few days would allow killing the males and would increase the propensity of females to blood-feed instead of sugar-feed, resulting in a higher egg production.

The work carried out on *Ae. albopictus* enables us to draw optimistic conclusions as to the use of the SIT against this species on Reunion Island. The logical next step appears to be a small-scale pilot trial, in order to adapt the technology, train staff for the various steps of an SIT program, and to test the sterile mosquitoes in natural conditions. This field phase would allow identifying any logistic and technological issues and the most suitable procedures for the release. The evaluation of the efficiency of this technique in a small-scale trial is essential prior to launching any operational phase.

Further implication of this work

As an alternative to the classical SIT, transgenic methods are being developed which allow genetic sex-separation and/or sterilisation. Although the use of transgenics for mosquito population elimination or replacement faces considerable criticism from a large part of the human population, there is an increasing number of projects on-going studying their potential implementation. The main argument put forward to support transgenic over classical SIT is the reduction of male competitiveness ascribed to the irradiation process (Alphey 2002). However, though an alteration of survival and mating competitiveness due to irradiation is undeniable, this work showed that it can be counteracted by adapting the release process, and does not prevent accomplishing a good reduction of the population fertility. The sterilization does not appear detrimental for the implementation of SIT against *Ae. albopictus*, and previous studies suggest that the same conclusion could be drawn for *An. arabiensis* (Helinski & Knols 2008).

The mating study carried out on *Ae. albopictus*, has brought interesting information but raised even more thrilling questions. Understanding the mechanisms of sperm transfer and storage in the female spermathecae, and the use of the sperm for the egg fertilization, would bring invaluable knowledge on the biology of this model and may have great implications for new vector control strategies such as the ones involving transgenics. Furthermore, we noticed different insemination patterns according to the female availability and to the strain; a better understanding of these discrepancies would be invaluable.

The SIT programmes in which this work has been involved, aim to bring additional opportunities for controlling populations of vectors of serious diseases. It has been a great challenge to play a part in a research project that intends to develop quintessential solutions for these urgent issues.

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Études biologiques et comportementales de deux espèces de moustiques (*Aedes albopictus* et *Anopheles arabiensis*) vectrices de maladies en vue du développement de la technique de l'insecte stérile contre ces vecteurs à l'île de la Réunion.

Les femelles moustiques peuvent être vectrices de nombreux agents infectieux (virus, protozoaires, helminthes) pour l'Homme, qui peuvent être la cause de maladies graves comme le paludisme et la dengue. Ces maladies menacent respectivement 50 et 40% de la population mondiale; le paludisme étant responsable de près d'un million de décès par an. Les méthodes de lutte anti-vectorielle destinées à limiter les populations vectrices et stopper la transmission de maladies, se heurtent au développement incessant de résistances de la part des moustiques et des agents infectieux vis-à-vis des traitements employés. Bien que certaines régions du monde aient réussi à stopper efficacement la transmission de certaines de ces maladies, une grande partie des régions tropicales reste menacée. De plus l'expansion rapide de certaines espèces vectrices, telles qu'*Aedes albopictus*, accroît les risques sanitaires dans de nouvelles régions du globe. La technique de l'insecte stérile (TIS), qui a permis l'éradication ou la suppression des populations de nombreux insectes nuisibles aux cultures et à l'Homme, représente un moyen de lutte prometteur contre les moustiques. Cette technique s'appuie sur le lâcher en masse de mâles stérilisés par rayonnements ionisants qui, en transférant un sperme stérile aux femelles sauvages, vont permettre une diminution progressive de la population cible. Suite à l'épidémie de chikungunya à l'île de la Réunion en 2005 et face aux menaces permanentes de recrudescence de la dengue et du paludisme, les services de lutte anti-vectorielle réunionnais mettent en place d'importants moyens de lutte contre les populations de moustiques concernées. Toutefois, ces mesures ne permettant pas une diminution durable des densités de vecteurs, une étude de faisabilité est en cours quant à l'utilisation de la TIS pour diminuer et contrôler les populations d'*Ae. albopictus*, vecteur de la dengue et du chikungunya, et d'*Anopheles arabiensis*, vecteur du paludisme. Ce travail de thèse s'inscrit dans le cadre du projet TIS Réunion, dans le but d'étudier la biologie et le comportement des souches destinées aux lâchers de mâles stériles. Dans un premier temps, cette étude s'intéresse à la comparaison entre les souches d'élevage d'*An. arabiensis* et les souches sauvages, ainsi qu'aux modalités de stérilisation des mâles de la souche à sexage génétique. Une seconde partie est consacrée à l'étude de l'effet de l'irradiation sur les mâles d'*Ae. albopictus*, en étudiant plus particulièrement leur stratégie de reproduction, leur capacité d'insémination en laboratoire, ainsi que leur compétitivité sexuelle et longévité face aux mâles sauvages en conditions semi-contrôlées.

Mots clés : Technique de l'Insecte Stérile – Ile de la Réunion – *Aedes albopictus* – *Anopheles arabiensis* – stérilisation – reproduction

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Biological and behavioral studies of two disease-transmitting mosquito species (*Aedes albopictus* and *Anopheles arabiensis*) with the aim of developing the sterile insect technique against these vectors on Reunion Island.

Mosquito females are potential vectors of numerous pathogens (viruses, protozoa, helminths), which can cause serious diseases such as malaria and dengue in humans. These two infectious diseases are threatening 50 and 40% of the world population respectively. Malaria is responsible for nearly one million deaths per year, and is considered by many experts as the most important insect-transmitted disease. Anti-vectorial control methods, intended to limit the vector populations and to stop the disease transmission have to face many challenges such as the development of mosquitoes' and pathogens' resistance to the treatments employed to control them. Although various regions of the world have succeeded in efficiently stopping the transmission of some diseases, most of the tropical regions remain under threat. In addition, the rapid expansion of some vector species, such as *Aedes albopictus*, increases the risks in previously safe areas of the world. The sterile insect technique (SIT) has allowed the eradication or suppression of various insect pest populations threatening crops, animal, and human health, and could offer a promising control tool against mosquitoes. The classical SIT relies on the mass releases of males sterilized by ionizing radiation; they transfer sterile sperm to wild females, which results in a progressive reduction of the target population. Following the chikungunya outbreak in Reunion Island in 2005 and considering the constant threat of a recrudescence of dengue and malaria, the anti-vectorial services in Reunion Island are deploying important means to control the relevant mosquito populations. However, these measures do not confer a permanent, or long-lasting reduction of vector densities. A feasibility study is ongoing, evaluating the use of the SIT to diminish and control the populations of *Ae. albopictus*, a vector of dengue and chikungunya, and *Anopheles arabiensis*, a vector of malaria. This PhD work was developed in the context of the SIT Reunion project, with the aim of studying the biology and the behaviour of some strains intended for the sterile male releases. Firstly, this study endeavours to compare colonized and wild strains of *An. arabiensis*, and to determine the sterilisation procedures of the genetic sexing strain males. The second part of this work studies the effect of irradiation on male *Ae. albopictus*, and most notably their reproductive strategy, the insemination capacity in laboratory, and finally their sexual competitiveness and longevity against wild males under semi-field conditions.

Key words: Sterile insect technique – Reunion Island – *Aedes albopictus* – *Anopheles arabiensis* – sterilization – reproduction