Parassitologia 44: 131-135, 2002

An alternative focus in strategic research on disease vectors: the potential of genetically modified non-biting mosquitoes

M. Coluzzi, C. Costantini

Dipartimento di Scienze di Sanità Pubblica, Sezione di Parassitologia, Università "La Sapienza" di Roma, Italy, and Istituto Pasteur-Fondazione Cenci Bolognetti, Università "La Sapienza" di Roma, Italy.

Abstract. We examine the constraints and the feasibility of field experiments involving the release of genetically modified (GM) pathogen-resistant mosquitoes, and whether there are alternatives to the research line based on the production of refractory strains. The production of a GM mosquito strain characterized instead by obligate primiparous and parous autogeny and by disrupted host seeking and biting behaviour could make the release more acceptable by the general public. Genetic transformation should act in this case to reverse some of the essential steps of the evolutionary process that gave rise to hematophagy. The replacement strategy could be based on the mass release of both sexes in a well defined ecological niche made temporarily empty of the natural population, thus avoiding the problems related to the need of sexual competitiveness of the released material. This option is encouraged by the growing evidence that competitive exclusion mechanisms influence the pattern of distribution of different taxa within *Anopheles gambiae* s.s. and by the fact that the plesiomorphic characteristics of vitellogenesis without a blood meal (autogeny), which exploits fat body reserve accumulated during larval life and food other than blood in adult life, persist as genetic variants in various hematophagous insect groups, and it has been found secondarily fixed in others showing stable reversions to primiparous and parous autogeny. If this has been the result of natural selection, then the artificial production of non-biting mosquito strains, by selection and/or transgenesis, should be feasible.

Key words: mosquitoes, genetic transformation, refractoriness to pathogens, vector replacement, obligate autogeny.

Blood sucking (hematophagous) arthropods are responsible for tremendous burdens on humanity, particularly in tropical areas where vector-borne diseases severely affect the quality and duration of human life, as well as livestock productivity (Rodhain and Perez, 1985; Lane and Crosskey, 1993; Kettle, 1995). Moreover, under certain conditions the intense human-biting activity of some of these arthropods often produces significant nuisance leading to widespread applications (by the public and private sectors) of insecticides in domestic, peridomestic, rural and urban environments. This may generate various toxicological and ecological impacts, as well as relevant financial obligations also for communities of temperate areas and of industrialised countries.

Attempting to overcome the limitations of currently available vector control measures and to develop new tools to disrupt the stability of *Plasmodium falciparum* malaria transmission in sub-Saharan Africa, the World Health Organization Special Programme for Research and Training in Tropical Diseases (WHO/TDR) launched a strategic research agenda, that includes a Molecular Entomology Programme based on the proposal of genetic control originally formulated by Curtis (1968a, b). Research priorities for Molecular Entomology were set forth in a meeting of the WHO/TDR Working Group on Disease Vectors held in Tucson, Arizona, USA, in January 1991 (WHO, 1991). Vector control by genetic manipulation of mosquitoes was identified as the general long-term aim, and although many different approaches were proposed for using genetic tools to control disease transmission, the ultimate objective of most scientists has since been the production of mosquito strains refractory to pathogens, with the Afrotropical malaria vector *Anopheles gambiae* being the most important target of this programme (Collins *et al.*, 1999; James *et al.*, 1999; Powell *et al.*, 1999; and references therein).

Relevant progress in vector genetics and in the knowledge of *Plasmodium*-mosquito intimate relationship has been achieved since the Tucson meeting, especially during the last 6 years (Collins *et al.*, 2000; Alphey *et al.*, 2002; Morel *et al.*, 2002). The possibility of successfully transforming mosquitoes by introducing genes of interest has been recently established (Coates *et al.*, 1998; Jasinskiene *et al.*, 1998; Catteruccia *et al.*, 2000), and genes conferring resistance to parasite invasion have been identified (Ito *et al.*, 2002). Thus, the need is emerging to examine critically what are the conditions and the feasibility of field experiments involving the release of genetically modified non-vector strains, and whether there are alternatives to genetic vector con-

Correspondence: Professor Mario Coluzzi, Università degli Studi di Roma "La Sapienza", Dipartimento di Scienze di Sanità Pubblica, Sezione di Parassitologia, Piazzale Aldo Moro 5, 00185 Roma, Italy, Tel ++39 06 44 55 780, Fax ++39 06 49914653, e-mail: mario.coluzzi@uniroma1.it

trol based on mosquito strains refractory to pathogens, other than the well known sterile-insect technique (SIT).

Constraints to the release of transgenic strains refractory to pathogens

Once a refractory genotype is stabilised, ideally it should be introduced into natural vector populations by release of only male mosquitoes. The existing evidence from the few studies on mating behaviour and pre-copulatory barriers in *Anopheles* mosquitoes (Clements, 1999) suggests that mating recognition systems are under continuous selection and that it may be difficult to ensure equal sexual competitiveness of the released material, or at least to decide where, when and how many times genetically transformed males should be released. An important prerequisite to the spread of the refractory mechanisms in natural populations is their close association with an effective driving system which is expected to consist in a transposon or a transposon-like agent. Moreover, when dealing with the control of *An. gambiae* each taxon of the species complex represents a new separate challenge, i.e. new reproductive barriers to be overcome, although there is evidence from the genetic analyses of incipient speciation processes within *An. gambiae* that there are parts of the genome passing among the main vector species of the complex, presumably depending on their co-adaptive and selective value.

More serious constraints exist on socio-political and cultural grounds. Recent public debates on the opportunity to exploit genetically-modified organisms (GMOs) reveal that emotional reactions and extreme caution towards GMOs are widespread sentiments in the public opinion (for example, some African governments decline to accept donations of GM maize food for famine relief) and we would expect such scepticism to be accentuated when dealing with the field release of biting insects. A small group of health authorities from seven African countries (Benin, Burkina Faso, Madagascar, Mali, Tanzania, Uganda and Zanzibar) as well as African students of medicine and biology from Mali, Nigeria and Burkina Faso polled by one of us (MC) were not optimistic about the possibility of securing the informed consent that is required to initiate a release of transformed *Anopheles* strains in their respective countries. Their most significant concern referred to the danger that, while suppressing the vector competence for the target pathogen (e.g. *Plasmodium falciparum*) through genetic manipulation, unexpected transmission abilities may develop that could either amplify the spread of other vector-borne pathogens (yellow fever and dengue arboviruses were the most frequently mentioned) or even favour the transmission of pathogens currently not transmitted by vectors such as HBV and HIV. Although the vector-borne transmission of HBV is usually regarded as unlikely and HIV is absolutely unconceivable, potentially disastrous outcomes cannot be discarded on theoretical ground, especially considering the evidence that HBV survives long enough in mosquitoes (Hyams, 1989; Blow *et al.*, 2002) to be transmitted either by interrupted feeding or when haematophagous insects are crushed on the skin during blood-feeding.

Vigorous promotion of scientific information could alleviate concerns in groups of people who are more capable of understanding such issues by virtue of their profession or education (e.g. doctors, para-medical staff, students, etc.). Data can also be provided which allow to discard the HBV and HIV transmission by testing the material to be released. However, any consent obtained for the release trial would be vulnerable to criticism from the general public (who are less competent to assess the scientific issues – even if they are well aware of the information) and could be suddenly lost in the unfortunate event of an unrelated epidemic that might occur concomitantly to the experimental release. This scenario could be exacerbated if the perceived correlation between the release and the epidemic is supported by misleading messages promulgated by local mass media and/or if charged with adverse ideological or religious preconceptions. Accordingly, the risk of securing consent is likely to be considered unbearable by both local authorities and donor agencies, and consequently the objective of implementing vector control by the use of refractory strains is likely to remain at the laboratory stage of development – eventually losing financial support. In addition to the cultural and political issues voiced by representatives of disease-endemic countries, the intended products of this scientific endeavour (i.e. pathogen-resistant vectors) are not appealing to non-endemic countries or to the private sector in terms of economic returns. This could prove to be another major obstacle to obtaining the economic support needed for such a costly and high-risk research enterprise, whereby substantial scientific resources must be committed on a long-term basis. Thus, it looks like the long-term prospects for implementing vector control strategies by release of transgenic refractory strains are not encouraging.

From blood meal-induced to autogenous vitellogenesis

Only the presentation of a GM mosquito strain, characterized by obligate autogeny (i.e. ovarian maturation without a prior blood meal) and by disrupted host-seeking and biting behaviour, could make the release acceptable by the general public. Genetic transformation should act in this case to reverse some of the essential steps of the evolutionary processes that gave rise to haematophagy. Autogeny of female mosquitoes is usually limited to the first gonotrophic cycle, the following cycles being blood-meal dependent, i.e. anautogenous. Hence, for most

species of autogenous mosquitoes, this capacity does little to reduce vectorial capacity of females that generally survive after the initial autogenous oviposition and may blood-feed repeatedly. Thus, the purpose of this research line should also aim to develop genetic mechanisms to disrupt blood-feeding, and eventually result in the production of strains characterised by obligate autogeny of parous females.

Such a completely autogenous mosquito strain would not necessarily require to be sexually competitive with the natural vector population if the replacement strategy is based on mass release of both sexes into a well-defined biotope (ecological niche) where the natural anautogenous population of the target species has been suppressed by prior insecticide treatment or innundative release of sterilized male insects (SIT). Also the need for an effective driving system and for its close linkage to the non-biting genetic characteristics appear to be less compelling. The option of releasing transformed strains is encouraged by the growing evidence that competitive exclusion mechanisms influence the pattern of distribution of different taxa of the main malaria vector in tropical Africa, *Anopheles gambiae* and that larval niche partitioning (genetically modulated by chromosomal paracentric inversions) characterizes ecotypic differences and sympatric incipient species (Coluzzi *et al.*, 1979; Coluzzi, 1982; Coluzzi *et al.*, 2002; della Torre *et al.*, 2002). Once the transformed strain is established in the field, contact zones with the natural populations should be under intensive monitoring to evaluate possible isolating mechanisms and/or the extent of introgression and its impact on the wild-vector phenotype. Although this is undoubtedly a high-risk research line, several sources of evolutionary evidence support its feasibility as shown below.

Adaptation to blood feeding has arisen independently in various groups of arthropods, from lice and fleas to bedbugs and ticks. Hematophagy is particularly frequent in the order Diptera where it occurs in phylogenetically well separated groups such as mosquitoes (Culicidae), biting midges (Ceratopogonidae), blackflies (Simuliidae), sandflies (Phlebotominae), tsetse flies (Glossinidae), stable flies (Muscidae) and horse flies (Tabanidae). Such multiple independent events of convergent evolution imply that the reproductive advantage of access to blood is so high that it drives the selection of dramatic adaptive changes involving both structural and etho-physiological traits, e.g. specialised mouth parts, host-seeking behaviour, and metabolic pathways for effective blood sucking and digestion (Clements, 1999). The gonotrophic cycle of hematophagous insects is generally induced by the blood meal, and egg maturation is directly related to blood digestion. However, the plesiomorphic characteristic of vitellogenesis without a blood meal, which exploits fat body reserves accumulated during larval life or food other than blood in adult life (primiparous and parous autogeny respectively), persists in some hematophagous insect groups and is an important option for population stability in the absence of suitable hosts. Evidence of a reversion from blood feeding to exclusive autogenous reproduction is observed in various dipteran families and, in some cases, this reversion entails the regression of mouth parts which are then too weak and poorly shaped to be able to pierce the host's skin (Lane and Crosskey, 1993). In spite of a general structural adaptation to biting habits, the families Ceratopogonidae and Simuliidae commonly show autogeny and non-biting behaviour arising secondarily in most genera. The Mesozoic radiation of biting midges and blackflies was presumably associated with the spread of large reptiles and, when these hosts suddenly disappeared, the reversion to autogeny could have been a more successful adaptive option than shifting to completely new or relatively rare host species of birds or mammals.

Obligate mosquito autogeny and adaptation to blood substitutes as research targets for new strategies of pest control

Perhaps the most appropriate starting material for production of obligate autogenic strains can be found in mosquitoes of the *Culex pipiens* complex, including the tropical *Cx. quinquefasciatus*. These abundant mosquitoes have several important advantageous characteristics: they are distributed worldwide, and they represent important pest species in most urban and periurban areas, they are vectors of bancroftian filariasis and arboviruses, they host *Wolbachia* symbionts (i.e. a potential mechanism to drive foreign genes into natural populations; Curtis and Sinkins, 1998), they are easily bred in the laboratory, and strains characterised by primiparous autogeny are already available or can be easily selected; moreover, a germline transformation system has been recently made available (Allen *et al.*, 2002). Using *Culex pipiens*, some of the research objectives could be: (i) to identify the genetic and phenotypic determinants of primiparous and parous autogeny; (ii) to clone and characterise the expression of the major genetic factors responsible for these traits; (iii) to study the distribution of these genes within and outside the *Culex pipiens* complex; (iv) to test the capacity of the genes to be transferred and expressed in the genome of other mosquitoes; and (v) to elucidate the regulatory plasticity of the mouth parts development directed by the *hox* gene proboscipedia (*pb*) and to evaluate the possibilities to alter its expression.

A strain of *Cx. pipiens* characterised by stable primiparous autogeny could be utilized for investigations aimed at: (a) decreasing the fitness of those individuals that are dependent on a blood meal for the second (and subsequent) gonotrophic cycle(s), possibly making the blood meal unsuitable to the mosquito by manipulation of genes expressed in the

mouth parts, salivary glands or midgut, and associated with blood-feeding and/or blood digestion; (b) disrupting host-seeking behaviour by acting on the genetic determinants of the etho-physiological steps that control mosquito activation, orientation towards the host, or landing and probing; and (c) promoting etho-physiological mechanisms for gonotrophic cycles based on natural nutrients alternative to blood, or on artificial blood substitutes specifically produced and made available to the non-biting mosquito. The production, by selection or transgenesis, of mosquitoes structurally unable to pierce the host skin could represent an interesting alternative to (b) obviously leading to an obligate autogenous strain.

Further protocols can be proposed for species whose larval ecology is unfavourable to the development of preimaginal vitellogenic reserves (therefore less prone to autogeny and more dependent on blood sucking), such as the Afrotropical malaria vector *An. gambiae*. In this case we advocate selection for larval predation and/or adult food alternative to blood, since natural models exist in mosquitoes for both these adaptations. Koenraadt and Takken (2003) described predation of older larvae on younger larvae among members of the *An. gambiae* complex, while autogeny among mosquitoes includes many species of aedines, sabethines and at least one *Anopheles* (Russell, 1979). In the non-biting mosquito genus *Toxorhynchites,* the trophic resources for egg production are provided by the predatory activity of the larva, and the hook-shaped proboscis of the adult has adapted to feeding on nectar or other plant juices and is therefore unable to pierce any host's skin. The non-biting mosquitoes of the genus *Malaya* insert their specialised proboscis into the mouth of *Crematogaster* ants to suck regurgitated drops of honeydew and other secretions which the ants have obtained from plant aphids. Commonly *An. gambiae* predatory activity is optional depending on the availability of conspecific or heterospecific newly hatched larvae. This trait could be manipulated for laboratory strains maintaining them on a diet of first-instar larvae and selecting for efficiency of predation or cannibalism. The induction of vitellogenesis by feeding on blood substitutes should be attempted by exploiting selection and/or transgenesis. At the same time the possibility should be explored of inducing suitable changes in the mosquito mouth parts to avoid the utilisation of the structure for biting activity. Specific genetic modifications of mosquito host-seeking behaviour can be foreseen, determined by disruption of the very complex physioethological steps on which it is based. Once such genetically modified non-biting strains become available, release experiments can be safely planned, and further selection may be achieved by mass breeding in stringent natural or semi-natural conditions capable of inducing a negative selection of any attempt to biting and a positive selection for all traits enhancing reproductive capacity by primiparous and parous autogeny. The most critical issue remains the advantage of the wild hematophagous phenotype over the autogenous released material. The study of artificial diets which can induce vitellogenesis in anautogenous mosquitoes appears of crucial importance. Once a blood substitute is identified the challenge would be to obtain a shift in biting behaviour implying the mosquito adaptation to feed on artificial diet dispensers. Ideally the mosquito should react to a specific odour associated with the artificial diet dispenser and ignore human/animal odours. This would be obviously more reliable than simply transposing the factors governing zoophily versus anthropophily as proposed by Curtis *et al.* (1999), since host preference is not sufficiently fixed (e.g. Pates *et al.*, 2001) and haematophagous strains may inevitably bite humans to some extent.

Concluding remarks

The production of a non-biting strain of mosquitoes for replacement strategies is undoubtedly a complex enterprise – probably even more complex than the production of GMOs characterised by sexual competitiveness and by complete, multi-factorial, dominant refractoriness to parasites tightly linked to an efficient driving system. However, we believe that some preliminary attempts to implement some of our protocols are worthwhile. As natural selection has produced hundreds of cases of complete reversal from hematophagy to autogeny and very special mosquito adaptations – like those observed in the genus *Malaya* – then a GM mosquito adapted to feed on an artificial diet dispenser could be created to displace the natural blood-feeding conspecific population. Indeed, the recent advances in the molecular genetics of mosquitoes, spurred by the goal of developing parasite resistance, provide an optimistic framework in which to consider developing genetically-engineered autogeny and altered patterns of biting behaviour, or evaluating possible modification of the pattern of vector distribution with an impact on competitive exclusion. Perhaps more importantly, work toward this goal could raise an even wider interest in genetic control, since the GMOs would be instrumental in this case not only in the replacing strategies for disease vectors in endemic countries, but could also be used more generally against pest species. Thus, such an approach is more likely to become actively sponsored by industrialised countries and to stimulate the economic interests of the private sector. In conclusion, the expansion of the WHO/TDR Molecular Entomology programme to embrace the relevant objectives described in this paper should provide more realistic perspectives based on end-user demands, thus increasing the likelihood of long-term support to develop sufficiently stable know-how shared by a critical mass of scientists for implementing safe solutions to global problems.

Acknowledgements

The first draft of this paper was circulated among some friends during September-October 2000 and it was included in a report dated April 2001 to the World Health Organization, dealing with the activity of the WHO Collaborating Centre for Malaria Epidemiology and Control. It took nearly two years and discussion with various colleagues to convince ourselves that it was worthwhile to share the provocative dream of the non-biting mosquito with a wider audience and to release the paper as a formal publication. For comments (positive and negative), for suggestions of changes, for improvement of English and for encouragement we would like to express our gratitude to Frank Collins, Nora Besansky, Jeffrey Powell, Anthony James, Peter Atkinson, Adalgisa Caccone, Graham White, Carlos Morel, Yeya Tiemoko Touré, Fotis Kafatos, Boris Dobrokhotov, Bruno Arcà and Andrea Bosman. We acknowledge the support of WHO/TDR and Compagnia di San Paolo of Turin, Italy.

References

Allen ML, O'Brochta DA, Atkinson PW, Levesque CS (2001). Stable, germ-line transformation of *Culex quinquefasciatus* (Diptera: Culicidae). J Med Entomol 38: 701-710.

Alphey L, Beard CB, Billingsley P, Coetzee M, Crisanti A, Curtis C, Eggleston P, Godfray C, Hemingway J, Jacobs-Lorena M, James AA, Kafatos FC, Mukwaya LG, Paton M, Powell JR, Schneider W, Scott TW, Sina B, Sinden R, Sinkins S, Spielman A, Toure Y, Collins FH (2002). Malaria control with genetically manipulated insect vectors. Science 298: 119-121.

Blow JA, Turell MJ, Walker ED, Silverman AL (2002). Post-bloodmeal diuretic shedding of hepatitis B virus by mosquitoes (Diptera: Culicidae). J Med Entomol 39: 605-612.

Catteruccia F, Nolan T, Loukeris TG, Blass C, Savakis C, Kafatos FC, Crisanti A (2000). Stable germline transformation of the malaria mosquito *Anopheles stephensi*. Nature 405: 959-962.

Clements AN (1999). The Biology of Mosquitoes, vol 2, Sensory reception and behaviour. CABI Publishing, Wallingford, Oxon, UK, 1-740 pp.

Coates CJ, Jasinskiene N, Miyashiro L, James AA (1998). Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. Proc Natl Acad Sci USA 95: 3748-3751.

Collins FH, Kamau L, Ranson HA, Vulule JM (2000). Molecular entomology and prospects for malaria control. Bull Wld Hlth Org 78: 1412-1423..

Collins FH, Saunders RD, Kafatos FC, Roth C, Ke Z, Wang X, Dymbrowski K, Ton L, Hogan J (1999). Genetics in the study of mosquito susceptibility to *Plasmodium*. Parassitologia 41: 163-168.

Coluzzi M (1982). Spatial distribution of chromosomal inversions and speciation in Anopheline mosquitoes. In: Mechanisms of Speciation (Barigozzi C, ed). Alan Liss Inc, New York, pp 143-153.

Coluzzi M, Sabatini A, della Torre A, Di Deco MA, Petrarca V (2002). A polytene chromosome analysis of the *Anopheles gambiae* species complex. Science 298: 1415-1418.

Coluzzi M, Sabatini A, Petrarca V, Di Deco MA (1979). Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Trans R Soc Trop Med Hyg 73: 483-497.

Curtis CF, Pates HV, Takken W, Maxwell CA, Myamba J, Priestman A, Akinpelu O, Yayo AM, Jiang Ting Hu (1999). Biological problems with the replacement of a vector population by *Plasmodium*-refractory mosquitoes. Parassitologia 41: 479-481.

Curtis CF, Sinkins SP (1998). *Wolbachia* as a possible means of driving genes into populations. Parasitology 116 (Suppl): S111-S115.

Curtis CF (1968a). A possible genetic method for the control of insect pests, with special reference to tsetse flies (*Glossina* spp). Bull Entomol Res 57: 509-523.

Curtis CF (1968b). Possible use of translocations to fix desirable genes in insect pest populations. Nature 218: 368-369.

della Torre A, Costantini C, Besansky NJ, Caccone A, Petrarca V, Powell JR, Coluzzi M (2002). Speciation within *Anopheles gambiae*: the glass is half full. Science 298: 115-117.

Hyams KC (1989). Mosquito transmission of hepatitis B. Trop Geogr Med 41: 185-189.

Ito J, Ghosh A, Moreira LA, Wimmer EA, Jacobs-Lorena M (2002). Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. Nature 417: 452-455.

James AA, Beerntsen BT, Capurro M de L, Coates CJ, Coleman J, Jasinskiene N, Krettli AU (1999). Controlling malaria transmission with genetically-engineered, *Plasmodium*-resistant mosquitoes: milestones in a model system. Parassitologia 41: 461-471.

Jasinskiene N, Coates CJ, Benedict MQ, Cornel AJ, Rafferty CS, James AA, Collins FH (1998). Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the Hermes element from the housefly. Proc Natl Acad Sci USA 95: 3743-3747.

Kettle DS, ed (1995). Medical and Veterinary Entomology, 2nd edn. CABI Publishing, Wallingford, Oxon, UK, 1-725 pp.

Koenraadt CJM, Takken W (2003). Cannibalism and predation among larvae of the *Anopheles gambiae* complex. Med Vet Entomol (in press).

Lane RP, Crosskey RW, eds (1993). Medical Insects and Arachnids. Chapman & Hall, London, UK, 1-723 pp.

Morel CM, Toure YT, Dobrokhotov B, Oduola AMJ (2002). The mosquito genome: a breakthrough for public health. Science 298: 69.

Pates HV, Takken W, Curtis CF, Huisman PW, Akinpelu O, Gill GS (2001). Unexpected anthropophagic behaviour in *Anopheles quadriannulatus*. Med Vet Entomol 15: 293-298.

Powell JR, Petrarca V, della Torre A, Caccone A, Coluzzi M (1999). Population structure, speciation, and introgression in the *Anopheles gambiae* complex. Parassitologia 41: 101-113.

Rodhain F, Perez C (1985). Précis d'Entomologie médicale et vétérinaire. Maloine SA, Paris, France.

Russell RC (1979). A study of the influence of some environmental factors on the development of *Anopheles annulipes* Walker and *Anopheles amictus hilli* Woodhill and Lee (Diptera: Culicidae). Part 2: Influence of salinity, temperature and larval density during the development of the immature stages on adult fecundity. Gen Appl Entomol 11: 42-45.

WHO (1991). Prospects of malaria control by genetic manipulation of its vector. TDR/BCV/MAL-ENT/91.3 World Health Organization, Geneva, Switzerland.